CYTOTOXIC THIOL ALKYLATORS CONTAINING THE 1,5-DIARYL-3-OXO-1,4-PENTADIENYL PHARMACOPHORE

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in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in the College of Pharmacy and Nutrition
University of Saskatchewan
Saskatoon

By

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ABSTRACT

For the last several years curcumin has attracted considerable interest due to its ability to inhibit cancer cell proliferation *in vitro* and *in vivo* by targeting a number of different cell signaling and molecular mechanism pathways in cancer cells. These results prove its potential to be considered as a future anticancer drug candidate. However its metabolic instability, low oral bioavailability and high clearance have limited its use as a clinical drug candidate. Subsequently enormous efforts have been expended by medicinal chemists to modify the curcumin structure which has led to the development of novel cytotoxic curcuminoid analogs.

The replacement of the β-diketone moiety by a mono carbonyl group led to the discovery of the 1,5-diaryl-3-oxo-1,4- pentadienyl pharmacophore which was found to be metabolically stable and demonstrate high cytotoxic potencies. This class of compounds which possesses multiple alkylating sites are referred to as thiol alkylators based on their ability to interact preferentially with the thiol groups of macromolecules compared to the hydroxy and amino groups present in nucleic acids. The ability of this class of compounds to target multiple biochemical pathways has been considered to be an advantage to overcome multidrug resistance that is shown by many tumours to current anticancer drugs.

My current work in this report focuses on the development of novel curcuminoid analogs possessing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore as potent cytotoxic agents with the aspiration of identifying some lead molecules which can further be developed as anticancer drug candidates. In particular, the synthesis of novel curcuminoids based on the 3,5-bis(arylidene)-4-piperidone nucleus is pursued. One of the main objectives was to produce novel cytotoxins which will display selective toxicity towards malignant cells compared to normal cells. In order to obtain tumour-selective cytotoxins, the design of molecules was based on a theory of sequential cytotoxicity which states that an initial chemical attack on cellular constituents followed by a second chemical attack will cause more damaging effects in cancer cells than normal cells. The sequential alkylation reaction was proposed to take place on both of the olefinic carbon atoms of the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore in the molecules at the primary binding site whereas the other part of the molecule would interact at an auxiliary binding site which may confer preferential toxicity to tumours. Efforts have been made to improve the physicochemical properties of the molecules by introducing hydrophilic groups

such as phosphates onto the molecules. Bioevaluations of the novel molecules disclosed in the thesis revealed that many of these compounds display potent cytotoxic properties towards a wide range of neoplastic and transformed cells and show greater cytotoxic potencies to neoplasms than normal cells. Most of the molecules demonstrated higher cytotoxic potencies and greater tumour selectivity than melphalan, a reference alkylating anticancer drug. In general, increasing the number of thiol alkylating sites in the molecule has increased cytotoxic potencies and selective toxicities to tumours compared to normal cells. One of the major challenges in cancer treatment is the resistance shown by tumors towards a number of chemotherapeutic agents. The molecules designed in this report are chemically and structurally divergent from established anticancer drugs; therefore they are expected to display different modes of action and may be able to overcome drug resistance shown by tumours to contemporary anticancer agents. The ability of novel cytotoxic agents to modulate P-glycoprotein mediated drug resistance, a major form of drug resistance in cancers, was verified in a neoplastic cell transfected with the mdr1 gene. A number of molecules demonstrate remarkable multidrug resistance reversal properties in a neoplastic cell line and the aspiration is that one or more molecules can be developed as a potent multidrug resistance modulator. A striking feature of many of these curcuminoids is that using a dose level up to and including 300 mg/kg is well tolerated in mice and displays no significant toxicities. The modes of action of a number of representative potent cytotoxic molecules were evaluated which include apoptosis, caspase-3 activation, DNA fragmentation, PARP cleavage and cell cycle arrest. The mitochondrion is emerging as a potential target for anticancer agents and to evaluate this possibility, the effect of a number of potent cytotoxins on mitochondrial functions was verified. The compounds affected respiration and caused swelling in mitochondria. Some guidelines for future development of these molecules are suggested.

It is hoped that this eulogy of the importance of the conjugated dienone group will encourage researchers to consider incorporating this structural unit into candidate cytotoxins.

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Dedicated
to
My Parents and Family

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LIST OF ABBREVIATIONS

ADP: Adenosine diphosphate

AP-1: Activating protein-1

Apaf-1: Apoptotic peptidase activating factor 1

APC: Adenomatous polyposis coli

APO-1: Apoptosis antigen-1

ATCC: American type culture collection

ATP: Adenosine triphosphate

BBO: Broadband observe

CC_{50}: Concentration required to kill 50% of the cell

CD1: Cyclin D1

CD95: Cyclin D95

$CDCl_3$: Deuterated Chloroform

CHNS: Carbon hydrogen nitrogen sulfur

CNS: Central nervous system

COX-2: Cyclooxygenase-2

CYP3A4: Cytochrome P_{450} 3A4

DCE: Dichloroethane

DMEM: Dulbecco's Modified Eagle's medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DTT: Dithiothreitol

EDTA: Ethylenediaminetetraacetic acid

EGFR: Epidermal growth factor receptor

Egr-1: Early growth response protein 1

EGTA: Ethylene glycol tetraacetic acid

ErbB-2: Epidermal growth factor receptor in breast cancer

FAD: Flavin adenine dinucleotide

FADD: FAS-associated death domain protein

FAP: Familial adenomatous polyposis

FAR: Fluorescence activity ratio

FAS: Factor related apoptosis

FBS: Fetal bovine serum

FITC: Fluorescein isothiocyanate

5-FU: 5-Fluorouracil

HBA: Hydrogen bond acceptor

HBD: Hydrogen bond donor

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HER-2: Human epidermal growth factor Receptor 2

HGF: Human gingival fibroblast

HL-60: Human promyelocytic leukemia cells

h-NMT: Human N-myristoyl transferase

HPC: Human pulp cell

HPLF: Human periodontal ligament fibroblast

HSC: Human oral squamous cell carcinoma

HSG: Hysterosalpingogram

HUVEC: Human umbilical vein endothelial cells

IC_{50}: Inhibition concentration 50%

KCl: Potassium chloride

Ki-ras: Kirsten ras gene (found in the human lung carcinoma cell line)

KSRP: KH-type splicing regulatory protein

LogP: Logarithm of the partition coefficient

LOX: Lipoxygenase

MAPK: Mitogen-activated protein kinase

MDR: Multidrug resistance

MHz: Megahertz

MMP-9: Matrix metalloproteinase-9

MOPAC: Molecular orbital package

MR: Molecular refractivity

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MW: Molecular weight

NADPH: Nicotinamide adenine dinucleotide phosphate

NCI: National Cancer Institute

NF-κB: Nuclear factor-κB

NINDS: National Institute of Neurological Disorders and Stroke

NMR: Nuclear magnetic resonance

NOS: Nitric oxide synthase

ORTEP: Oak Ridge Thermal Ellipsoid Plot Program

PARP: Poly-ADP ribose polymerase

PBS: Phosphate buffered saline

PC-3: Prostate cancer cell line (human)

P-gp: p-Glycoprotein

PK: Pharmacokinetic

PM3: Parameterized model number 3

PMSF: Phenylmethanesulfonyl fluoride

PSE: Potency-selectivity expression

PTP: Permeability transitions pore

QSAR: Quantitative structure activity relationship

RB: Rotatable bonds

RCR: Respiratory control ratio

RHEL: Red hat enterprise linux

RP: Relative potency

RPMI: Roswell Park Memorial Institute

RT: Room temperature

SD: Standard deviation

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SI: Selectivity index

SPSS: Statistical Package for the Social Sciences

TLC: Thin layer chromatography

TNF: Tumor necrosis factor

TPSA: Total polar surface area

TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling

UGT: UDP-glucuronosyltransferase

uPA: Urokinase plasminogen activator

UV: Ultraviolet

INTRODUCTION

Cancer continues to be one of the most devastating diseases in the 21st century. In the last decades scientists have expended numerous efforts to discover novel strategies to treat cancers; however, in general the cure of cancer still remains elusive. Even with the evolving of newer treatment options arising from current technological advancements, success in cancer treatment often remains unsatisfactory. Besides various treatment strategies such as surgery and radiation therapy, chemotherapy is still considered to be as the mainstay in cancer treatment. The emergence of multidrug resistance in cancers and the lack of selectivity of contemporary anticancer drugs to cancers than normal tissue are posing serious challenges today that need to be addressed for the success of chemotherapy. Therefore, novel drug candidates that are structurally divergent from available anticancer drugs and display different modes of action are urgently required to treat drug-resistant tumours.

Over the last many decades, chemotherapeutic drugs used in the clinic have ranged from natural products to semisynthetic and synthetically produced chemical agents. The development of new anticancer agents by modifying the structure of a pharmacologically active natural product was considered the most viable approach in the past. In the last few years, curcumin, an ingredient of Curcuma longa, used as a spice in curry by Asian people, has attracted the attention of medicinal chemists owing to its association with a wide range of pharmacological activities, particularly anticancer properties. The clinical use of curcumin was limited due to a number of factors including low systemic stability, poor bioavailability and high clearance. To address these issues, the structure of curcumin has been modified in a number of ways keeping in mind the pharmacophores in the molecule that contribute to the anticancer properties. This endeavor has led to a diverse class of curcumin analogs, referred to as curcuminoids. One of the important molecular fragments that contributes toward the anticancer properties of curcumin is the α,β-unsaturated ketone (enone) motif which alkylates cellular thiol nucleophiles. The molecules possessing this fragment display an array of pharmacological activities. A strategy was evolved by Dimmock's group to expand an aryl enone group (Ar-CH=CH-CO-) to a diaryl dienone function (Ar-CH=CH-CO-CH=CH-Ar) to enhance the alkylating potential of the molecule with a view to enhance anticancer properties. This diaryl dienone pharmacophore resembles the diaryl 1,3-diketone (Ar-CH=CH-CO-CH_2-CO-CH=CH-Ar) structural unit of curcumin and has led to

the discovery of novel molecules with improved cytotoxic properties and better metabolic stability.

Perspectives of the Ph.D. project

To develop novel curcuminoid analogs possessing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore based on the following hypotheses and to evaluate their potential as cytotoxic and multidrug resistance agents.

1. Hypotheses

1.1 Incorporation of a 1,5-diaryl-3-oxo-1,4-pentadienyl (Ar-CH=CH-CO-CH=CH-Ar) group onto alicyclic and heterocyclic scaffolds will lead to potent cytotoxic agents.

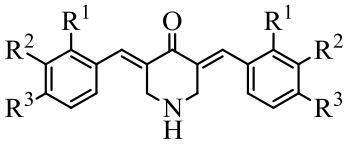
1.2 Sequential attack of cellular thiols onto the 1,5-diaryl-3-oxo-1,4-pentadienyl (Ar-CH=CH-CO-CH=CH-Ar) scaffold will be more detrimental to neoplasms compared to normal tissues.

1.3 Doubling the number of sites available for thiol alkylation in a candidate cytotoxin will increase cytotoxic potency by more than two-fold.

1.4 The design of cytotoxic molecules that are structurally divergent from contemporary anticancer agents may prove to be beneficial in overcoming drug resistance shown by tumours towards a number of anticancer agents.

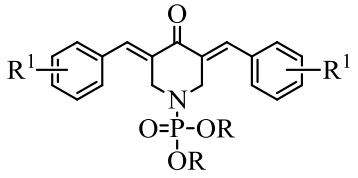
2. Research Objectives

2.1 3,5-bis(Benzylidene)-4-piperidones as building blocks for designing cytotoxic agents: A proof of concept.



R^1, R^2, R^3= H, alkyl, hydroxy, halo, nitro, amino, carboxy, carboethoxy, etc

2.2 Design and synthesis of N-phosphonate derivatives of 3,5-bis(benzylidene)-4-piperidones as a novel class of cytotoxic and multidrug resistance reversal agents.

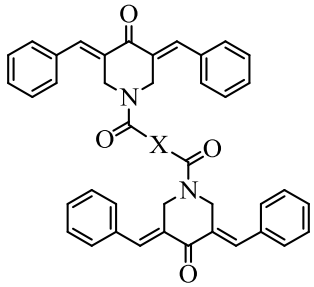


R= H, C_2H_5

R^1 = H, alkyl, alkoxy, halo, nitro

2.3 To examine the theory of sequential cytotoxicity using N-phosphonate derivatives of 3,5-bis(benzylidene)-4-piperidones.

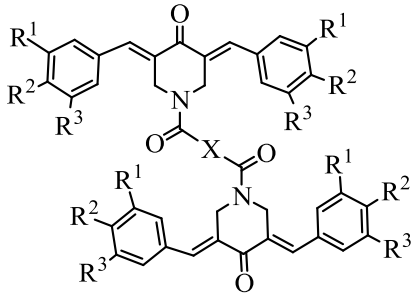
2.4 To develop 3,5-bis(benzylidene)-4-piperidones dimers as a novel class of thiol targeting cytotoxic agents- A proof of concept of cytotoxic synergism.



X= $(CH_2)_n$, $(CH{=}CH)_y$, C≡C , o,m,p-Ph

n = 0,1,2,3,6 y = 1, 2

2.5 To design and prepare aryl substituted 3,5-bis(benzylidene)-4-piperidone dimers as novel cytotoxic agents based on the lead compounds identified from the objective 2.4**.**



X= $(CH_2)_n$ n= 0, 1

R^1, R^2, R^3= H, alkyl, alkoxy, hydroxy, halo, nitro

2.6 To investigate the effect of cytotoxic agents possessing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore on mitochondrial functions. The question to be addressed is whether cytotoxicity shown by the lead potent cytotoxic agents is mediated by targeting mitochondria.

CHAPTER 1

Relationship of Chapter 1 to the objectives of this project

A thorough literature survey on cytotoxic molecules possessing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore (Ar-CH=CH-CO-CH=CH-Ar) was important in order to gain some insight for designing the novel molecules in this study.

Description

The review outlines a number of important sections : (i) the genesis of 1,5-diaryl-3-oxo-1,4-pentadiene as a cytotoxic pharmacophore, (ii) its selective affinity for thiols, (iii) acyclic and cyclic cytotoxic molecules possessing this pharmacophore, (iii) their modes of action for displaying cytotoxicity, and (iv) their multidrug resistance reversal properties.

Author Contribution

A literature survey on cytotoxic molecules possessing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore was conducted by me and the review was written by myself with the guidance of my supervisors Drs. J.R. Dimmock and D. K. J. Gorecki.

CHAPTER 1

CYTOTOXIC 1,5-DIARYL-3-OXO-1,4-PENTADIENES: A REVIEW OF THE LITERATURE

1. Introduction

The economic burden due to cancer is growing exponentially across the globe day by day. Despite evolving treatment strategies, cancer continues to be one of the most alarming diagnoses and the treatment remains problematical. Besides undesired toxicities and lack of sufficient effectiveness of currently available chemotherapeutic drugs, multidrug resistance (MDR) has emerged as a serious challenge for the successful treatment of cancers. The discovery of novel anticancer agents that can selectively target tumours compared to normal cells and show efficacy against MDR tumours are the need of the hour to combat the horrendous effects of cancers, reduce cancer deaths and to improve the quality of patients' lives.

In the last several years, many novel chemical structures have emerged from academia and pharmaceutical industries that display potent anticancer properties. However, the journey from discovering new chemical entities and developing them into clinically useful drugs is a complex, expensive and time-consuming process[1,2] especially when the attrition rate of anticancer compounds in the drug discovery pipeline is very high compared to finding drugs to treat other medical problems. There is a paradigm shift in anticancer drug discovery approach in the 20th century that traverse from non-target to target-specific strategies which is still heavily debated. It has resulted in some improvements in treating cancers but is still considered to be very little. Undoubtedly, cancer is a multifactorial disease caused by physiological and mechanistic deregulation of hundreds of genes and signaling cascades and therefore it appears that the treatment of cancer by a specific targeted approach is unlikely to bring success. Therefore, the chemotherapeutic agents that can target multiple intracellular components are of interest. The value of developing anticancer agents that interact at multiple sites[3-5] has been advocated in the literature and arguments have been made in favour of antineoplastic agents with multiple sites of action are more likely to overcome MDR shown by the tumours.

The therapeutic potential of natural products continues to attract the attention of drug discovery researchers and these medications comprise a substantial fraction of the current pharmacopeia.[6,7] Although there has been a substantial drop in the discovery of natural product–based drugs between 2001 and 2008[7] due to the evolution of synthetic drugs, a large number of

natural product–based drugs are in clinical studies. Enormous efforts have been expended by medicinal chemists over many years to modify the structures of bioactive natural products to improve their toxicological and pharmacological properties. In recent years one such natural product, namely curcumin 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadien-3,5-dione, which is obtained from the rhizome of *Curcuma longa* and is also known as "Indian saffron" in Europe, has drawn the attention of numerous researchers. Curcumin possesses potent anticancer and chemopreventive properties[8-11] and other biological activities such as anti-inflammatory[12] antibacterial[13] and antioxidant properties.[14] The cytotoxic potential of curcumin stems from its ability to suppress tumour initiation, promotion and metastasis by targeting multiple pathways, such as inhibiting the expression of COX2, LOX, NOS, MMP-9, uPA, TNF, chemokines, cyclin D1, downregulating the transcription factors NF-kB, AP-1, Egr-1, and growth factor receptors (such as EGFR and HER2), inhibition of kinases (protein tyrosine kinases, c-Jun N-terminal kinase, and protein serine/threonine kinases) and angiogenesis.[15] The usefulness of curcumin as a chemotherapeutic and chemopreventitive agent which emerges from its ability to target multiple biochemical pathways has triggered enormous interest of medicinal chemists to develop novel curcuminoid analogues as future anticancer drug candidates.

2. Genesis of the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore

Despite the promising bioactivities associated with curcumin, its clinical development has been hampered by its poor aqueous solubility, low *in vivo* efficacy and unfavourable and highly variable pharmacokinetics.[15] These drawbacks are attributed to its extensive first-pass metabolism[16] and some degree of intestinal metabolism, particularly glucuronidation and sulfation.[17] Curcumin undergoes metabolic reduction to form dihydrocurcumin, tetrahydrocurcumin, hexahydrocurcumin and octahydrocurcumin, which are subsequently converted into monoglucuronide conjugates.[17] Curcumin faces challenges of P-glycoprotein (Pgp) mediated drug resistance when administered via the oral route. The bioavailability of curcumin increases by 154 and 2000 % in rats and humans, respectively, when administered with l-piperoylpiperidine (piperine) which is an inhibitor of UGT, CYP3A4 and Pgp.[18]

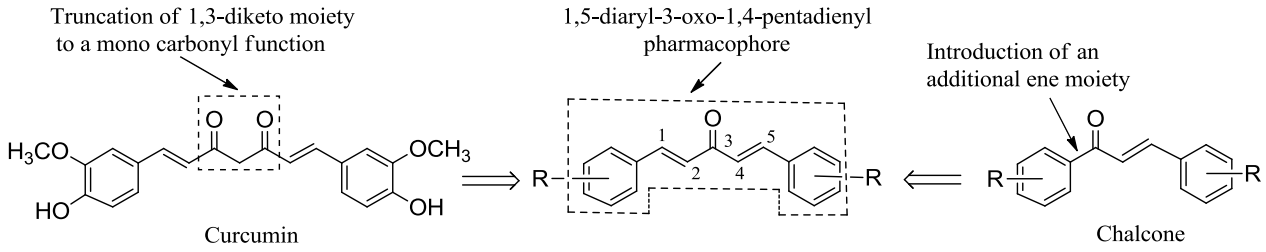


Figure 1: Development of the 1,5-diaryl-3-oxo-1,4-pentadiene pharmacophore

There has been much research devoted over the last few years to developing and understanding the structure-activity relationships (SAR) which are responsible for the pharmacological properties and metabolic instability of curcumin. Attempts have been made to produce curcumin analogues with increased potencies while retaining its low toxicity. In order to produce such molecules, molecular modifications of three regions of the curcumin structure have been undertaken, i. e., the aromatic ring, the β-diketone moiety and the two conjugated α,β-unsaturated keto groups. Curcumin is a symmetrical bidentated ligand containing two α,β-unsaturated diketone moieties flanked by 4-hydroxy-3-methoxyphenyl groups. The study carried out by Wang et al suggests that the instability of curcumin in aqueous solution is mainly due to the central diketone motif and the hydroxyl groups in the two terminal phenyl rings.[19] The α, β-unsaturated keto motifs in curcumin were also found to be crucial for displaying anticancer properties. Therefore the next generation of curcuminoid analogues retains these motifs while the central β-diketone moiety was truncated to a mono-carbonyl group to produce 1,5-diaryl-3-oxo-1,4-pentadienes (Figure 1).

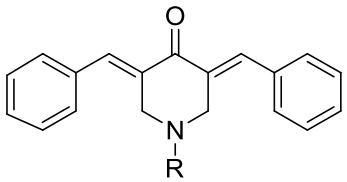
The development of 1,5-diaryl-3-oxo-1,4-pentadienes as cytotoxic agents may have been influenced by their structural similarity to chalcones (1,3-diaryl-2-propenones) which possess an array of biological activities including potent cytotoxic properties.[20,21] This argument was made on the basis that the bioactivities displayed by the chalcones are due to the α,β-unsaturated keto motif which acts as a Michael acceptor for cellular nucleophiles and the introduction of an additional ene group produces a dienone which will enhance the alkylating potential and lead to enhanced cytotoxic potencies. This new class of compounds possess potent cytotoxic activities and the results to date do not indicate significant toxicities in rodents.

Since the awareness of the utility of the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore in cancer chemotherapy, numerous efforts have been expended by medicinal chemists to develop

novel 1,5-diaryl-3-oxo-1,4-pentadienes as cytotoxic agents. Progress has been made to elucidate the mode of action of this class of compounds by which cytotoxicity is mediated and a number of molecules display promising *in vivo* efficacy. With such a success story, it has become necessary to collate the current developments in this field for the benefit of people who are researching in this area. The aim of this review is to emphasize the potential utility of 1,5-diaryl-3-oxo-1,4-pentadienes as future chemotherapeutic and multidrug resistance modulating agents and to outline recent developments of their molecular targets.

3. 1,5-Diaryl-3-oxo-1,4-pentadienes as thiol alkylators

A number of studies demonstrate that depletion of thiol concentrations prior to treatment with various anticancer drugs increase toxicities to cancer cells compared to the use of the drug alone.[22-26] Since the rate and extent of thiol depletion in tumours differ from normal cells,[27] the development of cytotoxic agents that target cellular thiols may prove to display selective toxicities to neoplasms compared to normal cells. Various cytotoxic α,β-unsaturated ketones demonstrate marked selective affinity for cellular thiols as compared to amino or hydroxy groups.[28,29] Therefore it was proposed that the use of thiol alkylators in cancer chemotherapy may avoid the problem of genotoxic properties that are inherent with a number of anticancer drugs.[30,31] The potential utility of thiol alkylators in cancer chemotherapy was reviewed a few years ago.[32] Based on the above observations it was proposed that the compounds bearing multiple sites for nucleophilic attack, e.g. arylidene dienones by cellular thiols, would prove to be potent cytotoxins. This phenomenon was verified using a novel group of cytotoxic compounds **1a**,**b** which displayed IC_{50} values of 16 and 134 picomolar towards P388/MRI cells, respectively.[33] **1a** and **1b** reduced hepatic GSH concentrations by 19 and 29%, respectively, when a dose of 0.87 mmol/kg of these compounds was administered to mice, suggesting that an increase in additional thiol alkylating sites enhances thiol depletion.



1a: R=H; **1b**: R= -CO-CH=CH-COOH

Further Dimmock and coworkers[34] formulated a hypothesis of sequential cytotoxicity which states that "a chemical insult prior to a subsequent chemical attack on cellular thiol constituents may produce greater toxicity to cancers than normal cells". In other words, an initial reaction with a cellular thiol by cytotoxic agents will sensitize the malignant cells preferentially and a further interaction with another thiol will cause greater damage to malignant cells than normal cells (Figure 2).

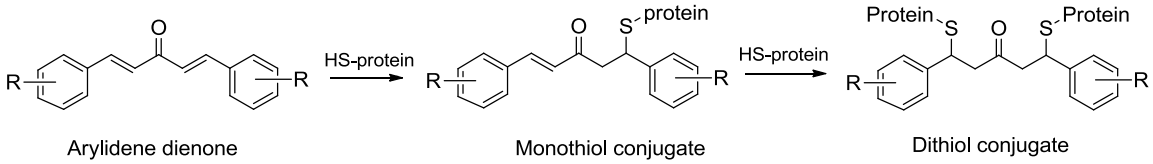


Figure 2: Sequential Michael reaction of protein thiols with an arylidene dienone electrophile

Sun and coworkers[35] investigated the reactivity of GSH and other thiol containing dipeptides with the symmetrical dienones **2** and **3** which possess cytotoxic, anti-angiogenic and antitumor activities. Conjugation of **2** and **3** with 2.1 equivalents of L-glutathione in aqueous acetonitrile at ambient temperature gave only the corresponding bis-adducts namely **2**-$(GSH)_2$ and **3**-$(GSH)_2$ whereas a 1.0 equivalent of L-glutathione with **3** delivered a mixture of mono- and bis-adducts, **3**-(GSH) and **3**-$(GSH)_2$, with the mono-adduct as the major product as identified by LC/MS. Conjugation of GSH with **3** takes place instantaneously while conjugation with **2** takes several hours to complete. The thiol alkylating ability of the dienones **2** and **3** along with several other cysteine-containing dipeptides such as Cys-Phe and Cys-Gly gave the corresponding conjugated adducts. The conjugates of **3** with Cys-Gly instantaneously form the **3**-$(Cys\text{-}Gly)_2$ adduct while **2** reacts with Cys-Gly to yield the bis-adduct at a much slower rate which takes at least 7 h at room temperature. Conjugation with higher mass dipeptides, for instance Cys-Phe, proceeds much more slowly relative to Cys-Gly. These observations suggest that the reactivity of the thiol is very much dependent on the electrophilicity of the arylidene carbon atoms that is influenced by the nature of the aryl ring, the electronic effect of the aryl substituents and the molecular size of the thiol group. From these observations it was proposed that the anticancer effects of **2** and **3** are mediated in part by redox-mediated induction of apoptosis. Investigation of the cytotoxic activities of the conjugates **2**-$(GSH)_2$ and **3**-$(GSH)_2$ demonstrated potent cytotoxicity with IC_{50} values of 1.5 μM and 1.0 μM against MDA-MB-435 human breast cancer cells which was very similar to the cytotoxic potencies of the parent compounds **2** and **3**.

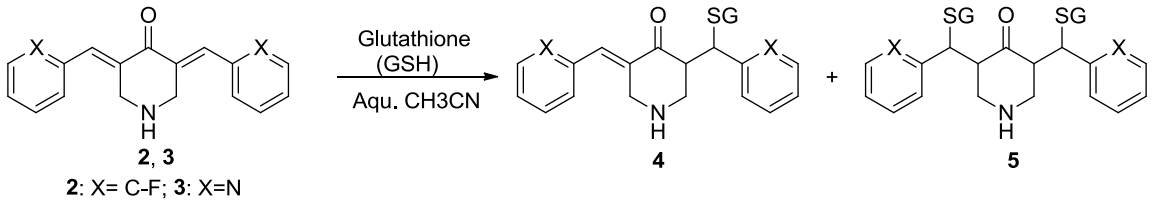


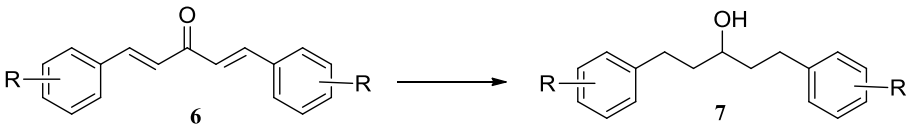
Figure 3: Formation of glutathione adducts

Another interesting phenomena observed from this study was that the reaction of **2**-$(GSH)_2$ and **3** in a molar ratio of 1:1 resulted in the formation of **2**-GSH, **3**-GSH and **3**-$(GSH)_2$ along with unreacted **3** and **2**-$(GSH)_2$ after 8h as indicated by LC/MS analysis. While extending the reaction period to 48 h gave no evidence of **3**, only **2**-$(GSH)_2$, **3**-$(GSH)_2$ and a small amount of **2** were noticed. These results demonstrate the possibility that the retro-Michael addition of **2**-$(GSH)_2$ takes place releasing free **2** and glutathione followed by GSH reacting with **3** leading to the formation of the corresponding mono- and bis-adducts. These results again confirm that the coupling of **3** with GSH is much faster than **2**. The glutathione conjugates represent a promising new series of soluble antitumor prodrugs.

4. Cytotoxic 1,5-diaryl-3-oxo-1,4-pentadienes

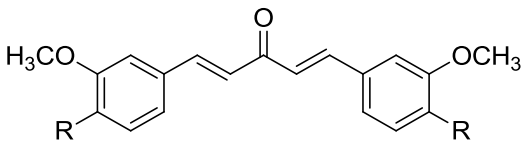
A number of 1,5-diaryl-3-oxo-1,4-pentadienyl acyclic and cyclic analogs have been developed which display potent growth-inhibiting properties against a variety of tumours. The cyclic analogs are based on cycloalkanone, β-tetralone and piperidone scaffolds. Various symmetrical and unsymmetrical dienone derivatives have been developed based on the hypothesis of sequential cytotoxicity and remarks are now made in terms of their selective toxicities towards neoplasms compared to normal cells.

4.1. Acyclic 1,5-diaryl-3-oxo-1,4-pentadienes

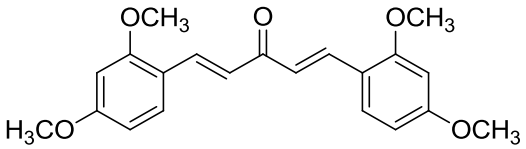


R= 2-OH, 2-OAc, 3-OH, 4-OH, 2-F, 2-OCH_3

A number of aryl-substituted acyclic diaryl dienones **6** display potent cytotoxicity.[36] The reduced analogs **7** were bereft of cytotoxicity which reveals the importance of the dienone motif which acts as Michael acceptors for cellular thiol nucleophiles. This result further supports the belief that enones and dienones are alkylators capable of binding to intracellular components bearing sulfhydryl groups such as GSH and thioredoxin-1.

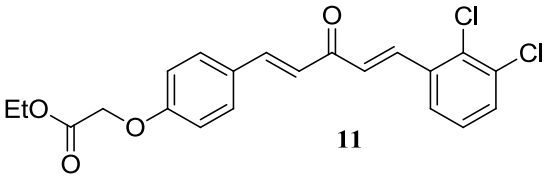


8 R= OH
9 R= OCH_3

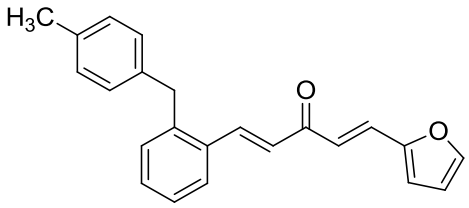


10

The acyclic derivatives **8-9** were evaluated against androgen-independent (PC-3) and androgen-dependent (LNCaP) human prostate cancer cell lines.[37] Both **8** and **9** exhibit excellent growth-inhibiting properties towards both these cell lines with IC_{50} values in the 1.4-3.8 μM range. The importance of 3,4-disubstitution on the aryl rings in exhibiting marked potencies was stressed. The methoxy group located at the meta position was found to be critical in governing cytotoxicity. Cytotoxic potencies were lowered 6-fold when the position of the methoxy group was changed to the ortho postion of the aryl ring as in **10**.

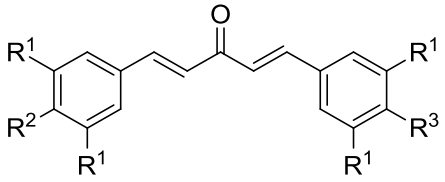


11



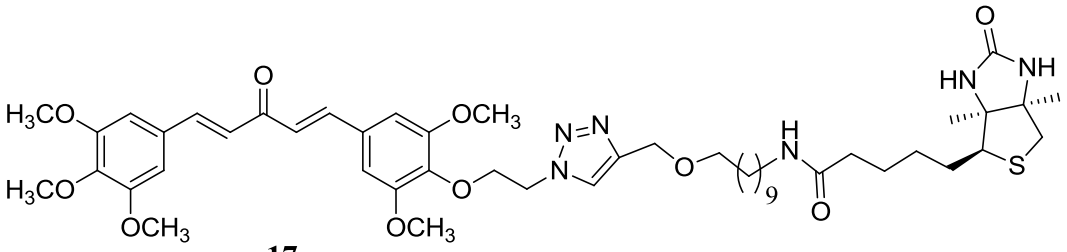
12

Song et al reported a novel series of cytotoxic arylidene dienone derivatives.[38] The most potent compounds **11** and **12** displayed IC_{50} values ranging from 6.6–8.6 μM against PC-3, BGC-823 and Bcap37 cells. The principal mechanisms by which **11** displayed cytotoxic properties is by inducing apoptosis triggered by DNA laddering.



13 $R^1=R^2=R^3=OCH_3$
14 $R^1=OCH_2OCH_3$, $R^2=R^3=H$
15 $R^1=OCH_3$, $R^2=R^3=OCH_2OCH_3$
16 $R^1=OCH_3$, $R^2=OCH_3$, $R^3=OCH_2CH_2N_3$

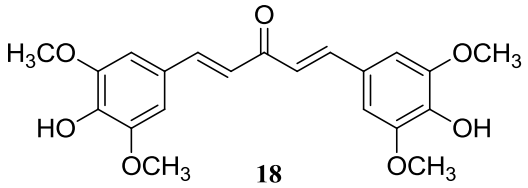
The structure-activity relationship of a number of bis(benzylidene)ketones has been reported and conclusions have been made for further development of their use in cancer chemotherapy. In *in vitro* cytotoxicity studies, the compounds **13-16** exhibited potent growth inhibition with a GI_{50} value of 0.3 μM against HCT-116 cells.[39,40] Various observations were noted from the SAR studies. (1) The 1,5-diaryl-3-oxo-1,4-pentadienyl group serves as a promising skeleton as the 3-oxo-1,4-pentadienyl structure is essential for eliciting cytotoxicity. (2) Symmetry between the two aryl rings is not an essential requirement for these compounds to display cytotoxicity. (3) The para-positions in the aryl rings allow the introduction of additional functional groups for developing novel cytotoxins. (4) Compounds possessing the 3,4,5-substitution in the aryl rings display the highest potency. Moreover, **14** exhibited *in vivo* chemopreventive activity in the familial adenomatous polyposis (FAP) mouse screen without apparent toxicity.[41]



17
GO-Y086

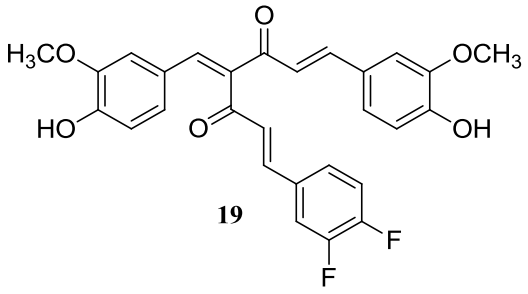
A biotinylated dienone **17**, referred to as GO-Y086, is a potent cytotoxic agent which specifically interacts covalently with the nuclear protein KSRP/FUBP2.[42] The KSRP/FUBP2 protein is involved in a variety of cellular processes, including splicing in the nucleus, mRNA decay, maturation of miRNA, and transcriptional control of proto-oncogenes such as c-myc.[43-46]

17 markedly suppresses the expression of the c-Myc protein, which plays an important role in cellular proliferation and whose expression is regulated by KSRP/FUBP2.



18

A further investigation of **8**, **14** and a related analog **18** demonstrated high cytotoxic potencies against the human colorectal cancer cell lines HCT-116, HT-29 and SW480 with IC_{50} values ranging between 0.51-4.48 μM compared to curcumin (IC_{50}: 10.26-13.31 μM).[47] In these cell lines **8**, **14** and **18** induce apoptosis as evidenced by cleavage of PARP and caspase-3, partly suggesting the way cytotoxicity is mediated by these compounds. Additionally, **8** and **18** displayed low toxicity to WI-38 normal human lung fibroblasts (IC_{50} : >1,000 μM).

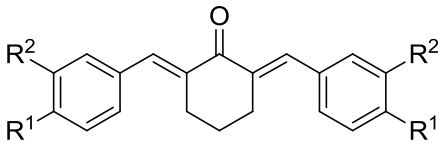


19

A novel curcuminoid difluoro analogue **19**, exhibits greater systemic and pancreatic tissue bioavailability compared to curcumin and has attracted considerable interest as a drug modality to improve the treatment outcome of patients diagnosed with pancreatic cancers.[48] Mechanistic investigations reveal that compound **19** down regulates Akt, cyclooxygenase-2, prostaglandin E2, vascular endothelial growth factor, and NF-κB as well as possessing DNA binding activity. The cytotoxicity displayed by **19** is mainly due to induction of apoptosis. A copper dichloride complex of **19** was developed to examine whether it improves cytotoxicity; however, it was found to be inactive in these screens.

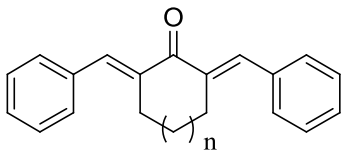
4.2. Cyclic 1,5-Diaryl-3-oxo-1,4-pentadienes

4.2.1 Cycloalkanone analogs



20

R^1, R^2 = H, Cl, CH_3, OCH_3, F, Br, OCH_2O, OCH_2CH_2O, OC_6H_5, $OCH_2C_6H_5$, $O(CH_2)_3N(CH_3)_2$

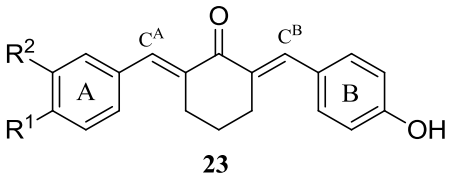


21 n = 0
22 n = 2

A series of bis(benzylidene)cyclohexanones **20** were screened against murine L1210 cells and human T-lymphocytes (Molt, CEM). The cytotoxic potencies (IC_{50}) vary from 0.2 to >50 μM.[49] The most potent compound **20** [$R^1 = O(CH_2)_3N(CH_3)_2$, R^2 = H] showed an IC_{50} value of 0.2 μM revealing 2-fold higher potencies than melphalan. Moreover **20** has average IC_{50} values of 1.55 μM against a number of human tumour cell lines from different neoplastic conditions using the NCI screen. As the alicyclic scaffold controls the relative positions of the olefinic carbon for interaction with cellular thiol nucleophiles, two analogs of **20** ($R^1 = R^2$ = H), (referred to subsequently as **20a**) namely **21** and **22** were prepared and their cytotoxic potencies were compared against **20a** in order to investigate whether a variation in the size of the cyclic ring which changes the relative location of the olefinic carbon atoms influences cytotoxic potencies. The IC_{50} values were in the order of **20a** (4.4 μM)> **21** (7.5 μM)> **22** (16 μM). These results suggest that a six-membered cyclic structure is the preferred over 5 and 7-membered rings. A negative correlation between the Hammett sigma and IC_{50} values suggests that the placement of strongly electron-withdrawing groups in the aryl rings enhances cytotoxic potencies.

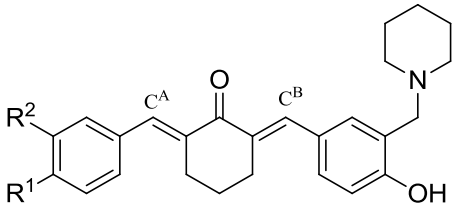
In order to evaluate the hypothesis of sequential cytotoxicity, a series of asymmetric 2,6-bis(benzylidene)cyclohexanones **23-27** were designed.[50,51] The compounds in these series display good tolerability in mice up to and including a dose level of 300 mg/kg.[50,51] In series **23**, the electronic environment of two olefinic carbon C^A and C^B vary considerably and hence a sequential attack of cellular thiols on the olefinic carbon atoms can be achieved which may cause greater damage to malignant tissues. Both series **23** and **24** demonstrated comparable cytotoxic potencies as melphalan and IC_{50} values ranged from 3-6 μM.[50] As the environment of certain tumours is more acidic than the corresponding normal cells,[52] the piperidinyl analogs **24** could display preferential cytotoxicity of tumours in at least two different ways. (a) In the more acidic

milieu, there will be a higher percentage of the molecules in the ionized form, i.e. , the piperidinyl nitrogen atom carries a positive charge. Hence the electron density on the olefinic carbon atoms will be lowered and enhanced electrophilic attack with cellular thiols can occur. (b) The protonated heterocycle may undergo elimination leading to a quinone methide which is predicted to be a powerful alkylating agent. A linear correlation between the Hammett σ values and the atomic charges on the C^A atoms was noted ($p < 0.05$).



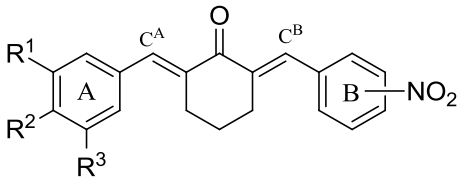
23

R^1, R^2= H, Cl, CH_3, OCH_3, OH



24

R^1, R^2= H, Cl, OCH_3

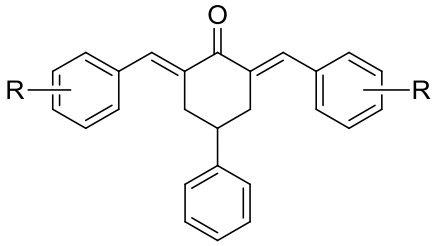


25: 2-NO_2; **26**: 3-NO_2; **27**: 4-NO_2

R^1, R^2, R^3= H, CH_3, Cl, F, OCH_3, $N(CH_3)_2$

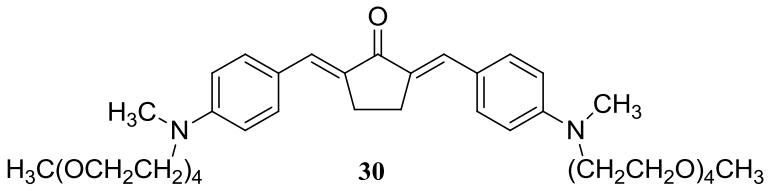
In series **25-27**, a **s**trong electron-withdrawing nitro group was introduced into the ortho, meta or para locations in ring B while the ring A possessed substituents with diverse electronic effects (σ values) in order to create a differential in the electronic charges at the olefinic carbons C^A and C^B.[51] The compounds exhibited average IC_{50} values of 1-10 μM against Molt 4/C8, CEM and L1210 cell lines. The potency order of the series **25-27** was in order of **25** (ortho)> **26**(meta) > **27** (para). The most potent compound **25** (R^1=R^2=R^3=OCH_3) displayed an average IC_{50} figures of 0.88 μM against the three cell lines. Due to the strong electron-withdrawing effect of nitro groups, a polarization of the π electrons occurred towards the olefinic carbon C^B in the 1,5-diaryl-3-oxo-1,4-pentadienyl group suggesting that an initial thiol alkylation takes place at C^A. A negative linear correlation between cytotoxic potencies and electronic charges at the olefinic carbon C^A also support this observation. A substantial change in the average θ_A figures (the torsion angle between ring A and the C^A atoms) of the series **25** (76.8º) as compared to **26** (51.3º) and **27** (51.1º) suggests that the design of new series of analogs with larger θ_A values such as the formation of congeners with large Taft E_S constants should be considered. The cytotoxic potency

was not influenced by the hydrophobicity of the molecules as the average log P figures of series **25**, **26** and **27** are very similar.

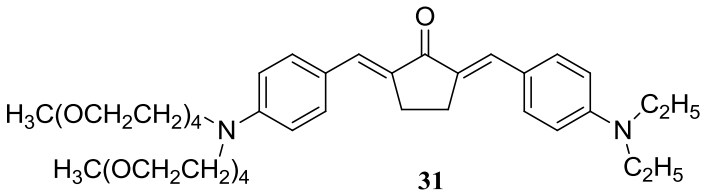


28 R= 3,4,5- $(OCH_3)_3$
29 R= 4-NO_2

Lee et al reported a series of 2,6-bis(benzylidene)-4-phenylcyclohexanones which displayed IC_{50} values in the low micromolar range against murine B16 melanoma and L1210 leukemia cell lines. Two potent cytotoxic molecules **28** and **29** were identified possessing average IC_{50} values of 0.51 and 0.35 μM against B16 and L1210 cell lines, respectively.[53]



30

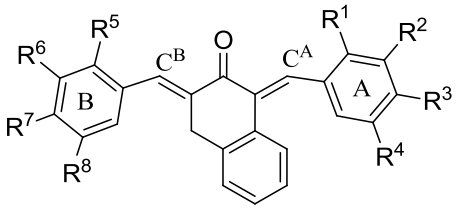


31

Photodynamic therapy (PDT) is a promising noninvasive treatment for cancer which offers the advantage of high selectivity to malignant targets *vs*. normal cells.[54] It uses light to activate a tumor-localized photosensitizer *via* the *in situ* generation of highly cytotoxic reactive oxygen species (ROS).[55] Due to the limited tissue penetration, the applications of PDT are mainly confined to the superficial tissues of an organ, such as in esophageal cancer and skin diseases.[56] In recent years two-photon excited PDT (TPE-PDT) offers the significant benefit of deep tissue penetration since two photons of infrared light rather than one photon of visible light are absorbed by the photosensitizer which helps resolve the penetration problem.[57,58]

A series of polyethylene glycol-functionalized benzylidene cyclopentanones with varying lipid/water partition coefficients were developed as photosensitizer cytotoxic agents.[59] Among them, two analogs **30** and **31** exhibited good solubility in PBS (>2 mg/ml, which is sufficient for clinical venous injection) and produced high ROS, large two-photon absorption and low dark toxicity under the therapy dosage. They were absorbed efficiently by human rectal cancer HRC1116 cells and demonstrated strong two-photon excited PDT activity in *in vitro* cell experiments. Both **30** and **31** accumulate mainly in the mitochondria.

4.2.2 β-Tetralone analogs

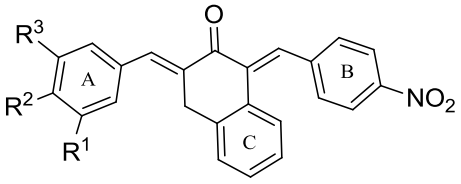


32

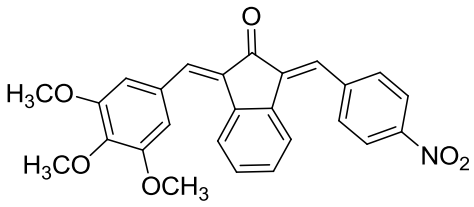
R^1-R^8: H, Cl, F, Br, CH_3, OCH_3, NO_2, CF_3, $COOCH_3$, $OCOCH_3$, $OCOCH{=}CHC_6H_5$, $N(CH_3)_2$, OH, $COOC_2H_5$

In view of the objective of achieving successive thiol reactions on arylidene olefinic carbons, a series of 1,3-bis(benzylidene)-3,4-dihydro-1*H*-naphthalen-2-ones **32**[60] was created in which carbon atoms C^A and C^B are in different electronic environments. All of the diaryldienones **32** displayed potent cytotoxicity with IC_{50} figures generally in the low micromolar range against human lymphocytes (Molt 4/C8, CEM) and murine leukemic (L1210, P388) cancer cell lines. Mechanistic investigations on some of the representative compounds in series **32** revealed that this class of compounds interfere with macromolecular biosynthesis by inhibiting DNA, RNA and protein biosynthesis while no binding with calf thymus DNA was noted. Further investigations revealed the promising cytotoxic potencies of **33-35** which are clearly lead molecules for further development. The IC_{50} values of **33-35** are in the range of 1.0-1.6 μM towards Molt 4/C8 and CEM cells.[61] In the NCI screen, compound **33** demonstrated promising cytotoxic potencies with an average GI_{50} of <1 μM against a panel of tumour cell lines which warrants further development of this analog as a candidate anticancer agent. Using a

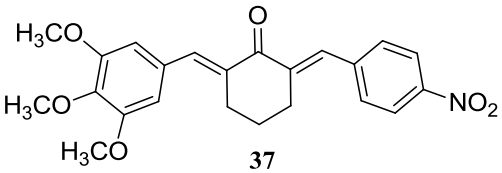
concentration of 1 μM, **33** induced apoptosis in human HepG2 liver cancer cells suggesting that apoptosis is an important way whereby cytotoxicity is mediated.



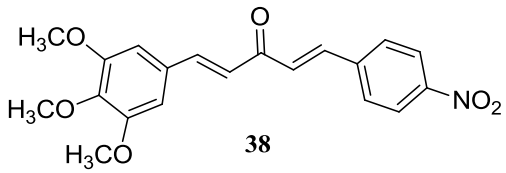
33: $R^1 = R^2 = OCH_2O$; R^3= H
34: $R^1 = R^2 = R^3 = OCH_3$
35: $R^1 = R^2 = OCH_3$; R^3= H



36

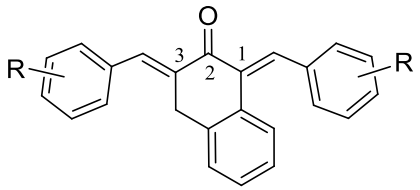


37

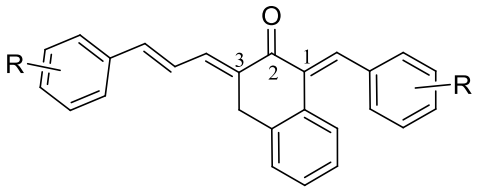


38

A number of structural analogs of **34** were designed and screened against various malignant and non-malignant cell lines.[62] Many of these analogs displayed high cytotoxic potencies and selective toxicities (SI) towards a number of tumorous cells such as oral squamous carcinomas (HSC-2, HSC-3, HSC-4) and promyelocytic leukemic HL-60 cells compared to various normal cells such as the HGF gingival fibroblasts, HPC pulp cells and HPLF periodontal ligament fibroblasts. Compound **34** emerged as the most promising selective cytotoxin (average CC_{50}:1.75 μM, SI: 400) which was comparable to the clinically used drug doxorubicin. The compounds **36-38** were prepared to develop a structure-activity relationship. These analogs displayed considerably less potency and selectivity than **34** revealing that (1) the aryl ring C in **34** may offer an additional hydrophobic binding at the receptor site which enhances the cytotoxicity of the molecule and (2) the relative locations of different functional groups in these compounds are very important in influencing bioactivity.



39



40

Jha et al prepared a number of arylidene β-tetralone derivatives **40** in which an extended conjugation was introduced between the aryl ring and C-3 olefinic group in **39** and compared

against the corresponding 1,3-bisarylidene-β-tetralone analogs **39**. A comparison of cytotoxic potencies of **40a** (R=H) vs **39a** (R=H)[63] against Molt 4/C8, CEM and L1210 cancer cell lines revealed that **40a** is on average nine times less potent than **39a** (average IC_{50}: 4.68 μM). These results further reconfirm that the relative location of aryl rings strongly influence cytotoxic potencies and an increase in the flexibility of the molecule by enhancing the number of rotatable bonds negatively influence bioactivity.

4.2.3 4-Piperidone analogs

The initial development of these compounds as cytotoxins was based on two important considerations. A number of acyclic Mannich bases display cytotoxic and anticancer properties.[64] However, they cause respiratory depression in low doses when administered to mice.[65] Unwanted toxicities associated with the acyclic enones may be considered to be due to (i) the flexibility of the acylic Mannich bases enabling them to assume a variety of different shapes and (ii) the rapid deamination of Mannich bases which release dienones which are the active molecules that sequester indiscriminately. Therefore while a conjugated arylidene keto group along with a basic centre beta to the carbonyl function was retained, molecular flexibility was reduced by the incorporation of most of the molecule into a rigid piperidine ring as illustrated in Figure 3. In addition, the high reactivity of the enone group was reduced by incorporating a phenyl group at the β-carbon. These modifications led to the genesis of arylidene diones **41** which possess potent cytotoxic activities; for example, **41a** (R^1=R^2=H) displayed an IC_{50} value of 920 picomoles towards murine P388D1 cells.[33] Second, administration of doses up to and including 240 mg/kg of this compound to mice on five successive days did not cause any mortalities.[33]

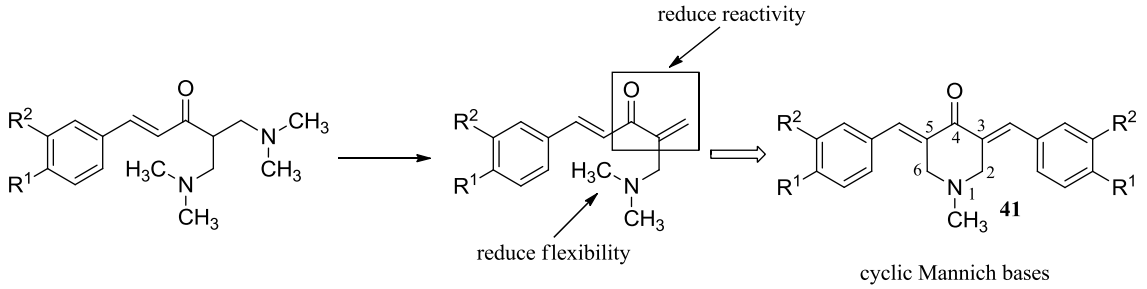
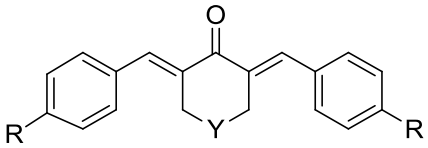


Figure 4. The design of 1-methyl-3,5-bis(benzylidene)-4-piperidones as candidate cytotoxic agents

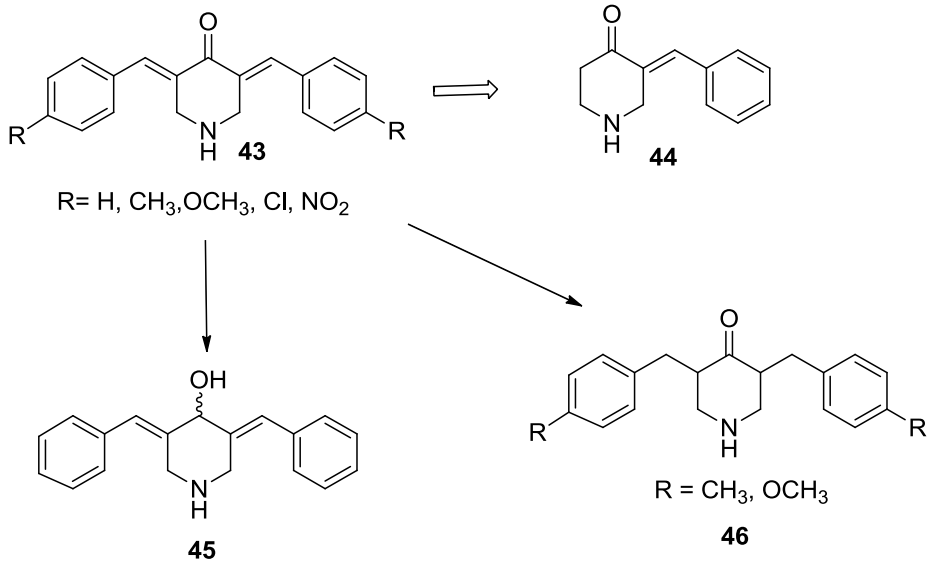


42

Y= NH, NCH_3, $^{+}N(CH_3)_2\ X^{-}$

X=Br, I ; R= H, $N(CH_3)_2$

Subsequently a series of bis-arylidene-4-piperidones **42** possessing a dimethylamino group and the corresponding des-methyl and quaternized derivatives were developed by Dimmock et al and their cytotoxic potencies evaluated against P388/MRI murine leukemia cells.[33] The des-methyl derivative **42a** (R=H, Y=NH) and the quaternary ammonium compound **42b** (R= $N(CH_3)_2$, Y= $^{+}N(CH_3)_2$ Γ) demonstrated 57- and 95-fold higher potencies, respectively than the N-methyl adduct **42c** (R= H, X=$N(CH_3)$). X-ray crystallography studies on some of these compounds demonstrated that the olefinic double bonds adopt the *E* stereochemistry and no coplanarity exists between that the aryl rings and the adjacent olefinic linkages. This phenomenon is due to nonbonded interactions between the equatorial protons at positions 2 and 6 of the piperidine ring and the ortho hydrogen atoms of the aryl rings. No correlation between cytotoxic potencies and torsion angles between the aryl rings and the olefinic bonds was noted.



43

R= H, CH_3,OCH_3, Cl, NO_2

44

45

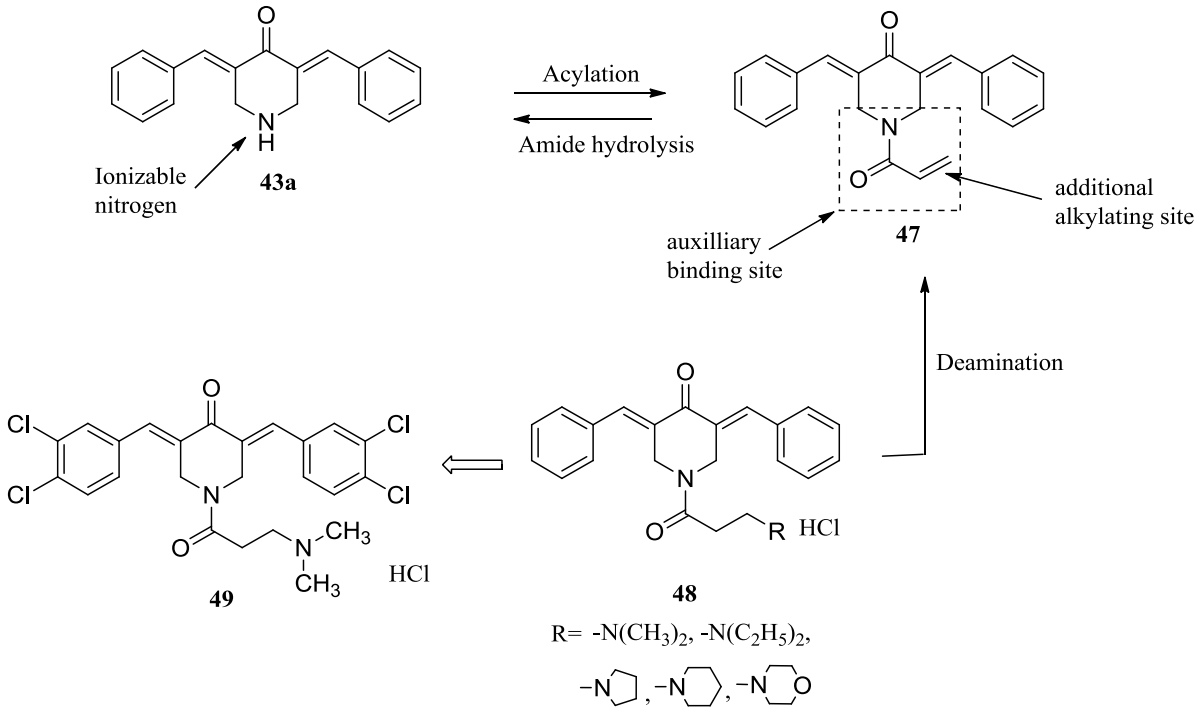
R = CH_3, OCH_3

46

Scheme 1: 3,5-bis(benzylidene)-4-piperidones **43** and the reduced analogs

In order to evaluate the importance of the dienone motifs in eliciting cytotoxicity, two molecular probes of **43a** (R=H) were prepared, one by reducing the carbonyl group and the

other by reducing the enone olefinic bond. Both the analogs **45**[66] and **46**[67] display substantial reduction of cytotoxic potencies which suggests that the integrity of the 1,5-diaryl-3-oxo-1,4-pentadienyl group should be retained. The monoarylidene analog **44**[66] showed a 14-fold reduction in potency over **43a** which reveals that the removal of a potential alkylation site in the molecules reduces cytotoxicity.

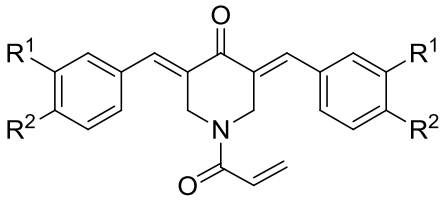


Scheme 2. 3,5-bis(benzylidene)-4-piperidones **43a** and some acylated analogs.

With the aim of developing potent cytotoxins, additional thiol alkylating sites were introduced into the arylidene dienone **43a** that led to *N*-acryloyl-3,5-bis(benzylidene)-4-piperidone **47**.[68] It was also hypothesized that while the 1,5-diaryl-3-oxo-1,4-pentadienyl group aligns at a primary binding site, the N-acryloyl group would interact at an auxiliary binding site that may lead to an increase in cytotoxic potency of **47** compared to **43a**. The amide **47** may act as a prodrug liberating the 4-piperidones by amidic hydrolysis. As ions experience difficulty in penetrating cell membranes, the *N*-acryloyl analog being an uncharged molecule may display increased membrane permeability as compared to **43a** which will ionize under physiological conditions.[69] The *N*-acryloyl analog **47** (IC_{50}: 0.8μM) displayed 5-fold greater potency compared to 3,5-bis(benzylidene)-4-piperidone **43a (**IC_{50} : 2.5 μM) against the L1210 leukemic

cell line.[68] These results again reconfirm that an increase in thiol alkylating sites leads to enhancement in cytotoxic potency.

The N-acylated analogs **48a** (R= $N(CH_3)_2$), which may be referred to as a prodrug of the N-acryloyl derivatives **47** and a double prodrug of **43a** since it can undergo deamination *in situ* to produce the parent compounds. This possibility was supported by the observation that incubation of **48b** (R=$N(C_2H_5)_2$) and **48e** (R=morpholine) in a mixture of deuterated phosphate buffered saline and dimethylsulfoxide-d_6 for 48 hours and 37º C led to the liberation of **43a** along with other decomposition products as observed in ^{1}H NMR spectroscopy. Various amines were introduced into the N-acyl chain of **48** in order to investigate the influence of the pKa of amines on cytotoxicity. The most potent analog **48b** (IC_{50}: 0.5μM) displayed a 1.6-fold higher cytotoxic potency compared to **47** whereas the dimethylamine and pyrrolidine analogs displayed very similar potencies to **47**. The placement of electron-withdrawing groups such as chloro atoms on to the aryl rings of **48a** in order to increase the electrophilicity of the olefinic carbons considerably led to a potent cytotoxin **49** which displayed more than 4-fold and 13-fold higher potency compared to the corresponding unsubstituted analog **47** and the parent precurser **43a**, respectively.

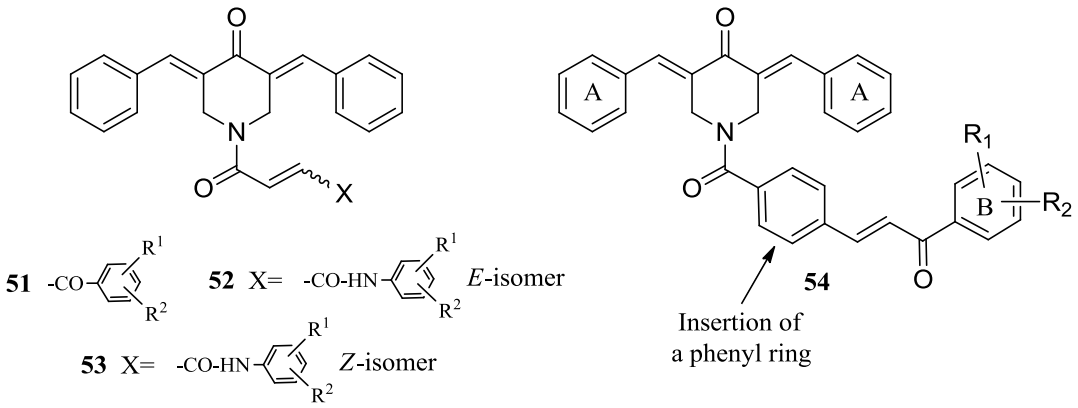


50

R^1=H, R^2= F, Cl, OCH_3, NO_2
R^1=R^2=Cl

In view of potent cytotoxicity displayed by the N-acryloyl analog **47**, a number of acryloyl substituted analogs **50** were developed and the relative cytotoxic potencies were compared against the parent 3,5-bis(benzylidene)-4-piperidones.[70] The aryl substitutions were chosen with varying electronic and hydrophobic properties. The *N*-acryloyl derivatives of **50** are, on average, 62, 30, 3 and 17 times more potent than the corresponding 3,5-bis(benzylidene)-4-piperidones against human Molt 4/C8 and CEM T-lymphocytes as well as murine P388 and L1210 leukemic cells. The average IC_{50} figures of compound **47** and the five compounds in series **50** were 0.96 (Molt 4/C8), 1.13(CEM), 0.28 (P388) and 4.71(L1210) μM, respectively. Furthermore, **47** displayed remarkable cytotoxic potency against a panel of approximately 56

human tumour cell lines from different groups of tumours, namely leukemia, melanoma and colon, renal, small cell lung, non-small cell lung, central nervous system and ovarian cancers in the NCI screen with an average IC_{50} figure of 0.16 μM. This compound demonstrated ~164-fold higher potency than a reference alkylating cytotoxic drug melphalan (IC_{50}: 26.3 μM). The SAR studies revealed that introduction of one or more strongly electron-withdrawing groups in the aryl ring, the insertion of substituents of different sizes in the ortho position of the aryl rings in order to increase the torsion θ values and employing groups which have lower redox potentials should be considered for further designing analogs with a view to increasing cytotoxic potencies. In addition, the N-acryloyl derivatives display potent selective cytotoxic activities towards a number of neoplasms compared to normal cell lines. Selective cytotoxicity is represented by selective index (SI) values which is derived by dividing the average CC_{50} values of the normal cells with the CC_{50} values of neoplastic cells and a SI value of 10 was considered to denote noteworthy selectivity. Far greater cytotoxic potencies and selectivities were observed with these compounds compared to melphalan.[71] Thus the compounds in series **50** emerge as lead molecules and warrant further development.



51-54 R^1, R^2= H, CH_3, Cl, OCH_3, NO_2

Figure 5. Structures **51-54**

Further some related N-acryloyl analogs **51-54** were developed with the rationale that the olefinic double bond in the *N*-acyl group would not only provide an additional site for thiol alkylation but offer rigidity to the molecules thereby controlling the location of terminal aryl ring B relative to the 3,5-bis(benzylidene)-4-oxopiperidinyl moiety.[72,73] A number of compounds

possessed submicromolar IC_{50} values against human Molt 4/C8 and CEM T-lymphocytes and murine L1210 leukemic cells.[72] The compounds in series **51** were less potent than **53** and **47**. However introducing an amide group between the olefinic group and the aryl ring in the side chain of **51** provided a novel series of potent cytotoxins **53**. In general, the compounds in series **53** were more potent than the N-acryloyl derivatives **47** and **43a**. A number of compounds displayed IC_{50} values less than 5 μM and were more potent than melphalan towards the T-lymphocytes. Series **52**, the corresponding *E*-analogs of the series **53**, was accompanied by a reduction in potency[73] suggesting that stereochemistry plays an important role in cytotoxic potencies. Introduction of a chalcone motif into the N-acyl side chain led to series **54** which showed very similar potencies as the series **51**. The series **51**-**54** display good tolerability in mice in short-term toxicity studies up to and including the dose level of 300 mg/kg.

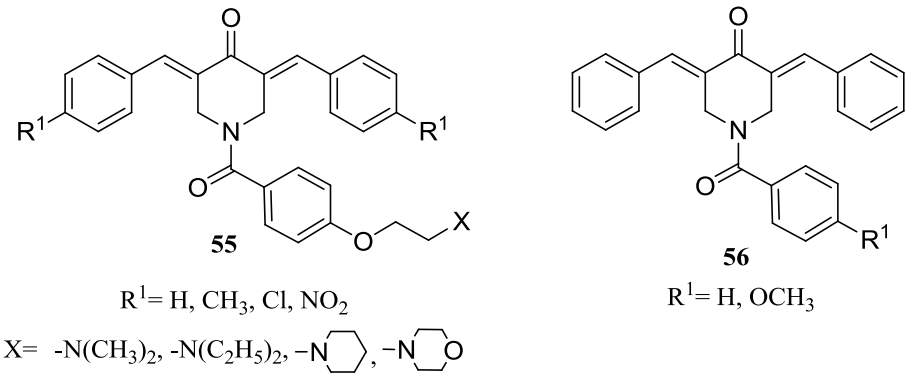
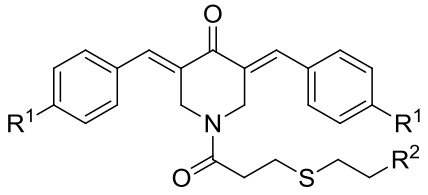


Figure 6. The *N*-aroyl derivatives **55** and **56**

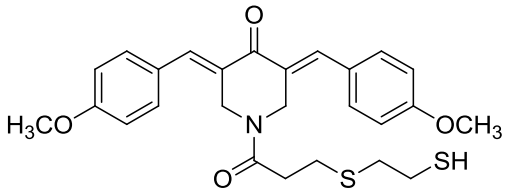
A novel series of N-aroyl analogs of 3,5-bis(arylidene)-4-piperidone namely **55** and **56** was developed by Das et al as cytotoxic agents.[74] Incorporation of the *N*-[4-(2-alkylaminoethoxy)phenylcarbonyl] structural fragment onto the piperidyl nitrogen of **43** provided the compounds in series **55** with remarkable cytotoxic potencies.[74] Structure-activity relationships were elucidated by placing substituents with divergent σ and π values in the arylidene aryl rings and incorporating a number of different amines which differed in size and basicity in the N-acyl side chain (Figure **6**). Among the aryl substituents, the nitro derivatives of **55** displayed very high cytotoxic potencies. In general, the greatest cytotoxic potencies are found when the R^1 substituent is either nitro or methyl and the dimethylamino, diethylamino and

1-piperidyl groups are present in the *N*-aroyl side chain. Compound **56** (R^1=H) displayed similar cytotoxic potencies as the parent compound **43a** suggesting that the 4-(2-aminoethoxy) fragment contributes considerably towards the remarkable cytotoxicity of the compounds in series **55**. From this result it is very likely that the N-[4-(2-aminoethoxy)phenylcarbonyl] side chain enables additional binding at an auxiliary receptor site thereby increasing potencies. A number of the compounds in series **55** demonstrated potent cytoxicity against a panel of approximately 49 human tumour cell lines in the NCI screen. In particular, **55** (R^1=Cl, X= morpholine) was a lead compound displaying an IC_{50} value of <5 nM against the colon cancer HCC-2998 cell line. The compounds in series **50** demonstrated differential toxicities towards certain tumour cell lines in the NCI screen compared to others. This observation suggests that N-acyl-3,5-bis(benzylidene)-4-piperidones may cause preferential lethality to neoplastic cells compared to normal cells. Hence series **55** were evaluated against a number of malignant cell lines and normal cell lines which demonstrated that the compounds in series **55** are potent selective cytotoxins.[75] Additionally, these compounds are well tolerated in mice up to and including a dose of 300 mg/kg without significant toxicities.[76]



R^1= **a**: H; **b**: CH_3; **c**: OCH_3; **d**: Cl; **e**: NO_2

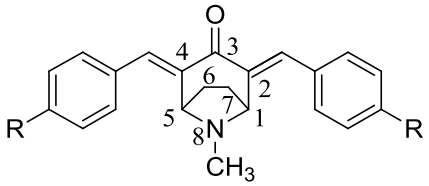
57 R^2= SO_3H **58** R^2= OH



59

A novel series of mercaptosulphonic acid adducts **57** was developed as tumour-selective cytotoxins with the hypothesis that mercaptoethanesulfonic acid will be released upon dethiolation of **57** under physiological conditions.[77] The sodium salt of mercaptoethanesulfonic acid (mesna) is used as a chemoprotectant and is administered on occasions with various anticancer drugs in order to protect normal cells from the toxic effects of chemotherapeutic agents while permitting the antineoplastic properties of the drugs.[78-80] However very surprisingly the compounds in series **57** displayed poor cytotoxic potencies against a number of cancer cell lines including L1210, HL-60 and T-lymphocytes (Molt, CEM)[81] and various squamous cell carcinomas.[77] The relatively weak potencies of **57** may be due to the presence of the highly

polar sulfonic acid group which impedes the penetration of the compounds through cell membranes. In a stability study, a solution of a representative compound **57a** revealed no dethiolated adduct after incubation at 37º C for 24h. In order to overcome these difficulties the sulphonic acid group in the series **57** was replaced by a hydroxyl group which led to a novel series of compounds **58** with remarkable cytotoxic potencies with IC_{50} values in the low submicromolar range against a number of malignant cells.[81] The most potent compound **58e** displays an average GI_{50} value of 0.28 µM against a panel of 49 tumour cell lines in the NCI screen and demonstrates >90–fold higher potency compared to 5-FU and melphalan. **58e** demonstrates most promising toxicities against a number of colon cancer cell lines (IC_{50}: 0.06-0.28 µM). By replacing the hydroxy group of a representative compound **58c** with a mercapto group leading to **59**, potency was reduced. This observation may be due to the differences in the sizes of the hydroxyl and thiol groups which have molar refractivity values of 2.85 and 9.22, respectively.[81a] In other words, the hydroxyl group may align more favourably at a binding site. This observation again reaffirms the probability that the side chain of **53** binds to an auxiliary binding site which influences cytotoxicity. Further evaluation of the series **57-59** against a number of malignant and non-malignant cells reveals that the series **58** are a promising group of selective cytotoxins towards a number of neoplasms compared to normal cells.[81]

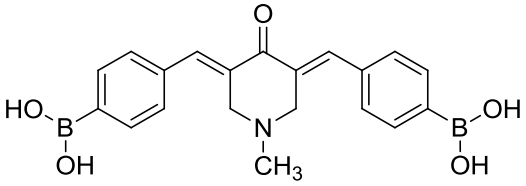


60

a: R = H; **b**: R = Cl; **c**: R = NO_2; **d**: R = CH_3; **e**: R = OCH_3

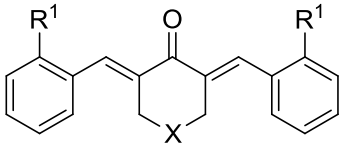
More conformationally rigid analogs of 3,5-bis(benzylidene)-1-methyl-4-piperidones were prepared and a comparison of the cytotoxic potencies of **60** with the corresponding analogs in series **41** was made using Molt 4/C8, CEM and L1210 cancer cell lines.[82] The introduction of a dimethylene bridge between the C-2 and C-6 atoms of **41a** (R^1=R^2=H) led to a decrease in cytotoxic potencies which was attributed to (i) a steric impedance to alignment at one or more binding sites; (ii) variation in hydrophobicity and (iii) changes in membrane transportation properties. The most potent compounds in both the series are the unsubstituted analogs **60a** and **41a** which display average IC_{50} values of 9.77 and 4.69 µM, respectively. These results suggest

that para substitution exerts an unfavourable steric effect at binding sites.[83] The compounds were examined against HSC-2, HSC-3, HSC-4 and HL-60 neoplasms as well as HGF, HPC and HPLF normal cells. The compounds **60a** (>15), **41b** (R^1=Cl, R^2=H) (>19) and **43c** (R^1=NO_2, R^2=H) (>11) displayed high selective cytotoxicities (SI values are in parenthesis).



61

A novel 3,5-bis-(4-boronic acid-benzylidene)-1-methyl-4-piperidone **61** exhibited potent growth-inhibitory activity in HCT-116 cells with IC_{50} values of 1.5 and 0.6 μM in the MTT and colony formation assays, respectively.[84] Compound **61** displays preferential toxicity to cells with wild-type p53 expression and a combination of this compound with ionizing radiation (IR) significantly enhanced the cell-killing activity of IR in both wild-type p53 and p53-null cells. **61** induces apoptosis in HCT-116 neoplasms which might contribute partly to the mode of action of the compound.

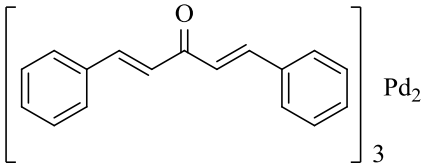


62 X= NCH_3, R^1=2-OH
63 X= NH.OAC, R^1=2-F
64 X= O, R^1= 2-OH
65 X=O, R^1= 2-F
66 X=CH_2, R^1= 2-OH

Adams et al developed a number of cytotoxic ortho-substituted cyclic arylidene dienones **62-66.**[36] Among them **63, 64** and **66** displayed remarkable cytotoxic activities against a panel of cancer cell lines in the NCI screen and compounds **63** and **64** demonstrate high selectivity towards leukemia cell lines.[36] These compounds exert an impressive blockade of endothelial cell proliferation and in particular, **63**, **64**, and **66** were potent inhibitors of cord formation and cell migration, thereby proving their ability as potent antiangiogenic agents. In the NCI anti-angiogenesis assays, **63** was just as potent as TNP-470, a well known antiangiogenic agent.

Compound **63** also effectively induced the regression of large number of human breast tumors in mice and demonstrated little toxicity during the treatment. In view of these results **63** emerged as a promising lead for clinical development.

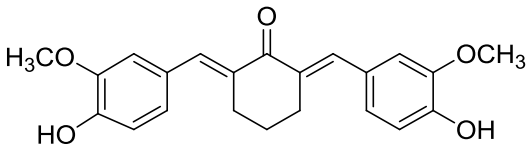
5. In vivo anticancer activity



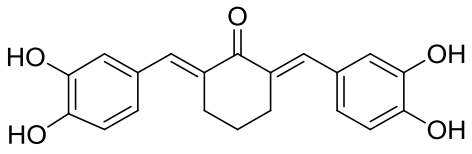
67

A bis-arylidene palladium metal complex **67** which was designed as an angiogenesis inhibitor displayed potent antitumour activities *in vitro*.[85,86] A 10 µg/mL concentration of **67** resulted in a 99% and 96% decrease in murine B16 and human A375 melanoma cell counts, respectively. This compound also reduced the tumour volume in murine (B16) and human (A375) melanoma xenograft models by 97% and 65%, respectively, by administering **67** (40 mg/kg/d) three times per week for 15 days as compared to control.[85,86]

1,5-bis(3,4-Dimethoxyphenyl)-1,4-pentadiene-3-one **9** and 2,6-bis((3-methoxy-4-hydroxyphenyl)-methylene)-cyclohexanone **68** displays a 2-3 fold higher potency than curcumin towards melanoma and breast cancer cell lines (RPMI 7951 and MDA-MB-231, respectively).[36] Both these compounds also inhibit tumour growth *in vivo*.[87] **69** which is a demethylated analog of **68** reduced the growth of T-364 L mammary tumours in mice revealing its anticancer properties *in vivo*.



68

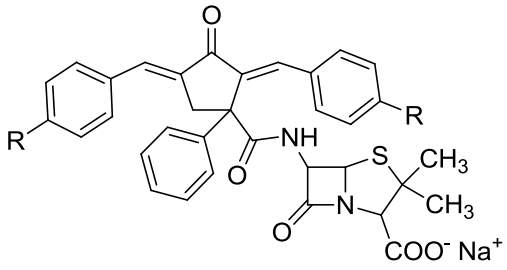


69

A bis-arylidene 4-piperidone analog **63**, displays very promising *in vivo* anticancer activity against the HCT-116 colon cancer xenograft tumour.[88] An intraperitoneal dose (0.2 mg/kg) of **63** exhibited significant tumour regression and increased survival rates without any significant toxicity. **63** also demonstrates a 70% reduction in tumour growth in breast tumour xenografted athymic mice when a dose of 20 mg/kg was administered subcutaneously.[36] The 100 mg/kg dose did not cause any liver, kidney or spleen toxicity and the animals had normal

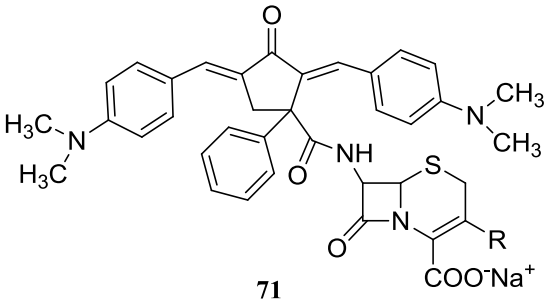
weight gain. The dose administered is a much lower dose than the lethal dose of 400 mg/kg. The oral and intraperitoneal bioavailability of **63** in male CD2F1 mice was 60% and 35%, respectively, which is a significant improvement over curcumin.[88] **63** is absorbed very rapidly with a t_{max} of 3 minutes and exhibits high plasma protein binding (>98%). About 40-50% of the compound is eliminated via metabolism ($t_{1/2}$: 73.6min).[89]

As membrane-bound receptor tissue factor (TF) is expressed on the surface of tumours and also on the endothelial cells of the tumour vasculature, a novel drug delivery strategy was devised to deliver a cytotoxic drug to the surface of tissue factor-expressing cancer cells and to the angiogenic cells that carry nutrients to the growing solid tumor by attaching the cytotoxic drug to a ligand that has high affinity for TF. An enzyme serine protease fVIIa displays high binding affinity towards TF. Tripeptide chloromethyl ketones, such as phenylalanine–phenylalanine–arginine–chloromethyl ketone (FFRck), are irreversible inhibitors of fVIIa.[90a] In addition, the FFRck-fVIIa conjugate displays 5-fold high binding affinity for TF than fVIIa alone. Therefore, a complex of compound **63**-FERck-fVIIa was developed which displays greater growth inhibition of TF expressing breast (MDA-MB-231) and melanocyte (RPMI-7951) cell lines and shows a minimal effect on non-TF expressing breast and melanocytes compared to **63.**[90b,91] This complex shows a reduction of a primary breast tumour xenograft and inhibition of angiogenesis in the rabbit corneal model.[92]



70

a: R = OCH_3
b: R = $N(CH_3)_2$



71

a: R = CH_2OCOCH_3
b: R = CH_3

The diaryldienones β-lactam derivatives **70** and **71** inhibit the growth of murine S-180 and S-37 cells.[93] The survival times of mice inoculated with Ehrlich's ascitic carcinoma increases by 25 and 100% upon treatment with **70a** and **70b**, respectively, whereas **71a** and **71b** did not show any efficacy in this model suggesting that **70b** is a promising lead for further evaluations. These compounds have a very good lethal dose (LD_{50}) window which ranges from

1250-3000 mg/kg in mice.[93] In addition, these molecules display high growth-inhibiting activities against *Staphylococcus aureus* and *Staphylococcus albus*. This observation illustrates the usefulness of the 1,5-diaryl-3-oxo-1,4-pentadienyl based compounds in various pathological conditions.

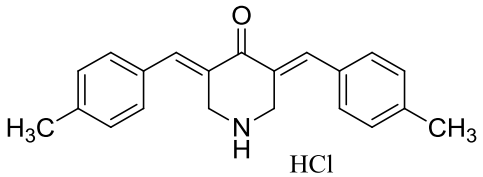
6. Mechanisms of Action

Some of the molecular mechanisms by which certain diaryldienones exert their cytotoxic properties have been discussed previously. Further comments revealing the array of different molecular targets of these compounds are now presented.

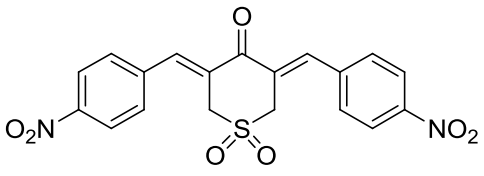
6.1 Induction of Apoptosis

Apoptosis which refers to programmed cell death is very much suppressed in tumours compared to normal cells. A number of anticancer agents inhibit the growth of cancer cells by activating apoptotic pathways through two important routes: (1) formation of caspase-8 via binding of Fas (CD95/APO-1)-associated death domain (FADD) to procaspase 8 and (2) cytochrome c release from the mitochondria leading to a complex formation with Apaf-1 which binds to procaspase-9 and activates various caspases including caspase-9.

The cytotoxic diaryldienones **13**-**15** impair DNA synthesis in human HCT116 colon cancer cells as confirmed by a cell cycle analysis.[39] Both **14** and **15** increase the sub-G_1 cell population indicating that the cytotoxicities displayed by these compounds are mediated through induction of apoptosis. Co-treatment of **14** and **15** with a Z-DEVD-fmk, an inhibitor of caspase-3 and caspase-8 in HCT116 cells, reduces the sub-G_1 population suggesting that apoptosis occurs via the caspase route. Furthermore **14** and **15** downregulate a number of oncoproteins, including ErbB-2, c-Myc, cyclin D1 and Ki-ras.



43d



72

A number of representative compounds from a series of bisarylidene-4-piperidones **43,** their N-acyl analogs **50** and **55** and a bis-arylidene sulfoxide derivative **72** display cytotoxicity via induction of apoptosis in different cancer cell lines. **43c** (R^1=Cl) and its corresponding N-

acryloyl analog **50c** (R^1=R^2=Cl) inhibit RNA and protein biosyntheses as well as inducing apoptosis in human Jurkat T cells.[70] The acryloyl derivative **50e** (R^1=H, R^2=NO_2) is a potent inhibitor of DNA, RNA and protein biosyntheses in L1210 murine leukemic cells with an average IC_{50} of 3.90 µM whereas its bis-arylidene-4-piperidone analog **43e** (R^1=NO_2) displays 7-fold less potency than **50e**.[70] In a TUNEL assay, **55** (R= Cl, X=morpholine) triggers apoptotic cell death in human HepG2 liver cancer cells.[74] Both **43d** (R=CH_3) and **72** display potent cytotoxicity and induce apoptosis via a Bcl-2-dependent but apoptosome-independent pathway of caspase activation in E1A/C9 cells[94] which is not commonly followed by many anticancer agents.[95] Both these compounds inhibit ubiquitin isopeptidases, thereby impeding ubiquitin-dependent protein degradation.[96]

6.2 DNA adducts

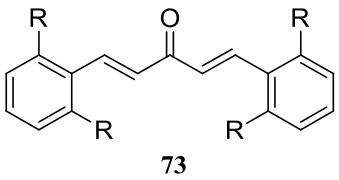
Treatment of two analogs of **42** (R = $N(CH_3)_2$; X = $+N(CH_3)_2$ $^-$I) and **42** (R = $N(CH_3)_2$; X = NCH_3) with various synthetic DNAs, namely poly[d(AT)], poly da·poly dT and poly [d(TG)]poly[d(CA)] revealed that the quaternary ammonium compound caused a rise in the melting temperature of all three DNAs but no changes were noted with the non-quaternary ammonium compound.[33] The behaviour of the quaternary ammonium compound indicates AT selectivity and is consistent with binding in the minor groove of DNA. Further experimentation with three unsubstituted compounds **42** (R =H, X= NH.HCl, CH_2 and $+N(CH_3)_2^-Br$) using poly[d(AT)] revealed that binding occurred only with the quaternary ammonium compound. A further study with related 3,5-bis(benzylidene)-1-methyl-4-piperidone methoiodides which are toxic to murine L1210 cells revealed that they too increased the melting temperature of poly[d(AT)].[97]

6.3 Angiogenesis

Angiogenesis is a process of forming new capillaries which provide a continuous supply of nutrients and oxygen to the rapidly dividing tumour cells[98] and thus plays an important role in the proliferation of tumour cells.[99] Tumour angiogenesis is controlled by a balance between angiogenic activators[100] and inhibitors.[101,102] The vascular endothelial growth factor (VEGF) is one of the angiogenic activators secreted from growing tumors to stimulate angiogenesis. Inhibition of VEGF reduces angiogenesis and suppresses tumor growth *in* vivo[103] and clinical

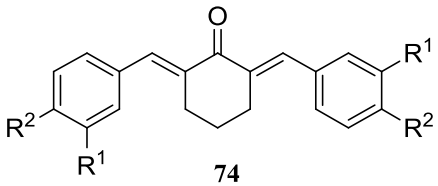
trials employing VEGF-targeting therapies have shown efficacy toward advanced-stage cancers.[104] Among various isoforms of VEGF, VEGF-A is the most widely studied member of the VEGF family which is required for the normal development of vasculogenesis and angiogenesis, as even one missing allele can be lethal to animals.[105] VEGF-A is overexpressed in most human solid tumours and thus emerges as a very good target for antitumourigenic agents.[106,107]

Various acyclic and cyclic bis-arylidene analogs **62, 63, 73a, 73b, 74a** and **74b** inhibit endothelial cell growth *in vitro.*[36,108] Except for **73b**, all of the other compounds displayed >90% inhibition of SVR endothelial cells, an established murine model of angiogenesis[109] at a concentration of 3 mg/mL. Further evaluations revealed that **63** exhibits a reduction in cord formation and cell migration in human umbilical vein endothelial cells (HUVEC) at a concentration of approximately 1 μM.[36]



73

a: R = H ; **b**: R = Cl



74

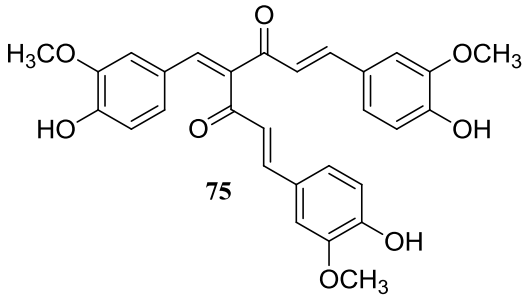
a: $R^1 = R^2 = H$

b: $R^1 = OH$, $R^2 = OCH_3$

The arylidene palladium complex **67** inhibits VEGF expression in B16 and A375 cells *in vitro.*[85,86] Treatment of 5 μg /mL of **67** with SVR cells for 48 hours decreased the cell viability by 97%.

6.4 Tubulin polymerisation

The microtubule network in eukaryotic cells is an essential component of the cytoskeleton and plays a pivotal role in a variety of cell signaling events.[110] A large number of antimitotic drugs bind tubulin or microtubules at one of the three characterized tubulin ligand sites: taxol, colchicine, and vinca binding sites.[111] Each site can accommodate compounds with very different structures.[112]



75

An arylidene derivative **75** is a potent inhibitor of tubulin polymerization which displays a half-maximum polymerization inhibition value of 16±1 μM, whereas the half-maximum polymerization inhibition value of curcumin was 20 ± 1 μM.[113] Curcumin and its analogue **75** inhibited the proliferation of A549 and HeLa cells in a concentration-dependent manner. Compound **75** was found to be more effective than curcumin and displays IC_{50} values of 7 ± 0.4 μM, and 6 ±0.8 μM against A549 and HeLa cells, respectively.

6.5 Human N-myristoyltransferase

The enzyme myristoyl-CoA:protein N-myristoyl transferase (HMT) catalyzes the attachment of the myristoyl group onto the N-terminal glycine residues of certain polypeptides.[114] In particular the cysteine-169 residue of human NMT (hNMT) is believed to be involved in the myristoylation process[115] and the activity of this enzyme is higher in some colorectal tumours than in the corresponding normal tissues.[116] hNMT may therefore be considered a molecular target in cancer chemotherapy.[117]

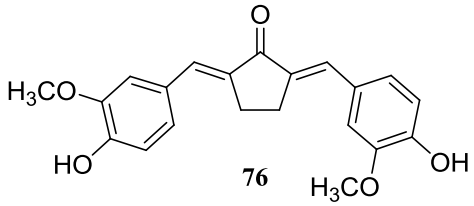
Two representative compounds in series **52** (R^1=3-Cl, R^2=4-Cl and R^1=4-CH_3, R^2=H) both inhibited the action of hNMT by 52% using concentrations of 100 and 50 μM, respectively.[73] A COMPARE program[118] of a number of compounds in series **52** revealed another possible mode of action of these compounds is inhibition of tyrosine kinases.

hNMT-1 is a potential target for the cytotoxic palladium metal complex **67**.[86] This molecule was designed as an angiogenesis inhibitor.[86] **67** inhibits purified hNMT in a concentration-dependent manner with maximal inhibition at a concentration of 2.5±0.97 μmol/L and half-maximal inhibition at 1.0 ± 0.26 μmol/L.[85] Gene chip analysis of A375 cells shows that NMT-1 was downregulated by the treatment of **67** and potent NMT-1 inhibitory activity was observed with an EC_{50} of 1 μM.[86] **67** inhibits the phosphorylated forms of MAPK and Akt signaling pathways in B16 cells and S6 kinase and Stat-3 downstream in A375 cells.[85,86] Since

NMT is required for the activation of upstream activators including Stat-3, it was hypothesized that inhibition of NMT activity could lead to decreased levels of NMT messages as a downstream consequence.

6.6 Topoisomerases

Topoisomerases (topos) play important roles in nuclear metabolic processes, such as replication and transcription and govern DNA topology through transient DNA cleavage, strand passing and religation.The two isoforms topo I and II act differently on DNA. Topo I acts by forming a transient single strand break through which the other DNA strand passes to achieve relaxation and topo II is able to do so with the two strands creating a DNA-linked protein gate. This gate allows another intact duplex to pass through.[119] Topo poisons or inhibitors are an important group of anticancer agents that include many clinically used drugs such as doxorubicin, etoposide and topotecan. Topo II is a sulfhydryl rich protein and considered as a very popular target for many anticancer agents. Topo II poisons are believed to act via alkylation of an exposed cysteine residue of topo II that leads to lethal DNA breaks.[120]

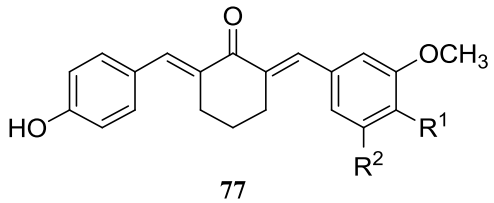


76

Topo I is a potential target for the arylidene dienone **76** which displays potent growth inhibitory activities against a number of human tumour cell lines with IC_{50} values in the low micromolar range. It interferes with DNA relaxation and DNA single strand breaks of topo I by binding to AT-rich sites in the DNA minor groove, not by intercalation.[121] More importantly, **76** does not show any cross-resistance against camptothecin-resistant cell lines. It induces caspase-3 mediated apoptosis and arrests the G2/M phase in the cell cycle of KB cells. Furthermore **76** activates the tumour suppressor gene p53 and displays no effect on the tumour activator oncogene bcl-2 in KB cells.[122] This compound also inhibits tubulin polymerization and causes apoptosis in KB cells whereby activation of caspase-3 was observed.

6.7 Oxidoreductases

Both glutathione reductase and thioredoxin reductase are homodimeric enzymes belonging to the same family of FAD dependent oxidoreductases.[123] They utilize NADPH as the electron donor. In both enzymes, similar FAD, NADPH, and interface domains are present.[124] Both glutathione reductase and thioredoxin reductase contain a dithiol/disulfide catalytic site close to the N-terminus. However, thioredoxin reductase possesses a flexible and exposed peptide containing SH/Se motif at the C-terminus that allows the enzyme to react with a variety of electrophilic and chemically unrelated compounds. Thioredoxin reductase catalyzes NADPH-dependent reduction of the disulfide group in thioredoxin. Both thioredoxin and thioredoxin reductase contain a critical cysteine molecule which plays an important role in the redox regulation of different signaling factors. Hence this enzyme appears to regulate pro-survival signaling factors in response to oxidative stress. A number of tumours have elevated concentrations of thioredoxin reductase[125] and thus molecules which inhibit this enzyme may lead to products which are useful in cancer chemotherapy.



77

a: $R^1 = R^2 = OCH_3$
b: $R^1 = OH$; $R^2 = H$

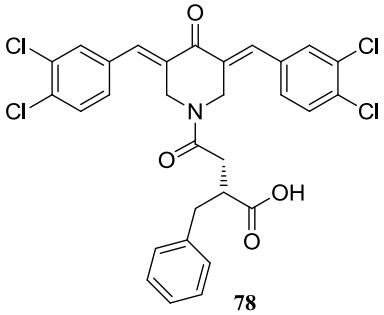
In view of the ability of curcumin to inhibit rat thioredoxin reductase,[126] a number of curcuminoid analogs including **77a** and **77b**[127] were evaluated for their ability to inhibit TRxR1. The IC_{50} values of **77a** and **77b** towards thioredoxin reductase are 24.9 and 5.1 μM, respectively. This results suggest that **77b** is a lead TRxR1 inhibitor and this mechanism of action may be a contributor to its cytotoxic properties.

6.8 Nuclear type II site

Nuclear type II sites are located in the nuclear matrix which assist in DNA synthesis and replication.[128,129] Ligands which interefere with this receptor inhibit cellular growth and proliferation. Compounds **68** and **69** bind to type II sites in rat uterine cells with very high affinity.[87] A correlation was noted between the extent of binding to the nuclear type II site and the percentage inhibition of the growth of human MCF-7 breast cancer cells.[87]

6.9 Proteosome inhibition

The proteasome is an essential component of the ATP-dependent proteolytic pathway in eukaryotic cells and is responsible for the breakdown of most cellular proteins. Proteasomes are mainly located in the nucleus and cytosol of eukaryotic cells including some associated with the endoplasmic reticulum and the cytoskeleton. Another essential function of the proteasome is to eliminate highly abnormal proteins that are produced by mutation or post synthetic damage. The ubiquitin dependent proteasomal degradation is crucial for both normal and malignant cells. The higher demand for metabolic/catabolic activity associated with the malignant phenotype renders the ubiquitin-proteasome pathway a suitable tool for cancer treatment.[130,131] Proteasome inhibitors may be particularly efficacious for certain cancer types with critical pathways that are dependent upon proteolytic degradation. Proteasome inhibitors may have the utility in the treatment of cervical and other HPV-related cancers.[132-134] Proteasome inhibitors preferentially kill tumor cells both *in* v*itro* and *in* vivo.[135-138] Furthermore, the induction of the NF-κB survival pathway by DNA damaging agents is dependent on proteosomal degradation of IκB. Thus, proteasome inhibitors block NF-κB activation that leads to the sensitization of cells to radiation and chemotherapy.[137]



78

A novel bisarylidene-N-phenylalanine carboxamide **78**, referred to as RA-1 displays potent growth inhibitory capacity in the human HeLa (+HPV18) and CaSki (+HPV16) cervical cancer cell lines with IC_{50} values of 0.32 and 1.5 μM, respectively.[139] In HeLa cells, **78** produces an increase in the levels of polyubiquitinated proteins similar to bortezomib, a FDA approved proteasome inhibitor.[140] Compound **78** is capable of inhibiting equally the three catalytic sites of the proteasome (IC_{50}=5 μM). The replacement of the phenylalanine amino acid residue by tyrosine or leucine decreases proteasomal activity. The rapid accumulation of polyubiquitinated proteins observed following exposure to **78** is consistent with impairment of proteasomal

functions. Taken together, these results suggest that the toxicity exerted by **78** on cervical cancer cells is associated with *in vivo* proteasomal inhibition. **78** inhibits proteasomal activity and has improved dose-dependent antiproliferative and proapoptotic properties in cervical cancer cells containing a human papilloma virus. Further, it induces synergistic killing of cervical cancer cell lines when tested in combination with an FDA approved proteasome inhibitor. Exploration of the potential mechanism of proteasomal inhibition by **78** using *in silico* docking studies suggests that the carbonyl group of its oxopiperidine moiety is susceptible to nucleophilic attack by the γ-hydroxythreonine side chain within the catalytic sites of the proteasome.

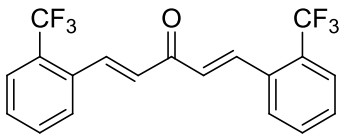
The diaryldienones **61** and the palladium complex **67** inhibit the activity of the chymotrypsin-like activity of the 20S proteasome *in vitro*, leading to a significant accumulation of ubiquitinated p53 and other cellular proteins in whole cells.[84] *In vitro* studies showed that **61** did not significantly disrupt the interaction of p53 and murine double minute 2 protein. The IC_{50} value of proteasome inhibition by **67** is approximately 1 μM.[84] These results indicate that **61** and **67** induce significant cytotoxic effects in cancer cells through the inhibition of the cellular proteasome and provide a rationale for the further development of this class of compounds as novel cancer chemotherapeutic agents.

Based on the observation that curcumin is a potent proteasome inhibitor in colon cancer HCT-116 and metastatic SW-480 cell lines,[141] the difluoro compound **19** was evaluated for its inhibitory effects on purified rabbit 20S proteasome or cellular 26S proteasome and found to be a potent proteasome inhibitor as tested *in vitro* and in HCT116 cells *in vivo*, and inhibited the proliferation of both colon and pancreatic cancer cells by inducing apoptotic cell death.[142] **19** displays potent growth inhibiting potential and is a good apoptosis inducer in BxPC-3 pancreatic cancer cells.[142] **19** was the most potent inhibitor compound showing 24%, 39% and 68% inhibition on cell proliferation at 10, 20 and 30 μM, respectively, compared to curcumin with 24%, 53% and 49% inhibition at the same concentrations in HCT-116 cells. **19** displayed higher potency over other fluoro substituted curcumin analogs.

6.10 NFkB pathway

Nuclear factor-kappa B (NF-κB) is a transcription factor that is activated by DNA damage[143,144] leading to its nuclear localization and promotes the expression of target genes that regulate a number of processes including cell growth, differentiation, and apoptosis,[145] while

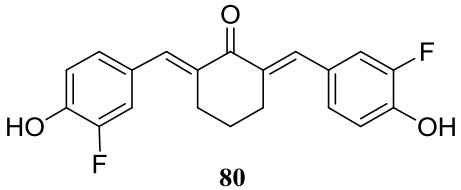
inhibition of NF-κB activation exhibits therapeutic responses. In a number of tumours, NF-κB activation protects malignant cells from apoptosis.[146] NF-κB activation resulted from IKK kinase catalyzed phosphorylation of the inhibitory factor IκB that promotes degradation of the IκB protein.[145] NF-κB inhibitors sensitize cancer cells to both radiation and chemotherapy,[147-150]which establish NF-κB inhibition as a potential chemotherapeutic strategy to treat cancers. There are many potential strategies to inhibit NF-κB activation with a most promising target being the IKK kinase.[145] NF-κB activation pathway can also be used as an alternate strategy to make normal tissue cells more resistant to the deleterious effects of radiation or chemotherapy. This strategy is supported by the fact that a polypeptide drug CBLB502 protects normal tissues against radiation by activating the NF-κB pathway.[151]



79

A number of cytotoxic acyclic and cyclic diarylidene dienones **6a** (R= 2-OH), **68,** and **79**[152] which possess average IC_{50} value of approximately 4.5 μM, inhibit TNFα-induced activation of NFκB in cells stably infected with NFκB-dependent reporter construct using the Panomics Reporter Stable Cell Line.

6.11 Mitochondria



80

Mitochondria is an important cellular target of compounds containing the 1,5-diaryl-3-oxo-1,4-pentadienyl group since a number of thiol alkylators react with mercapto groups in mitochondria.[153] In particular, these compounds have the capacity to increase the rates of respiration in these organelles. Compound **80** increases respiration rates in mitochondria and causes a collapse of the membrane potential.[154] This effect was reversed by 6-ketocholestanol

which is a recoupling agent suggesting that **80** is an uncoupler. This compound inhibits the production of superoxide and oxidizes certain thiol groups which lead to swelling of mitochondria and the release of cytochrome c, which are events associated with the induction of apoptosis by PTP opening. The induction of cell death in tumours triggered by treatment of uncouplers is well documented.[155] Furthermore **76** did not alter the mitochondrial membrane potential.

The effect of some of the representative bis-arylidene dienones from the series **55** such as **55a** (R^1=H, X=N$(CH_3)_2$ and **55** (R^1=H, X=morpholine) on mitochondrial functions were investigated. Both the compounds stimulate respiration in rat liver mitochondria.[76] A similar observation was also noted for a tropinone derivatives **60a** which stimulates mitochondrial respiration and produces rapid swelling of this organelle.[82] Thus interference with mitochondrial function appears to be one way in which cytotoxicity is caused and influences the magnitude of the cytotoxic potencies.

The cytotoxins **25d** (R^1=R^2=R^3=OCH_3) and **27d** (R^1=R^2=R^3=OCH_3) stimulate the rate of respiration in rat liver mitochondria using a concentration of 10 µM while **27d** had no effect at the same concentration.[51] The average IC_{50} figures of **25d**, **26d** and **27d** in the three bioassays are 0.88, 43.8 and 6.00 µM, respectively. While the variation in cytotoxicity is likely multifactorial, the different effects on mitochondrial function may contribute to the differences in the observed potencies.

7. Multidrug resistance

The resistance shown by tumours to a variety of chemotherapeutic drugs, referred to as multidrug resistance (MDR) possess a severe challenge in treating cancers.[156,157] Besides several biochemical pathways which are responsible for drug resistance, the most widely studied ones are the cell membrane transporter proteins[158] which act as extrusion pumps. Several families of pumps are present in mammals and microorganisms that use different energy sources.[159-161] Overexpression of a number of these transporter proteins accelerates the efflux of chemotherapeutic drugs that result in a decrease in drug concentrations in cells. The best known extrusion pumps are the ABC superfamily of transporters such as P-glycoprotein (P-gp), the MRP family and BCRP[162] that use ATP as their energy source. The human P-gp is encoded by a mdr1 gene. Inhibition of the functions of P-gp and sister proteins are considered to be a realistic

therapeutic strategy to reverse MDR, and drug candidates possessing P-gp inhibitory properties have been sought.[163-166] Inhibiting P-gp as a way of reversing MDR has been extensively studied for more than two decades.

Many agents that modulate the function of P-gp have been identified, including calcium channel blockers, calmodulin antagonists, steroidal agents, protein kinase C inhibitors, immunosuppressive drugs, antibiotics, and surfactants. The coadministration of a non-toxic "MDR-modulating" compounds in combination chemotherapy may significantly improve the outcome of cancer treatment. In addition, a cytotoxic agent possessing MDR reversal properties may be helpful in treating drug resistant tumours. Several efforts have been made to analyze the chemical features involved in P-glycoprotein modulating properties.[167,168] A number of general features of MDR reversal agents have been identified such as the amphiphilic nature of the molecules, the presence of aromatic rings, a basic nitrogen and the positive charge at neutral pH,[169] but no precise conclusions on the molecular features characterizing MDR reversal are yet established. However, this knowledge may prove to be very useful for the design and/or the selection of novel compounds to be tested in the clinical setting as MDR modulators.

A number of N-aroyl-diaryldienones in series **55** are potent revertants of P-gp associated multidrug resistance.[170] In the rhodamine 123 fluoresence assay, these compounds modulate P-gp expression in murine L-5178 lymphoma cells transfected with the human MDR1 gene.[171] The concentrations of rhodamine 123 in treated and untreated transfected and parental cells were measured and the fluorescence activity ratio (FAR) values of greater than 1 indicate the ability of the compound to reverse MDR. At concentrations of 4 μg/mL, the FAR figures of compounds in series **55** ranges from 46-179 which are substantially higher than a reference P-gp modulator verapamil. In series **55**, the piperidine group in the N-aroyl side chain displayed greater P-gp reversal properties compared to other amines such as dimethylamine, diethylamine and morpholine which possess different pKa values while the 4-methyl and 4-chloro aryl substituents were the preferred the groups to exhibit higher potency. The 3,5-bis(arylidene)-4-piperidones **43** which are the parent precursors for generating the compounds in series **55** were bereft of MDR reversal activity expect the 4-methylaryl substituted analog had weak activity. These results reveal that although the structural fragment *N*-[4-(2-alkylaminoethoxy)phenylcarbonyl] is virtually bereft of any MDR reversal properties, it contributes significantly in conferring MDR reversal properties of the compounds in series **55**. These observations again establish that

incorporation of a side chain linker onto the 4-piperidyl nitrogen atom enhances bioactivity of the molecule by likely interacting with an important auxiliary binding site.

Some of the other arylidene dienones such as **37** and a related analog **27** (R^1 = R^2 = OCH_3; R^3 = H) have FAR values of 16 and 20 respectively, in the L-5178 P-gp reversal screen[62] while some of the β–tetralone analogs demonstrate remarkable P-gp reversal properties. In particular **34** and **35** are potent P-gp modulators displaying FAR values of 134 and 44, respectively at a concentration of 4 μg/mL.[62]

8. Concluding Remarks

Numerous studies have established that curcumin possesses significant potential for the prevention and therapy of various cancers. No natural product has yet been known which modulates such a diverse array of signal transduction pathways as curcumin does. Cancer molecular biologists believe that tumour cells use multiple pathways to escape host defense mechanisms. Therefore, the drug that targets a specific molecular pathway may not be effective to treat cancers, in particular when tumours become resistant to chemotherapeutic drugs. Developing a drug that targets multiple pathaways and yet is pharmacologically safe is not an easy task for medicinal chemists. In view of this, curcumin's multitargeted mechanisms of action and its safe use merits its potential as a promising chemotherapeutic agent against a number of drug-resistant tumours. However, its poor bioavailability continues to be the most contentious issue for its further development as a clinical drug. In order to overcome this problem and not to compromise its pharmacological and toxicological properties, structural modifications of curcumin led to the development of the 1,5-diaryl-3-oxo-1,4-pentadienes, a novel class of curcuminoid analogs as emerging antineoplastics. Interesting features of many of these compounds are the remarkable selective toxicities towards neoplasms compared to normal cells and some have promising multidrug resistance reversal properties. Its multitargeted mechanisms of action may be the reason why these compounds display potent MDR reversal properties. Thus novel compounds which overcome MDR may find use as dual agents (possessing anticancer and MDR-revertant properties) or as compounds to be co-administered with established anticancer drugs. In addition, many of these compounds display good tolerability in mice and demonstrate *in vivo* anticancer properties. The possibility of diaryldienones serving as Michael acceptors and permitting the sequential alkylation of cellular thiols has been suggested as the reason for the

greater toxicities of this class of compounds towards malignant cells compared to normal cells. Reduction of either the keto or ethylene group of the α,β-unsaturated keto motif in arylidene dienones lowers cytotoxic potencies substantially suggesting that alkylation is the principal mechanism by which cytotoxicity of arylidienones is mediated. Substantial evidence has been envisaged in support of diarydienones as thiol alkylators. The diaryldienones exert their cytotoxicities by interacting with multiple signal transduction pathways as curcumin does; however, in a number of cases the molecular mechanisms of action are quite distinct from curcumin. For example, **63**, but not curcumin, disrupts normal mitosis, interphase microtubule organization and stabilizes cellular microtubules.[172] Finally, this literature review advocates the potential utility of 1,5-diaryl-3-oxo-1,4-pentadienes as emerging future anticancer drug candidates and presents the importance in pursuing this class of compounds.

9. References

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CHAPTER 2

Chapter 2 is a copy of an article which will be submitted to the *European Journal Medicinal Chemistry*.

Relation of Chapter 2 to the objectives of this project

To support the first hypothesis as mentioned in the introduction that "Incorporation of a 1,5-diaryl-3-oxo-1,4-pentadienyl (Ar-CH=CH-CO-CH=CH-Ar) group onto alicyclic and heterocyclic scaffolds will lead to potent cytotoxic agents".

Description

In view of this hypothesis, a series of 3,5-bis(arylidene)-4-piperidones which possess the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore was developed as cytotoxic agents. The influence of aryl substituents on cytotoxic potencies of the compounds was examined by introducing a number of substituents possessing diverse electronic, hydrophobic and steric properties onto the aryl rings. Some potential water soluble O-phosphorylated analogs of 3,5-bis(4-hydroxybenzylidene)-4-piperidones were prepared. Also, the mono trimethylammonium as well as the bis trimethylammonium quaternized salts of 3,5-bis(4-dimethylaminobenzylidene)-4-piperidone were synthesized. All compounds were evaluated against human Molt 4/C8 and CEM T-lymphocytes as well as murine leukemic L1210 cells. Some of these compounds were also assessed against a panel of approximately 55 human tumor cell lines by the National Cancer Institute (NCI). All the compounds were assessed for short term toxicity in mice by the National Institute of Neurological Disorders and Stroke (NINDS). These screens identified a number of lead molecules.

Author Contributions

My contributions to this publication were undertaking a literature review of the prepared compounds, designing the synthetic chemical routes, synthesizing the compounds, determining and interpreting the ^{1}H and ^{13}C NMR spectra of the compounds in series **2.** Elemental analysis was done by me using the CHNS analyzer. The coauthors on this paper are Inci Gul, who synthesized some of the compounds in series **2**, U. Das, supervised the synthesis of the compounds and undertook the QSAR and molecular modeling studies, E. De Clercq and J. Balzarini supervized the cytotoxic assays against human Molt 4/C8 and CEM T-lymphocytes and murine leukemic L1210 cells, the laboratory of J. P. Stables performed the murine toxicity

screen in rodents, and overall guidance and supervision was provided by my supervisors J. R. Dimmock and D. K. J. Gorecki. The experimental in the manuscript was written by me while Dr. Dimmock gave final shape to the manuscript with some inputs given from my end. The figures and schemes were drawn by me using ChemBioOfficeUltra, version 12.0.

3,5-bis (Benzylidene)-4-piperidones: building blocks for developing potent cytotoxins

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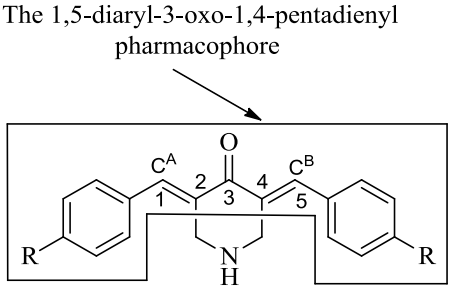
Abstract

A number of 3,5-bis (benzylidene)-4-piperidones **2a-n** were prepared in the quest to find analogs with potent cytotoxic properties which will serve as cytotoxic warheads in the subsequent development of these compounds. Most of the compounds have IC_{50} values less than 10 μM when evaluated against human Molt4/C8 and CEM T-lymphocytes and for five of these compounds; some of these figures are submicromolar. More than half of these compounds are more potent than melphalan in these two bioassays. The biodata suggest that potencies are greater when substituents are placed in the 2 and 3 positions of the aryl rings rather than in the 4 location. The IC_{50} values of eight of these compounds towards four human leukemic cell lines and seven human colon cancer cells are mainly in the 10^{-7} to 10^{-8} M range. A representative compound caused apoptosis in HT29 cells and caused arrest of the cell cycle at the Sub G1 phase.

Keywords: Conjugated unsaturated ketones, cytotoxicity, structure-activity relationships, flow cytometry

Introduction

The principal interests in this laboratory are the design and syntheses of various conjugated ketones as candidate antineoplastics. These molecules have a marked affinity for thiol groups and little or no capacity for reacting with amino and hydroxy groups [1]. Thus these compounds may spare nucleic acids and hence be devoid of the mutagenic and carcinogenic properties of a number of alkylating agents used today in cancer chemotherapy [2]. Furthermore certain tumours are more susceptible to an initial chemical interaction than normal cells and in these cases a subsequent attack is more detrimental to the neoplasms than non-malignant cells [3, 4]. Hence recent studies have involved the preparation of compounds capable of undergoing sequential alkylation of cellular thiols. Specifically the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore has been mounted on heterocyclic and cycloaliphatic scaffolds[5]. In these compounds as illustrated in series **1** in Figure 1, an initial attack can occur at the olefinic carbon atom C^A followed by a further interaction with cellular thiols at C^B.



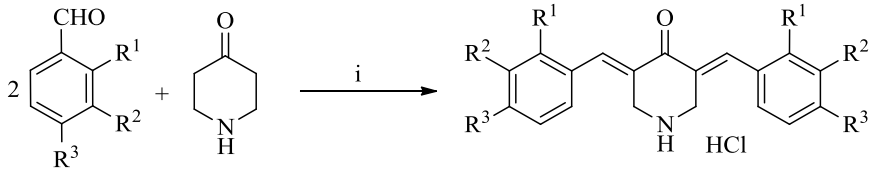
1

Figure 1: Series **1**

The unsubstituted compound **1a** (**1**, R= H) has IC_{50} values in the low micromolar range against three cell lines as indicated in Table 1. However when substituents such as chloro, nitro, fluoro, methoxy and dimethylamino were placed in the para locations of the aryl rings, in virtually all cases a statistically significant reduction in cytotoxic potencies was observed[6]. Potencies could be enhanced however by placing various substituents on the piperidyl nitrogen atom thereby creating more complex molecules [7, 8].

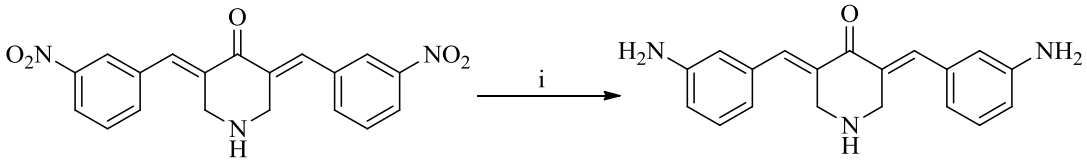
The objectives of the present study are as follows. First, this study was designed to find which substituents can be placed in the aryl rings to create 3,5-bis (benzylidene)-4-piperidones

with submicromolar IC_{50} values. Second, the aspiration was made that the results generated will provide guidelines for future directions so that further increases in potencies will be achieved. The longterm plan is to obtain one or more cytotoxic warheads, i. e., highly potent 3,5-bis(benzylidene)-4-piperidones. These molecules can be structurally modified to obtain optimal characteristics of anticancer efficacy. For example, various substituents could be placed on the piperidyl nitrogen atom to achieve an appropriate hydrophilic-hydrophobic balance.



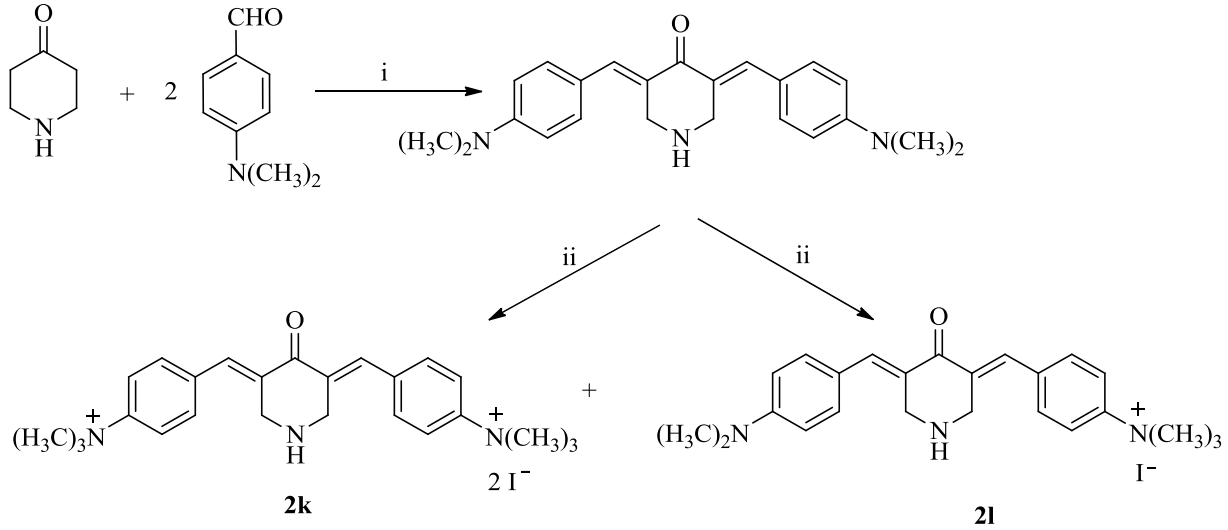
2a,b, d-j

Scheme 1. Synthesis of **2a**, **b**, **d-j**. Reagent: i= HCl. The nature of the aryl substituents is presented in Table 1.



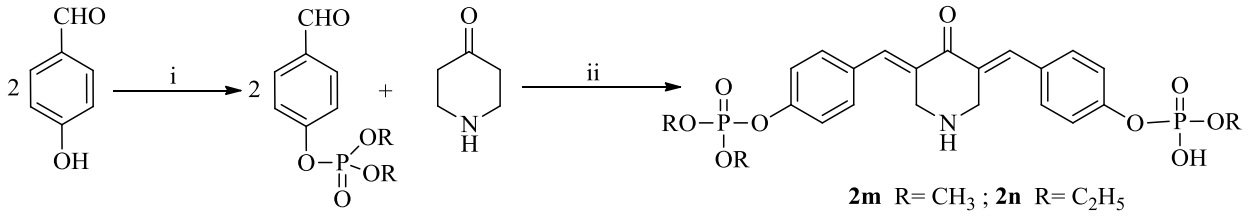
2c

Scheme 2. Synthesis of **2c**. Reagent: i= $SnCl_2$



2k **2l**

Scheme 3. Synthesis of **2k**, **l**. Reagent: i= HCl; ii= CH_3I.



Scheme 4. Synthesis of **2m**, **n**. Reagents: i= $ClP(O)(OR_2)_2/K_2CO_3$; ii= HCl. The nature of the ester group is portrayed in Table 1.

The compounds proposed are presented in schemes **1-4**. The groups in the aryl rings of **2a-n** have varied electronic, hydrophobic and steric properties and were chosen for the following reasons. Since the placement of substituents in the 4-position of the aryl rings in series **1** lowered cytotoxic potencies in general as illustrated for **1b** (**1**, R= Cl) in Table 1, an E_4 effect [9] was suspected, i.e., an unfavourable steric effect of 4-substitution. This theory was explored by placing the chloro atom into the 2 and 3 positions (**2a, b**). Other groups were placed in the 3 location of the aryl rings (**2c-e**) and as a null hypothesis, different groups were inserted in the 4-position of the aryl rings (**2e-j**). The thiol alkylating capacity of series **1** would be enhanced by the presence of a strongly electron-withdrawing group in the aryl rings such as in **2k** since the σ_p value of the trimethylammonium group is 0.82 [10]. Finally a comparison of the bioactivities of the 4-hydroxy analog **2f** with the candidate prodrugs **2m, n** was planned.

2. Results

Compounds **a, b, d-j** were prepared by condensation between the appropriate aryl aldehyde and 4-piperidone (Scheme 1). Reduction of the nitro group of 3, 5-bis(3-nitrobenzylidene)-4-piperidone led to the formation of **2c** (Scheme 2). The reaction between 4-dimethylamino benzaldehyde and 4-piperidone afforded 3, 5-bis(4-dimethylaminobenzylidene)-4-piperidone. Condensation of this compound with a slight molar excess of methyl iodide afforded **2l** initially while the addition of more methyl iodide to the mother liquor and prolonged heating led to the isolation of **2k** (Scheme 3). Reaction of 4-hydroxybenzaldehyde with either dimethylchlorophosphate or diethylchlorophosphate afforded the corresponding 4-dialkylphosphoryloxybenzaldehydes. Condensation of both aldehydes with 4-piperidone led to the formation of the corresponding unsaturated ketones **2m, n** (Scheme 4).

Table 1: Evaluation of **1a**, **b** and **2a-n** against human Molt 4/C8 and CEM T-lymphocytes and murine L1210 cells

Compound	Aryl Substituents	IC_{50} (μM)[a]		
		Molt 4/C8	CEM	L1210
1a[b]	H	1.67±0.15	1.70±0.02	7.96±0.11
1b[b]	4-Cl	13.4±4.0	8.63±0.48	41.5±0.3
2a	2-Cl	0.540±0.36	0.245±0.01	1.82±0.23
2b	3-Cl	0.466±0.19	0.333±0.01	4.48±1.38
2c[c]	3-NH_2	0.439±0.11	0.396±0.09	4.24±0.73
2d	3-OH	0.242±0.04	1.54±0.32	7.56±0.42
2e	3,4-$(OH)_2$	7.66±1.59	8.51±0.76	11.7±1.4
2f	4-OH	10.5±1.6	8.24±0.05	73.6±3.0
2g	4-Br	7.70±0.81	1.70±0.04	31.1±11.0
2h	4-CF_3	1.68±1.21	0.418±0.053	4.72±1.79
2i	4-COOH	168±51	464±51	275±10
2j[c]	4-$COOC_2H_5$	1.81±0.13	5.84±0.90	8.13±0.85
2k	4-$N(CH_3)_3I$	4.72±1.93	5.14±3.12	41.2±1.3
2l	4-$N(CH_3)_3I$/4-$N(CH_3)_2$	4.04±2.87	5.26±3.76	28.2±17.5
2m	4-$OP(O)(OCH_3)_2$/ 4-$OP(O)(OH)(OCH_3)$	7.70±2.04	31.8±5.6	78.7±25.2
2n	4-$OP(O)(OC_2H_5)_2$/ 4-$OP(O)(OH)(OC_2H_5)$	42.0±1.9	38.0±5.3	211±24
Melphalan[b]	----	3.24±0.56	2.47±0.21	2.13±0.02

[a]The IC_{50} value is the concentration of the compound required to inhibit the growth of the cells by 50%. [b]The data has been reported previously [6]. [c]The compound was prepared as the free base.

All of the compounds were evaluated against human Molt4/C8 and CEM T-lymphocytes as well as murine L1210 lymphoid leukemia cells. These data are presented in Table 1. In addition **1a, 2a-d, h, j-l** were screened against approximately 55 human tumour cell lines. These

results are portrayed in Table 2. The effect of a representative compound on the cell cycle of HT29 cells is summarized in Table 3.

3. Discussion

The synthesis of **2a-j** proceeded in a straightforward manner. However the first product isolated when 3, 5-bis (4-dimethylaminobenzylidene)-4-piperidone reacted with methyl iodide was the monoquaternary salt **2l** (Scheme 3). The trimethylammonium group in **2l** is a strongly electron-attracting substituent and will delocalize the lone pair of electrons on the nitrogen atom of the dimethylamino group of **2l** thereby hindering the reaction with methyl iodide. Under forcing conditions the desired product **2k** was obtained. In the process of reacting the 4-dialkylphosphoryloxybenzaldehydes with 4-piperidone, O-dealkylation of one of the alkoxy groups occurred leading to **2m, n**. The partial dealkylation of a diethoxyphosphorylalkyl group attached to the nitrogen atom of some 3, 5-bis(benzylidene)-4-piperidones has been recorded previously [11]. The ^{1}H NMR spectra of **2a-n** revealed that the compounds were isomerically pure and were designated the *E*, *E* isomers [6]. This observation is in accordance with X-ray crystallographic evidence which assigned the *E* configuration to various related 3, 5-bis (benzylidene)-4-piperidones [12].

All of the compounds in series **2** were evaluated against human Molt4/C8 and CEM T-lymphocytes in order to estimate the potencies of these compounds towards human transformed cells. In addition, a number of anticancer drugs are cytotoxic towards murine L1210 cells [13] and hence this assay, which may identify lead molecules, was also utilized. The data are presented in Table 1.

Two general comments upon the results are as follows. First, the objective of finding compounds with IC_{50} values in the submicromolar range in some of the bioassays was achieved by **2a-d, h** which is clearly lead molecules. Second, in order to obtain some concept of how these biodata relate to an established alkylating agent used in cancer chemotherapy, a comparison was made between the potencies of melphalan and the compounds in series **2**. The following compounds have statistically significant lower IC_{50} values than melphalan (fold increase in potency in parentheses) namely **2a** (6.0), **2b** (7.0), **2c** (7.4), **2d** (13.4), **2j** (1.8) in the Molt4/C8 assay, **2a** (10.1), **2b** (7.4), **2c** (6.2), **2d** (1.6), **2g** ((1.5), **2h** (5.9) in the CEM test and **2a** (1.2) in the L1210 screen. In addition **2h**, **2k**, **2l** (Molt4/C8 assay) and **2k**, **2l** (CEM screen) are

equipotent with melphalan. Thus over half of the compounds in series **2** are more potent or equipotent with melphalan in the case of the evaluation against Molt4/C8 and CEM T-lymphocytes. In particular, **2a** and **2d** having greater than a 10-fold increase in potency than melphalan towards CEM and Molt4/C8 cells are noteworthy.

Some specific comments pertaining to the biodata in Table 1 are now presented. A question requiring resolution is whether substituents could be placed in the aryl rings of **1a** which would lead to statistically significant potency increases. The results indicate that **2a-c** are more potent than **1a** in all three screens. It is of interest that **2a-c** have substituents in the 2 and 3 positions of the aryl rings and not in the para location. This observation was noted in other comparisons made. Thus in regard to the ortho (**2a**), meta (**2b**) and para (**1b**) chloro isomers, the average IC_{50} values of these compounds towards Molt4/C8, CEM and L1210 cells are 0.87, 1.76 and 21.2 μM, respectively. Furthermore one may note from Table 1 the substantially greater potency of the 3-hydroxy analog **2d** (average IC_{50}= 3.11 μM) than the 4-hydroxy structural isomer **2f** (average IC_{50} = 30.8 μM). These observations suggest that an E_4 effect is indeed operating and further investigations with a range of other aryl substituents should be undertaken to evaluate this hypothesis. For example, the 3-amino analog **2c** displays excellent potency towards the T-lymphocytes and the preparation of the ortho and para structural isomers for bioevaluations is warranted.

The cytotoxic potencies of **2g-k** were compared with the data for **1a**. The aryl rings in **2g-k** contain electron-withdrawing groups which means that the atomic charges on the olefinic carbon atoms should be lower than in **1a** and thus enhance the interaction with cellular thiols. However only **2h** had greater potency than **1a** in the CEM and L1210 screens while equipotency with **1a** was demonstrated in the following cases (bioassay in parentheses) namely **2g** (CEM), **2h**,**j** (Molt4/C8) and **2j** (L1210). These data afford a further intimation that an E_4 effect is in operation and placement of the same aryl substituents in the 2 and 3 locations should be undertaken. The high IC_{50} values of **2i** (average IC_{50} figure is 302 μM) may have been due to the polarity of the carboxy group impeding the penetration of cell membranes. Esterification of the acidic groups of **2i** leading to **2j** (average IC_{50} value of 5.26 μM) led to a 57-fold increase in potency. The average IC_{50} figure of 3,5-bis(4-dimethylaminobenzylidene)-4-piperidone towards Molt4/C8, CEM and L1210 cells was shown previously to be 209 μM [6]. The bis and mono quaternary ammonium iodides **2k** and **2l** have much lower average IC_{50} values namely 17.0 and

12.5 μM, respectively, i.e., potency increases of approximately 15 were noted. In general, the phosphoryl analogs **2m, n** were substantially less potent than the 4-hydroxy analog **2f**.

In summary **2a-d**, **h** have IC_{50} values in the submicromolar range and are lead molecules. The structure-activity relationships noted in series **2** suggest that greater cytotoxic potencies are found in the 2 and 3 positions of the aryl ring as an E_4 effect is likely. These observations afford useful directions in amplifying the project.

In order to explore further the potential of the lead compounds **2a-d**, **h** and related analogs, and evaluations against a substantially increased number of cell lines was undertaken. Thus **2a-d**,**h**,**j-l** as well as **1a** were examined using approximately 55 human tumour cell lines from nine different neoplastic conditions namely leukemia and melanoma as well as non-small cell lung, colon, central nervous system, ovarian, renal, prostate and breast cancers [15]. The compounds were examined in the concentration range of 10^{-4} to 10^{-8} M. In virtually all cases IC_{50} values were obtained but for a few assays (which are indicated in the Experimental section), these figures are outside this range. Hence in considering the data for all cell lines, the term GI_{50} is employed. Examination of the mean graphs [16] revealed that leukemic and colon cancer cell lines are particularly sensitive to these compounds. The cytotoxic potencies towards all cell lines as well as various leukemic and colon cancer cell lines are presented in Table 2.

The average GI_{50} values presented in Table 2 confirm that **2a-d, h** are lead molecules. These compounds as well as **2h, j** are more potent than **1a** while the two analogs which contain quaternary ammonium groups have higher GI_{50} values than the unsubstituted compound **1a**. All of the enones are more potent than melphalan, e.g., the average GI_{50} value of **2d** is 71 times lower than the figure for melphalan.

Compounds which vary in their potencies towards different neoplastic cell lines are not general biocidal agents. Thus it is conceivable that such differing toxicities may indicate compounds which are preferentially cytotoxic to neoplasms rather then normal cells. Hence selectivity index (SI) figures were generated which are the ratios of the highest and lowest IC_{50} values of the compounds. The enones **2a**, **b**, **h** have impressive SI figures thereby confirming their designation as prototype molecules for analog development.

Table 2. Evaluation of **1a, 2a-d, h, j-l** against a panel of human tumour cell lines

Compound	All cell lines		Leukemic cells, $IC_{50}(\mu M)$[a]					Colon cancer cells, $IC_{50}(\mu M)$[a]							
	Ave. GI_{50} (μM)[b]	SI[c]	CCRF-CEM	K562	RPMI 8226	HL60 (TB)	Ave. IC_{50} (μM)	COLO 205	HCC 2998	HCT 116	HCT 15	HT 29	KM 12	SW 620	Ave. IC_{50} (μM)
1a	1.62	93.3	1.62	0.49	0.31	0.36	0.70	1.55	2.00	0.18	0.63	0.58	0.79	0.58	0.90
2a	0.42	>3312	0.05	0.02	0.03	---	0.03	4.17	0.17	0.04	0.21	0.77	0.10	2.57	1.15
2b	0.66	>3162	0.24	---	0.23	---	0.24	0.50	0.20	0.03	0.19	1.98	0.26	0.04	0.46
2c	0.53	60.3	0.29	0.55	0.22	0.30	0.34	0.25	0.43	0.21	0.21	0.21	0.21	0.20	0.25
2d	0.38	195	0.01	0.04	0.05	0.15	0.06	1.45	1.62	0.23	0.04	2.63	0.22	0.16	0.91
2h	1.07	>1995	0.04	0.12	0.02	0.66	0.21	1.48	1.45	0.17	0.15	0.67	6.76	0.11	1.54
2j	0.45	>347	0.18	0.13	0.08	0.22	0.15	---	1.02	0.13	0.20	0.21	0.21	0.17	0.32
2k	9.55	>339	2.63	1.35	---	1.20	1.73	1.91	6.31	3.55	>100	6.92	3.47	7.08	>18.5
2l	7.41	347	1.58	1.15	0.29	0.48	0.87	1.70	4.68	3.63	>100	5.62	2.69	6.03	>17.8
Melphalan[d]	26.9	118	6.17	43.7	66.1	2.04	29.5	66.1	41.7	30.2	36.3	46.8	43.7	38.9	43.4
5-Fluorouracil	12.0	10,000	14.1	35.5	1.70	---	17.1	14.8	<0.25	1.91	2.29	28.2	<0.25	22.4	<10.0

[a]The term IC_{50} refers to the concentrations of the compounds which are required to inhibit the growth of the cells by 50%. [b]The term GI_{50} indicates the 50% growth-inhibiting concentrations in most cases. When this value fell outside of the concentrations utilized, e.g., > 10^{-4} M, the figure of 10^{-4} was used in calculating the average GI_{50} data. [c]The letters SI refer to the selectivity index which is the ratio of the highest and lowest IC_{50} values of the compound to all cell lines. [d]The data for melphalan is reported previously.[7]

The data presented in Table 2 reveal that enones **1a**, **2a-d**, **h**, **j-l** demonstrate remarkable potencies towards four leukemic cell lines in which 81% of the IC_{50} values are submicromolar. Nine of the IC_{50} figures are in the double digit nanomolar range (10^{-8} M) and the average IC_{50} values of **2a** and **2d** are 32 and 62 nM, respectively. The compounds are far more potent than melphalan which is used in treating chronic lymphocytic leukemia [17]. In particular, the average IC_{50} values of **2a** and **2d** are 922 and 476 times lower than the figure for melphalan.

All of the compounds were evaluated against seven human colon cancer cell lines. The data in Table 2 reveal that 58% of the IC_{50} values **2a-d, j, k-l** are submicromolar or if one considers only the non-quaternary ammonium compounds namely **2a-d, h, j**, the figure rises to 75%. Of particular note are the significant potencies of **2a, b** towards HCT116 cells, **2b** against SW-620 neoplasms and **2d** versus the HCT116 tumour. 5-fluorouracil is used in treating colon cancers [18] and all of the compounds **1a, 2a-d, h, j-l** have lower IC_{50} values than 5-fluorouracil towards HT29 and SW-620 cells.

Table 3: Effect of **2a** on the cell cycle of HT29 cells

Conc. of **2a** (μM)	Sub G1[a]	G1[a]	S[a]	G2/M[a]
0.00	0.62	80.3	11.4	7.62
0.5	23.6	47.8	13.9	14.4
1.0	29.8	44.2	11.9	13.7
5.0	54.1	27.9	8.42	8.57

[a]The figures are the percentage of the cells in different phases of the cell cycle.

In order to gain some idea as to how these compounds exert their antineoplastic properties, the effect of a representative enone **2a** on the cell cycle using the HT29 neoplasm was undertaken. The results presented in Table 3 reveal that **2a** caused a concentration dependent arrest of the cell cycle at the sub G1 phase. Thus **2a** causes an increase in the percentage of the cells undergoing apoptosis and this mode of action likely applies to other compounds in series **1** and **2**.

Finally in considering the possible development of a series of cytotoxins, some appreciation of their mammalian tolerability should be obtained. Hence most of the members of series **2** were examined in a short term toxicity test in mice. Doses of 30,100 and 300 mg/kg of **2a-k, n** were administered intraperitoneally to mice and the animals examined after 0.5 and 4 hours for lethality as well as neurotoxicty using the rotorod test [19]. No deaths were noted

among **2a-k** while one of the two animals which had received 300 mg/kg of **2n** was dead at the end of four hours. Neurotoxicity was noted in some of the animals which had received the maximum dose of 300mg/kg of **2c, i, j, k** details of which are given in the Experimental Section. One may conclude that the antineoplastics **2** are well tolerated in mice which enhance their potential utility for development as cytotoxic warheads.

4. Conclusions

Structural modifications of the lead compound **1a** led to **2a-d**, **h** which are more potent in some or all of the Molt4/C8, CEM and L1210 bioassays. Over half of the compounds in series **2** have either lower IC_{50} values than melphalan or are equipotent with this antineoplastic drug. The biodata generated for **2a-n** suggest that in the future the placement of substituents in the 2 or 3 locations of the aryl rings will give the greatest potencies. The excellent cytotoxic potencies of **2a-d**, **h** noted from the initial screens were confirmed using a large number of human tumour cell lines. These compounds, along with **2j-l**, are more potent than melphalan when the average GI_{50} data are concerned. In addition, **2a**, **b**, **h** have excellent SI values. The enones **2a-d**, **h**, **j-l** demonstrate excellent growth-inhibiting properties towards human leukemic cell lines and in many cases towards human colon cancer cells. The mode of action of the compounds in series **2** likely includes apoptosis and in general, these enones are well tolerated in mice.

5. Experimental

5.1 Chemistry

Melting points were determined on a Gallenkamp instrument and are uncorrected. ^{1}H NMR spectra were obtained using a Bruker Avance spectrometer 500 MHz machine equipped with a BBO probe. Chemical shifts (δ) are reported in ppm. Elemental analyses were undertaken using an Elementer CHNS analyzer.

*5.1.1 Synthesis of 3,5-bis(arylidene)-4-piperidones (**2a,b,d-j**)*

A mixture of 4-piperidonehydrochloride (0.005 mol) and an appropriate aryl aldehyde (0.01 mol) in acetic acid (15 ml) was saturated with anyhydrous hydrogen chloride and stirred at room temperature for 10-12 h. The precipitate was filtered, washed with acetone (2×10 ml) and dried. The crude hydrochloride salt was purified by crystalization from chloroform-methanol.

*5.1.1.1. 3, 5-bis (2-Chlorobenzylidene)-4-piperidone hydrochloride (**2a**).* Yield 72%; mp 221 ^{o}C; ^{1}H NMR (DMSO-d_6): δ 9.78 (s, 2H, $^{+}$NH2), 7.43 (s, 2H, 2×=CH), 6.62 (m, 8H, Ar-H), 3.66 (s, 4H, 2×NCH_2). Anal.calcd for $C_{19}H_{16}Cl_3NO.H_2O$: C 59.94; H 4.24; N 3.68 %, found: C 59.87; H 4.22; N 3.62%.

*5.1.1.2 3, 5-bis (3-Chlorobenzylidene)-4-piperidone hydrochloride (**2b**).* Yield 74%; mp 250 ^{o}C; ^{1}H NMR (DMSO-d_6): δ 9.78 (s, 2H, +NH_2), 7.85 (s, 2H, 2×=CH), 7.65 (s, 2H, Ar-H), 7.56 (m, 4H, Ar-H), 7.49 (d, J=7.89Hz, 2H, Ar-H), 4.48 (s, 4H, 2×NCH_2). Anal.calcd for $C_{19}H_{16}Cl_3NO$: C 59.94; H 4.24; N 3.68 %, found: C 59.78; H 4.00; N 3.61%.

*5.1.1.3. 3,5-bis (3-Hydroxybenzylidene)-4-piperidone hydrochloride (**2d**).* Yield 61%; mp 300 ^{o}C; ^{1}H NMR (DMSO-d_6): δ 9.89 (s, 2H, +NH_2), 9.76 (s, 2H, 2×OH), 7.77 (s, 2H, 2×=CH), 7.31 (t, 2H, Ar-H), 6.90 (m, 6H, Ar-H), 4.45 (s, 4H, 2×NCH_2). Anal.calcd for $C_{19}H_{18}ClNO_3$: C 66.38; H 5.28; N 4.07 %, found: C 65.85; H 5.16; N 3.98%.

*5.1.1.4. 3, 5-bis (3,4-Dihydroxybenzylidene)-4-piperidone hydrochloride (**2e**).* Yield 66%; mp 254 ^{o}C; ^{1}H NMR (DMSO-d_6): δ 9.82 (brs, 2H, 2×OH), 9.58 (brs, 2H, 2×OH), 9.42 (s, 2H, +NH_2), 7.67 (s, 2H, 2×=CH), 6.92 (s, 2H, Ar-H), 6.86 (q, 4H, Ar-H), 4.41 (s, 4H, 2×NCH_2). Anal.calcd for $C_{19}H_{18}ClNO_5$.0.5 H_2O: C 59.30; H 4.97; N 3.73 %, found: C 58.20; H 5.02; N 3.46%.

*5.1.1.5. 3, 5-bis (4-Hydroxybenzylidene)-4-piperidone hydrochloride (**2f**).* Yield 63%; mp >300 ^{o}C; ^{1}H NMR (DMSO-d_6): δ 10.31(s, 2H, 2×OH), 9.66 (s, 2H, +NH_2), 7.77(s, 2H, 2×=CH), 7.39 (d, J=8.45Hz, 4H, Ar-H), 6.92 (s, 4H, J=8.45Hz, Ar-H), 4.44 (s, 4H, 2×NCH_2). Anal.calcd for $C_{19}H_{18}ClNO_3$.0.25H_2O: C 65.46; H 5.16; N 4.02 %, found: C 65.24; H 5.58; N 4.14%.

*5.1.1.6. 3,5-bis (4-Bromobenzylidene)-4-piperidone hydrochloride (**2g**).* Yield 74%; mp 280 ^{o}C; ^{1}H NMR (DMSO-d_6): δ 9.75 (s, 2H, +NH_2), 7.90 (s, 2H, 2×=CH), 7.84(s, 4H, Ar-H), 7.57 (s, 4H, Ar-H), 4.45 (s, 4H, 2×NCH_2). Anal.calcd for $C_{19}H_{16}Br_2ClNO$: C 48.60; H 3.43; N 2.98 %, found: C 48.27; H 3.18; N 2.92%.

*5.1.1.7. 3,5-bis(4-Trifluoromethylbenzylidene)-4-piperidone hydrochloride (**2h**).* Yield 67%; mp 244 ^{o}C; ^{1}H NMR (DMSO-d_6): δ 9.92 (s, 2H,+NH_2), 7.95 (s, 2H, 2×=CH), 7.89 (d, J=8.20Hz, 4H, Ar-H), 7.75 (d, J=8.10Hz, 4H, Ar-H), 4.49 (s, 4H, 2×NCH_2). Anal.calcd for $C_{21}H_{16}ClF_6NO$: C 56.33; H 3.60; N 3.13 %, found: C 56.03; H 3.45; N 3.06%.

*5.1.1.8. 3,5-bis(4-Carboxybenzylidene)-4-piperidone hydrochloride (**2i**).* Yield 74%; mp >300 ^{o}C; ^{1}H NMR (DMSO-d_6): δ 13.20 (brs, 2H, 2×COOH), 9.76 (brs, 2H, +NH_2), 8.05 (d, J= 7.95Hz, 4H, Ar-H), 7.92 (s, 2H, 2×=CH), 7.65 (d, J=7.90Hz, 4H, Ar-H), 4.50 (s, 4H, 2×NCH_2). Anal.calcd for $C_{21}H_{18}ClNO_5$: C 63.08; H 4.54; N 3.50 %, found: C 63.32; H 4.68; N 3.49%.

*5.1.1.9. 3,5-bis [4-(Ethoxycarbonyl)benzylidene]-4-piperidone hydrochloride (**2j**).* Yield 62%; mp >300 ^{o}C; ^{1}H NMR (DMSO-d_6): δ 9.78 (brs, 2H, +NH_2), 7.93 (s, 2H, 2×=CH), 8.07 (d, J=7.85Hz, 4H, Ar-H), 7.67 (d, J=7.85Hz, 4H, Ar-H), 4.50 (s, 4H, 2×NCH_2), 4.34 (q, 4H, 2×OCH_2), 1.34 (t, 6H, 2×CH_3). Anal.calcd for $C_{25}H_{26}ClNO_5$: C 65.86; H 5.75; N 3.07 %, found: C 65.50; H 5.72; N 2.53%.

*5.1.2. Synthesis of 3,5-bis (3-aminobenzylidene)-4-piperidone (**2c**).* Stannous chloride (0.03 mol, 5.7 g) was added to a suspension of 3,5-bis (3-nitrobenzylidene)-4-piperidone [20] (0.05 mol, 1.82 g) and aqueous potassium carbonate in ethyl acetate (10%, 25ml). The mixture was stored at room temperature for 12 h. The precipitate was collected by filtration, washed with ethyl acetate (2×5 ml) and suspended in water (15 ml). The solid was collected by filtration, dried and recrystallized from chloroform-methanol. Yield 43%; mp >300 ^{o}C; ^{1}H NMR (DMSO-d_6): δ 7.56(s, 2H, 2×=CH), 7.13 (t, 2H, Ar-H), 6.63(m, 6H, Ar-H), 5.30 (s, 4H, 2×NH_2), 4.19 (s, 4H, 2×NCH_2). Anal.calcd for $C_{19}H_{19}N_3O$. $3H_2O$: C 63.44; H 5.28; N 11.68 %, found: C 63.06; H 5.30; N 11.49%.

*5.1.5. Synthesis of quaternary salts **2k** and **2l**.*

To a solution of 3,5-bis(4-dimethylaminobenzylidene)-4-piperidone [6] (0.005 mol, 1.9 g) in chloroform (30 ml) was added methyl iodide (0.0125 mol, 1.77 gm) and stirred at room temperature overnight. The volume of chloroform was reduced to ~15 ml under reduced pressure at room temperature and left for crystallization. The solid crystallized out from the solvent was

filtered off after 48hrs. The solid was washed with chloroform cooled at ~2 ^{0}C and dried. The solid was characterized as the mono quaternized adduct **2l** and the mother liquor contained a mixture of mono and the corresponding bis adduct **2k** as established by ^{1}N NMR. The mother liquor obtained after filtering the mono adduct was added in excess quantities of methyl iodide (0.01mol, 1.5 gm) and heated to ~ 40 ^{0}C for 12 hrs using a water condenser circulated with cold water (~ 5 ^{0}C). The solvent was removed under reduced pressure at room temperature and the solid obtained was crystallized from ethanol to produce the analytically pure bis adduct **2k**.

*5.1.5.2. 3,5-bis(4-(N,N,N-Trimethylammonium)iodide)benzylidene)-4-piperidone (**2k**)*. Yield 32%, mp >300 ^{0}C. ^{1}H NMR (DMSO-d_6): δ 7.91 (s, 2H, 2×=CH), 7.39 (d, J=8.75Hz, 2H, Ar-H), 6.81 (d, J=8.75Hz, 2H, Ar-H), 4.89 (s, 4H, 2×NCH_2), 3.21 (s, 6H, 2×$^+NCH_3$), 3.03 (s, 12H, 4×$^+NCH_3$). Anal.calcd for $C_{25}H_{33}I_2N_3O.H_2O$: C 45.22; H 5.27; N 6.33 %, found: C 45.29; H 4.94; N 6.20%.

*5.1.5.1. 3-(4-(N,N,N-Trimethylammonium)iodide)benzylidene)-5-(4-dimethylaminobenzylidene)-4-piperidone (**2l**).* Yield 42%, mp >300 ^{0}C. ^{1}H NMR (DMSO-d_6): δ 7.91 (s, 2H, 2×=CH), 7.39 (d, J=8.55Hz, 4H, Ar-H), 6.81 (d, J=8.70Hz, 4H, Ar-H), 4.89 (s, 4H, 2×NCH_2), 3.24 (s, 6H, 2×NCH_3), 3.03 (s, 9H, 3×NCH_3). Anal.calcd for $C_{24}H_{30}IN_3O$: C 57.26; H 6.01; N 8.35 %, found: C 57.27; H 6.18; N 7.98%.

*5.1.3. Synthesis of 3-(4-((dimethylphosphoryl)oxy)benzylidene)-5-(4-((methoxy(hydroxyl)-phosphoryl)oxy)benzylidene)4-piperidone hydrochloride (**2m**)*

Step I: Synthesis of 4-((dimethoxyphosphoryl)oxy)benzaldehyde

A mixture of 4-hydroxybenzaldehyde (0.05 mol, 6.1 gm), dimethylchlorophosphate (0.065 mol, 9.4 gm), anhydrous potassium carbonate (0.05 mol, 6.9 gm) and a catalytic amount of potassium iodide (20 mg) in acetone was refluxed for 4 hrs. The mixture was filtered to remove inorganic salts, washed with acetone and the filtrate was evaporated under vacuum at room temperature. The crude product was dissolved in ethyl acetate, washed with 5% potassium carbonate, dried over sodium sulphate and evaporated to yield a thick liquid, which was characterized by ^{1}H NMR. This product was taken for reaction in step II without further purification. ^{1}H NMR

($CDCl_3$): δ 9.98 (s, 1H, CHO), 7.91 (d, J=8.45Hz, 2H, Ar-H), 7.40 (d, J=8.40Hz, 2H, Ar-H), 3.92 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3).

Step II: A mixture of 4-piperidone hydrochloride (0.0065 mol, 1gm) and 4-((dimethoxyphosphoryl)oxy)benzaldehyde (0.013 mol, 3gm) in acetic acid (20 ml) was saturated with anhydrous hydrogen chloride and stirred at room temperature for 12 hrs. The solvent was removed under reduced pressure at 50-55 ^{0}C and the crude thick mass obtained was triturated with acetone. The solid obtained was filtered and washed with acetone. The crude product **2m** was purified by refluxing in methanol, the solid filtered, washed with methanol (2×5 ml) and dried. Yield 54%; mp 216 ^{o}C (dec); ^{1}H NMR (DMSO-d_6): δ 9.85 (brs, 2H, $+NH_2$), 7.85 (d, J=9.8Hz, 2H, 2×=CH), 7.60 (d, J=7.95Hz, 2H, Ar-H), 7.48 (d, J=7.75Hz, 2H, Ar-H), 7.37 (d, J=8.10Hz, 2H, Ar-H), 7.27 (d, J=8.05Hz, 2H, Ar-H), 4.47 (s, 4H, 2×NCH_2), 3.83 (d, J=11.25Hz, 6H, 2×OCH_3), 3.56 (d, J=10.90Hz, 3H, OCH_3). Anal.calcd for $C_{22}H_{26}ClNO_9P_2$: C 48.41; H 4.80; N 2.57 %, found: C 48.76; H 5.04; N 2.65%.

*5.1.4. Synthesis of 3-(4-((diethylphosphoryl)oxy)benzylidene)-5-(4-((ethoxy(hydroxyl)-phosphoryl)oxy)benzylidene)4-piperidone hydrochloride (**2n**)*

Step I: Synthesis of 4-((diethoxyphosphoryl)oxy)benzaldehyde

It was prepared following the same process as described above by using 4-hydroxybenzaldehyde (0.05 mol, 6.1 gm), diethylchlorophosphate (0.06 mol, 10.35 gm) and anhydrous potassium carbonate (0.05 mol, 6.9 gm) and taken for reaction in the second step without further purification. Yiled: 73%. ^{1}H NMR ($CDCl_3$): δ 9.86 (s, 1H, CHO), 7.85 (d, J=8.65Hz, 2H, Ar-H), 7.51 (d, J=8.62Hz, 2H, Ar-H), 4.17 (p, 4H, 2×OCH_2), 1.27 (t, 6H, 2×CH_3).

Step II: It was prepared following same process as described above for **2m** by using 4-piperidone hydrochloride (0.004 mol, 0.62 gm) and 4-((ethoxyphosphoryl)oxy)benzaldehyde (0.008 mol, 2.1gm). Crude yield: 65%. The crude product **2n** was purified by column chromatography by using the eluent chloroform/methanol (82:18). Yield 48%; mp 210 ^{o}C (dec); ^{1}HNMR (DMSO-d_6): δ 7.78(d, J=11.72Hz, 2H, 2×=CH), 7.58 (d, J=8.40Hz, 2H, Ar-H), 7.39(d, J=8.3Hz, 2H, Ar-H), 7.34 (d, J=8.22Hz, 2H, Ar-H), 7.21 (d, J=8.32Hz, 2H, Ar-H), 4.38 (s, 4H,

2×NCH_2), 4.19 (p, 4H, 2×OCH_2), 3.76 (p, 2H, OCH_2), 1.29 (t, 6H, 2×CH_3), 1.08 (t, 3H, CH_3). Anal.calcd for $C_{25}H_{32}ClNO_9P_2.5H_2O$: C 44.25; H 4.72; N 2.06 %, found: C 44.41; H 4.86; N 1.8%.

5.2. Bioevaluations

5.2.1. Cytotoxicity Assays.

The Molt 4/C8, CEM and L1210 screens were undertaken using a literature procedure [21]. The evaluation of **1a, 2a-d, h, j-l** towards a panel of 55±4 human tumor cell lines utilized a reported methodology [15]. The concentrations of compounds were 10^{-4} to 10^{-8} and the number of IC50 values obtained/number of cell lines employed are as follows, namely **1a**: 59/59; **2a**: 53/54; **2b:** 52/53 ; **2c** : 55/55 ; **2d** : 58/58 ; **2h**: 57/58; **2j** : 52/53 ; **2k** : 51/55 ; **2l** : 52/56. The small number of IC_{50} figures outside of the range of 10^{-4} to 10^{-8} M were in excess of 10^{-4} M in the case of **2b**, **k, l** and lower than 10^{-8} for **2a, h, j**. The range of concentration employed for melphalan was $10^{-3.6}$ to $10^{-7.6}$ and IC_{50} values were found for all 57 cell lines. For 5-fluorouracil, 58 cell lines were assayed using concentrations of $10^{-2.6}$ to $10^{-6.6}$ M. The IC_{50} figures for two cell lines were in excess of $10^{-6.6}$ M while in four of the assays, the IC_{50} values were lower than $10^{-6.6}$ M.

5.2.2. Cell Cycle Analysis

The effect of **2a** on the cell cycle of HT29 cells was undertaken by a procedure which has been described in detail recently [22]. In brief, HT29 cells were incubated for 24h in a humidified incubator at 37 °C with 5 % carbon dioxide. The compound was added to the cells and incubated at 37 °C in a humidified incubator with 5 % carbon dioxide for 48 h along with untreated cells. After the addition of propidium iodide (20 μg/ml) and RNase (300 μg/ml) for 20 minutes, the samples were analysed by a FACScaliber (BD) flow cytometer. Cells were gated to include the subG1, G1, S and G2/M population of cells.

5.2.3. Toxicity evaluations.

Compounds **2a-k, n** were administered to mice by intraperitoneal route. This experimentation was undertaken by the National Institute of Neurological Disorders and Stroke according to their protocols [23]. Neurotoxicity was noted after administration of the following compounds to mice (number of animals displaying neurotoxicity/total number of animals evaluated, dose in mg/kg,

time of observation in h) viz, **2c** (1/4,300, 0.5; 1/2, 300, 4), **2i** (1/2, 300, 4), **2j** (1/4, 300, 0.5) and **2k** (1/4, 300, 0.5). The animals were housed, handled and fed in accordance with the procedures published in the document of the National Research Council entitled "Guide for the Care and Use of Laboratory Animals". Euthanasia of the rodents was undertaken using the procedures of the Institute of Laboratory Resources.

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CHAPTER 3

Chapter 3 consists of copies of two articles: one was published in *ChemMedChem* in 2009.*† and the other one in *Bioorganic and Medicinal Chemistry Letters* in 2010.**#

Relationship of Chapter 3 to the objectives of this project

The objectives of this study were to develop cytotoxic water soluble prodrugs of 3,5-bis(arylidene)-4-piperidone derivatives by introducing phosphoryl groups onto the piperidone nitrogen atom and to examine the theory of sequential cytotoxicity using these series of compounds in order to explore the hypothesis that (i) the prodrugs display greater cytotoxic potencies over their precursor enones; (ii) sequential thiol alkylation will be more detrimental to tumours than non-malignant cells and also to evaluate their potential as multidrug resistance modulators.

Description

A series of ethyl esters of 3,5-bis(arylidene)-4-piperidone-N-phosphonates and the N-phosphonic acid derivatives were synthesized from their corresponding 3,5-bis(arylidene)-4-piperidone precursors for evaluation of their cytotoxic and multidrug resistance reversal properties. The stereochemistry of one of the representative compounds was examined by X-ray crystallography. All compounds were evaluated against human Molt 4/C8 and CEM T-lymphocytes as well as murine leukemic L1210 cells. Some of these compounds were assessed against a panel of approximately 55 human tumor cell lines by the NCI. The influence of aryl substituents possessing diverse electronic, hydrophobic and steric properties on the cytotoxic potencies of the compounds was also assessed. To evaluate the theory of sequential cytotoxicity, the compounds were screened against a number of malignant HSC-2, HSC-3, HSC-4 and HL-60 cell lines and compared against the non-malignant HGF, HPC and HPLF cell lines. The modes of action of representative compounds by which cytotoxicity is mediated were investigated.

Author Contributions

My contributions to the *ChemMedChem* publication were: (i) undertaking a literature review of the prepared compounds, designing the synthetic chemical routes, synthesizing the compounds, determining and interpreting ^{1}H and ^{13}C NMR spectra of the compounds in series **3-5**, (ii) the mitochondrial studies, (iii) writing of experimental section of the manuscript, and (iv) elemental analysis was done by me using the CHNS analyzer. The coauthors on this paper are U.

Das, who supervised the synthesis of the compounds and undertook the QSAR and some molecular modeling studies, P. Selvakumar undertook the cell cycle analysis of **5g** under the supervision of R.K. Sharma, E. De Clercq and J. Balzarini supervized the cytotoxic assays using human Molt 4/C8 and CEM T-lymphocytes and murine leukemic L1210 cells, J. Serly and Z. Barath conducted the multidrug resistance reversal assay under the supervision of J. Molnar, B. Bandy guided the mitochondrial work, G. Schatte elucidated the X-ray crystal structure of **4g**, and the project was guided and supervised by J. R. Dimmock and D. K. J. Gorecki. The manuscript was written by J. R. Dimmock with some inputs from me.

My contribution to the publication in *Bioorg. Med. Chem. Lett.* was the undertaking of the literature review of the prepared compounds, designing the synthetic chemical routes, synthesizing the compounds, determining and interpreting the ^{1}H and ^{13}C NMR spectra of the compounds in series **3-5**. The coauthors on this paper are U. Das, who supervised the synthesis of the compounds and undertook the QSAR and molecular modeling studies, K. Hashimoto undertook the cytotoxic studies against some malignant and non-malignant cell lines and performed the caspase activation and DNA fragment studies on **1c** under the supervision of H. Sakagami and M. Kawase, and the project was guided and supervised by J. R. Dimmock and D. K. J. Gorecki. The manuscript was written by J. R. Dimmock with some suggestions from the coauthors.

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† Das et al. *ChemMedChem* **2009**, 4, 1831-1840.

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3,5-Bis(benzylidene)-4-oxo-1-phosphonopiperidines and Related Diethyl Esters: Potent Cytotoxins with Multi-Drug-Resistance Reverting Properties

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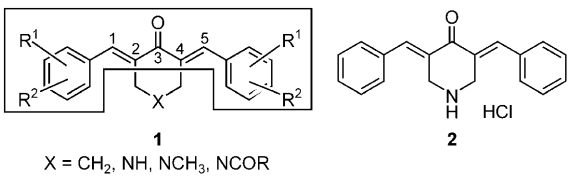
A series of 3,5-bis(benzylidene)-4-piperidones **3** were converted into the corresponding 3,5-bis(benzylidene)-1-phosphono-4-piperidones **5** via diethyl esters **4**. The analogues in series **4** and **5** displayed marked growth inhibitory properties toward human Molt 4/C8 and CEM T-lymphocytes as well as murine leukemia L1210 cells. In general, the *N*-phosphono compounds **5**, which are more hydrophilic than the analogues in series **3** and **4**, were the most potent cluster of cytotoxins, and, in particular, 3,5-bis-(2-nitrobenzylidene)-1-phosphono-4-piperidone **5g** had an average IC_{50} value of 34 nM toward the two T-lymphocyte cell lines. Four of the compounds displayed potent cytotoxicity toward a panel of nearly 60 human tumor cell lines, and nanomolar IC_{50} values were observed in a number of cases. The mode of action of **5g** includes the induction of apoptosis and inhibition of cellular respiration. Most of the members of series **4** as well as several analogues in series **5** are potent multi-drug resistance (MDR) reverting compounds. Various correlations were noted between certain molecular features of series **4** and **5** and cytotoxic properties, affording some guidelines in expanding this study.

Introduction

A major interest in our research groups is the design of antineoplastic agents that contain the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore. A number of reasons for the inclusion of this group into the structures of candidate cytotoxins have been collated recently.[1] Two important considerations are as follows: First, conjugated unsaturated ketones are thiol alkylators with little or no capacity to interact with amino or hydroxy groups, which are found in nucleic acids.[2] Thus these molecules should be free of the mutagenic effects elicited by certain alkylating agents used in cancer chemotherapy.[3] Second, the concept of sequential cytotoxicity states that successive alkylations of cellular constituents may be more detrimental to malignant cells than to the corresponding normal tissues.[4] This theory is based on the observation that an initial chemical insult caused by a bifunctional alkylator, for example, may be greater in neoplasms than in the corresponding normal cells.[5,6] Thus selective toxicity to tumors may result when the 1,5-diaryl-3-oxo-1,4-pentadienyl group is present in candidate cytotoxins, as illustrated in Figure 1.



1 2

X = CH_2, NH, NCH_3, NCOR

Figure 1. Structures of series **1** (the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore is boxed) and compound **2**.

The excellent cytotoxic properties of various groups of compounds possessing the general structure **1** have been reported.[1] In particular, when X is a secondary amino group in series **1**, in a number of cases the IC_{50} values toward various transformed and malignant cells are in the low micromolar and sub-micromolar range.[7,8] For example, the free base of **2** has an IC_{50} value of 7.96 μM toward murine L1210 leukemic cells.[7] However, when assessment of **2** was made using this cell line passaged in mice, there was no increase in the life span of the animals.[8] A possible reason for this observation is the lipophilicity of 3,5-bis(benzylidene)-4-piperidones; for example, the

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log *P* value for the free base of **2** is 3.38.[9] Hence, the conversion of these amines into the corresponding 1-phosphono derivatives was considered, as the π value of the phosphono groups is −1.59.[10] We therefore decided to embark on a synthetic strategy leading to the compounds of series **3**–**5** as indicated in Scheme 1 in order to explore the hypothesis that cytotoxic potencies are greater in **5a**–**i** than in the precursor enones **3a**–**i**.

The choice of aryl substituents was made on the basis of the considerable differences in their electronic, hydrophobic, and steric characteristics. One or more of these properties may correlate with cytotoxic potencies. The aryl groups in series **3**–**5** are identical and, hence, if the compounds bearing the same substituents in the aryl rings have identical IC_{50} values, their potencies could be ascribed to the 1,5-diaryl-3-oxo-1,4-pentadienyl group. On the other hand, variations in potencies, for example between **3c**, **4c**, and **5c**, would point to a contribution to the magnitude of the bioactivity by the substituent on the piperidyl nitrogen atom. In addition, a comparison of the IC_{50} values of the 4-piperidones in series **4** and **5** may give some indication of whether masking of the acidic groups present in the 1-phosphono analogues is beneficial in terms of cytotoxic potencies.

A previous study revealed that while a small series of 3,5-bis(benzylidene)-4-piperidones with an average log *P* value of 3.90 had little or no capacity to reverse P-glycoprotein-associated multi-drug resistance (MDR), conversion into the corresponding amides with an average log *P* value of 5.52 led to clusters of potent MDR reverting agents.[9] Hence the decision was made to examine whether the phosphoramidates **4** and **5** possess this important biological property and whether lipophilicity affects the potencies of these compounds.

In summary, the objectives of the present study included examining the compounds in series **3**–**5** for cytotoxic properties as well as **4a**–**i** and **5a**–**i** as candidate MDR reverting agents. In addition, experiments were designed to find some of the reasons for any variation in potencies observed in the different biological evaluations.

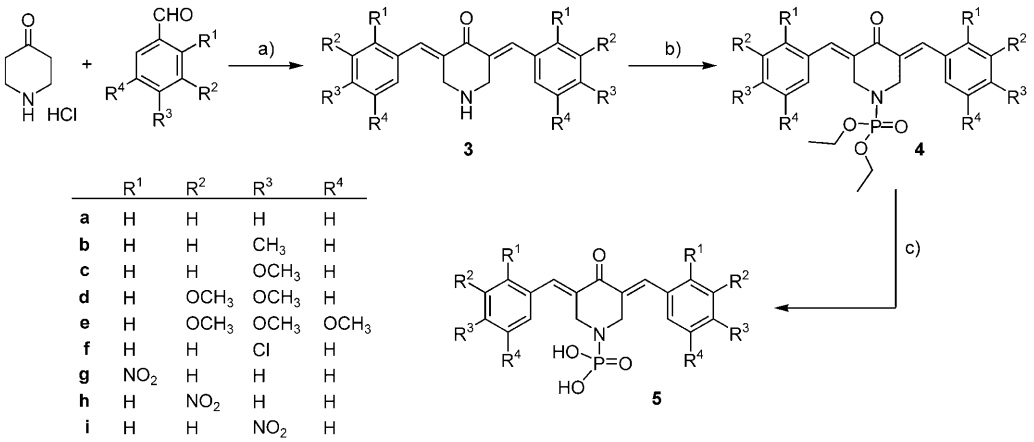
Results

The synthetic route for the preparation of the 4-piperidones **3**–**5** is presented in Scheme 1. The compounds in series **3** were prepared by acid-catalyzed condensation between a variety of aryl aldehydes and 4-piperidone. Reaction of **3a**–**i** with diethyl chlorophosphonate led to the formation of the corresponding amides **4**, which were hydrolyzed with trimethylsilyl bromide to yield the phosphonic acids **5a**–**i**. The Clog *P* values of the 4-piperidones in series **4** and **5** were computed and are listed in Table 1. The X-ray crystallographic structure of **4g** is presented in Figure 3 below.

All of the compounds in series **4** and **5** were evaluated against human Molt 4/C8 and CEM T-lymphocytes as well as murine leukemia L1210 cells. These data are presented in Table 1. The biological data from these three assays were reported previously for **3a**,**c**,**f**,**i**[7] and also for **3b**.[11] Thus **3d**,**e**,**g**,**h** were prepared, and their growth inhibiting properties from the Molt 4/C8, CEM, and L1210 assays are listed in Table 1. The 4-piperidones **4a**,**c**,**d** and **5c** were examined by NCI against a panel of 58–59 human tumor cell lines, and these results are presented in Table 5 below. Two mode-of-action studies used human colon cancer HT29 cells: First, the effect of **5g** on these cells was examined by flow cytometry, and the results are shown in Figure 4. Second, evaluations of **5d** and **5g** on respiration in HT29 cells were undertaken, and the effects are illustrated in Figure 5. All of the compounds in series **4** and **5** were examined as candidate MDR reverting agents and the results are presented in Table 1.

Discussion

X-ray crystallography of a number of compounds having the general structure **3**[7,8,12] and related *N*-acyl derivatives[7,13,14] revealed that the olefinic double bonds adopt the *E* configuration. In addition, a representative compound prepared in this study, namely **4g**, is the *E*,*E* geometrical isomer as revealed by X-ray crystallography. Hence, the compounds in series **3**–**5** are considered to be the *E*,*E* isomers.



	R^1	R^2	R^3	R^4
a	H	H	H	H
b	H	H	CH_3	H
c	H	H	OCH_3	H
d	H	OCH_3	OCH_3	H
e	H	OCH_3	OCH_3	OCH_3
f	H	H	Cl	H
g	NO_2	H	H	H
h	H	NO_2	H	H
i	H	H	NO_2	H

Scheme 1. Synthesis of series **3**–**5**: a) HCl, CH_3COOH; b) $(C_2H_5O)_2P(O)Cl$, K_2CO_3, KI; c) $(CH_3)_3SiBr$.

All of the compounds in series **4** and **5** were evaluated against human Molt 4/C8 and CEM T-lymphocytes in order to determine whether cytotoxic properties would be exhibited toward human transformed cells. A number of anticancer drugs display growth inhibiting properties in the L1210 bioassay,[15] and this assay was also used to detect promising lead compounds. These data, along with the results of evaluating **3d**,**e**,**g**,**h**, which have not been assessed previously against these cell lines, are presented in Table 1.

The biological evaluations reveal that both series **4** and **5** demonstrate potent cytotoxicity toward human T-lymphocytes. No fewer than 94% of the IC_{50} values of **4a–i** and **5a–i** in the Molt 4/C8 and CEM screens are $<10\ \mu M$, and 61% of these are in the sub-micromolar range. In particular, the high potency of **5g**, with IC_{50} values of 34 ± 2 nM toward both T-lymphocyte lines should be noted. This establishes this compound as a lead molecule. The marked potencies of these compounds toward Molt 4/C8 and CEM cells is confirmed when comparisons are made between these biological data and the results for melphalan, which is an alkylating agent used in cancer chemotherapy. In series **4**, the 4-piperidones **4a**,**d–i** are more potent than melphalan in both assays, that is, in 78% of the comparisons made. Furthermore, **5a**,**b**,**d–i** and **5b–i** have statistically significantly lower IC_{50} values than melphalan in the Molt 4/C8 and CEM screens, respectively, that is, in 89% of the data for series **5**. In particular, **5g** has 90-fold greater potency than this reference drug toward Molt 4/C8 cells, and is 76-fold more potent than melphalan in the CEM test. While the murine L1210 cells are more refractory to the 4-piperidones in series **4** and **5**, 78% of the IC_{50} values are $<10\ \mu M$, and both **5d** and **5e** possess sub-micromolar IC_{50} values.

The next part of the biological data analysis involved comparison of the potencies of the compounds in series **3**–**5**. The approach involved dividing the IC_{50} value of a compound in series **3** by that of the analogue in series **4** or **5** having the same aryl substituents. This procedure gave rise to a number of $\Delta_{3/4}$ and $\Delta_{3/5}$ values in of each of the Molt 4/C8, CEM, and L1210 screens which are presented in Tables 2 and 3.

The results in Table 2 indicate that **4c**,**f**,**h**,**i** are more potent than **3c**,**f**,**h**,**i** in all three bioassays, that is, in 44% of the comparisons made. Three (11%) of the $\Delta_{3/4}$ values (indicated as footnote [b] in Table 2) denote equal potency. In the remaining cases (45%), higher potency was observed for the analogues in series **3**. Thus overall there was neither an increase nor decrease in potencies, although in some cases such as **4c** and **4h**, the IC_{50} values were considerably lower than for **3c** and **3h**, respectively. In addition, the fact that the compounds in series **4** are potent cytotoxins suggests that analogue development should be pursued vigorously, such as the preparation of a variety of related esters.

Table 1. Cytotoxicity of series **3**, **4**, and **5** compounds and Clog*P* values and fluorescence activity ratio (FAR) data for **4a–i** and **5a–i**.

Compd	IC_{50} [μM][a] Molt 4/C8	CEM	L1210	Clog *P*[b]	FAR[c]
3d	0.25±0.13	0.37±0.05	1.33±0.37	–	–
3e[d]	0.31±0.02	0.30±0.02	0.53±0.34	–	–
3g[d]	0.37±0.03	0.36±0.11	1.17±0.37	–	–
3h	1.64±0.81	4.90±2.33	33.0±2.20	–	–
4a	1.36±0.20	1.52±0.19	8.46±0.18	4.01±0.43 (0.72)	51.6
4b	12.60±1.3	24.0±3.00	49.40±4.9	4.86±0.48 (0.76)	17.1
4c	2.90±1.07	5.82±0.07	38.5±3.9	3.95±0.58 (0.70)	49.6
4d	0.27±0.03	0.85±0.01	2.00±0.20	3.52±0.75 (0.69)	57.9
4e	0.45±0.02	0.51±0.05	3.00±1.01	3.38±1.09 (0.76)	52.9
4f	0.83±0.10	0.99±0.11	5.20±0.00	5.11±0.34 (0.65)	23.6
4g	1.58±0.08	1.91±0.01	7.12±0.54	3.75±0.40 (0.70)	1.86
4h	0.19±0.01	0.16±0.01	1.49±0.11	3.81±0.42 (0.68)	17.5
4i	1.02±0.06	1.30±0.06	8.63±0.04	3.76±0.32 (0.69)	11.2
5a	0.46±0.41	1.88±0.34	6.20±1.49	2.21±0.92 (−1.08)	3.66
5b	0.45±0.09	0.99±0.03	9.89±0.07	2.95±0.99 (−1.15)	13.3
5c	5.22±3.24	2.00±0.09	47.10±3.9	2.08±0.95 (−1.17)	2.53
5d	0.16±0.03	0.19±0.02	0.73±0.31	1.83±0.94 (−1.00)	60.5
5e	0.11±0.07	0.25±0.03	0.16±0.11	1.48±1.18 (−1.14)	43.7
5f	0.47±0.11	1.25±0.35	10.3±1.80	3.28±0.87 (−1.18)	1.51
5g	0.03±0.03	0.03±0.03	1.41±0.28	1.90±0.87 (−1.15)	2.86
5h	0.36±0.06	0.41±0.08	2.01±0.66	1.97±0.90 (−1.16)	1.45
5i	0.13±0.08	0.91±0.04	6.92±0.25	1.93±0.89 (−1.14)	0.99
melphalan[e]	3.24±0.79	2.47±0.30	2.13±0.03	–	–

[a] Concentration required to inhibit cell growth by 50%. [b] Values in parentheses are the differences in Clog*P* values from the respective analogue in series **3** that possesses the same aryl substituent; a negative value indicates lower hydrophobicity for the molecule than the series **3** analogue. [c] FAR values are the ratios of the fluorescence intensities of rhodamine 123 in treated versus untreated murine L-5178Y cells transfected with the human *mdr1* gene; compound concentration is 20 μM; the reference compound verapamil has a FAR value of 8.23 when 22 μM of this drug is used. [d] Evaluated as the HCl salt. [e] These data were reported previously in reference [36].

The $\Delta_{3/5}$ values are listed in Table 3. In 78% of the comparisons, **5a–i** are more potent than the analogues in series **3**, whereas in 19% of the cases equal potency was observed. The only case in which greater potency is displayed in series **3** is the IC_{50} value of **3b**, which is lower than that of **5b** in the L1210 screen. The $\Delta_{4/5}$ values were also computed and are listed in Table 3. In 70% of the comparisons, the analogues in series **5** have lower IC_{50} values than **4a–i**, whereas in 15% of the cases equal potency was noted. Thus, not only are series **4** compounds a group of promising cytotoxins, but hydrolysis of the ester groups of **4a–i** led to a highly potent cluster of cytotoxic molecules, namely series **5**.

To guide future expansion from these initial groups of compounds in series **4** and **5**, different approaches were adopted, including QSAR studies and molecular modeling. The magnitudes of the electronic, hydrophobic, and steric proper-

Table 2. Comparison of the potencies of **4a–i** with respective analogues in series **3** that have the same aryl substituents.

Compd	Ar Group	$\Delta_{3/4}$[a] Molt 4/C8	CEM	L1210
4a	H	1.2[b]	1.1[b]	0.9
4b	4-CH_3	0.1	0.1	0.2
4c	4-OCH_3	99	28	6.3
4d	3,4-$(OCH_3)_2$	0.9[b]	0.4	0.7
4e	3,4,5-$(OCH_3)_3$	0.7	0.6	0.2
4f	4-Cl	16	8.7	8.0
4g	2-NO_2	0.2	0.2	0.2
4h	3-NO_2	8.8	30	23
4i	4-NO_2	8.1	3.4	3.8

[a] The designation $\Delta_{3/4}$ refers to the quotient of the IC_{50} value of a compound in series **3** divided by that of the analogue in series **4** which bears the same aryl substituent. [b] No statistical difference in the IC_{50} values when standard deviations are taken into account.

Table 3. Comparison of the potencies of **5a–i** with compounds in series **3** and **4** that have the same substituents on the aryl rings.

Compd	Ar Group	Molt 4/C8 $\Delta_{3/5}$[a]	Molt 4/C8 $\Delta_{4/5}$[a]	CEM $\Delta_{3/5}$[a]	CEM $\Delta_{4/5}$[a]	L1210 $\Delta_{3/5}$[a]	L1210 $\Delta_{4/5}$[a]
5a	H	3.6	2.9	0.9[b]	0.8[b]	1.3	1.4
5b	4-CH_3	3.8	28	1.7	24	0.9	5.0
5c	4-OCH_3	55	0.6[b]	82	2.9	5.2	0.8
5d	3,4-$(OCH_3)_2$	1.6[b]	1.7	2.0	4.5	1.8[b]	2.7
5e	3,4,5-$(OCH_3)_3$	2.7	4.0	1.2	2.1	3.3[b]	19
5f	4-Cl	28	1.8	6.9	0.8[b]	4.0	0.5
5g	2-NO_2	10	44	11	59	0.8[b]	5.1
5h	3-NO_2	4.6	0.5	12	0.4	16	0.7[b]
5i	4-NO_2	62	7.7	4.9	1.4	4.8	1.3

[a] The $\Delta_{3/5}$ and $\Delta_{4/5}$ values are the quotients of the IC_{50} values of a compound in series **3** divided by that of either a respective series **5** ($\Delta_{3/5}$) or series **4** ($\Delta_{4/5}$) compound having the same substituents on the aryl rings. [b] No statistical difference in the IC_{50} values when standard deviations are taken into account.

ties of aryl substituents are indicated by the Hammett σ values (and Taft σ* values for *ortho* substituents), Hansch π constants and molecular refractivity (MR) values, respectively. Linear and semilogarithmic plots were made between these constants and the IC_{50} values of **4a–i** and **5a–i** in the Molt 4/C8, CEM, and L1210 screens. In addition, logarithmic plots were made between the MR values and the IC_{50} values. The following correlations ($p < 0.05$) or trends to significance ($p < 0.1$) were noted in series **5**. Negative correlation values were observed between the IC_{50} values of **5a–i** and the σ/σ* constants in the Molt 4/C8 screen ($p < 0.1$) as well as the MR values in the CEM assay ($p < 0.05$) and L1210 test ($p < 0.1$). A positive correlation with regard to the π constants in the Molt 4/C8 screen ($p < 0.1$) was also noted. No other correlations were observed in either series **4** or **5** ($p > 0.1$). Thus in developing these compounds, large electron-withdrawing groups of low hydrophobicity should be placed in the aryl rings.

The calculated log*P* values of the esters **4** and phosphonic acids **5** are listed in Table 1. The differences between the hydrophobicity of these compounds and the analogues **3a–i** are also indicated in Table 1. Thus, on average, series **4** is more hydrophobic than **3a–i** by 0.71 log*P* units, while the phosphonic acids **5** are more hydrophilic than **3** by 1.13 log*P* units. It is conceivable that a physicochemical parameter which contributes to the IC_{50} values of series **5** being lower than those of series **3** and **4** is their greater hydrophilic properties. In addition, we investigated whether the cytotoxic potencies of each of the series **3–5** compounds are influenced by the log*P* values. Thus linear, semilogarithmic, and logarithmic plots were constructed between the IC_{50} values and the Clog*P* data. Positive correlations were observed between the IC_{50} values in the L1210 screen and the Clog*P* values of series **3** ($p < 0.1$) and **5** ($p = 0.05$). This observation is in agreement with the recommendation made earlier of decreasing the magnitude of the π values of the aryl substituents. Hence, as a general rule, a variety of hydrophilic groups should be included in the future expansions of the compounds in series **3–5**.

In some cases biological potencies are influenced by the torsion angles (θ) between an aryl ring and the adjacent unsaturated group.[16] Hence the torsion angles θ_1 and θ_2, as indicated in Figure 2, were calculated by molecular modeling, and the

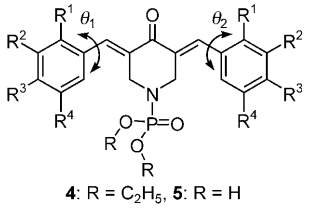


Figure 2. Designation of the torsion angles θ_1 and θ_2 in series **4** and **5**.

data for the compounds in series **3–5** are presented in Table 4. The θ_1 and θ_2 angles were calculated in a clockwise fashion and revealed that rings A and B rotate in opposite directions. In each series, the greatest torsion angles are found in the *ortho*-nitro analogues, namely **3g**, **4g**, and **5g**. Because **5g** is the most potent compound toward both T-lymphocytes among the 4-piperidones examined in this study, the placement of substituents of varying size at one or both of the *ortho* locations of rings A and B may establish whether a correlation is present between the magnitude of the torsion angles and cytotoxic potencies. In general, the torsion angles in series

Table 4. Torsion angles created between the aryl rings and the adjacent olefinic group in compound series **3–5**, **a–i**.

Compd	θ_1 [°] 3	4	5	θ_2 [°] 3	4	5
a	60.03	56.65	58.43	122.10	122.88	124.29
b	56.37	55.87	55.02	125.43	127.09	129.68
c	55.50	54.96	54.11	127.05	126.88	125.46
d	68.39	53.57	54.07	107.15	131.07	127.84
e	62.02	54.03	55.49	122.40	126.18	124.22
f	63.81	55.67	57.44	117.16	123.76	125.35
g	98.87	70.56	78.22	104.74	82.21	81.15
h	56.50	62.33	61.12	128.07	123.31	121.50
i	73.26	58.07	61.21	107.73	126.15	122.40

5 are not substantially different from those found in **3a–i** and **4a–i**, and hence θ values *per se* are unlikely to be the principal reason for the greater cytotoxic potencies of the analogues in series **5**.

Linear, semilogarithmic, and logarithmic plots were constructed between the θ_1 and θ_2 values in series **3**–**5**, and the IC_{50} values in each of the Molt 4/C8, CEM, and L1210 screens. Negative correlations were observed in the plots between the θ_1 values in series **5** and the IC_{50} values obtained in the Molt 4/C8 ($p < 0.1$) and CEM ($p < 0.05$) assays. On the other hand, positive correlations were observed from the plots between the θ_2 values in series **5** and the IC_{50} values toward both Molt 4/C8 ($p < 0.1$) and CEM ($p < 0.05$) cells. No other correlations were found ($p > 0.1$). This observation is important for the development of series **5**, whereby further analogues should ensure that θ_1 values are large and conversely θ_2 values are small. This objective can be achieved by placing large groups in the *ortho* position of ring A, and having ring B either unsubstituted or possessing small groups at the *para* position of the aryl ring.

A representative compound **4g** was examined by X-ray crystallography, and an ORTEP diagram[17] is presented in Figure 3.

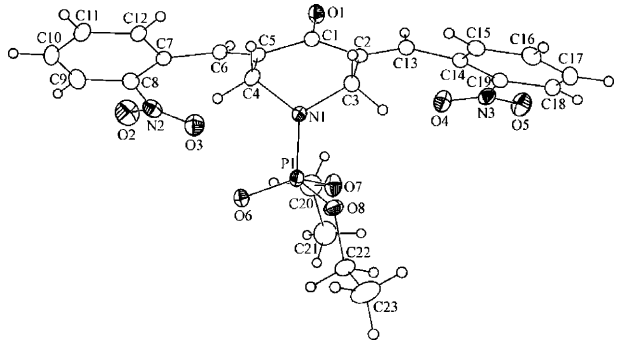


Figure 3. ORTEP diagram of **4g**.

The torsion angles C2–C13–C14–C19 (θ_1) and C5–C6–C7–C8 (θ_2) are 141.7(3) and −145.0(2), respectively. The piperidone ring adopts a half-chair conformation. The two aryl rings are orientated in an almost perpendicular fashion toward the piperidone ring. The nitro groups point away from both the heterocycle and the phosphonate moieties.

The biological data summarized in Table 1 for the potencies of various clusters of compounds containing the 1,5-diaryl-3-oxo-1,4-pentadienyl group toward Molt 4/C8, CEM, and L1210 cells are encouraging. Thus an important question is whether cytotoxicity toward a greater number and variety of neoplasms can be demonstrated. Hence, four compounds, namely **4a**,**c**,**d** and **5c**, were evaluated against 58–59 human tumor cell lines which originated from nine different neoplastic conditions: leukemia, melanoma, non-small-cell lung carcinoma, and colon, CNS, ovarian, renal, prostate, and breast cancers.[18] The results of these evaluations are presented in Table 5. When considering the toxicity toward all cell lines, the term GI_{50} rather than IC_{50} is used, because the average potencies listed include IC_{50} values that are greater than the maximum concentration used. The biological data reveal that **4a**,**c**,**d** and **5c** are potent cytotoxins, especially **4d**, which has a sub-micromolar average GI_{50} value, and is 21-fold more potent than melphalan. A positive feature of a candidate anti-neoplastic agent is that it displays varying toxicity toward different cell lines, which may be reflected in causing greater damage to tumors than the corresponding normal cells. Notably, the very high selectivity index (SI) values displayed by **4c** establish it as a lead molecule. Examination of the mean graphs[19] revealed that in general, colon cancers and leukemic cells are particularly sensitive to these compounds. In the case of the colon cancer cell lines, 64% of the IC_{50} values of **4a**,**c**,**d**, and **5c** are sub-micromolar, and 18% possess double-digit nanomolar values. Where specific data are available, the IC_{50} values of 5-fluorouracil (5-FU), which is a drug used in treating colon cancer, are in general substantially higher than the data obtained for **4a**,**c**,**d** and **5c** against colon cancer cells. In regard to anti-leukemic properties, the data in Table 5 reveal that **4a**,**c**,**d** and **5c** have high potencies: 63% of the IC_{50} figures are sub-micromolar. In particular, the IC_{50} values of <10 nM and 32 nM displayed by **4a** and **4c**, respectively, toward RPMI 8226 cells are impressive. The average IC_{50} values reveal that **4a**,**c**,**d** and **5c** possess 58-, 35-, 93-, and 15-fold greater potency than melphalan, which is used clinically in treating various types of leukemia. The data in Table 5 afford ample evidence to pursue series **4** and **5** as excellent leads for the future development of candidate anti-neoplastic agents.

A further issue to be addressed is the way in which the compounds prepared in this study exert their cytotoxic activity. Ex-

Table 5. Evaluation of **4a**,**c**,**d** and **5c** against a panel of 59 human tumor cell lines

Compd	All cell lines		Colon cancers, IC_{50} [μM]								Leukemic cell lines, IC_{50} [μM]					
	GI_{50} [μM][a]	SI[b]	COLO 205	HCT 116	HCT 15	HT29	KM12	SW 620	HCC 2998	Avg. IC_{50}	HL60 (TB)	K-562	RPMI 8226	SR	CCRF CEM	Avg. IC_{50}
4a	1.20	>381	1.62	0.21	1.26	0.37	0.20	0.23	2.14	0.86	1.12	2.29	<0.01	0.19	0.32	0.79
4c	1.58	>3152	1.41	0.05	0.81	0.04	0.04	0.04	0.07	0.35	4.90	–	0.03	0.06	0.16	1.29
4d	0.93	240	1.55	0.39	1.10	0.42	0.27	0.33	2.09	0.88	0.91	0.49	0.30	0.44	0.29	0.48
5c	3.47	>355	2.04	0.34	2.24	0.72	0.32	0.68	2.00	1.19	3.72	3.63	0.28	5.25	2.19	3.01
melphalan	19.1	513	32.4	39.8	36.3	70.8	57.5	26.9	52.5	45.2	0.38	195	28.2	3.24	0.39	45.4
5-FU	12.0	>10000	14.80	1.91	2.29	28.2	<0.25	22.4	<0.25	<10.0	–	35.5	1.70	2.09	14.1	13.4

[a] Average GI_{50}; GI_{50} is used instead of IC_{50}, as the average potencies listed include IC_{50} values greater than the maximum concentration used. [b] Selectivity index: quotient of the IC_{50} values from the most refractory versus most sensitive cell lines for a given compound.

periments to monitor the effects on both cell cycle and respiration using HT29 human colon cancer cells were undertaken. The IC_{50} value of **5g** after incubation with HT29 cells for 96 h is 4.25 μM. The effect of this compound at 5 μM on the cell cycle is illustrated in Figure 4, which reveals that the sub-G_1 phase

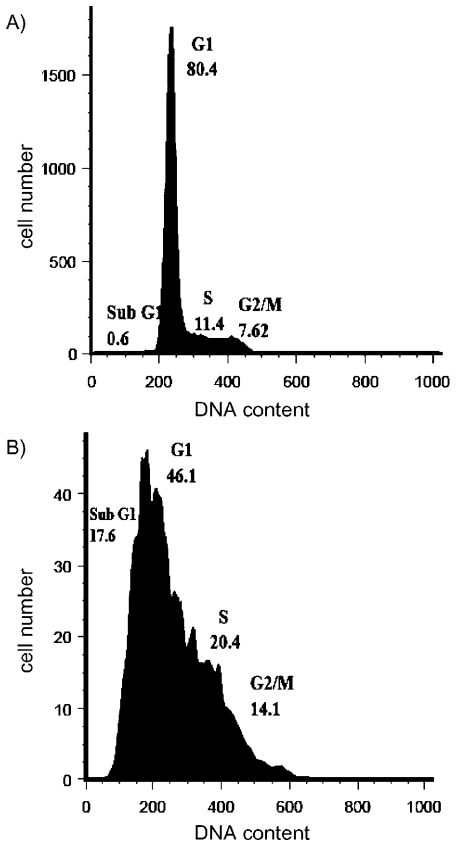


Figure 4. The effect of compound **5g** on the cell cycle of HT29 cells: A) control; B) **5g** at 5 μM.

has increased 29-fold, indicating that apoptosis has occurred. Previous work from our research groups has revealed that various compounds containing the 1,5-diaryl-3-oxo-1,4-pentadienyl group cause stimulation of respiration in rat liver mitochondria.[20–22] In the present investigation, two of the potent cytotoxins, **5d** and **5g**, as well as 5-FU were examined for their effects on respiration in HT29 cells. A concentration of 25 μM was chosen, which is close to the IC_{50} value of 5-FU toward this cell line. The results are presented in Figure 5, which reveals that only inhibition of respiration was observed. Hence, interference with mitochondrial respiration is one way in which the cytotoxicity of **5d** and **5g**, and presumably analogues of these compounds, is mediated. The significant inhibition of respiration by 5-FU suggests that this is an important mode of action for this anticancer drug.

The final question is whether the compounds in series **4** and **5** have MDR reverting properties or not. The assays for P-glycoprotein MDR reversal employed murine L-5178Y lymphoma cells transfected with the human *mdr1* gene. The concentrations of the dye rhodamine 123 in treated and untreated trans-

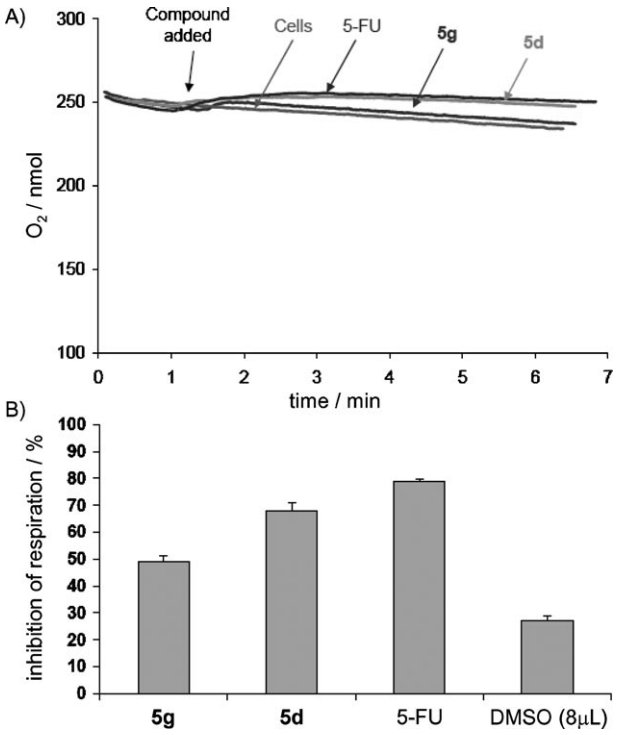


Figure 5. Effect of **5d**, **5g**, and 5-fluorouracil (25 μM each) on respiration in colon HT29 cancer cells: A) kinetics of oxygen consumption; B) percent inhibition of respiration. Error bars indicate the standard deviations from three replicates.

fected and parental cells were measured, and the relative fluorescence intensities are referred to as the fluorescence activity ratio (FAR) values. A FAR value of >1 indicates MDR reversal has occurred. These data are presented in Table 1. In general, MDR reversal is more pronounced in series **4** than in **5**, as revealed from the following observations: First, the average FAR values for series **4** and **5** are 32 and 15, respectively. Second, with the exception of **4d** and **4g**, for the same substituent in the aryl rings, the analogues in series **4** have the higher FAR values. A number of MDR reversal agents have high lipophilicity.[23,24] Because the average Clog*P* values in series **4** and **5** are 4.02 and 2.18, respectively, the greater hydrophobicity of **4a–i** than **5a–i**, in general, may contribute to the higher MDR reverting properties of the compounds in series **4**. The following 4-piperidones possess FAR values in excess of 20 and are lead molecules, namely **4a**,**c–f** and **5d**,**e**. Notably, the two compounds in each of series **4** and **5** with the highest MDR reverting properties, i.e., **4d**,**e** and **5d**,**e**, have the same aryl substituents, namely 3,4-dimethoxy and 3,4,5-trimethoxy groups. Hence the placement of a number of methoxy and related alkoxy substituents at various locations on the aryl rings may be worth pursuing in future searches for novel MDR reverting agents. Furthermore, to determine whether MDR reversal is governed by one or more of the physicochemical properties of the aryl substituents, linear and semilogarithmic plots were made between the σ/σ^*, π, and MR constants of the groups in the aryl rings and the FAR values in both series **4** and **5**. A negative correlation was observed between the FAR values of **4a–i** and the σ/σ^* constants ($p < 0.01$). In addition, a positive corre-

lation was found between the MR values of the aryl substituents of the compounds in series **5** and the FAR data ($p = 0.01$). A trend toward a negative correlation ($p < 0.1$) was observed between the FAR values of **5a–i** and the σ/σ^* constants of the aryl substituents. No other correlations were noted ($p > 0.1$). One may conclude that when developing the compounds in series **4** and **5** as candidate MDR reverting agents, strongly electron-releasing substituents should be placed in the aryl rings. In the case of series **5**, increasing the size of the aryl substituents will likely increase the magnitude of MDR reversal. An intriguing question is whether any correlation exists between the FAR values and the IC_{50} values generated for series **4** and **5** in the Molt 4/C8, CEM, and L1210 assays. Hence linear, semilogarithmic, and logarithmic plots were constructed, and a negative correlation was observed for **5a–i** in the murine L1210 screen ($p < 0.05$). No other correlations were found ($p > 0.1$). Consequently the design of analogues **5a–i** for greater cytotoxic potencies should be accompanied by increased MDR reversal.

Conclusions

A series of 3,5-bis(benzylidene)-1-phosphono-4-piperidones **5** and the related diethyl esters **4** were synthesized. These compounds display potent cytotoxicity toward human Molt 4/C8 and CEM T-lymphocytes as well as murine leukemia L1210 cells. In general, greater potencies are observed for series **5** than for the more hydrophobic analogues **4a–i**. In particular, **5g**, with an average IC_{50} value of 34 nM toward the T-lymphocyte lines, is clearly a lead molecule. Many of the compounds are more potent than the anticancer drug melphalan. Various physicochemical properties were shown to influence the magnitude of the IC_{50} values generated. Four of the 4-piperidones, namely **4a,c,d** and **5c**, are substantially more potent than melphalan and 5-fluorouracil toward nearly 60 human tumor cell lines. In this biological evaluation, approximately two-thirds of the IC_{50} values toward several colon cancer cell lines and leukemic cells are sub-micromolar, and several are in the double-digit nanomolar range. The modes of action of representative compounds include the induction of apoptosis and interference with cellular respiration. Most of the compounds in series **4** as well as **5b,d,e** have significant MDR reverting properties. Thus this study has disclosed the discovery of two novel series of cytotoxic compounds, some of which have pronounced MDR reverting properties. A number of guidelines for expanding this project have been made.

Experimental Section

Chemistry

Synthesis of 3d,e,g,h, 4a–i, and 5a–i: Melting points were determined on a Gallenkamp instrument and are uncorrected. ^1H and ^{13}C NMR spectra were recorded at 500 and 125 MHz, respectively, on a Bruker Avance spectrometer equipped with a 5 mm BBO probe. Chemical shifts (δ) are reported in ppm. Elemental analyses were conducted with an Elementer analyzer. Mass spectra were measured using a Micromass Quattro II mass spectrometer.

Synthesis of 3,5-bis(arylidene)-4-piperidones (3a–i): The syntheses of **3a–c,f,i** were reported previously.[7,11] Compounds **3d,e,g,h** were prepared following the same procedure.

3,5-Bis-(3,4-dimethoxybenzylidene)-4-piperidone (3d): Yield: 67%; mp: 162 °C; ^1H NMR (DMSO): $\delta = 7.56$ (s, 2H, 2×=CH), 7.07 (d, 6H, Ar-H, $J = 14.74$ Hz), 4.03 (s, 4H, 2×NCH_2), 3.82 (s, 12H, 4×OCH_3); Anal. calcd for $C_{23}H_{25}NO_5$: C 69.86, H 6.37, N 3.54, found: C 69.76, H 6.14, N 3.32.

3,5-Bis-(3,4,5-trimethoxybenzylidene)-4-piperidone hydrochloride (3e): Yield: 68%; mp: 251 °C; ^1H NMR (DMSO): $\delta = 9.5$ (br d, 2H, $^+NH_2$), 7.84 (s, 2H, 2×=CH), 6.86 (s, 4H, Ar-H), 4.58 (s, 4H, 2×NCH_2), 3.84 (s, 12H, 4×OCH_3), 3.73 (s, 6H, 2×OCH_3); Anal. calcd for $C_{25}H_{30}ClNO_7$: C 61.04, H 6.15, N 2.85, found: C, 60.78, H 6.10, N 2.75.

3,5-Bis-(2-nitrobenzylidene)-4-piperidone hydrochloride (3g): Yield: 52%; mp: 218 °C; ^1H NMR (DMSO): $\delta = 9.15$ (br s, 2H, $^+NH_2$), 8.28 (d, 2H, Ar-H, $J = 8.15$ Hz), 8.14 (s, 2H, 2×=CH), 7.90 (t, 2H, Ar-H), 7.76 (t, 2H, Ar-H), 7.57 (d, 2H, Ar-H, $J = 7.45$ Hz), 4.19 (s, 4H, 2×NCH_2); Anal. calcd for $C_{19}H_{16}ClN_3O_5$: C 56.74, H 3.98, N 10.45, found: C, 56.45, H 3.96, N 10.25.

3,5-Bis-(3-nitrobenzylidene)-4-piperidone (3h): Yield: 68%; mp: 214 °C; ^1H NMR (DMSO): $\delta = 8.34$ (s, 2H, Ar-H), 8.27 (d, 2H, Ar-H, $J = 8.20$ Hz), 7.96 (d, 2H, Ar-H, $J = 7.71$ Hz), 7.77 (t, 2H, Ar-H), 7.72 (s, 2H, 2×=CH), 4.06 (s, 4H, 2×NCH_2); Anal. calcd for $C_{19}H_{15}N_3O_5$: C 62.46, H 4.14, N 11.50, found: C, 62.34, H 3.99, N 11.59.

Synthesis of [3,5-bis(arylidene)-4-oxo-1-yl]phosphonic acid diethyl esters (4a–i). *General procedure:* A mixture of **3a–i** (0.01 mol), diethylchlorophosphate (2.07 g, 0.012 mol), anhydrous K_2CO_3 (2.07 g, 0.015 mol), and a catalytic amount of KI (0.166 g, 0.001 mol) in acetone (30 mL) was held at reflux for 2–3 h. Reaction progress was monitored by TLC (solvent: MeOH/$CHCl_3$ 5:95 *v/v*). The solvent was evaporated under vacuum at 40–45 °C. An aqueous solution of K_2CO_3 (5% *w/v*, 50 mL) was added to the crude mass and stirred for 2 h. The solid was removed by filtration, dried, and crystallized from a suitable solvent.

[3,5-Bis(benzylidene)-4-oxo-1-yl]phosphonic acid diethyl ester (4a): Yield: 61%; mp: 127 °C (acetone); ^1H NMR ($CDCl_3$): $\delta = 7.86$ (s, 2H, 2×=CH), 7.44 (m, 10H, Ar-H), 4.50 (d, 4H, 2×NCH_2, $J = 7.86$ Hz), 3.96 (m, 4H, 2×OCH_2), 1.20 (t, 6H, 2×CH_3); ^{13}C NMR ($CDCl_3$): $\delta = 187.02$, 136.99, 134.77, 132.59, 132.55, 130.46, 129.40, 128.97, 128.79, 62.74, 62.69, 46.28, 46.26, 16.02, 15.98; MS (ESI): *m/z* 450.01 $[M+K]^+$, 434.13 $[M+Na]^+$, 412.15 $[M+H]^+$; Anal. calcd for $C_{23}H_{26}NO_4P \cdot 0.25\,H_2O$: C 66.35, H 6.25, N 3.36, found: C, 66.37, H 6.32, N 3.20.

[3,5-Bis-(4-methylbenzylidene)-4-oxo-1-yl]phosphonic acid diethyl ester (4b): Yield: 66%; mp: 151 °C (*i*PrOH); ^1H NMR ($CDCl_3$): $\delta = 7.82$ (s, 2H, 2×=CH), 7.34 (d, 4H, Ar-H, $J = 8.03$ Hz), 7.26 (d, 4H, Ar-H, $J = 7.96$ Hz), 4.49 (d, 4H, 2×NCH_2, $J = 7.56$ Hz), 3.96 (m, 4H, 2×OCH_2), 2.42 (s, 6H, 2×Ar-CH_3), 1.21 (t, 6H, 2×CH_3); ^{13}C NMR ($CDCl_3$): $\delta = 187.08$, 139.78, 136.91, 132.04, 131.88, 131.84, 130.60, 129.53, 62.68, 62.64, 46.31, 46.29, 21.48, 16.06, 16.01; MS (ESI): *m/z* 440.16 $[M+H]^+$; Anal. calcd for $C_{25}H_{30}NO_4P \cdot 0.25\,H_2O$: C 67.57, H 6.75, N 3.15, found: C, 67.77, H 6.85, N 3.06.

[3,5-Bis-(4-methoxybenzylidene)-4-oxo-1-yl]phosphonic acid diethyl ester (4c): Yield: 64%; mp: 144 °C (MeOH); ^1H NMR ($CDCl_3$): $\delta = 7.81$ (s, 2H, 2×=CH), 7.40 (d, 4H, Ar-H, $J = 8.70$ Hz), 6.98 (d, 4H, Ar-H, $J = 8.71$ Hz), 4.49 (d, 4H, 2×NCH_2, $J = 7.31$ Hz), 3.98 (m, 4H, 2×OCH_2), 3.88 (s, 6H, 2×Ar-OCH_3), 1.21 (t, 6H, 2×CH_3); ^{13}C NMR ($CDCl_3$): $\delta = 186.92$, 160.56, 136.51, 132.47, 130.72, 130.68, 127.58,

114.31, 62.68, 62.64, 55.40, 55.32, 46.30, 46.27, 16.08, 16.03; MS (ESI): *m/z* 472.27 $[M+H]^+$; Anal. calcd for $C_{25}H_{30}NO_6P{\cdot}0.5\,H_2O$: C 62.43, H 6.24, N 2.91, found: C, 62.24, H 6.34, N 2.81.

[3,5-Bis-(3,4-dimethoxybenzylidene)-4-oxo-1-yl]phosphonic acid diethyl ester (4d): Yield: 58%; mp: 120 °C (EtOH); ^{1}H NMR ($CDCl_3$): $\delta = 7.79$ (s, 2H, 2×=CH), 7.05 (dd, 2H, Ar-H, $J = 1.52$, 8.32 Hz), 6.97 (d, 2H, Ar-H, $J = 1.53$ Hz), 6.95 (d, 2H, Ar-H, $J = 8.35$ Hz), 4.51 (d, 4H, 2×NCH_2, $J = 7.70$ Hz), 4.02 (m, 4H, 2×OCH_2), 3.95 (s, 6H, 2×Ar-OCH_3), 3.93 (s, 6H, 2×Ar-OCH_3), 1.22 (t, 6H, 2×CH_3); ^{13}C NMR ($CDCl_3$): $\delta = 186.75$, 150.27, 148.93, 136.79, 130.99, 130.96, 127.82, 123.97, 113.85, 111.17, 62.69, 62.65, 55.99, 55.98, 46.31, 46.28, 16.11, 16.05; MS (ESI): *m/z* 532.25 $[M+H]^+$; Anal. calcd for $C_{27}H_{34}NO_8P{\cdot}0.25\,H_2O$: C 60.44, H 6.34, N 2.61, found: C, 60.31, H 6.40, N 2.48.

[3,5-Bis-(3,4,5-trimethoxybenzylidene)-4-oxo-1-yl]phosphonic acid diethyl ester (4e): Yield: 54%; mp: 129 °C (MeOH); ^{1}H NMR ($CDCl_3$): $\delta = 7.78$ (s, 2H, 2×=CH), 6.66 (s, 4H, Ar-H), 4.52 (d, 4H, 2×NCH_2, $J = 8.40$ Hz), 3.97 (m, 4H, 2×OCH_2), 3.93 (s, 6H, 2×Ar-OCH_3), 3.91 (s, 12H, 4×Ar-OCH_3), 1.23 (t, 6H, 2×CH_3); ^{13}C NMR ($CDCl_3$): $\delta =$ 186.66, 153.26, 139.43, 137.17, 131.89, 131.86, 130.27, 107.89, 62.73, 62.69, 61.01, 56.27, 56.20, 46.27, 46.24, 16.12, 16.06; MS (ESI): *m/z* 592.24 $[M+H]^+$; Anal. calcd for $C_{29}H_{38}NO_{10}P{\cdot}0.25\,H_2O$: C 58.38, H 6.37, N 2.34, found: C, 58.01, H 6.44, N 2.21.

[3,5-Bis-(4-chlorobenzylidene)-4-oxo-1-yl]phosphonic acid diethyl ester (4f): Yield: 67%; mp: 132 °C (MeOH); ^{1}H NMR ($CDCl_3$): $\delta =$ 7.78 (s, 2H, 2×=CH), 7.44 (d, 4H, Ar-H, $J = 8.42$ Hz), 7.36 (d, 4H, Ar-H, $J = 8.45$ Hz), 4.45 (d, 4H, 2×NCH_2, $J = 7.54$ Hz), 3.97 (m, 4H, 2×OCH_2), 1.21 (t, 6H, 2×CH_3); ^{13}C NMR ($CDCl_3$): $\delta = 186.57$, 135.73, 135.57, 133.12, 132.87, 132.83, 131.64, 129.13, 62.84, 62.79, 46.19, 46.17, 16.07, 16.02; MS (ESI): *m/z* 480.10 $[M+H]^+$; Anal. calcd for $C_{23}H_{24}Cl_2NO_4P{\cdot}0.25\,H_2O$: C 56.92, H 4.95, N 2.88, found: C, 56.91, H 5.05, N 2.78.

[3,5-Bis-(2-nitrobenzylidene)-4-oxo-1-yl]phosphonic acid diethyl ester (4g): Yield: 42%; mp: 177 °C (EtOH); ^{1}H NMR ($CDCl_3$): $\delta = 8.23$ (d, 2H, Ar-H, $J = 8.22$ Hz), 8.11 (s, 2H, 2×=CH), 7.73 (t, 2H, Ar-H), 7.60 (t, 2H, Ar-H), 7.41 (d, 2H, Ar-H, $J = 7.61$ Hz), 4.18 (d, 4H, 2×NCH_2, $J = 9.67$ Hz), 3.95 (m, 4H, 2×OCH_2), 1.21 (t, 6H, 2×CH_3); ^{13}C NMR ($CDCl_3$): $\delta = 185.49$, 147.93, 134.64, 133.75, 133.09, 133.06, 130.89, 130.82, 129.78, 125.35, 62.87, 62.82, 16.04, 15.99; MS (ESI): *m/z* 502.30 $[M+H]^+$; Anal. calcd for $C_{23}H_{24}N_3O_8P{\cdot}0.25\,H_2O$: C 54.51, H 4.77, N 8.20, found: C, 54.55, H 4.74, N 8.30.

[3,5-Bis-(3-nitrobenzylidene)-4-oxo-1-yl]phosphonic acid diethyl ester (4h): Yield: 52%; mp: 147 °C (EtOH); ^{1}H NMR ($CDCl_3$): $\delta = 8.29$ (d, 2H, Ar-H, $J = 8.71$ Hz), 8.27 (s, 2H, Ar-H), 7.88 (s, 2H, 2×=CH), 7.76 (d, 2H, Ar-H, $J = 7.69$ Hz), 7.66 (t, 2H, Ar-H), 4.51 (d, 4H, 2×NCH_2, $J = 7.88$ Hz), 4.00 (m, 4H, 2×OCH_2), 1.22 (t, 6H, 2×CH_3); ^{13}C NMR ($CDCl_3$): $\delta = 185.89$, 136.14, 135.79, 134.56, 134.51, 130.00, 124.62, 123.98, 63.03, 62.98, 46.19, 46.17, 16.08, 16.03; MS (ESI): *m/z* 524.23 $[M+Na]^+$, 502.37 $[M+H]^+$; Anal. calcd for $C_{23}H_{24}N_3O_8P$: C 55.09, H 4.82, N 8.38, found: C, 54.79, H 4.76, N 8.33.

[3,5-Bis-(4-nitrobenzylidene)-4-oxo-1-yl]phosphonic acid diethyl ester (4i): Yield: 55%; mp: 188 °C (EtOH); ^{1}H NMR ($CDCl_3$): $\delta = 8.33$ (d, 4H, Ar-H, $J = 8.64$ Hz), 7.86 (s, 2H, 2×=CH), 7.58 (d, 4H, Ar-H, $J = 8.57$ Hz), 4.48 (d, 4H, 2×NCH_2, $J = 8.37$ Hz), 3.98 (m, 4H, 2×OCH_2), 1.21 (t, 6H, 2×CH_3); ^{13}C NMR ($CDCl_3$): $\delta = 185.98$, 147.82, 140.79, 135.12, 135.08, 134.66, 130.86, 124.05, 63.05, 63.01, 46.21, 46.18, 16.10, 16.04; MS (ESI): *m/z* 500.59 $[M-H]^-$; Anal. calcd for $C_{23}H_{24}N_3O_8P$: C 55.09, H 4.82, N 8.38, found: C, 55.13, H 4.87, N 8.13.

Synthesis of [3,5-bis(arylidene)-4-oxo-1-yl]phosphonic acids (5a–i): *General procedure:* $Si(CH_3)_3Br$ (7.65 g, 0.05 mol) was added to a solution of **4a–i** (0.01 mol) in CH_3CN (30 mL) under N_2 atmosphere at room temperature, and the reaction was allowed to continue for 12–15 h. Reaction progress was monitored by TLC (solvent: MeOH/$CHCl_3$ 10:90 *v/v*). The solvent was evaporated under reduced pressure at 45–50 °C. H_2O (30 mL) was added to the crude mass and stirred for 2–3 h. The solid was filtered off, dried, and crystallized from $CHCl_3$/MeOH (2:8 *v/v*).

[3,5-Bis(benzylidene)-4-oxo-1-yl]phosphonic acid (5a): Yield: 41%; mp: 258 °C (dec.); ^{1}H NMR (DMSO): $\delta = 9.39$ (br s, 1H, OH), 9.33 (br s, 1H, OH), 7.91 (s, 2H, 2×=CH), 7.55 (m, 10H, Ar-H), 4.54 (s, 4H, 2×NCH_2); ^{13}C NMR (DMSO): $\delta = 182.84$, 139.79, 134.15, 131.04, 130.63, 129.47, 128.26, 44.58, 44.55; MS (ESI): *m/z* 276.29 $[M-HPO_3+H]^+$; Anal. calcd for $C_{19}H_{18}NO_4P{\cdot}3\,H_2O$: C 55.70, H 4.39, N 3.42, found: C, 55.57, H 4.48, N 3.40.

[3,5-Bis-(4-methylbenzylidene)-4-oxo-1-yl]phosphonic acid (5b): Yield: 46%; mp: 254 °C (dec.); ^{1}H NMR (DMSO): $\delta = 9.29$ (br s, 2H, 2×OH), 7.87 (s, 2H, 2×=CH), 7.45 (d, 4H, Ar-H, $J = 8.04$ Hz), 7.36 (d, 4H, Ar-H, $J = 7.97$ Hz), 4.53 (s, 4H, 2×NCH_2), 2.39 (s, 6H, 2×Ar-CH_3); ^{13}C NMR (DMSO): $\delta = 182.72$, 140.82, 139.76, 131.40, 131.19, 130.09, 127.40, 44.63, 21.53; MS (ESI): *m/z* 304.33 $[M-HPO_3+H]^+$; Anal. calcd for $C_{21}H_{22}NO_4P{\cdot}1.25\,H_2O$: C 55.70, H 4.39, N 3.42, found: C, 55.57, H 4.48, N 3.40.

[3,5-Bis-(4-methoxybenzylidene)-4-oxo-1-yl]phosphonic acid (5c): Yield: 51%; mp: 256 °C; ^{1}H NMR (DMSO): $\delta = 9.29$ (br s, 2H, 2×OH), 7.86 (s, 2H, 2×=CH), 7.53 (d, 4H, Ar-H, $J = 8.72$ Hz), 7.11 (d, 4H, Ar-H, $J = 8.73$ Hz), 4.52 (s, 4H, 2×NCH_2), 3.85 (s, 6H, 2×Ar-OCH_3); ^{13}C NMR (DMSO): $\delta = 182.52$, 161.31, 139.43, 133.28, 126.73, 125.98, 115.05, 55.97, 44.65; MS (ESI): *m/z* 336.14 $[M-HPO_3+H]^+$; Anal. calcd for $C_{21}H_{22}NO_6P{\cdot}0.5\,H_2O$: C 59.38, H 5.18, N 3.29, found: C, 59.09, H 5.35, N 3.18.

[3,5-Bis-(3,4-dimethoxybenzylidene)-4-oxo-1-yl]phosphonic acid (5d): Yield: 53%; mp: 249 °C; ^{1}H NMR (DMSO): $\delta = 9.29$ (br s, 2H, 2×OH), 7.87 (s, 2H, 2×=CH), 7.17 (s, 2H, Ar-H), 7.14 (d, 4H, Ar-H, $J = 7.90$ Hz), 4.57 (s, 4H, 2×NCH_2), 3.85 (s, 6H, 2×Ar-OCH_3), 3.83 (s, 6H, 2×Ar-OCH_3); ^{13}C NMR (DMSO): $\delta = 182.44$, 151.15, 149.20, 139.86, 126.91, 126.07, 124.85, 114.86, 112.29, 56.19, 56.16, 44.67; MS (ESI): *m/z* 396.25 $[M-HPO_3+H]^+$; Anal. calcd for $C_{23}H_{26}NO_8P{\cdot}3\,H_2O$: C 52.13, H 4.91, N 2.64, found: C, 52.32, H 5.05, N 2.53.

[3,5-Bis-(3,4,5-trimethoxybenzylidene)-4-oxo-1-yl]phosphonic acid (5e): Yield: 61%; mp: 245 °C; ^{1}H NMR (DMSO): $\delta = 9.29$ (br s, 2H, 2×OH), 7.87 (s, 2H, 2×=CH), 6.87 (s, 4H, Ar-H), 4.63 (s, 4H, 2×NCH_2), 3.86 (s, 12H, 4×Ar-OCH_3), 3.75 (s, 6H, 2×Ar-OCH_3); ^{13}C NMR (DMSO): $\delta = 182.65$, 153.42, 140.07, 139.79, 129.65, 127.46, 108.98, 60.69, 56.66, 44.64; MS (ESI): *m/z* 456.22 $[M-HPO_3+H]^+$; Anal. calcd for $C_{25}H_{30}NO_{10}P{\cdot}0.5\,H_2O$: C 55.09, H 5.50, N 2.57, found: C, 55.06, H 5.66, N 2.52.

[3,5-Bis-(4-chlorobenzylidene)-4-oxo-1-yl]phosphonic acid (5f): Yield: 62%; mp: 264 °C (dec.); ^{1}H NMR (DMSO): $\delta = 9.34$ (br s, 2H, 2×OH), 7.89 (s, 2H, 2×=CH), 7.62 (d, 4H, Ar-H, $J = 8.58$ Hz), 7.58 (d, 4H, Ar-H, $J = 8.64$ Hz), 4.52 (s, 4H, 2×NCH_2); ^{13}C NMR (DMSO): $\delta =$ 182.67, 138.53, 135.35, 133.01, 132.80, 129.50, 128.81, 44.49; MS (ESI): *m/z* 344.14 $[M-HPO_3+H]^+$; Anal. calcd for $C_{19}H_{16}Cl_2NO_4P{\cdot}4\,H_2O$: C 45.94, H 3.22, N 2.82, found: C, 45.59, H 3.31, N 2.58.

[3,5-Bis-(2-nitrobenzylidene)-4-oxo-1-yl]phosphonic acid (5g): Yield: 34%; mp: 259 °C (dec.); ^{1}H NMR (DMSO): $\delta = 9.35$ (s, 2H, 2×OH), 8.42 (d, 2H, Ar-H, $J = 8.15$ Hz), 8.27 (s, 2H, 2×=CH), 7.89 (t,

2H, Ar-H), 7.76 (t, 2H, Ar-H), 7.58 (d, 2H, Ar-H, $J=7.60$ Hz), 4.31 (s, 4H, $2\times NCH_2$); ^{13}C NMR (DMSO): $\delta=182.50$, 146.53, 137.64, 137.11, 134.34, 129.80, 128.51, 128.13, 124.52, 44.46; MS (ESI): *m/z* 365.99 $[M-HPO_3+H]^+$; Anal. calcd for $C_{19}H_{16}N_3O_8P\cdot0.5\,H_2O$: C 50.18, H 3.52, N 10.39, found: C, 50.45, H 3.87, N 10.76.

[3,5-Bis-(3-nitrobenzylidene)-4-oxo-1-yl]phosphonic acid (5h): Yield: 52%; mp: 254 °C; ^1H NMR (DMSO): $\delta=9.35$ (br s, 2H, $2\times$OH), 8.38 (s, 2H, Ar-H), 8.35 (d, 2H, Ar-H, $J=8.20$ Hz), 8.03 (s, 2H, $2\times$=CH), 8.01 (d, 2H, Ar-H, $J=8.30$ Hz), 7.85 (t, 2H, Ar-H), 4.61 (s, 4H, $2\times NCH_2$); ^{13}C NMR (DMSO): $\delta=182.55$, 148.55, 137.69, 137.13, 135.67, 131.00, 130.35, 125.13, 124.89, 44.38; MS (ESI): *m/z* 366.18 $[M-HPO_3+H]^+$; Anal. calcd for $C_{19}H_{16}N_3O_8P\cdot0.5\,H_2O$: C 50.18, H 3.52, N 10.39, found: C, 50.22, H 3.76, N 10.03.

[3,5-Bis-(4-nitrobenzylidene)-4-oxo-1-yl]phosphonic acid (5i): Yield: 42%; mp: 204 °C; ^1H NMR (DMSO): $\delta=9.36$ (br s, 2H, $2\times$OH), 8.37 (d, 4H, Ar-H, $J=8.70$ Hz), 8.00 (s, 2H, $2\times$=CH), 7.83 (d, 4H, Ar-H, $J=8.68$ Hz), 4.55 (s, 4H, $2\times NCH_2$); ^{13}C NMR (DMSO): $\delta=182.61$, 148.15, 140.55, 137.63, 132.05, 131.09, 124.35, 44.49; MS (ESI): *m/z* 366.06 $[M-HPO_3+H]^+$; Anal. calcd for $C_{19}H_{16}N_3O_8P\cdot0.5\,H_2O$: C 50.18, H 3.52, N 10.39, found: C, 50.32, H 3.67, N 10.48.

Determination of Clog P values

The Clog *P* values of the compounds in series **3**–**5** were determined with a commercial software package.[25] The Clog *P* values for the compounds in series **3** are as follows: **3a**: 3.29 ± 0.43; **3b**: 4.10 ± 0.55; **3c**: 3.25 ± 0.60; **3d**: 2.83 ± 0.73; **3e**: 2.62 ± 1.22; **3f**: 4.46 ± 0.39; **3g**: 3.05 ± 0.47; **3h**: 3.13 ± 0.54; **3i**: 3.07 ± 0.41.

Determination of QSARs

The σ, π, and MR values were obtained from Hansch and Leo,[26] whereas the σ^* value was taken from Taft.[27] Linear, semilogarithmic, and logarithmic plots were made with SPSS v. 14.0.0.[28]

Molecular modeling

Models of the compounds in series **4** and **5** were constructed using BioMedCache v. 6.1 software.[29] The lowest-energy conformations were generated with the MOPAC system and were optimized by PM3 parameters.

X-ray crystallography of 4g

Apart from the structure factors, CCDC 733192 (**4g**) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Cytotoxicity assays

The evaluation of **3d**,**e**,**g**,**h**, **4a**–**i**, and **5a**–**i** as candidate cytotoxins using human Molt 4/C8 and CEM T-lymphocytes as well as murine L1210 cells was carried out by following published procedures.[30] Briefly, various concentrations of compounds were incubated with cells in RPMI 1640 medium for 72 h at 37 °C (Molt 4/C8 and CEM T-lymphocytes), whereas a 48 h incubation was used in the L1210 assay. The methodology with which **4a**,**c**,**d**, **5c**, melphalan, and 5-fluorouracil were assayed by using 58 or 59 human tumor cell lines was described previously.[18] The compounds were evaluated at concentrations of 0.10 mM–10 nM (**4a**,**c**,**d**, and **5c**), 0.25 mM–25 nM (melphalan), and 2.5 mM–250 nM (5-fluorouracil). The number of cell lines for which IC_{50} values lay outside the range of concentrations employed are: 1/58 (**4a**), 1/59 (**4c**), 0/59 (**4d**), 4/58 (**5c**), 0/59 (melphalan) and 6/58 (5-fluorouracil).

Determination of MDR reverting properties

The ability of compounds **4a**–**i** and **5a**–**i** to reverse MDR was evaluated by a published procedure,[31] which was summarized recently.[9] Briefly, the compounds were dissolved in DMSO, added to L-5178 MDR and parental cells, and incubated at room temperature for 10 min. After the addition of a solution of rhodamine 123 in DMSO, the cells were incubated at 37 °C for 20 min. The fluorescence was measured in treated MDR cells (F1), untreated MDR cells (F2), treated parental cells (F3), and untreated parental cells (F4), and the FAR values were calculated from the equation: FAR = (F1/F2)/(F3/F4). In these experiments, the FAR value of DMSO was 0.89.

Evaluation of 5g on cell proliferation and HT29 cell cycle

HT29 cells were obtained from the American Type Culture Collection (ATCC) and grown in DMEM and 10% fetal calf serum. Cell cultures were maintained at 37 °C under an atmosphere of humidified air and 5% CO_2.[32] The cells were subsequently dissociated from culture flask surfaces with a solution of trypsin (2.5 g L^{-1}) and resuspended in DMEM to give a concentration of 1×10^5 cells mL^{-1}. The cells were added to 96-well plates (9000 cells per plate) and allowed to attach for 24 h, after which time various concentrations of **5g** were added. After incubation for 96 h, cell proliferation was estimated by the MTT assay using a microplate reader ($\lambda=$ 540 nM).[33]

For cell cycle studies, HT29 cells were plated and grown for 48 h to reach 50–60% confluency.[34] Cells were treated with various concentrations of **5g**, and after 48 h the cells were treated with trypsin, washed with PBS, and fixed overnight in 70% EtOH at 4 °C. At the time of harvest, the cultures were 70–90% confluent. After removing the EtOH by centrifugation, the cells were resuspended in buffer containing Tris (10 mM, pH 7.5), sucrose (125 mM), $MgCl_2$ (2.5 mM), NP40 (0.185%), RNase A (0.02 mg mL^{-1}), sodium citrate (0.05%), and propidium iodide (25 μg mL^{-1}). After incubation on ice for 1 h, the cells were subjected to DNA content analysis using a FACScan cytometer (Becton Dickinson).

Effect of 5d and 5g on respiration in HT29 cells

The effect of **5d**, **5g**, and 5-fluorouracil on oxygen consumption in human HT29 colon cancer cells was measured by polarography[35] of 1×10^5 cells in air-saturated DMEM at 37 °C.

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Sequential cytotoxicity: A theory examined using a series of 3,5-bis(benzylidene)-1-diethylphosphono-4-oxopiperidines and related phosphonic acids

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ABSTRACT

The concept of sequential cytotoxicity, which states that successive chemical attacks on cellular constituents can be more deleterious to neoplasms than normal cells, was evaluated using a series of 3,5-bis(benzylidene)-1-diethylphosphono-4-oxopiperidines **1** and related phosphonic acids **2**, which were screened against a panel of malignant and normal cell lines. The compounds proved to be not only potent cytotoxins (71% of the CC_{50} figures are submicromolar) but to display greater cytotoxicity to the neoplastic cells. QSAR revealed that both cytotoxic potencies and selective toxicity were increased by a rise in the electron-withdrawing properties and a decrease in the hydrophobicity of the aryl substituents. Utilisation of the PL10 concept and evaluation of druglike properties revealed **1c** as the lead tumour-specific cytotoxin. This molecule activated caspase-3 in HL-60 cells but not in the HSC-2 cell line. While **1c** caused internucleosomal DNA fragmentation in HL-60 cells, it did not elicit this effect in either HSC-2 and HSC-4 cells. Clearly **1c** exerts its cytotoxic potencies by different mechanisms and such pleiotropy is likely the principal reason for the remarkable display of preferential toxicity towards malignant cells of the compounds in series **1** and **2**.

The theory of sequential cytotoxicity was originally defined as the successive release of two or more cytotoxic compounds thereby causing greater toxicity to neoplasms than normal cells.[1] In the present study, this hypothesis is simplified to refer to compounds which are designed to cause successive chemical attacks on cellular constituents. This concept is based on the observation that on occasions an initial chemosensitisation followed by a subsequent chemical attack is more deleterious to tumours than non-malignant tissues.[2,3]

In order to examine this hypothesis further, a series of 3,5-bis (benzylidene)-1-diethylphosphono-4-oxopiperidines **1** and related phosphonic acids **2** were designed for the following reasons. First, conjugated enones have a marked affinity for thiols in contrast to amino and hydroxyl groups.[4,5] Hence interactions with the amino and hydroxyl groups of nucleic acids, which may lead to genotoxic effects,[6] should be absent in these compounds. Second, series **1** and **2** contain the dienone motif which permits sequential interactions at the olefinic carbon atoms with cellular thiols. Third, previous studies revealed that various cytotoxic 3,5-bis(benzylidene)-4-piperidones **3** demonstrated greater toxicity to certain neoplasms than various non-malignant cell lines.[7] N-acylation of series **3** with acryloyl chloride led to series **4** which was accompanied by increases in both potency and selective toxicity to malignant cells.[7] However the hydrophobicity of **4** increases compared to **3** which may lead to pharmacokinetic and formulation problems. Hence the acryloyl group of **4** was replaced by the hydrophilic phosphono substituent leading to series **2**.[8] Since there are two acidic protons in **2**, which may impede penetration of cell membranes, the corresponding diethyl esters **1** were synthesised.[8] The structures of the compounds in series **1**–**4** are presented in Figure 1.

A preliminary investigation revealed that most of the compounds in series **1** and **2** display potent cytotoxicity towards several malignant and transformed cell lines.[8] The aims of the present investigation are (1) to determine whether the theory of sequential cytotoxicity is validated from the biodata generated in series **1** and **2**, (2) to identify a lead molecule that possesses potent cytotoxicity and selective toxicity to neoplasms compared to normal cells for future in vivo pharmacokinetic and pharmacodynamic evaluations, (3) to investigate the mode of action of the lead molecule in order to gain some insight into the possible ways bioactivity is mediated and (4) to develop suitable QSAR models which will enable the design of potent, tumour-selective cytotoxins for future development.

All of the compounds in series **1** and **2** were evaluated against four human neoplastic cell lines namely HL-60 promyelocytic

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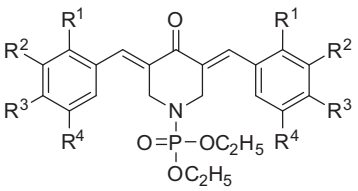

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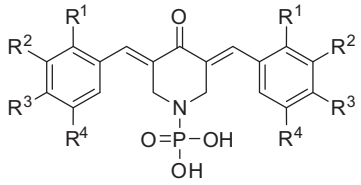

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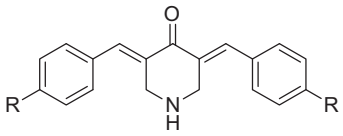

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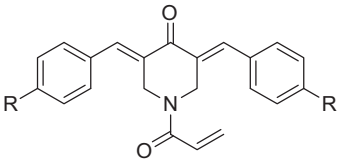

4

1 and **2** **a**: $R^1=R^2=R^3=R^4=H$; **b**: $R^1=R^2=R^4=H$; $R^3=CH_3$; **c**: $R^1=R^2=R^4=H$; $R^3=OCH_3$; **d**: $R^1=R^4=H$; $R^2=R^3=OCH_3$; **e**: $R^1=H$, $R^2=R^3=R^4=OCH_3$; **f**: $R^1=R^2=R^4=H$, $R^3=NO_2$; **g**: $R^1=R^2=R^4=H$; $R^3=Cl$; **h**: $R^1=NO_2$, $R^2=R^3=R^4=H$; **i**: $R^1=R^3=R^4=H$, $R_2=NO_2$

3 and **4** R=H, Cl, NO_2, CH_3

Figure 1. Structures of the compounds in series **1**–**4**.

leukemic cells as well as HSC-2, HSC-3 and HSC-4 oral squamous cell carcinomas. In addition, these dienones were screened against three human normal cells viz HGF gingival fibroblasts, HPC pulp cells and HPLF periodontal ligament fibroblasts.[9] These data are presented in Table 1.

The biodata displayed in Table 1 indicate that the compounds in series **1** and **2** are potent cytotoxins. In fact, 71% of the CC_{50} values of **1a–i** and **2a–i** are submicromolar while **1i**, **2e**, **h**, **i** have double digit nanomolar CC_{50} figures towards HL-60 cells. The potencies of virtually all of the compounds towards the three squamous cells carcinomas compare very favourably with melphalan, for example, **2h** possesses 51, 78 and 320 times the potency of this drug towards HSC-2, HSC-3 and HSC-4 cell lines, respectively.

In order to address the issue of whether the compounds display greater toxicity to neoplasms than normal cells, selectivity index (SI) figures were calculated for **1a–i** and **2a–i**. Under clinical conditions, tumours are surrounded by a number of different types of cells and in order to simulate in vivo situations, the average CC_{50}

Table 1
Evaluation of **1a**–**i**, **2a**–**i** and melphalan against human tumour and normal cell lines

	Human tumour cell lines[a] (CC_{50}, μM)								Human normal cell lines[a] (CC_{50}, μM)			
	HL-60	SI[b]	HSC-2	SI[b]	HSC-3	SI[b]	HSC-4	SI[b]	HGF	HPC	HPLF	Ave[c]
1a	0.61 ± 0.05	15	2.4 ± 0.13	3.8	2.5 ± 0.11	3.7	1.1 ± 0.11	8.4	11 ± 0.55	6.5 ± 0.91	10 ± 0.2	9.2
1b	3.3 ± 0.54	6.7	4.9 ± 0.05	4.5	4.1 ± 0.19	5.4	4.6 ± 0.09	4.8	28 ± 7.50	18 ± 1.9	21 ± 0.7	22
1c	1.9 ± 0.18	25	0.92 ± 0.08	51	0.66 ± 0.07	71	1.3 ± 0.32	36	53 ± 3.20	41 ± 0.5	46 ± 3.2	47
1d	0.75 ± 0.12	8.4	1.7 ± 0.23	3.7	1.4 ± 0.38	4.5	0.50 ± 0.01	13	7.4 ± 2.60	6.2 ± 1.9	5.3 ± 0.5	6.3
1e	0.29 ± 0.03	6.2	0.33 ± 0.02	5.5	0.43 ± 0.09	4.2	0.16 ± 0.03	11	2.0 ± 0.07	1.3 ± 0.38	2.2 ± 0.11	1.8
1f	0.14 ± 0.02	17	0.33 ± 0.02	7.3	0.38 ± 0.09	6.3	0.72 ± 0.22	3.3	3.1 ± 0.75	1.9 ± 0.35	2.2 ± 0.35	2.4
1g	0.60 ± 0.04	6.7	1.5 ± 0.25	2.7	2.0 ± 0.05	2.0	0.64 ± 0.19	6.3	4.3 ± 0.16	2.8 ± 0.4	4.8 ± 0.22	4.0
1h	0.38 ± 0.04	37	1.6 ± 0.24	8.8	1.2 ± 0.01	12	1.8 ± 0.26	7.8	18 ± 1.40	8.3 ± 1.9	15 ± 4.9	14
1i	0.05 ± 0.01	54	0.76 ± 0.07	3.3	0.74 ± 0.18	3.4	0.26 ± 0.04	9.6	2.2 ± 0.05	1.5 ± 0.18	3.9 ± 4.9	2.5
2a	0.46 ± 0.11	12	0.77 ± 0.54	7.4	0.98 ± 0.03	5.8	0.57 ± 0.14	10	6.9 ± 0.81	3.2 ± 0.50	7.0 ± 1.8	5.7
2b	1.1 ± 0.23	4.6	0.76 ± 0.09	6.7	0.95 ± 0.05	5.4	0.96 ± 0.05	5.3	5.9 ± 1.70	3.3 ± 0.92	6.1 ± 0.25	5.1
2c	5.6 ± 1.30	12	1.7 ± 0.18	40	2.2 ± 0.15	31	3.9 ± 1.20	17	107 ± 7	54 ± 6.5	44 ± 6.30	68
2d	0.26 ± 0.01	8.1	0.40 ± 0.11	5.3	0.59 ± 0.11	3.6	0.42 ± 0.04	5.0	2.6 ± 0.45	1.3 ± 0.56	2.5 ± 0.05	2.1
2e	0.06 ± 0.01	12	0.13 ± 0.01	6.2	0.27 ± 0.01	3.0	0.12 ± 0.03	6.7	0.88 ± 0.04	0.59 ± 0.36	1.0 ± 0.05	0.8
2f	0.14 ± 0.03	21	0.18 ± 0.02	17	0.23 ± 0.02	13	0.13 ± 0.00	23	4.1 ± 0.61	1.6 ± 0.11	3.4 ± 0.85	3.0
2g	0.46 ± 0.02	9.8	0.42 ± 0.18	11	0.41 ± 0.10	11	0.72 ± 0.08	6.3	5.9 ± 0.50	2.6 ± 0.05	5.1 ± 0.23	4.5
2h	0.07 ± 0.02	30	0.17 ± 0.05	13	0.32 ± 0.18	6.9	0.10 ± 0.01	22	1.4 ± 0.57	2.1 ± 0.06	3.0 ± 0.09	2.2
2i	0.09 ± 0.01	30	0.20 ± 0.04	13	0.18 ± 0.05	14	0.14 ± 0.03	19	2.3 ± 1.20	2.2 ± 0.11	3.3 ± 0.83	2.6
Melphalan	1.4 ± 1.2	150	8.7 ± 4.20	24	25 ± 7.70	8.4	32 ± 8.80	6.6	161 ± 27	269 ± 153	199 ± 60	210

[a] The CC_{50} values are the concentrations of the compounds required to kill 50% of the cells.
[b] The letters SI refer to the selectivity index. These numbers are the quotients of the average CC_{50} value of the compound towards normal cells and the CC_{50} figure generated for each neoplastic cell line.
[c] These figures are the average CC_{50} values of the compounds towards HGF, HPC and HPLF cell lines.

value of the compounds towards the three normal cell lines was divided by the CC_{50} figure generated using a specific neoplasm. These data are presented in Table 1. All of the compounds have SI figures of greater than 1 which indicates that the theory of sequential cytotoxicity is worthy of further investigation.

An effort was made to identify lead molecules using the PL10 concept.[10] This approach seeks to identify *P*romising *L*ead compounds which have CC_{50} values of 10 μM or less and the SI figure is 10 or more. No less than 43% of the results obtained indicate the PL10 criteria has been achieved namely **1a**, **c**, **f**, **h**, **i**, **2a**, **c**, **e**, **f**, **h**, **i** (HL-60 screen), **1c**, **2c**, **f–i** (HSC-2 assay), **1c**, **h**, **2c**, **f**, **g**, **i** (HSC-3 test) and **1c–e**, **2a**, **c**, **f**, **h**, **i** (HSC-4 screen). Of particular note are **1c**, **2c**, **f**, **i** which have PL10 status when assayed against all four tumour cell lines. The average SI values for these four compounds (average CC_{50} figures in μM against the four neoplastic cell lines are presented in parentheses) are 46(1.20), 25(3.35), 19(0.17) and 19(0.15), respectively, indicating their being important lead molecules, especially **1c**.

The next phase of the study was aimed at discerning any physicochemical properties of the aryl substituents which control the magnitude of the CC_{50} figures towards the HL-60, HSC-2, HSC-3 and HSC-4 malignant cell lines. Accordingly linear and semilogarithmic plots were made between the σ/σ^* and π constants of the aryl substituents of **1a–i**.[11] In addition, linear, semilogarithmic and logarithmic plots were constructed between the CC_{50} values and the molecular refractivity (MR) figures of the aryl groups. The process was then repeated for the compounds in series **2**. The following correlations (p <0.05) and trends towards significance (p <0.1) were noted. The CC_{50} figures of **1a–i** correlate negatively with the σ constants in the HL-60 screen and positively with the π values in the HSC-3 assay. Positive trends towards significance were noted between the π values in the HL-60 and HSC-2 screens and a negative trend with the MR constants in the HSC-4 test. In the case of series **2**, negative correlations were noted between the σ constants in all four bioassays. No other correlations or trends to significance were noted (p >0.1). Thus future development should include the placement of substituents in the aryl rings of both series **1** and **2** which are more electron-withdrawing while in series **1**, these groups should also be more hydrophilic.

In a further attempt to discern correlations between various physicochemical parameters and the cytotoxic potencies of the compounds in series **1** and **2**, multilinear regression analyses were undertaken.[12] Excellent correlations were noted using the biodata generated in the HSC-2 and HSC-3 assays as indicated in Eqs. 2 and 3, respectively, and the studied descriptors. However modest correlations were obtained for HL-60 and HSC-4 cell lines. Efforts to improve the statistical quality of the Eqs. 1 and 4 by changing or omitting the studied descriptors did not give any good results.

$$\begin{aligned}\mathrm{Log}_{10}(CC_{50\ \mathrm{HL-60}}) &= -1.93(\pm0.86) - 1.12(\pm0.31)\sum\sigma - 0.94(\pm0.76)\sum\pi + 0.66(\pm0.34)\\ &\quad \log P + 0.73(\pm0.49)I_{\mathrm{Et}}\\ &\quad n = 18, r = 0.795, r_{\mathrm{adj}} = 0.518, s = 0.425, F = 5.57, p = 0.008 \qquad (1)\\ \mathrm{Log}_{10}(CC_{50\ \mathrm{HSC-2}}) &= 1.05(\pm0.86) - 0.97(\pm0.49)\sum\sigma + 1.65(\pm0.88)\sum\pi - 0.62(\pm0.34)\\ &\quad \log P - 1.55(\pm0.58)I_{\mathrm{Et}} - 0.09(\pm0.03)\sum\mathrm{MR} + 0.011(\pm0.009)\mathrm{TPSA}\\ &\quad n = 18, r = 0.906, r_{\mathrm{adj}} = 0.722, s = 0.236, F = 8.37, p = 0.001 \qquad (2)\\ \mathrm{Log}_{10}(CC_{50\ \mathrm{HSC-3}}) &= 1.51(\pm0.84) - 0.31(\pm0.17)\sum\sigma + 0.90(\pm0.51)\sum\pi - 0.46(\pm0.27)\\ &\quad \log P - 0.95(\pm0.37)I_{\mathrm{Et}} - 0.05(\pm0.02)\sum\mathrm{MR}\\ &\quad n = 18, r = 0.863, r_{\mathrm{adj}} = 0.637, s = 0.234, F = 6.94, p = 0.003 \qquad (3)\\ \mathrm{Log}_{10}(CC_{50\ \mathrm{HSC-4}}) &= 0.23(\pm0.22) - 0.60(\pm0.19)\sum\sigma - 0.05(\pm0.01)\sum\mathrm{MR} + 0.32(\pm0.17)I_{\mathrm{Et}}\\ &\quad n = 18, r = 0.764, r_{\mathrm{adj}} = 0.494, s = 0.35, F = 6.53, p = 0.005 \qquad (4)\end{aligned}$$

In these equations, n is the number of determinations, r is the correlation coefficient, r_{adj} is the adjusted π value, s is the standard deviation of the regression equation, F is related to the F—statistic analysis (Fisher test) and I_{Et} is an indicator variable which is assigned a value of 1 or 0 depending upon the ethyl group being present or absent, respectively.

From these statistical analyses guidelines for expansion of this study have been achieved. Thus by inserting the appropriate physicochemical constants into Eqs. 1–4, a prediction of analogs with increased cytotoxic potencies can be made. The fact that quite different equations were generated depending on the cell line under consideration reinforces the conclusion that the compounds in series **1** and **2** have pleiotropic properties which give rise to the remarkable SI values observed.

In order to evaluate whether certain of the physicochemical properties of the aryl substituents in series **1** and **2** influence the SI values, the following statistical analysis was undertaken. Linear and semilogarithmic plots were made between the σ, π and MR constants and the SI figures. In the HL-60 screen a positive trend to significance with the σ constants and a negative correlation with the π values of **1a–i** were noted. For series **2**, a positive correlation between the σ and SI figures was observed in the HL-60 and HSC-4 screens and a negative correlation with the π constants in these screens. No other correlations were noted (p >0.1). Thus in considering analog development, greater cytotoxic potencies and selective toxicity to neoplasms are predicted to occur by placing substituents with increased electron-withdrawing and hydrophilic substituents in the aryl rings of series **1** and **2** such as forming the 3-nitro-4-acetoxy and 2-nitro-4-carboxy analogs.

A study was conducted to determine if any of the lead compounds identified by using the PL10 concept have favourable druglike properties. Hence **1c, 2c, f, i** were examined in terms of certain physicochemical parameters which govern intestinal absorption[13] as well as their predicted capacity for inducing various toxic symptoms. The results are portrayed in Table 2 which reveals that the most favourable ratings appear with **1c** and **2c**. However, **1c** is considered the primary lead molecule due its higher SI value and greater potency towards neoplastic cell lines than **2c** (Table 1).

A number of cytotoxic agents exert their bioactivity, at least in part, by inducing apoptosis.[14] There are two apoptotic mechanisms, namely one which operates via the intrinsic pathway in the mitochondria and also the extrinsic pathway involving death receptors on the cell surface.[15] Both mechanisms of action utilise the proteolytic enzymes known as caspases. In particular caspase-3 is an effector caspase. The lead compound **1c** was evaluated for its ability to activate caspase-3 using concentrations which are ×1, ×2 and ×4 of the CC_{50} values for the cells. The result is portrayed in Figure 2.[16] Clearly **1c** causes a concentration-dependent activation of caspase-3 in HL-60 cells not in HSC-2 cells. Therefore the cytotoxicity of this compound to HSC-2 cells is regulated by an alternate mechanism. This finding is further substantiated by the observation that **1c** induced internucleosomal DNA fragmentation[17] as revealed in Figure 3. In contrast, using the same concentrations of **1c** as indicated in Figure 3 did not lead to any DNA fragmentation in HSC-2 and HSC-4 cells. The fact that **1c** causes cell death by different mechanisms depending on the specific cell line is probably the reason for the remarkable SI values of this compound and others in series **1** and **2**.

In conclusion, this study was designed to explore the theory of sequential cytotoxicity and is validated when the 3,5-bis(benzylidene)-1-diethylphosphono-4-oxopiperidines **1** and related phosphonic acids **2** are considered. In addition the submicromolar CC_{50} values of many of the compounds are noteworthy. Series **1** and **2** are novel clusters of molecules which should be developed in three ways, namely (1) analog development based on the QSAR, (2) further explorations as to the mechanisms of action of these compounds, and (3) in vivo pharmacokinetic and pharmacodynamic evaluations of the lead compound **1c**.

Table 2
Evaluation of **1c**, **2c**, **f**, **i** for druglike properties

Compound	Physicochemical properties[a]						Toxicity[b]				
	Log *P*	MW	HBA	HBD	RB	TPSA	M	T	I	R	Ratings[c]
1c	4.09	471.49	7	0	9	74.32	–	–	–	–	10
2c	2.78	415.38	7	2	5	96.30	–	–	–	–	10
2f	2.59	445.32	11	2	5	169.48	+	+	–	–	6
2i	2.54	445.32	11	2	5	169.48	–	–	–	–	8
Druglike compound	<5	<500	<10	<5	<10	<140	–	–	–	–	10

[a] The physicochemical properties considered are the logarithm of the partition coefficient (log *P*), molecular weight (MW), the number of hydrogen bond acceptors (HBA), hydrogen bond donors (HBD) and rotatable bonds (RB) as well as total polar surface area (TPSA). These figures were obtained using the molinspiration Web explorer.[21]
[b] The possible induction of certain toxic effects are mutagenicity (M), tumour-induction (T), irritant effects (I) and impairment of reproduction (R). The assessment for toxicity used the Osiris Property Explorer tool.[22]
[c] One point was allocated for each positive result, that is, a favourable physical property or the absence of one of the toxic symptoms. There is a maximum of 10 points. The toxicity effects are classified as toxic (+), less toxic (±) and no toxicity (−).

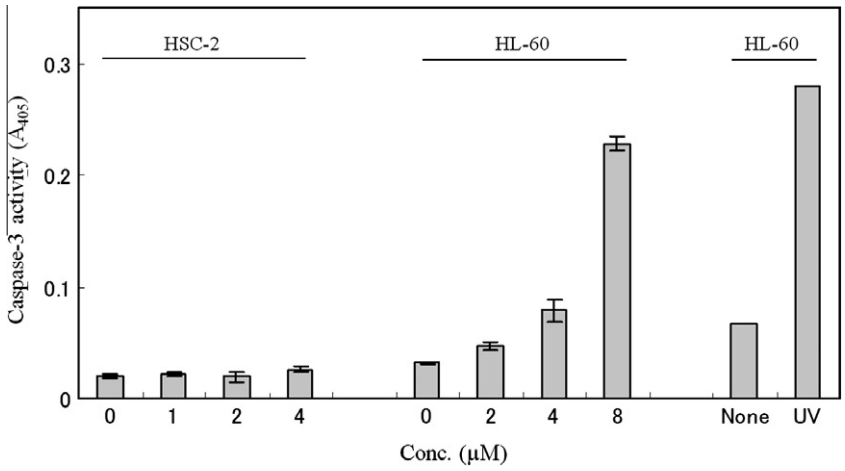

Figure 2. Evaluation of **1c** to activate caspase-3. Cells were incubated for 6 h and then harvested for caspase-3 activity. The bars are the mean determinations accompanied by standard deviations ($n = 3$).

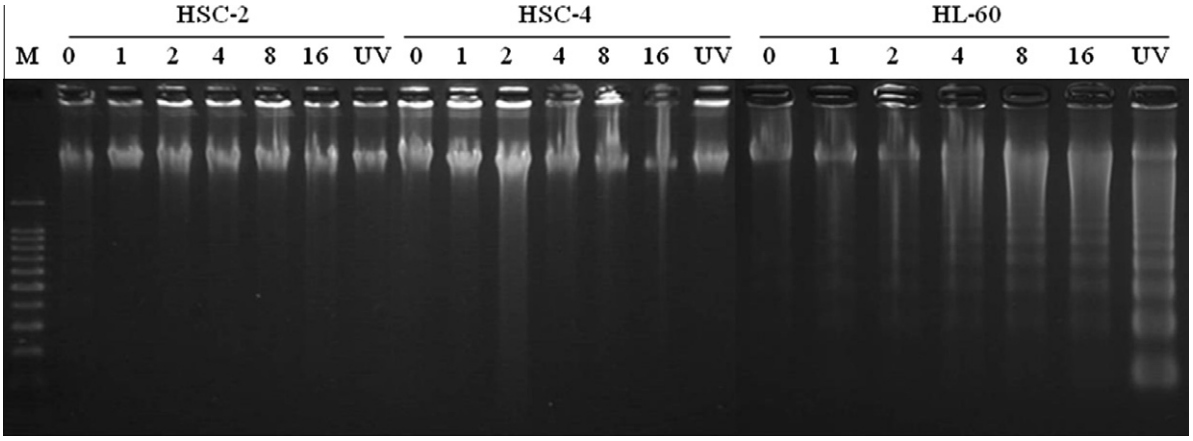

Figure 3. The effect of **1c** on internucleosomal DNA fragmentation in HSC-2, HSC-4 and HL-60 cells. The cells were incubated with different concentrations (0, 1, 2, 4, 8, 16 μM) of **1c** for 6 h and then harvested for DNA fragmentation. As a positive control, cells were exposed to UV irradiation for 1 min. followed by 3.5 h incubation. M is a 100 bp DNA ladder marker.

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CHAPTER 4

Chapter 4 consists of copies of two articles: one published in ChemMedChem in 2011*† and other one in the European Journal of Medicinal Chemistry**#.

Relation of Chapter 4 to the objectives of this project

The aim was to investigate the hypothesis of cytotoxic synergism, namely that compounds capable of multiple cellular interactions in neoplasms exert a synergistic effect. In other words, an increase in the thiol alkylating sites will lead to increase in cytotoxic potencies, and selective tumour toxicity compared to normal cells.

Description

In order to probe this theory of cytotoxic synergism, a series of dimeric 3,5-bis(arylidene)-4-piperidones **1** containing two 1,5-diaryl-3-oxo-1,4-pentadienyl groups was synthesized. In this case, the inclusion of two such groups into a molecule may more than double the potency of a related 3,5-bis(benzylidene)-4-piperidone which contains only one dienone moiety. The relative location of dienone groups may be very important for multiple thiol alkylations to take place which in turn can influence cytotoxic potencies substantially. Therefore in series **1**, a linker group containing varying carbon chain lengths was introduced to develop a structure-activity relationship and to find out the most optimal distance between two dienone motifs for highest cytotoxic potencies. All the compounds were evaluated against human Molt 4/C8 and CEM T-lymphocytes as well as murine leukemic L1210 cells. The ability of the compounds to inhibit non-adherent cancer cells which diffuse *in vivo* very rapidly that leads to tumour metastasis was assessed by using a number of non-adherent human CEM, JURKAT, and SUP-T1 and murine EL-4 T-cell lymphomas, as well as human BJAB, Nalm-6, and Ramos B-cell lymphomas and compared against adherent human HeLa ovarian cancer cells, as well as two adherent non-malignant cell lines: human foreskin Hs27 and murine NIH-3T3 fibroblasts. Some of these compounds were assessed against a panel of approximately 55 human tumor cell lines by the NCI. To evaluate the selective tumour toxicity of the compounds in series **1**, they were screened against a number of malignant HSC-2, HSC-3, HSC-4 and HL-60 cell lines and compared against non-malignant HGF, HPC and HPLF cell lines. The modes of action of representative compounds were investigated which included cell cycle analysis, apoptotic cell death, caspase activation, DNA fragmentation and PARP cleavage.

Author Contributions

My contributions to the *ChemMedChem* publication were: (i) undertaking a literature review of the prepared compounds, designing the synthetic chemical routes, synthesizing the compounds, determining and interpreting ^{1}H and ^{13}C NMR spectra of the compounds in series **1**, and (ii) writing the experimental section of the manuscript. The coauthors on this paper are U. Das, who supervised the synthesis of the compounds and undertook the QSAR and molecular modeling studies, A. Varela-Ramirez and C. Lema undertook the cytotoxic assays against adherent and non-adherent cancer cell lines and performed the flow cytometry analysis of the cytotoxic effect of **1a** on non-adherent cancer cell lines in order to evaluate the mode of cell death under the supervision of R. J. Aguilera, S. G. Dimmock provided the statistical analysis to establish cytotoxic synergism, E. De Clercq and J. Balzarini supervised the cytotoxic assays against human Molt 4/C8 and CEM T-lymphocytes and murine leukemic L1210 cells, J. Serly evaluated the compounds for multidrug resistance reversal activity under the supervision of J. Molnár, and the project was guided and supervised by J.R. Dimmock and D. K. J. Gorecki. The manuscript was coordinated by J. R. Dimmock.

My contributions to the *Eur. J. Med. Chem.* publication were: (i) the synthesis and characterization of the compounds in series **1**, (ii) cell cycle analysis, and (iii) writing the experimental section of the manuscript. The coauthors on this paper are U. Das, who supervised the synthesis of the compounds and undertook the QSAR and molecular modeling studies, N. Umemura, S. Iwamoto and T. Matsuta undertook the cytotoxic studies on the compounds in series **1** against some malignant and non-malignant cell lines and also performed some modes of action investigations such as caspase activation, PARP cleavage and DNA fragmentation studies on **1c** under the supervision of H. Sakagami and M. Kawase, and the project was guided and supervized by J.R. Dimmock and D. K. J. Gorecki. The manuscript was written by J. R. Dimmock with some inputs given by me.

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Bis[3,5-bis(benzylidene)-4-oxo-1-piperidinyl]amides: A Novel Class of Potent Cytotoxins

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The principal objective of this study was the examination of the theory of cytotoxic synergism. In this exploratory study, we tested the hypothesis that doubling the number of sites available for thiol alkylation in a series of candidate cytotoxins increases potency more than two-fold. This concept was verified in one-third of our comparisons using human Molt 4/C8 and CEM T-lymphocytes and murine L1210 cells. In addition, the significant potencies of various members of our compound series justified further studies. Molecular modeling revealed that relative locations of the amidic groups correlate with cytotoxicity. A potent cytotoxic compound, 1,2-bis(3,5-dibenzylidene-4-oxo-piperidin-1-yl)ethane-1,2-dione (**1a**) inhibited the growth of a large number of human tumor cell lines and displayed greater toxicity toward certain non-adherent cells than toward adherent neoplasms or fibroblasts. The mode of action of **1a** includes induction of apoptosis and necrosis.

Introduction

The principal aim of this work was the design, syntheses, and biological evaluation of conjugated styryl ketones as candidate antineoplastic agents. A number of studies have revealed that these enones react readily with thiols.[1,2] In particular, conjugated styryl ketones react only with the thiol group in a variety of compounds containing other functional groups such as amino,[3] and hydroxy groups,[4] as well as with proteins containing one or more mercapto substituents.[5] This affinity of conjugated arylidene ketones for thiols, in contrast to other functional groups present in nucleic acids, indicates that the genotoxic properties displayed by various contemporary anticancer drugs[6] should be absent in these compounds. Furthermore, a number of different proteins contain thiol groups, leading to the possibility that these compounds have multiple molecular targets. The importance of such pleiotropy has recently been discussed.[7–9] Currently, emphasis has been placed on the inclusion of a 1,5-diaryl-3-oxo-1,4-pentadienyl group (ARCH=CHCOCH=CHAR), referred to hereafter as a dienone moiety, into candidate cytotoxins. This pharmacophore presents the possibility that sequential thiol alkylation can occur with the olefinic carbon atoms. Multiple studies have revealed that an initial lowering of the concentration of cellular thiols, followed by a second chemical attack, is more detrimental to malignant cells than normal tissues.[10,11] In addition, if this interaction occurs at two different sites, the effect may be far more detrimental to the neoplasm than reaction at only one site.[12]

The aim of the present investigation was to evaluate the hypothesis of cytotoxic synergism in cancer cells, which suggests that compounds capable of multiple cellular interactions in neoplasms exert a synergistic effect. In order to probe the viability of this theory, we designed a series of compounds (**1**) containing two dienone groups (Figure 1). In this case, the inclusion of two such groups into a molecule may more than double the potency of a related compound containing only one dienone moiety. Series **1** was designed to include compounds in which the locations of the dienone groups vary in

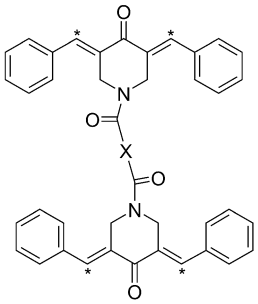

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Figure 1. General structure of series **1**. * indicates the olefinic carbon atoms that are capable of interacting with different thiol groups of a protein.

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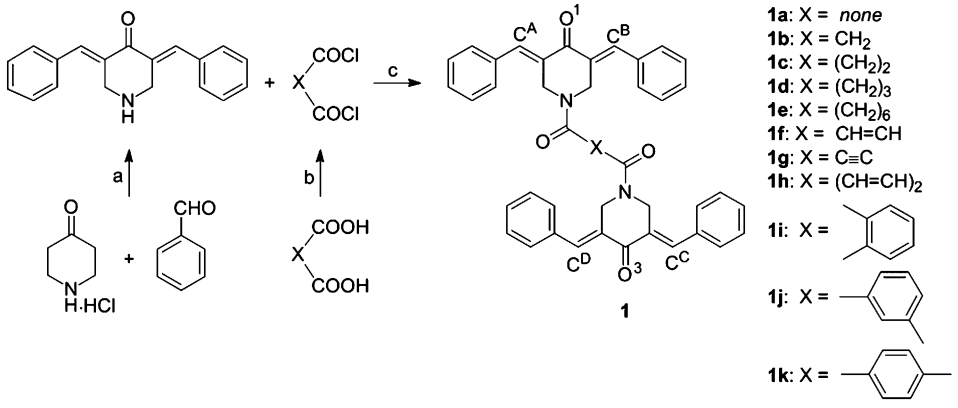
relation to each other. Molecular modeling was used as a means to determine the spatial relationship between the pharmacophores. Biological evaluations were undertaken using multiple cell lines in order to explore the generality of any trends in the relative potencies of the compounds.

Results

Compounds were synthesized using the procedure indicated in Scheme 1. All of the compounds in series **1** were evaluated against human Molt 4/C8 and CEM T-lymphocytes, as well as murine L1210 cells (Table 1). Various structural features of **1a**–**j** were examined by molecular modeling. The most active compounds, **1a** and **1b**, were evaluated against a large number of human tumor cell lines, and selected biological data from these studies is presented in Figure 4. In general, lead compound **1a** displayed greater potency toward non-adherent cells than toward either an adherent neoplasm or two fibroblasts. These results are illustrated in Figures 5 and 6 and Table 3. Cell-cycle analysis of **1a** revealed that this compound resulted in apoptosis in four neoplastic cell lines, and necrosis was also observed (Figure 7).



Scheme 1. Synthesis of series **1**. *Reagents and conditions*: a) AcOH, dry $HCl_{(g)}$, 10% aq K_2CO_3, RT, 12 h, 80%; b) $SOCl_2$, 60–65 °C, 4–5 h, 90%; c) Et_3N, ~20 °C, 12 h, 48–72%.

Discussion

^{1}H NMR spectra of **1a**–**k** indicate that the compounds are stereoisomerically pure, and absorbance of the olefinic protons in the region from 7.63–7.90 ppm indicates that the compounds have an *E* configuration.[13] In addition, X-ray crystallography of a number of 3,5-bis(benzylidene)-4-piperidones also confirmed that these compounds adopt the *E* stereochemistry.[13,14] While this study was in progress, syntheses of 2-fluoro analogues of **1a** and **1f** were described, and X-ray crystallography of the 2-fluoro analogue of **1a** showed that the four olefinic double bonds possess an *E* configuration.[15]

The compounds in series **1** were evaluated against human Molt 4/C8 and CEM T-lymphocytes in order to determine whether they demonstrate cytotoxic properties towards human transformed cells. A murine L1210 assay was employed, as a number of anticancer drugs display potency in this screen,[16] and it may, therefore, identify compounds of potential clinical value. The results presented in Table 1 show that a number of compounds in series **1** are potent cytotoxins. However, compound **1k** is virtually insoluble in multiple solvents, and the observed IC_{50} values of greater than 500 μM in the three assays is likely due to the insufficient solubility of **1k** in the media preventing penetration of the malignant cells. Hence, this compound has been removed from further discussion of the correlations between series **1** compounds and cytotoxic potencies. With regard to the IC_{50} values of **1a**–**j**, 47% are more potent than melphalan, 60% are below 5 μM, and six are in the sub-micromolar range. Of particular interest are **1a** and **1b**, which have average IC_{50} values towards Molt 4/C8 and CEM T-lymphocytes of 0.61 and 0.14 μM, respectively, and clearly emerge as lead molecules. The following compounds have IC_{50} values that indicate higher potency than melphalan, which is an alkylating agent used in cancer chemotherapy (the fold increase in relative potency as com-

Table 1. Evaluation of **1a**–**j** against Molt 4/C8, CEM, and L1210 cells, as well as a comparison of their potencies with **2**.[a]

Compd	Molt 4/C8 cells		CEM cells		L1210 cells	
	IC_{50} [μM]	RP[b]	IC_{50} [μM]	RP[b]	IC_{50} [μM]	RP[b]
1a	0.46±0.11	18	0.75±0.16	2.5	4.46±0.23	1.8
1b	0.07±0.01	115	0.20±0.17	9.3	1.23±0.38	6.5
1c	0.57±0.16	14	1.21±0.87	1.5	14.0±1.2	0.6
1d	1.61±0.05	5.0	2.03±0.48	0.9	11.5±0.3	0.7
1e	1.53±0.54	5.3	2.28±0.27	0.8	15.3±4.2	0.5
1f	4.40±2.95	1.8	7.75±0.07	0.2	28.9±2.0	0.3
1g	1.50±0.10	5.4	3.35±1.27	0.6	9.86±0.76	0.8
1h	25.1±15.8	0.3	39.2±5.4	0.1	79.0±10.2	0.1
1i	0.85±0.41	9.5	1.45±0.85	1.3	4.19±2.19	1.9
1j	12.5±1.8	0.7	36.0±7.6	0.1	150±65	0.1
2	8.07±0.45	–	1.86±0.08	–	7.97±0.75	–
Melphalan	3.24±0.79	–	2.47±0.30	–	2.13±0.03	–

[a] IC_{50} values were determined using a literature procedure and are the average of three independent experiments ±SD. [24] Cells were exposed to the compounds for 3 d (Molt 4/C8 and CEM screens) or 2 d (L1210 assay). [b] Relative potencies (RP) were obtained by dividing the IC_{50} values of **2** by the values for each of the compounds in series **1**.

pared with melphalan is given in parentheses): **1a** (7.0), **1b** (44), **1c** (5.7), **1d** (2.1), **1g** (2.2), and **1i** (3.8) in the Molt 4/C8 screen; **1a** (3.3), **1b** (12), and **1c** (2.0) in the CEM test; **1b** (1.7) towards L1210 cells.

For **1a–j**, IC_{50} values are lowest in the Molt 4/C8 screen and highest in the L1210 assay. This observation is confirmed by average IC_{50} values for **1a–j** in the Molt 4/C8, CEM, and L1210 tests of 4.86, 9.42, and 31.8 μM, respectively (Table 1). Variations in potency were displayed by each compound **1a–j** towards Molt 4/C8, CEM, and L1210 cells. For example, Molt 4/C8 T-lymphocytes are 25-times more sensitive to treatment with **1c** than are L1210 cells, and this differential toxicity may be also be observed between malignant and non-malignant cells, leading to greater adverse effects toward neoplasms.

The following observations were made pertaining to the effect of the nature of the spacer group (X in structure **1**) on average IC_{50} values of **1a–j** towards T-lymphocytes (these values are given in parentheses). The most potent compound is **1b** (0.14 μM) which has a single methylene group spacer. Removal of the spacer, leading to **1a** (0.61 μM), or insertion of additional methylene groups to **1b**, giving rise to **1c** (0.89 μM), **1d** (1.82 μM), and **1e** (1.91 μM), led to reduced potencies. A comparison of IC_{50} values for three compounds which have a two carbon atom spacer showed that **1f** (6.08 μM) and **1g** (2.43 μM) are less potent than **1c** (0.89). The addition of a further olefinic linkage to **1f** (6.08 μM), creating **1h** (32.2 μM), reduced potency five-fold. Upon introduction of an aryl ring spacer, the relative location of the substituents influences potency considerably, as observed by the substantial difference in IC_{50} values for **1c** (1.15 μM) and **1j** (24.3 μM).

If the hypothesis is valid that synergism occurs when each compound interacts at a different binding site, then one would expect to observe similar relative potencies using various biological assays. In order to evaluate this possibility, Kendall's coefficient of concordance[17] was applied to data generated in our three assays. Equation (1) used in this determination is given below, where W is Kendall's coefficient of concordance, i denotes the individual compound ($i=1$ for **1a**, $i=2$ for **1b**, etc.), n is the number of compounds, R_i is the average rank given to cell line i, m is the number of cell lines, T is the correction factor for ties and j refers to the individual cell line. Kendall's coefficient of concordance is a test for assessing the similarity of rankings. If the relative potency rank for each assay is identical, Kendall's coefficient of concordance will be 1. If there is no agreement in rankings, Kendall's coefficient of concordance will be zero. The coefficient of concordance for our biological data was found to be 0.924 ($p=0.03$), providing very strong evidence that the relative potency rankings are similar across the assays. One may conclude, therefore, that despite the difference in sensitivity of the three cell lines to **1a–j**, the molecular shapes may influence cytotoxic potencies in a similar fashion.

$$W = \frac{12\sum_{i=1}^{n}\left(R_i^2\right) - 3m^2n(n+1)^2}{m^2n(n^2-1) - m\sum_{j=1}^{m}\left(T_j\right)} \quad (1)$$

The hypothesis that synergism may occur with the presence of two dienone groups was examined by comparing the IC_{50} values of **1a–j** with that of piperidone **2**. This dienone was chosen because, like the compounds in series **1**, **2** is a 1-acyl-3,5-bis(benzylidene)-4-piperidone. Additionally, the hydrophobic 1-tetradecanoyl group ensures that **2** resembles series **1** with regard to their markedly lipophilic nature. For example, the log*P* values of **1e** and **2** are 8.58 and 9.02, respectively. If the hypothesis of cytotoxic synergism is valid, then the cytotoxic potencies of **1a–j** should be greater than twice the figures generated for **2**. Therefore, the IC_{50} value of **2** was divided by the corresponding value for each of analogues **1a–j** in the Molt 4/C8, CEM, and L1210 assays to give the relative potency (RP) figures (Table 1). RP values greater than two were obtained for **1a–e, g**, and **i** in the Molt 4/C8 screen, **1a** and **b** in the CEM assay, and **1b** in the L1210 test, that is, in one-third of the comparisons made. The RP figures for the Molt 4/C8 screen are particularly encouraging, and these results warrant further evaluation of the hypothesis.

Previous studies by our group involved the evaluation of several series of structurally related 1-acyl-3,5-bis(benzylidene)-4-piperidones for cytotoxic properties, including assessment using Molt 4/C8, CEM, and L1210 cells. Series **3–5** have, respectively, aroyl,[18] acryloyl,[13] and phosphono[19] groups attached to the nitrogen atom (Figure 2). In general, series **1** exhibits

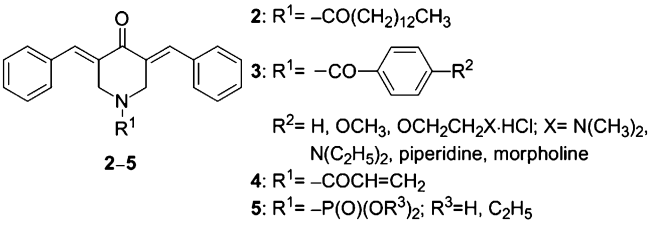


Figure 2. Structures of compounds in series **2–5**.

slightly weaker activities than **3–5**; however, removal of the two outliers (**1h** and **1j**) shows that the observed IC_{50} values for the remaining members of the series are comparable to series **3–5** (Table 2).

Further evaluation of the biological data presented in Table 1 was undertaken in order to examine the theory that the topography of **1a–j** controls cytotoxicity. While a substantial number of interatomic distances as well as various bond

Table 2. Potencies of series **1** compounds as compared to series **3–5**.

Compd	IC_{50}[a] [μM] Molt 4/C8	CEM	L1210
3	2.64	2.92	49.8
4[b]	1.42	1.48	8.69
5	0.91	1.70	7.33
1a–j	4.86	9.42	31.8
1a–g,i	1.37	2.38	11.2

[a] Values shown are the average of three independent experiments. [b] Data reported previously.[13]

and torsion angles could be determined, we focused primarily on the relative positions of the olefinic carbon atoms of both pharmacophores, followed by the piperidyl nitrogen atoms and the spacer group oxygen atoms. The generated figures are presented in table S1 of the Supporting Information.

The relative positions of the olefinic carbon atoms in both pharmacophoric groups were determined as follows: The four olefinic carbon atoms were designated C^A, C^B, C^C, and C^D (Figure 3a). Axes 1 and 2 were constructed (Figure 3b), and d_1, d_2, and ψ were measured. Linear and semi-logarithmic plots were generated using the average IC_{50} values of **1a–j** towards Molt 4/C8 and CEM T-lymphocytes and their d_1, d_2, and ψ data. No correlations ($p > 0.05$) nor trends to significance ($p > 0.1$) were found, although a negative trend to significance was nearly attained when using ψ values ($p = 0.11$). Thus, the possibility exists that the preparation of analogues of series **1** in which ψ values are increased may lead to molecules with greater cytotoxicity. Linear and semi-logarithmic plots were constructed using the average IC_{50} values of **1a–j** towards the two T-lymphocyte cell lines and the interatomic distances d_3 (N1—N2), d_4 (N1—O2) and d_5 (O2—O4) (Figure 3c). Positive correlations were noted between the IC_{50} values and d_3 ($p = 0.04$), d_4 ($p = 0.04$), and d_5 ($p = 0.02$), indicating that potency increases as the d_3–d_5 spans diminish. Expansion of this project should involve the design of molecules in which the spacer group (X) is either small or eliminated entirely. In addition, these results highlight the importance of the proximity of the piperidyl nitrogen atoms to the oxygen atoms. Further experimentation should be pursued, such as incorporating the CO—X—CO group into rigid heterocyclic rings with the goal of finding the optimal topography of these molecules to maximize cytotoxic properties.

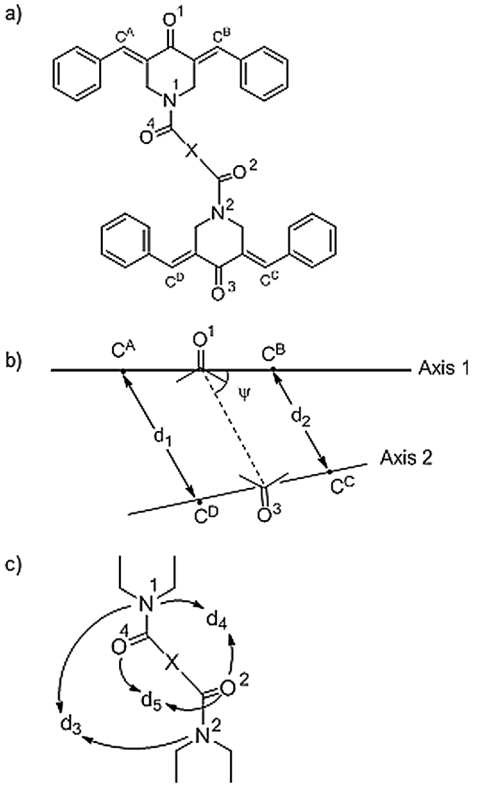


Figure 3. Various structural features of **1a–j** as determined by molecular modeling: a) olefinic carbon atoms C^A–C^D, piperidyl nitrogen atoms N1 and N2, and carbonyl oxygen atoms O1–O4; b) interatomic distances d_1 and d_2, and bond angle ψ; and c) interatomic distances d_3–d_5.

In light of the encouraging biological data displayed by **1a–g**, and **i**, we undertook further evaluations. First, it was necessary to select a lead compound. As shown in Table 1, both **1a** and **1b** exhibited sub-micromolar IC_{50} values toward human cancer cells. These molecules were, therefore, evaluated against a substantial number of human tumor cell lines,[20] with the cytotoxic effect of **1a** and **1b** against some of these neoplasms presented in Figure 4. The data presented shows that **1a** generally displays lower IC_{50} values than **1b**; consequently, **1a** was chosen for further studies. An additional noteworthy feature of **1a** and **1b** is the differential potencies both compounds display towards the cell lines. This observation strengthens the view expressed earlier that these compounds and analogues in series **1** may possess greater toxicity towards neoplasms than normal cells.

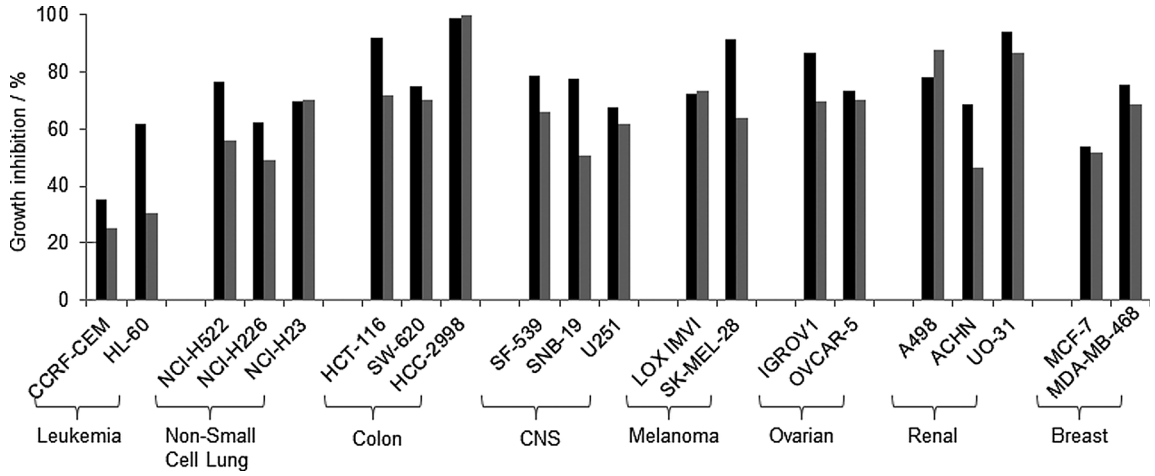


Figure 4. Growth inhibitory effect of 10 μM **1a** or **1b** toward a number of human tumor cell lines. Cells were exposed to **1a** (■) and **1b** (■) for 48 h using a previously described procedure.[20]

The ability of non-adherent cells to diffuse in vivo, leading to metastasis, led us to next address the discovery of novel prototypic molecules that inhibit the growth of non-adherent neoplasms. The potential of such compounds would be enhanced even further if greater toxicity could be demonstrated towards non-adherent neoplasms over either adherent or non-malignant cells. Compound **1a** was examined against the following non-adherent cell lines: human CEM, JURKAT, and SUP-T1 and murine EL-4 T-cell lymphomas, as well as human BJAB, Nalm-6, and Ramos B-cell lymphomas. This compound was also assessed against adherent human HeLa ovarian cancer cells, as well as two adherent non-malignant cell lines: human foreskin Hs27 and murine N1H-3T3 fibroblasts.

Figure 5 shows that, in general, greater toxicity was demonstrated toward non-adherent cells than toward either the adherent HeLa cell line or the two fibroblasts. CC_{50} values were

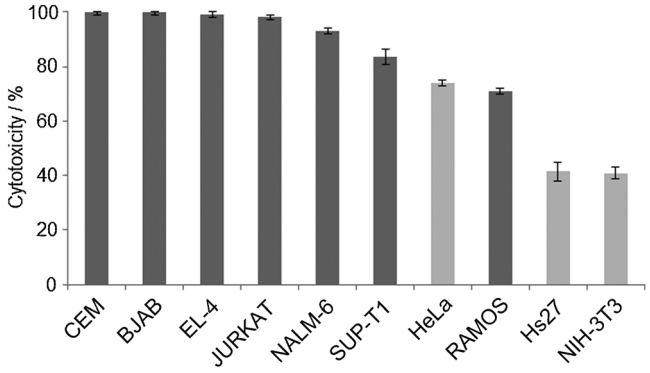


Figure 5. Cytocidal effects of **1a** (5 μM) as determined by flow cytometry using a previously reported method.[25] Cells are non-adherent with the exception of the adherent HeLa, Hs27, and N1H-3T3 cell lines. Each bar represents the average value of triple measurements with error bars showing standard deviations. Cells were exposed to **1a** for 22 h.

also determined in order to garner an appreciation of the differential toxicity between several non-adherent cells and the adherent NIH-3T3 fibroblast (Table 3). Selectivity index values are impressive and establish **1a** as an important lead molecule. Further experimentation was initiated to determine whether higher toxicity toward non-adherent over adherent cell lines is a common property of the more potent compounds in series **1**. Accordingly, **1b**–**g**, and **i** were evaluated against JURKAT and SUP-T1 non-adherent cells, as well as against normal (Hs27) and malignant (HeLa) adherent cell lines (Figure 6). The various

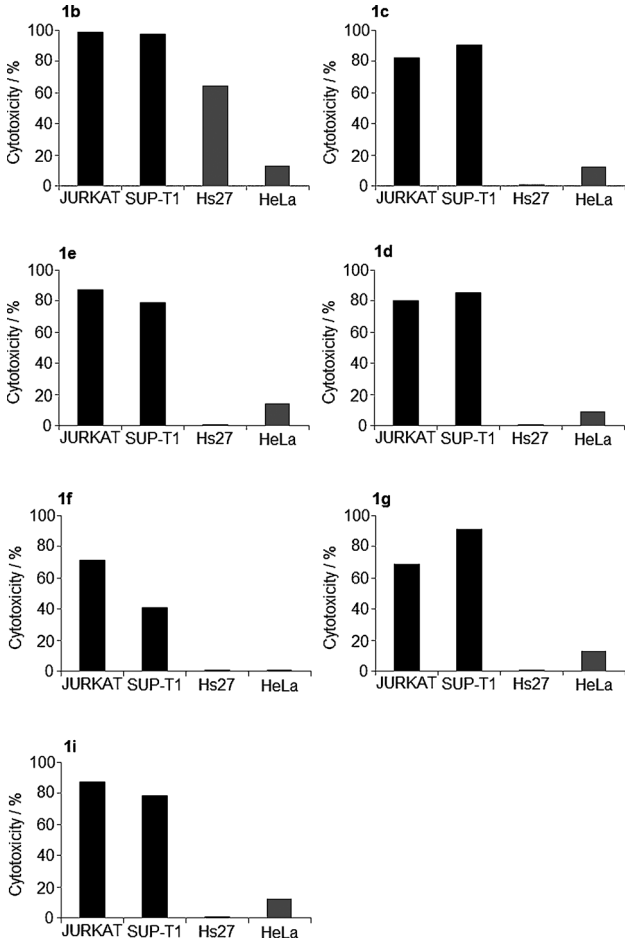


Figure 6. Evaluation of **1b**–**g**, and **1i** against non-adherent (JURKAT, SUP-T1) and adherent (Hs27, HeLa) cells. Concentrations of compounds used were 2.5 μM (**1f**, **g**, and **i**), 5 μM (**1e**), and 10 μM (**1b**–**d**). Cells were exposed to compounds for 22 h as previously described.[25]

concentrations of **1b**–**g** and **i** were chosen to emphasize the consistently greater cytotoxicity of these compounds in adherent versus non-adherent cells. The results provide unequivocal evidence that **1b**–**g** and **i**, similar to **1a**, display preferential toxicity against non-adherent cells. This observation strengthens the need for development of these compounds, which display antimetastatic potential in addition to their cytotoxic properties.

Flow cytometry was pursued, using four non-adherent cell lines, in order to gain an understanding of the ways in which **1a** mediates its cytotoxic properties (Figure 7). After 8 h incubation, an average of 19% of the cells were apoptotic, with this percentage doubling after 20 h. Necrosis was virtually absent after 8 h, while on an average of 9% of the cells were necrotic after 20 h. One may therefore conclude that **1a** causes cell death inter alia by apoptosis and, to a lesser

Table 3. Evaluation of **1a** against non-adherent cell lines and NIH-3T3 fibroblasts.

Cell line	CC_{50}[a] [μM]	SI[b]
CEM	0.034	376
EL-4	0.031	412
JURKAT	0.029	441
Nalm6	0.022	581
Sup-T1	0.384	33
NIH-3T3	12.78	–

[a] Cells were incubated with **1a** for 22 h as previously described.[25] Values shown are the average of three independent experiments. [b] Selectivity index (SI) figures are quotients of the CC_{50} values of **1a** toward NIH-3T3 fibroblasts and the data for each of the non-adherent cell lines.

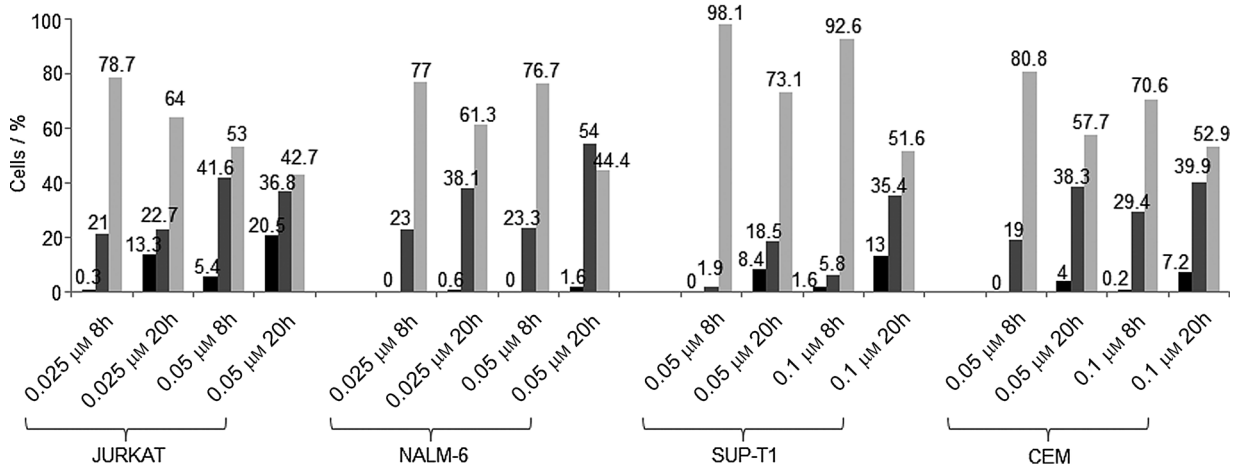


Figure 7. Flow cytometry analysis of the cytotoxic effect of **1a** on four non-adherent cell lines following incubation for 8 and 24 h.[25] The exact percentage of apoptotic (■), necrotic (■), and viable (■) cells is indicated at the top of each bar graph. Two concentrations of **1a** are shown on the x-axis. Note that two-fold higher concentrations were utilized for the most resistant cell lines, SUP-T and CEM.

degree, by necrosis. In a previous study, a cytotoxic 1-acyl-3,5-bis(benzylidene)-4-piperidone activated caspase-3, with the extent of activation dependent on the cell line under investigation.[21] Since caspases are involved in most apoptosis, it is likely that one mechanism by which **1a–j** may exert their cytotoxic properties is the activation of one or more caspases.

Conclusions

This study reveals that the unsaturated ketones in series **1** are, in general, potent cytotoxins. The theory that increasing the number of sites for thiol alkylation two-fold should more than double cytotoxic potency was validated in approximately half of the biological data generated using Molt 4/C8 and CEM T-lymphocytes, although there was little support when utilizing the murine L1210 screen. Extensions of this study should consider the design and evaluation of analogues of **1a–j** that contain more than two identical phamacophores. In addition, the synthesis of heterodimers should be pursued in which different substituents are appended to the aryl rings, thereby creating molecules with varying atomic charges on the olefinic carbon atoms. For compounds such as these, stepwise reactions with thiols should be enhanced, which may lead to greater increases in toxicity to neoplasms than to normal cells.[10,11] Molecular modeling was also used to emphasize the importance of the relative positions of the amide groups.

From this initial investigation, **1a** and **1b** emerged as lead molecules, displaying potent cytotoxicity against a wide range of human tumor cell lines. Further evaluations using **1a** revealed its increased toxicity toward non-adherent cell lines than toward either an adherent neoplasm or fibroblasts. Compound **1a** was shown to exert its toxic effects against certain cancer cells through apoptosis and necrosis. Sufficient evidence has been presented through these studies to warrant rapid expansion of evaluation of this compound series in order to further investigate their potential as candidate anticancer agents.

Experimental Section

Chemistry

Synthesis of 1a–k: Melting points were determined on a Gallenkamp instrument and are uncorrected. ^{1}H and ^{13}C NMR spectra were obtained using a Bruker Avance AMX 500 spectrometer equipped with a BBO probe. Chemical shifts (δ) are reported in ppm. Mass spectra were obtained using a quad tandem 4000 QTRAP mass analyzer. Elemental analyses were undertaken using a CHNS elemental analyzer (Vario EL III microanalyzer).

General procedure for the synthesis of 3,5-bis(benzylidene)-4-piperidone dimers (1a–k): A mixture of the corresponding dicarboxylic acid (0.005 mol) and thionyl chloride (0.02 mol, 2.4 g) was heated at 60–65 °C for 4–5 h. Excess thionyl chloride was removed at 45 °C in vacuo and moisture-free conditions. The resulting acid chloride was used for further reaction without purification.

The previously prepared acid chloride in 1,2-dichloroethane (DCE; 5 mL) was added slowly over a period of 30 min to a stirred suspension of 3,5-bis(benzylidene)-4-piperidone (0.009 mol, 2.75 g) prepared according to a literature method[13] in DCE (20 mL) containing Et_3N (0.11 mol, 1.12 g) at ≈20 °C. The reaction stirred at RT overnight, then the solvent was removed in vacuo at 45 °C. Aq K_2CO_3 (25 mL, 10% *w/v*) was added to the crude material and stirred for 2 h. The resulting solid was filtered, dried, and crystallized from a suitable solvent to yield pure product. In the case of **1a**, **b**, **d**, and **e**, the appropriate acid chlorides were procured from commercial sources.

1,2-Bis(3,5-dibenzylidene-4-oxo-piperidin-1-yl)ethane-1,2-dione (1a): Yield: 62%; mp: 246 °C ($CHCl_3$/MeOH); ^{1}H NMR (500 MHz, [D_6]DMSO): δ = 7.72 (s, 2H, 2×=CH), 7.56 (s, 2H, 2×=CH), 7.53 (t, 4H, Ar-H), 7.49 (d, J = 7.07 Hz, 2H, Ar-H), 7.45 (m, 6H, Ar-H), 7.39 (m, 8H, Ar-H), 4.48 ppm (d, J = 23.28 Hz, 8H, 4×NCH_2); ^{13}C NMR (125 MHz, [D_6]DMSO): δ = 184.7, 162.6, 137.9, 137.5, 134.4, 134.1, 131.4, 131.0, 130.9, 130.7, 130.2, 130.1, 129.3, 129.2, 46.4, 41.6 ppm; MS (ESI) *m/z*: 627 $[M+Na]^+$; Anal. calcd for $C_{40}H_{32}N_2O_4 \cdot H_2O$: C 77.17; H 5.14; N 4.50, found: C 77.05; H 4.87; N 4.42.

1,3-Bis-(3,5-dibenzylidene-4-oxo-piperidin-1yl)propane-1,3-dione (1b): Yield: 65%; mp: 201 °C (acetone); ^{1}H NMR (500 MHz, [D_6]DMSO): δ = 7.72 (s, 2H, 2×=CH), 7.57 (s, 2H, 2×=CH), 7.53 (d,

J=4.18 Hz, 8H, Ar-H), 7.47 (m, 12H, Ar-H), 4.62 (d, J=21.13 Hz, 8H, 4×NCH_2), 3.46 ppm (s, 2H, CH_2); ^{13}C NMR (125 MHz, [D_6]DMSO): δ=186.3, 165.9,136.6, 136.5, 134.7, 134.5, 132.6, 132.5, 131.0, 130.9, 130.1, 130.0, 129.3, 129.2, 47.0, 42.4 ppm; MS (ESI) m/z: 641 [M+Na]$^+$; Anal. calcd for $C_{41}H_{34}N_2O_4{\cdot}H_2O$: C 77.27; H 5.65; N 4.39, found: C 77.31; H 5.50; N 4.47.

1,4-Bis-(3,5-dibenzylidene-4-oxo-piperidin-1-yl)butane-1,4-dione (1c): Yield: 58%; mp: 188 °C ($CHCl_3$/MeOH); ^1H NMR (500 MHz, [D_6]DMSO): δ=7.68 (s, 4H, 4×=CH), 7.49 (m, 20H, Ar-H), 4.78 (d, J=10.95 Hz, 8H, 4×NCH_2), 2.29 ppm (s, 4H, 2×CH_2); ^{13}C NMR (125 MHz, [D_6]DMSO): δ=186.5, 170.4, 136.6, 136.5, 134.8, 134.5, 133.0, 132.8, 131.0, 130.0, 129.3, 46.3, 42.9, 27.16 ppm; MS (ESI) m/z: 655 [M+Na]$^+$; Anal. calcd for $C_{42}H_{36}N_2O_4{\cdot}0.5H_2O$: C 78.53; H 5.76; N 4.36, found: C 78.16; H 5.71; N 4.11.

1,5-Bis-(3,5-dibenzylidene-4-oxo-piperidin-1-yl)pentane-1,5-dione (1d): Yield: 43%; mp: 170 °C ($CHCl_3$/MeOH); ^1H NMR (500 MHz, [D_6]DMSO): δ=7.71 (s, 2H, 2×=CH), 7.66 (s, 2H, 2×=CH), 7.54 (m, 14H, Ar-H), 7.42 (m, 6H, Ar-H), 4.76 (d, 8H, 4×NCH_2, J=28.18 Hz), 2.02 (t, 4H, 2×CH_2), 1.42 ppm (p, 2H, CH_2); ^{13}C NMR (125 MHz, [D_6]DMSO): δ=186.6, 171.0, 136.6, 134.8, 134.5, 133.1, 133.0, 131.0, 130.3, 129.3, 46.4, 42.8, 31.3, 20.4 ppm; MS (ESI) m/z: 627 [M+Na]$^+$; Anal.calcd for $C_{43}H_{38}N_2O_4{\cdot}0.25H_2O$: C 79.22; H 5.91; N 4.30, found: C 79.18; H 5.64; N 4.08.

1,8-Bis-(3,5-dibenzylidene-4-oxo-piperidin-1-yl)octane-1,8-dione (1e): Yield: 64%; mp: 160 °C ($CHCl_3$/MeOH); ^1H NMR (500 MHz, [D_6]DMSO): δ=7.71 (s, 4H, 4×=CH), 7.51 (m, 20H, Ar-H), 4.81 (d, 8H, J=29.13 Hz, 4×NCH_2), 2.00 (t, 4H, 2×CH_2), 1.20 (m, 4H, 2×CH_2), 0.76 ppm (m, 4H, 2×CH_2); ^{13}C NMR (125 MHz, [D_6]DMSO): δ=186.6, 171.4, 136.8, 136.4, 134.8, 134.6, 133.2, 131.0, 130.0, 129.3, 46.5, 43.1, 32.4, 28.5, 24.7 ppm; MS (ESI) m/z: 711 [M+Na]$^+$; Anal.calcd for $C_{46}H_{44}N_2O_4{\cdot}0.5H_2O$: C 79.10; H 6.30; N 4.01, found: C 78.85; H 6.32; N 4.04.

1,4-Bis-(3,5-dibenzylidene-4-oxo-piperidin-1-yl)but-2-ene-1,4-dione (1f): Yield: 71%; mp: 220 °C (EtOH); ^1H NMR (500 MHz, [D_6]DMSO): δ=7.74 (s, 2H, 2×=CH), 7.66 (s, 2H, 2×=CH), 7.51 (m, 20H, Ar-H), 6.92 (s, 2H, 2×=CH), 4.83 ppm (d, J=8.54 Hz, 8H, 4×NCH_2); ^{13}C NMR (125 MHz, [D_6]DMSO): δ=186.2, 163.9, 136.9, 136.7, 134.7, 134.3, 132.6, 131.0, 130.1, 129.3, 46.9, 43.1 ppm; MS (ESI) m/z: 653 [M+Na]$^+$; Anal.calcd for $C_{42}H_{34}N_2O_4{\cdot}5H_2O$: C 69.92; H 4.71; N 3.88, found: C 69.85; H 4.79; N 3.57.

1,4-Bis-(3,5-dibezylidene-4-oxo-piperidin-1-yl)-but-2-yne-1,4-dione (1g): Yield: 48%; mp: 220 °C ($CHCl_3$/MeOH; dec.); ^1H NMR (500 MHz, [D_6]DMSO): δ=7.81 (s, 1H, =CH), 7.75 (d, 2H, 2×=CH, J=17.63 Hz), 7.68 (s,1H, =CH), 7.56 (m,20H, Ar-H), 4.79 (d, J=18.80 Hz, 4H, 2×NCH_2), 4.64 ppm (d, J=16.96 Hz, 4H, 2×NCH_2); ^{13}C NMR (125 MHz, [D_6]DMSO): δ=186.0, 185.7, 185.4, 162.4, 161.9, 149.9, 137.2, 134.5, 132.3, 131.8, 131.5, 131.0, 130.7, 130.4, 129.1, 128.7, 125.25, 95.25, 46.8, 42.7, 42.2 ppm; MS (ESI) m/z: 651 [M+Na]$^+$; Anal.calcd for $C_{42}H_{32}N_2O_4{\cdot}2H_2O$: C 75.82; H 4.81; N 4.21, found: C 75.48; H 4.74; N 4.01.

1,6-Bis-(3,5-dibenzylidene-4-oxo-piperidin-1-yl)-hexa-2,4-diene-1,6-dione (1h): Yield: 56%; mp: 199 °C ($CHCl_3$/MeOH); ^1H NMR (500 MHz, $CDCl_3$): δ=7.87 (s, 4H, 4×=CH), 7.42 (m, 20H, Ar-H), 6.94 (m, 2H, 2×=CH), 6.16 (m, 2H, 2×=CH), 4.98 (s, 4H, 2×NCH_2), 4.77 ppm (s, 4H, 2×NCH_2); ^{13}C NMR (125 MHz, [D_6]DMSO): δ=186.6, 164.7, 140.3, 137.5, 134.5, 131.5, 130.6, 130.0, 125.9, 125.6, 46.3, 44.1 ppm; MS (ESI) m/z: 679 [M+Na]$^+$; Anal.calcd for $C_{44}H_{36}N_2O_4{\cdot}2.5H_2O$: C 75.23; H 5.12; N 3.98, found: C 75.23; H 5.02; N 3.91.

1,2-Bis-[(3,5-dibenzylidene-4-oxo-piperidin-1-yl)-1-carbonyl]benzene (1i): Yield: 68%; mp: 240 °C ($CHCl_3$/MeOH; dec.); ^1H NMR (500 MHz, [D_6]DMSO): δ=7.77 (s, 2H, 2×=CH), 7.69 (s, 2H, 2×=CH), 7.57 (t, 10H, Ar-H), 7.27 (brs, 5H, Ar-H), 7.18 (brs, 5H, Ar-H), 6.89 (m, 2H, Ar-H), 6.78 (m, 2H, Ar-H), 4.92 (brs, 4H, 2×NCH_2), 4.49 ppm (s, 4H, 2×NCH_2); ^{13}C NMR (125 MHz, [D_6]DMSO): δ=186.1, 167.9, 137.3, 136.5, 134.8, 133.8, 132.6, 131.1, 130.3, 129.8, 129.4, 129.0, 126.4, 47.8, 43.4 ppm; MS (ESI) m/z: 703 [M+Na]$^+$; Anal.calcd for $C_{46}H_{36}N_2O_4{\cdot}H_2O$: C 78.99; H 5.43; N 4.0, found: C 79.09; H 5.42; N 4.02.

1,3-Bis-[(3,5-dibenzylidene-4-oxo-piperidin-1-yl)-1-carbonyl]benzene (1j): Yield: 63%; mp: 220 °C ($CHCl_3$/MeOH; dec.); ^1H NMR (500 MHz, [D_6]DMSO): δ=7.78 (s, 4H, 4×=CH), 7.53 (m, 10H, Ar-H), 7.31 (m, 10H, Ar-H), 7.11 (s, 1H, Ar-H), 7.06 (d, J=8.93 Hz, 2H, Ar-H), 6.75 (t,1H, Ar-H), 4.97 (brs, 4H, 2×NCH_2), 4.57 ppm (brs, 4H, 2×NCH_2); ^{13}C NMR (125 MHz, [D_6]DMSO): δ=186.03, 168.0, 137.2, 134.51, 132.6, 131.0, 130.1, 129.2, 128.9, 128.3, 125.7, 48.9, 45.6 ppm; MS (ESI) m/z: 703 [M+Na]$^+$; Anal.calcd for $C_{46}H_{36}N_2O_4{\cdot}0.5H_2O$: C 80.02; H 5.36; N 4.01, found: C 80.31; H 5.29; N 4.01.

1,4-Bis-[(3,5-dibenzylidene-4-oxo-piperidin-1-yl)-1-carbonyl]benzene (1k): Yield: 72%; mp: 220 °C (AcOH/H_2O; dec.); ^1H NMR (500 MHz, CF_3COOD): δ=10.44 (s, 2H, 2×=CH), 10.38 (s, 2H, 2×=CH), 9.81 (d, J=8.4 Hz, 10H, Ar-H), 9.63 (brs, 6H, Ar-H), 9.49 (brs, 4H, Ar-H), 9.16 (s, 4H, Ar-H), 7.47 (s, 4H, 2×NCH_2), 6.86 ppm (s, 4H, 2×NCH_2); ^{13}C NMR (125 MHz, CF_3COOD): δ=192.9, 174.1, 146.6, 144.2, 135.9, 135.4, 135.3, 133.4, 133.0, 132.6, 132.2, 132.0, 131.0, 130.9, 130.8, 129.0, 49.5, 47.12 ppm; Anal. calcd for $C_{46}H_{36}N_2O_4{\cdot}0.5H_2O$: C 80.02; H 5.36; N 4.01, found: C 79.67; H 5.25; N 3.99.

3,5-Bis(benzylidene)-1-tetradecanoyl-4-piperidone (2): Myristoyl chloride (0.011 mol, 2.7 g) in DCE (5 mL) was added slowly over a period of ≈30 min to a suspension of 3,5-bis(benzylidene)-4-piperidone (0.007 mol, 2 g), prepared according to a literature method,[13] in DCE (15 mL) containing Et_3N (0.016 mol, 1.7 gm) at ≈15 °C. The reaction stirred at RT overnight, then the solvent was removed in vacuo at 45 °C. Aq K_2CO_3 (25 mL, 10% w/v) was added to the crude, and the mixture was stirred for 2 h. The resulting solid was filtered, dried, and crystallized from EtOH. Yield: 90%; mp: 82 °C; ^1H NMR (500 MHz, $CDCl_3$): δ=7.91 (s, 1H, =CH), 7.85 (s, 1H, =CH), 7.53–7.40 (m, 10H, Ar-H), 4.96 (s, 2H, NCH_2), 4.87 (s, 2H, NCH_2), 2.14 (t, 2H, $COCH_2$), 1.45 (p, 2H, CH_2), 1.26 (m, 16H, Ar-H), 1.09 (m, 4H, 2×CH_2), 0.91 ppm (t, 3H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$): δ=186.9, 172.1, 138.6, 137.1, 134.7, 134.6, 132.1, 131.9, 130.7, 130.1, 129.6, 128.9, 128.8, 46.3, 43.6, 33.2, 32.0, 29.7, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 25.1, 22.7, 14.2 ppm; Anal.calcd for $C_{31}H_{39}NO_2$: C 81.60; H 8.92; N 2.88, found: C 81.27; H 9.29; N 2.83.

Computational experiments

Molecular modeling: Models were built using the SYBYL 8.0 program[22] on a Lenovo workstation with the RHEL 4.0 operating system. Energy minimizations were performed with the conjugate gradient method using the Tripos force field and Gasteiger–Huckel charges with a convergence criterion of 0.001 kcal mol^{-1} Å. Each structure was further subjected to simulated annealing for identifying the lowest energy conformation. The system was heated at 1000 K for 1 ps, and then cooled at 200 K for 1 ps. The exponential annealing function was used, and ten such cycles were run. The lowest energy conformer was used to calculate the distance between two points and bond angles as depicted in Figure 3.

Determination of log P values: The predicted log *P* values for **1e** and **2** were obtained using Molinspiration Chemoinformatics software online.[23]

Biology

Cytotoxicity assays: The compounds in series **1** were evaluated against Molt 4/C8, CEM, and L1210 cells using a previously reported procedure.[24] Briefly, various concentrations of the compounds were incubated with the appropriate cell line in RPMI 1640 medium at 37 °C for 72 h (Molt 4/C8 and CEM assays) or 48 h (L1210 screen). Numbers of cells were determined using a Coulter counter. The IC_{50} value given is the concentration required to inhibit cell proliferation by 50%. Data are expressed as the mean ± SD from the dose–response curves of at least three independent experiments.

Compounds **1a** and **1b** were examined by the US National Cancer Institute against 52 and 59 human tumor cell lines, respectively, as previously described.[20] Solutions of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M of **1a** or **1b** were added to the cells, which were grown in RPMI 1640 medium containing 5% fetal bovine serum (FBS) and 2 mM L-glutamine. After incubation at 37 °C in an atmosphere of air (95%), carbon dioxide (5%), and relative humidity of 100% for 48 h, the concentration required to inhibit growth by 50% was determined spectrophotometrically using sulforhodamine B. Compound **1a** was evaluated at a concentration of 5 μM against CEM, BJAB, EL-4, JURKAT, Nalm-6, SUP-T1, HeLa, Ramos, Hs27, and N1H-3T3 cell lines essentially as previously described.[25] Briefly, a solution of **1a** in DMSO was added to cells grown in RPMI (CEM, BJAB, EL-4, JURKAT, Nalm6, SUP-T1 and Ramos) or DMEM (for HeLa, Hs27 and N1H-3T3) media, followed by incubation for 22 h at 37 °C. The average cytotoxicity of three independent experiments, expressed as a percentage, was obtained by noting the disruption of the plasma membrane using flow cytometry with propidium iodide as described previously.[25] In cases where 100% cytotoxicity is indicated, no cell viability was observed. Several of the 4-piperidones were also examined against JURKAT, SUP-T1, Hs27 and HeLa cells (concentration used, in μM, shown in parentheses): **1b** (10), **1c** (10), **1d** (10), **1e** (5), **1f** (2.5), **1g** (2.5), and **1i** (2.5). Using a range of concentrations of **1a**, we also obtained CC_{50} values of this compound towards JURKAT, SUP-T1, CEM, EL-4, Nalm6, and N1H-3T3 as previously described.[25]

*Flow cytometry analysis of **1a***: Compound **1a** was incubated with JURKAT, Nalm-6, SUP-T1, or CEM cells in RPMI media. After 8 and 20 h, the percentage of apoptotic, necrotic, and viable cells were determined by flow cytometry using Annexin-V-FITC and propidium iodide.[26]

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Original article

Dimeric 3,5-bis(benzylidene)-4-piperidones: A novel cluster of tumour-selective cytotoxins possessing multidrug-resistant properties

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ABSTRACT

A series of bis[3,5-bis(benzylidene)-4-oxo-1-piperidinyl]amides **1** display potent cytotoxic properties towards a wide range of tumours. A number of the CC_{50} and IC_{50} values are in the range of 10^{-8} M. Specifically, these compounds have the following important properties. First, greater toxicity was demonstrated towards certain tumours than various non-malignant cells. Second, various compounds in series **1** are toxic to a number of human colon cancer and leukaemic cells. Third, these compounds reverse P-gp mediated multidrug resistance. Various prototypic molecules such as **1a**,**b** and **1i** were identified as lead molecules for further studies. A representative lead molecule **1b** induces apoptosis via internucleosomal DNA fragmentation and PARP cleavage in HSC-2 and HL-60 cells while flow cytometry revealed that this compound blocked the G2/M and S-phases in the cell cycle of human colon cancer HCT-116 cells.

1. Introduction

The principal aim of this laboratory is the synthesis of anti-neoplastic conjugated unsaturated ketones which are designed as thiol alkylators [1,2]. One of the reasons for the interest in these compounds is that α,β-unsaturated ketones often react readily with thiols but have little or no affinity for hydroxyl and amino groups [3,4]; these latter functionalities are found in nucleic acids. Hence the genotoxic effects associated with a number of alkylating agents used in cancer chemotherapy [5] may be avoided. Furthermore, one or more thiol groups are found in a variety of cellular constituents such as thioredoxin, glutathione and cysteine. Thus thiol alkylators have the potential to be multitargeted ligands and the perceived importance of such pleiotropy has been documented recently [1,6–8].

Initially a number of cytotoxins were designed possessing a single 1-oxo-2-propenyl group (–CH=CH–CO–) [9,10]. However a number of studies revealed that an initial lowering of the concentrations of cellular thiols followed by a second chemical attack was more detrimental to various neoplasms than normal cells [11,12]. Such observations led to the decision to prepare series **1**. These compounds have the potential to display dual functions, namely to cause greater chemosensitivity to neoplasms than normal cells and also to cause toxicity *per se*. For example, an initial reaction of **1a**–**j** with a chemoprotectant thiol at one olefinic carbon atom could sensitize cells to subsequent alkylation at the remaining olefinic carbon atoms. Alternatively, reactions at two or three olefinic carbon atoms may reduce the concentration of chemoprotectant thiols significantly causing cancer cells to be particularly vulnerable to subsequent chemical insults. The linker group between the amidic carbonyl groups was absent (**1a**) or consisted of saturated alkyl chains (**1b**–**e**), unsaturated alkyl groups (**1f**–**h**) and an aryl ring (**1i**, **j**). In addition, the nature of the linker itself may contribute to potency and selectivity. Thus the hypothesis to be evaluated is that the design of series **1** leads to tumour-selective cytotoxins. A preliminary investigation revealed that **1a**–**j** inhibited the growth of human Molt4/C8 and CEM T-lymphocytes as well as murine L1210 lymphoid leukaemic cells while a representative molecule **1a** was cytotoxic to a number of cancer cell lines [13].

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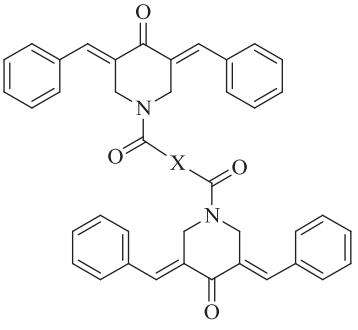
The aims of the present investigations are fourfold. First, the question needs to be addressed whether the compounds in series **1** display greater toxicity to neoplasms than non-malignant cells. Second, in view of the interest in our laboratories of compounds which inhibit the growth of colon cancer and leukaemic cells [14,15], the evaluation of representative compounds in series **1** against these neoplasms was planned. Third, further probing regarding the mode of action of one of the representative potent cytotoxins was considered of importance. Finally, a major problem in cancer chemotherapy is the development of multidrug resistance (MDR) in malignant cells. Hence compounds which are MDR-revertants may be co-administered with an anticancer drug or those which possess both MDR-revertant and antineoplastic properties have immense clinical potential. Since a number of cytotoxic 1-acyl-3,5-bis(benzylidene)-4-piperidone hydrochlorides are potent revertants of MDR [16], an evaluation of **1a**–**j** for this property was considered. Fig. 1

2. Results

The synthesis of series **1** was accomplished by acid-catalyzed condensation of 4-piperidone hydrochloride with benzaldehyde which led to the formation of 3,5-bis(benzylidene)-4-piperidone which in turn reacted with a variety of acid chlorides to form **1a**–**j** [13]. In addition, acylation of 3,5-bis(benzylidene)-4-piperidone with phthaloyl chloride produced the corresponding bisamide which is a structural isomer of **1i** and **1j**. However, its lack of adequate solubility in different solvents precludes any discussion of its cytotoxic properties.

All of the compounds **1a**–**j** were evaluated against the following neoplasms namely human HSC-2, HSC-3 and HSC-4 squamous cell carcinomas and HL-60 promyelocytic leukaemic cells and non-malignant HGF gingival fibroblasts, HPC pulp cells and HPLF periodontal ligament fibroblasts. These data are presented in Table 1. In addition, **1a**–**c**, **f**, **g**, **i**, **j** were examined against a number of colon cancer and leukaemic cell lines and the results are summarized in Table 2. A representative lead cytotoxin **1b** was examined for its



1

a: X= –
b: X= -CH_2-
c: X= -$(CH_2)_2$-
d: X= -$(CH_2)_3$-
e: X= -$(CH_2)_6$-
f: X= –C≡C–
g: X= –CH=CH–
h: X= –(CH=CH)$_2$–
i: X= 
j: X= 

Fig. 1. The structures of the compounds in series **1**.

Table 1
Evaluation of **1a**–**j** against various human neoplasms and human normal cells.

Compound	Human tumour cell lines, CC_{50} (μM)[a]									Human normal cells, CC_{50} (μM)[a]				
	HSC-2	SI[b]	HSC-3	SI[b]	HSC-4	SI[b]	HL-60	SI[b]	Average CC_{50}	Average SI[b]	HGF	HPC	HPLF	Average CC_{50}	PSE[c]
1a	0.085 ± 0.013	38.4	0.15 ± 0.023	21.7	0.20 ± 0.011	16.3	0.11 ± 0.009	29.6	0.136	26.5	3.8 ± 0.52	0.68 ± 0.073	5.3 ± 0.50	3.26	195
1b	0.050 ± 0.001	18.4	0.046 ± 0.026	20.0	0.084 ± 0.005	11.0	0.046 ± 0.009	20.0	0.057	17.4	1.1 ± 0.01	0.36 ± 0.10	1.3 ± 0.33	0.92	305
1c	0.099 ± 0.008	92.6	0.23 ± 0.027	39.9	0.24 ± 0.013	38.2	0.41 ± 0.20	22.4	0.245	48.3	10 ± 0.89	1.5 ± 0.075	16 ± 1.0	9.17	197
1d	0.071 ± 0.023	34.8	0.17 ± 0.004	14.5	0.19 ± 0.038	13.0	0.39 ± 0.10	6.33	0.205	17.2	2.9 ± 0.22	0.61 ± 0.058	3.9 ± 0.01	2.47	83.9
1e	0.17 ± 0.008	61.2	0.21 ± 0.025	49.5	0.62 ± 0.12	16.8	1.3 ± 0.12	8.00	0.575	33.9	13 ± 1.6	1.1 ± 0.041	17 ± 2.5	10.4	59.0
1f	0.19 ± 0.004	41.4	0.49 ± 0.008	16.1	0.93 ± 0.012	8.46	0.87 ± 0.16	9.05	0.620	18.8	8.5 ± 0.78	2.1 ± 0.29	13 ± 0.94	7.87	30.3
1g	0.078 ± 0.010	191	0.62 ± 0.052	24.0	0.58 ± 0.054	25.7	1.2 ± 0.05	12.4	0.620	63.3	17 ± 2.8	5.7 ± 0.35	22 ± 2.7	14.9	102
1h	2.6 ± 0.17	13.5	8.5 ± 0.34	4.12	6.4 ± 0.34	5.47	7.4 ± 0.77	4.73	6.23	7.00	39 ± 0.54	19 ± 0.10	47 ± 0.51	35.0	1.12
1i	0.026 ± 0.001	189	0.076 ± 0.001	64.7	0.082 ± 0.019	60.0	0.11 ± 0.017	44.7	0.074	89.6	5.7 ± 1.7	0.56 ± 0.049	8.5 ± 0.89	4.92	1211
1j	3.2 ± 0.49	11.3	6.3 ± 0.27	5.76	8.0 ± 0.92	4.54	6.6 ± 1.0	5.50	6.03	6.78	42 ± 0.81	21 ± 0.05	46 ± 3.0	36.3	1.12
Average	0.657	69.2	1.68	26.0	1.73	20.0	1.84	16.3	1.48	32.9	14.3	5.26	18.0	12.5	22.2
Melphalan	8.7 ± 4.2	24.1	25 ± 7.7	8.40	32 ± 8.8	6.56	1.4 ± 1.2	150	16.8	47.3	161 ± 27	269 ± 153	199 ± 60	210	2.82

[a] The CC_{50} values are the concentrations of compound required to kill 50% of the cells.
[b] The letters SI refer to the selectivity index which is obtained by dividing the average CC_{50} value of the compound towards the normal cells by the CC_{50} figure of the compound towards a specific cancer cell line.
[c] The letters PSE refer to the potency-selectivity expression. These figures are calculated by multiplying the reciprocal of the average CC_{50} value of the compound towards the four tumour cell lines by the average selectivity index of these neoplasms.

Table 2
Evaluation of **1a**–**c**, **f**, **g**, **i**, **j** against various human tumour cell lines.

Compound	All all cell lines		Colon cancer cells, IC_{50} (μM)								Leukaemic cells, IC_{50} (μM)				
	[a]GI_{50} (μM)	SI	COLO205	HCC2998	HCT116	HCT15	HT29	KM12	SW620	Ave	HL-60 (TB)	K-562	RPMI8226	SR	Ave
1a	0.31	95.5	0.36	0.19	0.03	0.30	0.17	0.04	0.07	0.17	0.20	0.20	0.25	0.02	0.17
1b	0.24	1191	0.16	0.17	0.03	0.17	0.05	0.13	0.05	0.11	0.29	0.05	0.13	0.03	0.13
1c	0.69	3316	0.39	0.23	0.14	0.38	0.24	0.30	0.26	0.28	0.27	0.22	0.06	0.05	0.15
1f	0.48	38.9	0.25	0.41	0.12	0.39	0.20	0.28	0.20	0.26	0.25	0.30	0.08	0.11	0.19
1g	1.12	11950	0.56	0.22	0.13	0.83	0.25	0.21	0.22	0.35	0.45	0.32	0.05	0.23	0.26
1i	0.36	135	0.21	0.20	0.03	0.35	0.17	0.21	0.30	0.21	0.21	0.21	0.06	0.03	0.13
1j	2.69	661	2.40	–	0.20	1.70	0.55	0.35	0.36	0.93	2.04	1.55	0.73	0.15	1.12
Average	0.84	484	0.62	0.24	0.10	0.59	0.23	0.22	0.21	0.33	0.53	0.41	0.19	0.09	0.31
5-Fluorouracil	>56.2	>2239	4.17	13.5	8.51	9.71	10.2	8.32	380	62.1	>2512	2.40	–	1.12	>839
Melphalan	26.9	1118	66.1	41.7	30.2	36.3	46.8	43.7	38.9	43.4	2.04	43.7	66.1	1.86	28.4

[a] The term GI_{50} rather than IC_{50} is used since in the case of **1g**, **1j** and 5-fluorouracil, 50% inhibition of the growth of a cell line was not achieved at the maximum concentration in a few instances.

ability to cause internucleosomal DNA fragmentation, caspase-3 activation and PARP cleavage in HSC-2 and HL-60 cells. In addition, **1b** was examined for its effect on the cell cycle of human HCT-116 colon cancer cells. These results are presented in Figs. 2–5. The unsaturated ketones **1a**–**j** were evaluated for their potential to reverse P-glycoprotein (P-gp) multidrug resistance in murine L-5178Y cells which were transfected with the human mdr1 gene. These results are summarized in Table 3.

3. Discussion

All of the compounds in series **1** were evaluated against HSC-2, HSC-3, HSC-4 and HL-60 neoplastic cells and these results are portrayed in Table 1.These data reveal that in general **1a**–**j** are a cluster of highly potent cytotoxins. In fact 75% of the CC_{50} values (the concentrations to kill 50% of the cells) are submicromolar and 28% are in the double digit nanomolar range. The average CC_{50} figures, which are listed in Table 1, are lowest towards HSC-2 cells while the other three cell lines are approximately one-third less sensitive. Comparisons were made between the potencies of **1a**–**j** and melphalan which is an alkylating agent used in cancer chemotherapy. All of the compounds have lower CC_{50} values than melphalan towards HSC-2, HSC-3 and HSC-4 cells while **1a**, **b**, **i** are more potent than melphalan in the HL-60 bioassay, i.e., in 83% of the comparisons made. The average CC_{50} figures of series **1** against HSC-2, HSC-3 and HSC-4 cells are 13, 15 and 19 times lower than the CC_{50} values of melphalan in these three bioassays. In many cases, the greater toxicity than melphalan is huge, e.g., **1i** is 335, 329 and 390 times more potent than this drug in the HSC-2, HSC-3 and HSC-4 screens, respectively.

Structure–activity relationships were noted based on the average CC_{50} values for **1a**–**j** against HSC-2, HSC-3, HSC-4 and HL-60 cells which are presented in Table 1. There is no spacer group X in **1a** which is a potent cytotoxin. The addition of one methylene group produced **1b** having 2.4 times the potency of **1a**. However, increasing the number of methylene groups in **1c**–**e** gave rise to analogues with higher average CC_{50} values than both **1a** and **1b**. A comparison of the CC_{50} figures of **1c** with the unsaturated analogues **1f** and **1g** revealed a 2.5-fold reduction in potencies. The addition of a second unsaturated linkage to **1g** forming **1h** results in a 10-fold drop in potency. A comparison between the CC_{50} values of the structural isomers **1i** and **1j** revealed an 82-fold difference in the average CC_{50} data. One may summarize the SAR by stating that in general the distance between the two amidic carbonyl groups should be small; increasing this span causes a reduction in potencies.

In addition to potency, an issue of major importance is whether compounds exert greater toxic effects towards neoplasms than normal cells. In order to address this issue, the compounds in series **1** were also evaluated against HGF, HPC and HPLF non-malignant cells and the results are presented in Table 1. Under clinical conditions, neoplasms are surrounded by different types of normal cells. Hence the selectivity index (SI) figures were computed by dividing the average CC_{50} value of the compound against the three normal cells by the CC_{50} figure generated towards a particular tumour cell line. These SI values are displayed in Table 1.

All of the compounds in series **1** have SI values greater than 1. In many cases the selectivity is enormous, e.g., the average SI figures for series **1** is 32.9 and for **1c**, **g**, **i** they are 92.6, 191 and 189, respectively, against HSC-2 cells. The enones **1c**, **g**, **i** display the

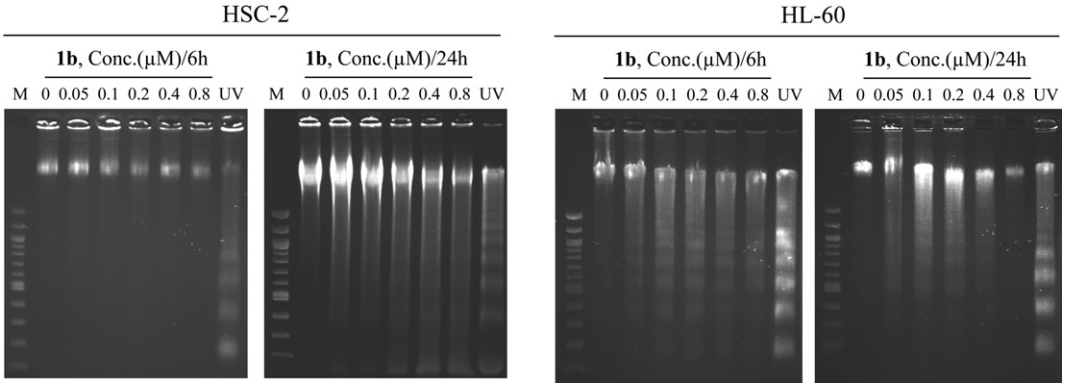


Fig. 2. The effect of **1b** on internucleosomal DNA fragmentation using HSC-2 and HL-60 cells. M is a 100 bp DNA ladder marker, the figures refer to the concentrations of **1b** in μM and cells irradiated with UV are the positive control.

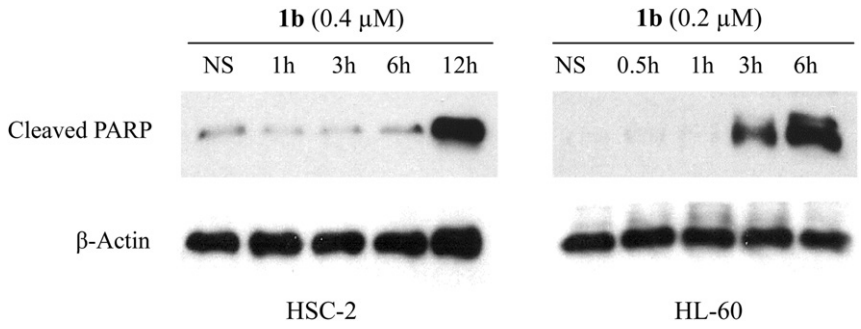


Fig. 3. A western blot analysis of the effect of **1b** on the cleavage of PARP in HSC-2 and HL-60 cells. NS means no stimulation.

highest average SI values of 48.3, 63.3 and 89.6, respectively, which are similar to or greater than the figure of 47.3 for melphalan. This observation indicates that **1c**,**g**,**i** are important lead molecules and it is of interest that there is a two carbon spacer between the amidic carbonyl groups in these three compounds. Other correlations between the average SI figures and the nature of the spacer group were noted. First, the degree of saturation and unsaturation in the two carbon spacer in **1c** (48.3), **1f** (18.8) and **1g** (63.3) have substantial effects on the extent of selectivity. Second, while the presence of one olefinic group in the spacer led to **1g** (63.3) with excellent selectivity, the placement of a second ethylenic moiety produced **1h** (7.00) which has a greatly reduced SI value. Third, there is a 13.2-fold difference between the average SI figures of the structural isomers **1i** (89.6) and **1j** (6.78). Thus one may conclude that series **1** is a novel group of tumour-selective cytotoxins.

Since both potency and selectivity are important features in identifying lead molecules, a potency-selectivity expression (PSE) was calculated for each compound. These values are the product of the reciprocal of the average CC_{50} values against four tumour cell lines and the average SI figures; these results are presented in Table 1. All of the compounds have higher PSE values than melphalan except **1h**, **j**. The compounds with the highest PSE figures are **1b** and **1i** which are 108 and 429 times greater than the value for melphalan. One may note that a short methylene chain is preferred (**1a**–**c** > **1d**, **e**) and the nature of the two carbon spacer affects the PSE figures considerably (**1c** > **1g** > **1f**).Furthermore, **1i** has a much more favourable PSE value than **1j**. From these data, **1a**–**c**, **g**, **i**, which have PSE figures in excess of 100, serve as prototypic molecules for further development.

The next phase of the investigation of the potential of these compounds as candidate anticancer drugs was the examination of representative members of series **1** for their ability to inhibit the growth of neoplasms of different somatic origins. Of particular interest was the question of whether cytotoxicity towards colon cancers and leukaemic cells would be observed. In order to address this issue, **1a**–**c**, **f**, **g**, **i**, **j** were examined against 59 ± 3 human tumour cell lines including not only colon cancers and leukaemia but also melanoma and non-small cell lung, central nervous system, ovarian, renal, prostate and breast cancers [17]. These data are presented in Table 2.

The biodata in Table 2 reveal that the compounds are potent cytotoxins to a wide variety of neoplasms. This conclusion may be drawn from the average GI_{50} values obtained for all cell lines which are submicromolar (**1a**–**c**, **f**, **i**) or in the low micromolar range (**1g**, **j**). In particular, the average GI_{50} figures for **1a**, **b**, **i** are impressive and serve as lead molecules. All of these compounds are substantially more potent than melphalan e.g., the average GI_{50} value of **1b** is 112 times lower than the figure of the established drug.

A review of the mean graphs [18], revealed that these compounds are particularly toxic towards colon cancers and leukaemic cells. In regard to colon cancers, all of the GI_{50} values are submicromolar except **1j** towards COLO 205 and HCT15 cells, i.e., in 95% of the GI_{50} values. In addition, double digit nanomolar values were obtained against HT-116 (**1a**, **b**, **i**), HT29 (**1b**), KM12 (**1a**) and SW620 (**1a**, **b**) cells. These observations establish **1a**, **b**, **i** as important lead molecules for further development of chemical agents against colon cancers. The anticancer drug 5-fluorouracil is used in treating colonic neoplasms and the huge differential in

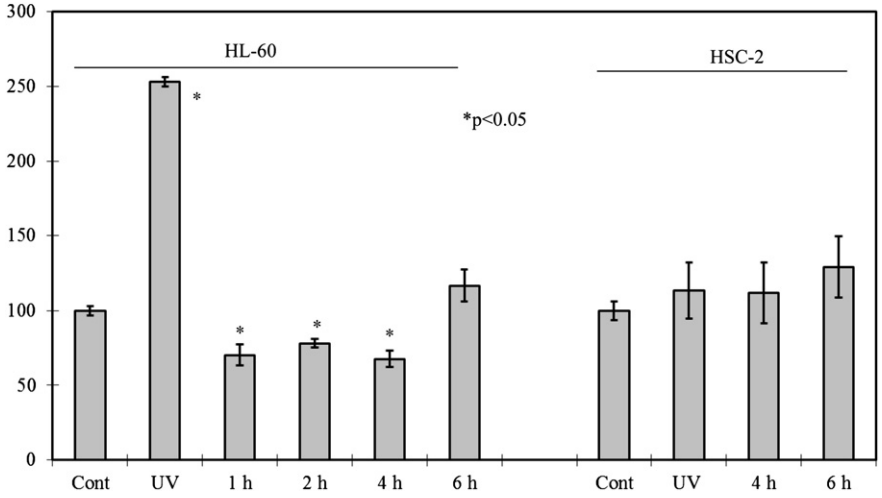


Fig. 4. Evaluation of **1b** as an activator of caspase-3. The bars are the mean determinations and the standard deviations ($n = 3$) are also indicated.

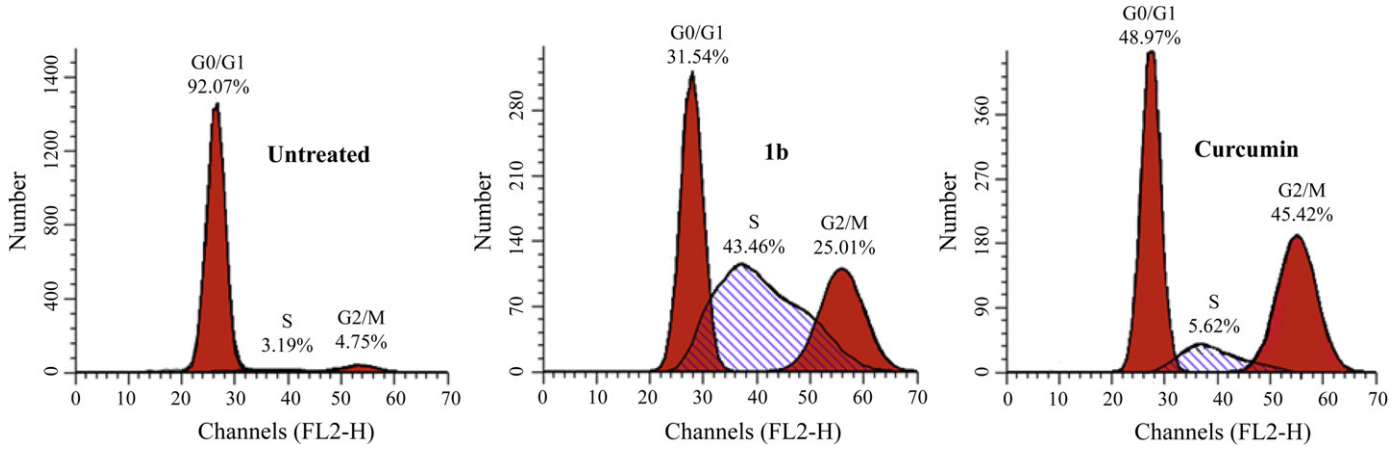


Fig. 5. Effect of **1b** (1 μM) and curcumin (16 μM) on the cell cycle of HCT-116 cells as determined by flow cytometry. Curcumin was taken as the positive control.

potencies between this drug and representative compounds in series **1** is most noteworthy. For example, the IC_{50} value of **1b** towards HCT 116 cells is 284 times lower than the figure for 5-fluorouracil.

The IC_{50} values of **1a**–**c**, **f**, **g**, **i**, **j** towards four leukaemic cell lines are presented in Table 2. These figures are submicromolar except for **1j** towards HL-60 (TB) and K-562 cells, i.e., in 93% of the determinations. Double digit nanomolar IC_{50} figures were noted in approximately one-third of the evaluations in the K-562 (**1b**), RPMI8226 (**1c**, **f**, **g**, **i**) and SR (**1a**–**c**, **i**) assays. The average IC_{50} data of these seven compounds towards the leukaemic cell lines are substantially greater than melphalan which is used in treating certain leukaemias. The biodata summarized in Table 2 reveals clearly that representative unsaturated ketones in series **1** inhibit the growth of a wide variety of human tumour cell lines especially colonic cancer and leukaemic cells.

An attempt was made to determine the mechanism of action of the most potent compound **1b**. Previously **1a** caused apoptosis in various neoplastic cells while very little necrosis was observed [13]. The structural similarity of **1a** and **1b** suggested that induction of apoptosis was an important way whereby **1b** also exerted its lethal effects. A characteristic feature of apoptosis is the formation of internucleosomal DNA fragmentation [19]. The unsaturated ketone **1b** caused DNA cleavage in both HSC-2 and HL-60 cells as indicated in Fig. 2. After 6 h, DNA fragmentation was noted in HL-60 cells but not in HSC-2 neoplasms while **1b** produced this effect in both cell lines after 24 h. This result indicates that apoptosis is one way in which **1b** displays its bioactivity and that this compound exerts its efficacy more rapidly in HL-60 than HSC-2 cells.

When DNA breaks, poly(ADP-ribose)polymerase 1 (PARP1) is activated almost immediately [20] since its principal action is the sensing and repair of DNA single-stranded breaks [21]. Hence compounds which cleave PARP1 may have an important role to play in cancer chemotherapy [22–24]. The results portrayed in Fig. 3 reveal that **1b** caused extensive PARP1 cleavage in HL-60 cells after 6 h and in HSC-2 cells after 12 h. These results correlate well with the DNA fragmentation experiments in which lower concentrations and shorter times of incubation with **1b** were noted using HL-60 cells.

Caspases, especially caspase-3, play important roles in apoptotic cell death induced by anticancer agents [25–27]. Compound **1b** was incubated with HSC-2 and HL-60 cells up to 6 h and the effect on caspase-3 activation is portrayed in Fig. 4. The data generated reveal that only a statistically insignificant activation of caspase-3 took place after 6 h; hence the cytocidal effect of **1b** on neoplastic cell lines is not principally due to caspase-3 activation. One may note that while in some cases PARP1 cleavage can be initiated by caspase-3 activation during apoptosis [28–30], the effect of **1b** on PARP1 cleavage is brought about by mechanisms other than caspase activation. In summary, the mode of action of **1b** includes apoptosis mediated by DNA fragmentation and PARP1 cleavage.

The effect of **1b** and curcumin on the cell cycle progression of HCT-116 cells was investigated by flow cytometry (Fig. 5). For cell cycle analysis, the cells were treated with 1 μM concentration of **1b** and 16 μM concentration of curcumin for 48 h. Curcumin, a potent cytotoxic agent [31] that possesses structural similarity with **1b** and known to display dose dependant G2/M phase arrest in HCT-116 cells [32] was considered as a positive control for the cell cycle analysis. **1b** induced an increase in the cell population in S-phase and G2/M phase by 40 and 20%, respectively as compared to the untreated cells. Upon treatment with **1b**, the cell population in G0/G1 phase reduced significantly to 32% as compared to 92% in untreated cells. These results suggest that **1b** displays growth inhibition of HCT-116 cells by inducing a G2/M and S block in the cell cycle. Our study also shows that curcumin induces G2/M phase arrest in HCT-116 cells which is in agreement with the previous report [32].

Finally, a major problem in cancer chemotherapy is the ability of various neoplasms to display multidrug-resistant (MDR) properties which reduces the efficacy of antineoplastic agents by different

Table 3
Fluorescence activity ratio values of **1a**–**j** in murine L-5178Y cells transfected with the human mdr1 gene.

Compound	FAR value[a]	
	4 μM	40 μM
1a	19.3	41.1
1b	35.3	53.3
1c	35.0	60.0
1d	50.2	73.4
1e	49.2	55.7
1f	42.0	45.7
1g	37.2	52.9
1h	48.6	44.3
1i	46.5	51.5
1j	38.9	61.3
Average	40.2	53.9

[a] The letters FAR refer to the fluorescence activity ratio. A reference drug verapamil has a FAR value of 8.71 when a concentration of 5.2 μM was employed.

means including accelerating the exodus of the anticancer drug by the membrane transporter P-glycoprotein (P-gp) [33]. Thus novel compounds which overcome MDR may find use as dual agents (possessing anticancer and MDR-revertant properties) or as compounds to be co-administered with established anticancer drugs. Previous work from this laboratory revealed that in addition to significant cytotoxic properties, various 1-acyl-3,5-bis(benzylidene)-4-piperidones reversed P-gp associated MDR [16]. Thus assessment of the compounds in series **1** for this property was considered appropriate.

The assay utilized employs murine L-5187Y lymphoma cells which have been transfected with the human mdr1 gene [34]. The concentrations of the dye rhodamine 123 in treated and untreated transfected and parental cells were obtained and the fluorescence measured. The results are expressed as fluorescence activity ratio (FAR) figures and a FAR value of greater than 1 indicates that reversal of MDR has occurred. The biodata will be discussed principally in terms of the FAR values obtained using a concentration of 4 μM since this figure is closer to the CC_{50} and IC_{50} cytotoxicity data. All of the compounds have MDR-revertant properties and are substantially greater than a reference MDR-modulator verapamil. With the exception of **1a**, the FAR values are in the range of 35–50. The compounds with the highest FAR values are **1d**, **e**, **h**, **i** which are clearly prototypic molecules for analogue development.

Molecular features which are found in a number of MDR-modulators are the presence of hydrogen bond acceptor atoms such as nitrogen and oxygen as well as hydrophobic aryl rings [35]. The compounds in series **1** have four oxygen atoms which permit hydrogen bonding to take place. The amidic groups *per se* may be important contributors to MDR-reversal since excision of the linker [–C(=O)–X–C(=O)–] leads to 3,5-bis(benzylidene)-4-piperidone which is virtually bereft of Pgp-mediated MDR-reversal [16]. Series **1** has four (**1a**–**h**) or five (**1i**, **j**) aryl rings and in order to determine if the hydrophobicity of the molecules was correlated with the extent of MDR-reversal, linear, semilogarithmic and logarithmic plots between the logP figures of **1a**–**j** and their FAR values were made. The semilogarithmic plot revealed a trend towards a positive correlation ($p = 0.12$) suggesting that MDR-revertant properties increase as the hydrophobicity of the molecules is raised.

The data in Table 3 indicate that, in general, by increasing the concentration of the compounds tenfold, the FAR values rise only by one-third approximately. This observation suggests that while MDR-reversal is enhanced as the concentration of the compounds is raised, biochemical processes which counteract MDR-revertant properties are activated. For example, the binding area of P-gp where these compounds interact may have become saturated. This phenomenon has been noted previously [16,36].

4. Conclusions

The bis[3,5-bis(benzylidene)-4-oxo-1-piperidinyl]amides **1a**–**j** are a cluster of potent cytotoxins which demonstrates greater toxicity to neoplasms than normal cells. This study revealed a number of promising lead molecules **1b**, **i** (in terms of potency and PCE figures) and **1c**, **g**, **i** (the most favourable SI values) for further development. Various compounds in series **1** inhibit the growth of a number of human colon cancer and leukaemic cells. Especially, three prototypic molecules were identified namely **1a**, **b**, **i.** An investigation of the mode of action of the most potent compound **1b** reveals that **1b** displays cytotoxicity by inducing apoptosis which acts through DNA damage and PARP cleavage in HSC-2 and HL-60 cells. **1b** also trigger G_2/M and S-phase arrest in cell cycle. Finally, the discovery of the MDR-revertant properties of series **1** indicates their being dual agents in the warfare against cancer insofar as they couple significant toxic effects towards neoplastic cells with inhibition of their extrusion from tumours by P-gp.

5. Experimental section

5.1. Synthesis of *1a–j*

The synthesis of **1a**–**j** has been described previously [13]. The details of synthesis and their characterization data are given in the supplementary section. In brief, 3,5-bis(benzylidene)-4-piperidone (0.02 mol) which was prepared using a literature procedure [37] was condensed with the appropriate dicarboxylic acid chloride (0.0 l mol) in the presence of triethylamine (0.01 mol) in dichloroethane at room temperature. After the reaction was complete, aqueous potassium carbonate solution was added to the mixture and the product was collected, dried and recrystallized from a suitable solvent.

5.2. Bioevaluations

5.2.1. Cytotoxic assays

The evaluation of **1a**–**j** against HSC-2, HSC-3, HSC-4, HL-60, HGF, HPC and HPLF cells was carried out using a literature method [38] except that the time of incubation was 48 h. The CC_{50} values were obtained from a dose–response curve.

The data in Table 2 was generated using a literature procedure [17]. The concentrations of each compound were 10^{-4} to 10^{-8} M (**1a**–**c**, **f**, **g**, **i**, **j**), $10^{-2.6}$ to $10^{-6.6}$M (5-fluorouracil) and $10^{-3.6}$ to $10^{-7.6}$M (melphalan). The compounds were evaluated against 50 (5-fluorouracil), 56 (**1j**), 57 (melphalan), 59 (**1b**) and 60 (**1a**, **c**, **f**, **g**, **i**) cell lines. The number of cell lines for which the maximum concentration of compound did not inhibit growth by 50% was 2/60 (**1g**), 5/56 (**1j**) and 6/50 (5-fluorouracil).

5.2.2. Internucleosomal DNA fragmentation

The evaluation of **1b** as an inducer of internucleosomal DNA fragmentation in HSC-2 and HL-60 cells was undertaken by a literature procedure [39]. In brief, cells were incubated with 0.05, 0.1, 0.2, 0.4 and 0.8 μM of **1b** for 6 h. As a positive control, cells were exposed to UV light (6J/m^2/min) for 1 min and incubated for 3 h. Subsequently the cells were harvested for evidence of DNA fragmentation.

5.2.3. Caspase-3 activation

The enone **1b** was examined for its ability to activate caspase-3 in both HSC-2 and HL-60 cells using a reported method [40]. In brief, cells were lysed and mixed with a substrate, namely DEVD-*p*-nitroanilide. After incubation at 37 °C for 2 h, the absorbance at 405 nm of the liberated chromophore *p*-nitroanilide was measured using a microplate reader.

5.2.4. PARP cleavage

HSC-2 and HL-60 cells were treated with 0.4 μM and 0.2 μM of **1b**, respectively. The cleavage of PARP was measured using a Promega PARP (Asp 214) human specific antibody [41]. In brief, cells were washed in ice-cold PBS, scraped, collected in lysis buffer (20 mM HEPES pH7.4, 1% Triton-X 100, 150 mM NaCl, 1.5 mM $MgCl_2$, 12.5 mM β-glycerophosphate, 2 mM EGTA, 10 mM NaF, 2 mM DTT, 1 mM Na_3VO_4, 1 mM PMSF plus 1x protease inhibitor).The cell lysates were applied to an 8% SDS-PAGE and the protein bands in the gels were transferred onto polyvinylidene difluoride membranes. The membranes blocked with 5% (w/v) nonfat dry milk were incubated with primary antibody [anti-cleaved PARP1 (Cell Signalling Technology, Beverly, MA), anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA)], and then with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies.

5.2.5. Cell cycle analysis

Cell Cycle analysis was carried out by modifying a literature procedure [42]. HCT-116 cells were maintained in McCoys'5A Modified media (ATCC) supplemented with 10% Foetal Bovine Serum (Fischer Scientific) and 1% antibiotic-antimycotic solution (Sigma). Cells (1×10^6 cells) were seeded in 75 cm^2 flasks and incubated for 24 h in a humidified incubator at 37 °C with 5% CO_2. Each flask containing cells was treated with the samples and untreated control for 48 h in a humidified incubator at 37 °C with 5% CO_2. Floating cells were collected and adherent cells were harvested with trypsin-EDTA (0.2%) and pooled. The samples were washed with cold PBS, fixed in 70% ethanol and left on ice for 2 h. Further samples were washed after 2 h with PBS and resuspended in PBS containing RNase (300 μg/ml). The samples were incubated for 20 min in the dark with propidium iodide (20 μg/ml) and RNase (300 μg/ml). The samples were analyzed by a FACScalibur (BD) flow cytotmeter. The data were analyzed using Modfit LT free trial version 3.3 available from Variety software house. Cells were gated to include G0/G1, S-phase, and G2/M populations.

5.2.6. Multidrug resistance reversal assay

The ability of **1a**–**j** to reverse MDR in murine L-5178Y cells transfected with the mdr1 gene followed a published procedure [34]. In brief, solutions of the compounds in dimethylsulfoxide were added to MDR and parenteral cells at room temperature. After 10 min, rhodamine 123 was added and the cells were incubated at 37 °C for 20 min. In these experiments, the FAR value of 1% dimethylsulfoxide is 0.82.

5.3. Determination of log P values

The logP values of **1a**–**j** which were determined using a software programme [43] are as follows, namely **1a** : 5.40, **1b** : 5.34, **1c** : 5.29, **1d** : 5.71, **1e** : 6.96, **1f** : 5.67, **1g** : 5.57, **1h** : 6.08, **1i**: 7.08 and **1j** : 7.08. Linear, semilogarithmic and logarithmic plots were made using a commercial statistical package [44].

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2012.02.042.

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SUPPLEMENTARY MATERIAL

Dimeric 3,5-bis(benzylidene)-4-piperidones: A novel cluster of tumour-selective cytotoxins possessing multidrug-resistant properties

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Experimental section†

Chemistry

Synthesis of 1a-j: Melting points were determined on a Gallenkamp instrument and are uncorrected. ^{1}H and ^{13}C NMR spectra were obtained using a Bruker Avance 500 spectrometer equipped with a BBO probe. Chemical shifts (δ) are reported in ppm. Mass spectra were obtained using a quad tandem 4000 QTRAP mass analyser. Elemental analyses were undertaken using an Elementer CHNS analyzer.

Experimental

Synthesis of 3,5-bis(benzylidene)-4-piperidone dimers (1a-j)

General procedure

A mixture of appropriate dicarboxylic acid (0.005 mol) and thionyl chloride (0.02 mol, 2.4 gm) was heated at 60-65 ^{o}C for 4-5 h. Excess thionyl chloride was removed at 45 ^{o}C under reduced pressure and moisture-free conditions. The acid chloride thus obtained was taken for further reaction without any purification.

To a stirred suspension of 3,5-bis(benzylidene)-4-piperidone (0.009 mol, 2.75 gm) prepared by a literature method [1] in DCE (20 mL) containing triethylamine (0.11 mol, 1.12 gm) at ~20 ^{o}C was added the above prepared acid chloride in DCE (5 mL) slowly over a period of 30 min. The reaction was stirred at room temperature overnight. The solvent was removed under reduced pressure at 45 ^{o}C. An aqueous solution of K_2CO_3 (25 mL, 10% w/v) was added to the crude mass and stirred for 2h. The solid obtained was filtered, dried, and crystallized from a suitable solvent to yield pure products.

In the case of **1a**,**b**,**d**,**e**, the appropriate acid chlorides were procured from commercial sources.

1,2-bis(3,5-Dibenzylidene-4-oxo-piperidin-1-yl)ethane-1,2-dione(1a)

Yield: 62%; mp (chloroform/methanol) 246 ^{o}C; ^{1}H NMR(500 MHz, DMSO-d_6): δ 7.72(s, 2H, 2×=CH), 7.56 (s, 2H, 2×=CH), 7.53(t, 4H, Ar-H), 7.49(d, J=7.07 Hz, 2H, Ar-H), 7.45(m, 6H, Ar-H), 7.39(m, 8H, Ar-H), 4.48(d, J=23.28Hz, 8H, 4×NCH_2; ^{13}C NMR (125 MHz, DMSO-d_6):184.7, 162.6, 137.9, 137.5, 134.4, 134.1, 131.4, 131.0, 130.9, 130.7, 130.2, 130.1,

129.3, 129.2, 46.4, 41.6; MS (ESI) m/z: 627 $(M+Na)^+$. Anal.calcd for $C_{40}H_{32}N_2O_4.H_2O$: C 77.17; H 5.14; N 4.50 %, found: C 77.05; H 4.87; N 4.42%.

1,3-bis-(3,5-Dibenzylidene-4-oxo-piperidin-1yl)propane-1,3-dione (1b)

Yield: 65%; mp (acetone) 201 ^oC; ^1H NMR (500 MHz, DMSO-d_6): δ 7.72(s, 2H, 2×=CH), 7.57(s, 2H, 2×=CH), 7.53(d, J=4.18Hz, 8H, Ar-H), 7.47 (m, 12H, Ar-H), 4.62(d, J=21.13Hz, 8H, 4×NCH_2), 3.46(s, 2H, CH_2); ^{13}C NMR (125 MHz, DMSO-d_6):186.3, 165.9,136.6, 136.5, 134.7, 134.5, 132.6, 132.5, 131.0, 130.9, 130.1, 130.0, 129.3, 129.2, 47.0, 42.4; MS (ESI) m/z: 641 $(M+Na)^+$. Anal.calcd for $C_{41}H_{34}N_2O_4.H_2O$: C 77.27; H 5.65; N 4.39%, found: C 77.31; H 5.50; N 4.47%.

1,4-Bis-(3,5-dibenzylidene-4-oxo-piperidin-1-yl)-butane-1,4-dione(1c)

Yield: 58 %; mp (chloroform/methanol) 188 ^oC; ^1H NMR(500 MHz, DMSO-d_6): δ 7.68(s, 4H, 4×=CH), 7.49(m, 20H, Ar-H), 4.78(d, J=10.95 Hz, 8H, 4×NCH_2), 2.29(s, 4H, 2×CH_2); ^{13}C NMR (125 MHz, DMSO-d_6):186.5, 170.4, 136.6, 136.5, 134.8, 134.5, 133.0, 132.8, 131.0, 130.0, 129.3, 46.3, 42.9, 27.16; MS (ESI) m/z: 655 $(M+Na)^+$. Anal.calcd for $C_{42}H_{36}N_2O_4.0.5\ H_2O$: C 78.53; H 5.76; N 4.36%, found: C 78.16; H 5.71; N 4.11%.

1,5-Bis-(3,5-dibenzylidene-4-oxo-piperidin-1-yl)-pentane-1,5-dione(1d)

Yield: 43 %; mp (chloroform/methanol) 170 ^oC; ^1H NMR(500 MHz, DMSO-d_6): δ 7.71(s, 2H, 2×=CH), 7.66(s, 2H, 2×=CH), 7.54(m, 14H, Ar-H), 7.42(m, 6H, Ar-H), 4.76(d, 8H, 4×NCH_2, J= 28.18 Hz), 2.02(t, 4H, 2×CH_2), 1.42(p, 2H, CH_2); ^{13}C NMR (125 MHz, DMSO-d_6):186.6, 171.0, 136.6, 134.8, 134.5, 133.1, 133.0, 131.0, 130.3, 129.3, 46.4, 42.8, 31.3, 20.4; MS (ESI) m/z: 627 $(M+Na)^+$. Anal.calcd for $C_{43}H_{38}N_2O_4.0.25\ H_2O$: C 79.22; H 5.91; N 4.30%, found: C 79.18; H 5.64; N 4.08%.

1,8-Bis-(3,5-dibenzylidene-4-oxo-piperidin-1-yl)-octane-1,8-dione (1e)

Yield: 64%; mp (chloroform/methanol) 160 ^oC; ^1H NMR(500 MHz, DMSO-d_6): δ 7.71(s, 4H, 4×=CH), 7.51(m, 20H, Ar-H), 4.81(d, 8H, J=29.13 Hz, 4×NCH_2), 2.00(t, 4H, 2×CH_2), 1.20(m, 4H, 2×CH_2), 0.76(m, 4H, 2×CH_2); ^{13}C NMR (125 MHz, DMSO-d6):186.6, 171.4, 136.8, 136.4, 134.8, 134.6, 133.2, 131.0, 130.0, 129.3, 46.5, 43.1, 32.4, 28.5, 24.7; MS

(ESI) m/z: 711 $(M+Na)^+$. Anal.calcd for $C_{46}H_{44}N_2O_4.0.5H_2O$: C 79.10; H 6.30; N 4.01%, found: C 78.85; H 6.32; N 4.04 %.

1,4-Bis-(3,5-dibenzylidene-4-oxo-piperidin-1-yl)-but-2-ene-1,4-dione(1f)

Yield: 71 %; mp (ethanol) 220 ^oC; ^1H NMR(500 MHz, DMSO-d_6): δ 7.74 (s, 2H, 2×=CH), 7.66(s, 2H, 2×=CH), 7.51(m, 20H, Ar-H), 6.92(s, 2H, 2×=CH), 4.83(d, J=8.54 Hz, 8H, 4×NCH_2); ^{13}C NMR (125 MHz, DMSO-d_6):186.2, 163.9, 136.9, 136.7, 134.7, 134.3, 132.6, 131.0, 130.1, 129.3, 46.9, 43.1; MS (ESI) m/z: 653 $(M+Na)^+$.Anal.calcd for $C_{42}H_{34}N_2O_4.5H_2O$: C 69.92; H 4.71; N 3.88 %, found: C 69.85; H 4.79; N 3.57%.

1,4-Bis-(3,5-dibenzylidene-4-oxo-piperidin-1-yl)-but-2-yne-1,4-dione(1g)

Yield: 48%; mp(chloroform/methanol) 220 ^oC (dec.); ^1H NMR (500 MHz, DMSO-d_6): δ 7.81(s, 1H, =CH), 7.75(d, 2H, 2×=CH, J=17.63 Hz), 7.68(s,1H, =CH), 7.56(m,20H, Ar-H), 4.79(d, J=18.80 Hz, 4H, 2×NCH_2), 4.64(d, J=16.96 Hz, 4H, 2×NCH_2); ^{13}C NMR (125 MHz, DMSO-d_6):186.0, 185.7, 185.4, 162.4, 161.9, 149.9, 137.2, 134.5, 132.3, 131.8, 131.5, 131.0, 130.7, 130.4, 129.1, 128.7, 125.25, 95.25, 46.8, 42.7, 42.2; MS (ESI) m/z: 651 $(M+Na)^+$. Anal.calcd for $C_{42}H_{32}N_2O_4.2H_2O$: C 75.82; H 4.81; N 4.21 %, found: C 75.48; H 4.74; N 4.01 %.

1,6-Bis-(3,5-dibenzylidene-4-oxo-piperidin-1-yl)-hexa-2,4-diene-1,6-dione(1h)

Yield: 56%; mp (chloroform/methanol) 199 ^oC; ^1H NMR(500 MHz, $CDCl_3$): δ 7.87(s, 4H, 4×=CH), 7.42(m, 20H, Ar-H), 6.94(m, 2H, 2×=CH), 6.16(m, 2H, 2×=CH), 4.98(s, 4H, 2×NCH_2), 4.77(s, 4H, 2×NCH_2); ^{13}C NMR (125 MHz, DMSO-d_6):186.6, 164.7, 140.3, 137.5, 134.5, 131.5, 130.6, 130.0, 125.9, 125.6, 46.3, 44.1; MS (ESI) m/z: 679 $(M+Na)^+$. Anal.calcd for $C_{44}H_{36}N_2O_4.2.5H_2O$: C 75.23; H 5.12; N 3.98 %, found: C 75.23; H 5.02; N 3.91%.

1,2-Bis-[(3,5-dibenzylidene-4-oxo-piperidin-1-yl)-1-carbonyl]-benzene(1i)

Yield: 68 %; mp (chloroform/methanol) 240 ^oC (dec.); ^1H NMR(500 MHz, DMSO-d_6): δ 7.77(s, 2H, 2×=CH), 7.69(s, 2H, 2×=CH), 7.57(t, 10H, Ar-H), 7.27(brs, 5H, Ar-H), 7.18 (brs, 5H, Ar-H), 6.89(m, 2H, Ar-H), 6.78(m, 2H, Ar-H), 4.92(brs, 4H, 2×NCH_2), 4.49(s, 4H, 2×NCH_2); ^{13}C NMR (125 MHz, DMSO-d_6):186.1, 167.9, 137.3, 136.5, 134.8, 133.8, 132.6,

131.1, 130.3, 129.8, 129.4, 129.0, 126.4, 47.8, 43.4; MS (ESI) m/z: 703 $(M+Na)^+$. Anal.calcd for $C_{46}H_{36}N_2O_4.H_2O$: C 78.99; H 5.43; N 4.0%, found: C 79.09; H 5.42; N 4.02%.

1,3-Bis-[(3,5-dibenzylidene-4-oxo-piperidin-1-yl)-1-carbonyl]-benzene(1j)

Yield: 63%; mp (chloroform/methanol) 220 ^oC (dec.); ^1H NMR(500 MHz, DMSO-d_6): δ 7.78(s, 4H, 4×=CH), 7.53(m, 10H, Ar-H), 7.31(m, 10H, Ar-H), 7.11(s, 1H, Ar-H), 7.06(d, J= 8.93 Hz, 2H, Ar-H), 6.75(t,1H, Ar-H), 4.97 (brs, 4H, 2×NCH_2), 4.57(brs, 4H, 2×NCH_2); ^{13}C NMR (125 MHz, DMSO-d_6):186.03, 168.0, 137.2, 134.51, 132.6, 131.0, 130.1, 129.2, 128.9, 128.3, 125.7, 48.9, 45.6; MS (ESI) m/z: 703 $(M+Na)^+$. Anal.calcd for $C_{46}H_{36}N_2O_4.0.5H_2O$: C 80.02; H 5.36; N 4.01%, found: C 80.31; H 5.29; N 4.01%.

Reference

[1] J.R. Dimmock, M.P. Padmanilayam, R.N. Puthucode, A.J. Nazarali, N.L. Motaganahalli, G.A. Zello, J.W. Quail, E.O. Oloo, H.B. Kraatz, J.A. Prisciak, T.M. Allen, C.L. Santos, J. Balzarini, E. De Clercq, E.K. Manavathu, *J. Med. Chem.* 44(2001) 586-593.

CHAPTER 5

Chapter 5 includes a copy an article that will be submitted to the European Journal of Medicinal Chemistry.

Relationship of Chapter 5 to the objectives of this project

This study aims at developing further analogs of 3,5-bis(benzylidene)-4-piperidone dimers based on the lead compounds obtained from the study in chapter 4 with a view to finding novel molecules with improved cytotoxic potencies. Two important hypotheses need to be evaluated: (1) the introduction of aryl groups with varying physicochemical properties may improve cytotoxic potencies significantly; (2) the presence of amidic groups are important for displaying potent cytotoxicity.

Description

As outlined in chapter 4, the dimeric 3,5-bis(arylidene)-4-piperidones **1a** and **1b** emerged as the most potent cytotoxic compounds. In particular, these two molecules demonstrated very high inhibitory activities against a panel of colon cancer cell lines in the NCI screen. Structural modifications were undertaken on **1a** and **1b** by introducing aryl substituents with diverse Hammet σ, or Taft σ*, Hansch π and molecular refractivity (MR) values that provided the novel series **2** and **3**, respectively. A representative molecule **4** was prepared in which the amidic carbonyl groups of **1b** were excised and the cytotoxic activity of **4** was compared against **1b** to delineate the contribution of the amide groups in exhibiting cytotoxicity.Series **1**-**3** and compound **4** were screened against two colon cancer cell lines HT29 and HCT-116. A structure-activity relationship was formulated to gain insights for further designing of the compounds. Flow cytometry analysis of the effect of a representative molecule on the cytotoxicity in HCT-116 cells was carried out.

Author Contributions

My contributions to this publication were (i) undertaking a literature review of the target compounds (ii) designing the synthetic chemical routes, synthesizing the compounds, and determining the ^{1}H and ^{13}C NMR spectra of the compounds in series **1**-**4** as well as undertaking the elemental analyses (iii) carrying out the QSAR studies (iv) performing the cytotoxicity assays and the flow cytometry analysis and (v) writing the manuscript. The coauthors on this paper are U. Das, who supervised the synthesis of the compounds and undertook the molecular

modeling studies, D. Michael assisted in performing the flow cytometry study, and this project was guided and supervised by J.R. Dimmock and D. K. J. Gorecki.

Novel 3,5-bis(arylidene)-4-piperidone dimers: Potent cytotoxins against colon cancer cells

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Abstract

Two novel series of dimeric 3,5-bis(arylidene)-4-piperidones **7** and **8** were prepared as cytotoxic agents. A specific objective of this study was the discovery of novel compounds displaying potent anti-proliferative activities against colon cancers. Most of the compounds demonstrate potent cytotoxicity against HCT116 and HT29 colon cancer cell lines in which the IC_{50} values range from low micromolar to nanomolar values. In general, the majority of the compounds showed greater cytotoxicity and some degree of selectivity towards HCT116 cells compared to HT29 cells. Compound **9** in which the amidic carbonyl groups were excised was substantially less potent than **8a** in both the cell lines suggested that the amide groups are important components of the molecule for exhibiting cytotoxicity. Virtually all the compounds were more potent than a reference drug 5-fluorouracil which is used in treating colon cancers as well as a related enone curcumin. QSAR studies were undertaken and some guidelines for amplification of the project have been formulated. Flow cytometry analysis of a representative compound **7f** revealed that it induced apoptosis in HCT116 cells.

Keywords: piperidone, α, β-unsaturated ketone, cytotoxicity, selective toxicity

1. Introduction

Colorectal cancer is the second leading cause of death in men and women among all cancers [1]. The low incidence of colorectal cancer in Asian countries has been suggested to be due *inter alia* to the regular use of curcumin in the diet [2, 3]. Curcumin **1** produces a profound cytotoxic effect on colon carcinogenesis in rats and mice, reduces a number of colon tumours in a mouse model with a mutation in the APC gene [4] and decreases the multiplicity of colon adenomas [5]. Curcumin was found to protect against the development of colon cancer induced by azoxymethane during both the initiation and the promotion stages in both rats and mice [6,7] and also demonstrates potent anticancer effects on intestinal cancer, stomach cancer, and hepatocellular carcinoma [8]. The α,β-unsaturated keto motif is considered to contribute significantly to the bioactivity observed. Subsequently a large number of curcuminoids have been described in the literature which display potent cytotoxic potencies against various cancers including colon cancers [9-11]. One of the reasons for the interest in these compounds is that α,β-unsaturated ketones display a selective affinity for thiols compared to hydroxyl and amino groups [12] which are present in nucleic acids. Hence these molecules should not elicit genotoxic properties which are associated with a number of alkylating agents used in cancer chemotherapy [13]. Cytotoxic α,β-unsaturated ketones are thiol alkylators [12] and their perceived importance as multitargeted ligands has been documented recently [14-16].

Our major interest is the development of conjugated unsaturated ketones as candidate cytotoxins with special emphasis on those compounds which inhibit the growth of colon cancers [17,18]. In view of these considerations, a bifunctional 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore was incorporated into a variety of cyclic and acyclic analogs that led to the discovery of a number of potent cytotoxins possessing the general structure **2** [14]. In particular, some 3,5-bis (arylidene)-4-piperidones demonstrate IC_{50} values in low micromolar to submicromolar values towards a number of malignant cell lines [19-20]. These compounds were designed based on the hypothesis of sequential cytotoxicity which states that successive chemical attacks may lead to greater damage in cancer cells compared to normal cells [19]. This theory was formulated on the observations that an initial lowering of the concentrations of cellular thiols followed by a second chemical attack would cause a greater deleterious effect to neoplasms than normal cells [21,22]. A N-acyl-3,5-bis(benzylidene)-4-piperidone **4a** displayed more than 8-fold greater cytotoxic potency than its precursor **3** towards P388/MRI cells [23]. An investigation of

the effect of these two compounds **3** and **4a** on hepatic glutathione levels in mice revealed reductions in the glutathione concentrations of 19 and 29%, respectively, suggesting that an increase in the number of thiol alkylating sites in the molecule contributed to an improvement in cytotoxic potency [23]. Based on this observation, the N-acryloyl-3,5-bis(arylidene)-4-piperidone **4b** and a number of aryl substituted analogs were developed which possess greater cytotoxic potencies over their 3,5-bis(arylidene)-4-piperidone precursors [19]. These observations unequivocally demonstrate the value of incorporating additional thiol alkylating groups in the molecule and led to a decision to prepare series **5.** In these compounds two molecules of **3** are attached by a linker with varying carbon chain lengths keeping in view that the relative location of one of the 1,5-diaryl-3-oxo-1,4-pentadienyl groups in series **5** with respect to the other one may be important for binding to the active sites of the proteins and may influence cytotoxicity substantially. The compounds in series **5** displayed potent cytotoxicity against human Molt4/C8 and CEM T-lymphocytes as well as murine L1210 lymphoid leukemic cells [24] while further investigations revealed the greater toxicity of these compounds to neoplasms than non-malignant cells [25]. From these studies two prototypic molecules **7a** and **8a** emerged which displayed remarkable cytotoxic potencies having average IC_{50} values of 0.17 and 0.11 µM, respectively, against a panel of seven different colon cancer cell lines [25]. These two compounds are clearly lead molecules for analog development.

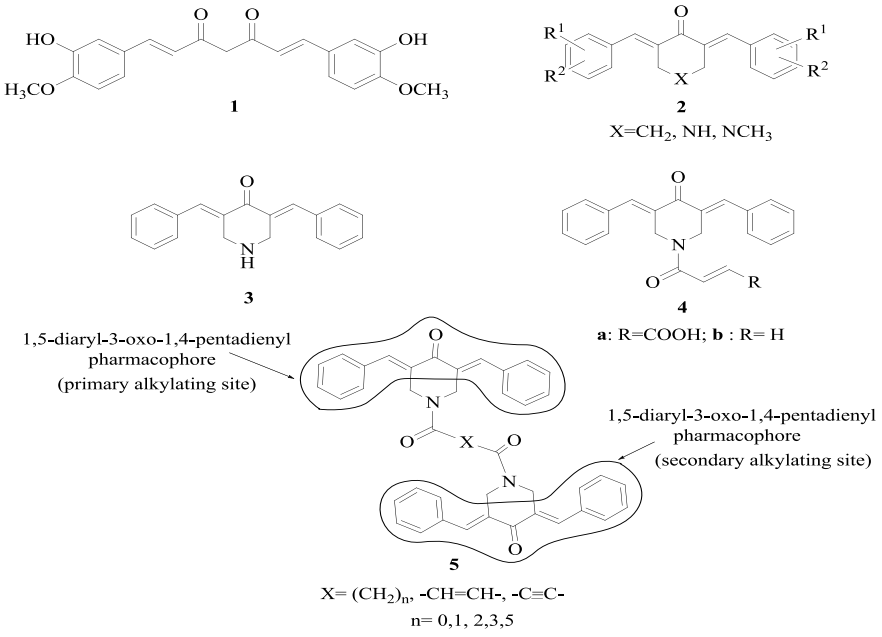
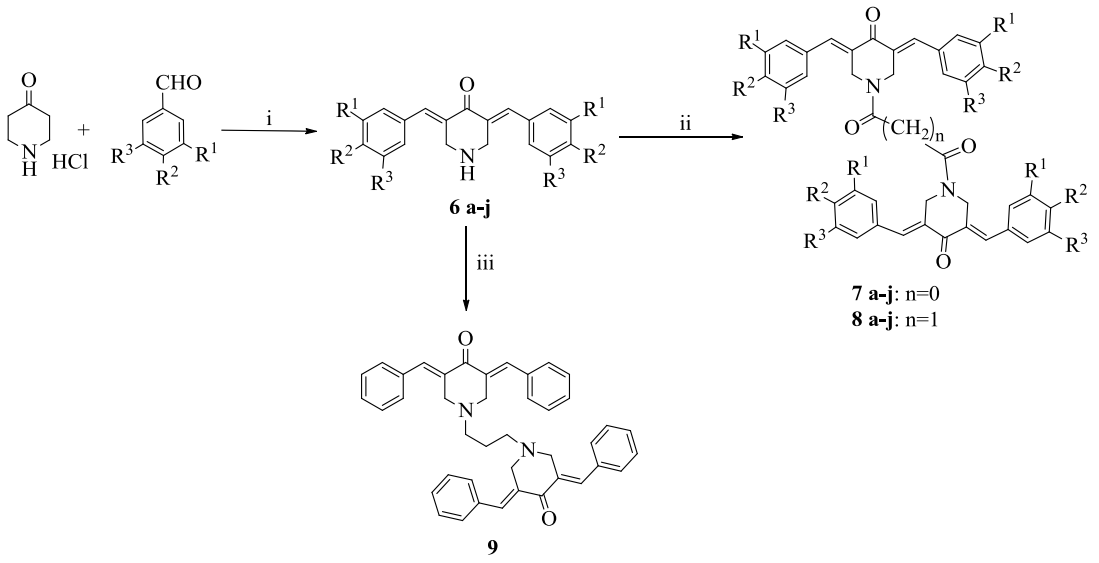


Figure 1. Structures of the compounds in series **1-5**

The aims of the present study are fourfold namely (i) examining the compounds in series **7** and **8** as candidate cytotoxins which are particularly effective against colon cancers, (ii) to evaluate the influence of the electronic, hydrophobic and steric properties of the aryl ring substituents in **7a-i** and **8a-j** on cytotoxic potencies and if possible to develop structure-activity relationships which will be helpful for designing further potent cytotoxic molecules, (iii) to establish the role of the amidic groups in governing cytotoxic properties of the molecules, and (iv) to investigate the mode action of a representative molecule whereby the cytotoxicity of this class of compounds is mediated.

2. Chemistry

The synthesis of compounds in series **6-9** is outlined in Scheme 1. Briefly, the 3,5-bis(benzylidene)-4-piperidones **6a-j** were synthesized by acid-catalyzed condensation of 4-piperidone hydrochloride with aryl aldehydes [19]. The dimers in series **7** and **8** were obtained from their corresponding 3,5-bis(benzylidene)-4-piperidones **6** by reacting with oxaloyl chloride or malonyl chloride, respectively. The synthesis of **9** was accomplished by a base catalyzed condensation of two mole equivalents of **6a** with one mole equivalent of 1,3-dibromopropane. The structures of all the compounds were established by ^{1}H NMR and elemental analysis.



Scheme 1. Syntheses of the compounds in series **7-9**. i = acetic acid/dry HCl; ii = oxaloyl chloride (for **7a-i**) and malonyl chloride (for **8a-j**); iii = 1,3-dibromopropane/K_2CO_3/KI. The aryl substituents are presented in Table 1.

The ^{1}H NMR spectra of **7a-i**, **8a-j** and **9** revealed that the compounds are isomerically pure and all four olefinic double bonds exhibit the *E*-configuration. The *E*-stereochemistry of these compounds was suggested based on the following observations. First, the olefinic protons of the dimers **7-9** appear at 7.39-7.79 ppm in the ^{1}H NMR spectra which is an indicative of the *E* geometry [19]. Second, a number of 3,5-bis(benzylidene)-4-piperidones **6** adopt the *E*-stereochemistry as established by X-ray crystallography [19, 23]. In addition, very recently an X-ray crystal structure of the 2-fluoro analog of **7** was shown to possess the *E*- geometry [26]. In order to evaluate the hypothesis that the amidic groups in the series **7-8** are essential for cytotoxicity, a representative molecule **9** was prepared which is bereft of both amidic carbonyl groups. Various physiochemical properties of the aryl substituents and the topography of the molecules were examined for correlations with the IC_{50} values.

3. Bioevaluations

The compounds in series **7-9** as well as two reference compounds 5-fluorouracil (5-FU) and curcumin were evaluated against two human colon cancer cell lines HCT116 and HT29. These biodata are reported in Table 1. Solubility considerations mandated that the highest concentration of **8i** that could be used is 25μM which was insufficient to generate an IC_{50} value. A representative lead cytotoxin **7f** was examined for its ability to cause apoptosis in HCT116 cell lines. This result is presented in Figure 2.

4. Results and Discussion

The compounds **7a-i** and **8a-j** were evaluated against human HCT116 and HT29 colon cancer cells. The biodata presented in Table 1 show remarkable cytotoxic potencies of all the compounds except **7d,i** and **8d,i.** The majority of the IC_{50} values were in the submicromolar to nanomolar range. The compounds displaying IC_{50} values < 1μM are considered to be highly cytotoxic. In series **7**, 78% of the IC_{50} values were <1 μM against both the cell lines and in series **8**, IC_{50} values of <1μM were observed in 80% of the compounds against HCT116 and HT29 cells.

In particular, **7a,e** and **8a,c,h** have double digit nanomolar IC_{50} values except in the case of **8a**, the IC_{50} figure is 3nM in the HT29 bioassay. The importance of the marked cytotoxic potencies of these compounds was confirmed when the IC_{50} values of **7-9** are compared with that

of 5-FU which is a drug used in treating colon cancers. Relative potency (RP) figures were generated by comparing the IC_{50} figures of the compounds in series **7-9** with that of 5-FU in these two screens. These data are presented in Table 1 which reveals that in general the compounds are far more toxic to these two neoplasms than the clinically useful drug. In addition, the RP figures indicate that in general the HCT116 cells are more sensitive to these compounds than the HT29 neoplasms. This difference between the RP values for each compound may reflect a capacity to display greater toxic effects to malignant cells than normal tissues. This possibility is enhanced by the variation in efficacy of the compounds to inhibit the growth of the HCT116 and HT29 cells, e.g. the 304-fold difference between **7b** and **7i** in the HCT116 screen.

Table 1: Evaluation of **7a-i**, **8a-j** and **9** against HCT116 and HT29 colon cancer cell lines

Compound	Aryl substituents			IC_{50} (μM)		RP[a]	
	R^1	R^2	R^3	HCT116	HT29	HCT116	HT29
7a	H	H	H	0.04±0.03	0.014±0.002	113	454
7b	H	CH_3	H	0.09±0.02	0.129±0.035	50	49
7c	H	Cl	H	0.03±0.01	0.78±0.13	151	8
7d	H	Cl	Cl	20.05±1.01	49.46±4.50	0.23	0.13
7e	H	F	H	0.02±0.01	0.03±0.001	226	212
7f	H	OCH_3	H	0.02±0.01	0.17±0.04	226	37
7g	H	OCH_3	OCH_3	0.03±0.01	0.568±0.265	151	11
7h	OCH_3	OCH_3	OCH_3	0.27±0.06	0.27±0.15	17	24
7i	H	$N(CH_3)_2$	H	27.37±3.68	18.56±2.30	0.17	0.34
8a	H	H	H	0.02±0.01	0.003±0.002	226	2117
8b	H	CH_3	H	0.41±0.25	0.20±0.02	11	32
8c	H	Cl	H	0.05±0.001	0.09±0.05	90	71
8d	H	Cl	Cl	26.73±2.35	6.05±0.10	0.17	1.1
8e	H	F	H	0.60±0.01	0.18±0.08	8	35
8f	H	OCH_3	H	0.06±0.01	0.77±0.05	75	8
8g	H	OCH_3	OCH_3	0.18±0.07	0.31±0.12	25	20
8h	OCH_3	OCH_3	OCH_3	0.02±0.003	0.04±0.02	226	159
8i	H	$N(CH_3)_2$	H	>25	>25	<1	<1
8j	H	OH	H	0.72±0.10	0.46±0.21	6	14
9	-	-	-	0.68±0.19	2.15±0.65	7	3
5-FU	-	-	-	4.52±0.54	6.35±1.12	1.00	1.00
Curcumin	-	-	-	11.54±4.45	13.20±2.08	0.39	0.48

[a]The relative potency (RP) of the compounds against a reference drug, 5-fluorouracil was calculated by dividing the IC_{50} value of 5-fluorouracil by the IC_{50} value of the compound against a particular cell line. Only the mean IC_{50} values were considered for calculating RP values.

In other words, the compounds in series **7** and **8** are not general biocidal agents and possibly are tumour-selective cytotoxins. Virtually all of the compounds in series **7**-**9** are substantially more potent than curcumin.

The following correlations were observed between the nature of the aryl substituents in series **7** and **8** and their cytotoxic potencies in both screens. First, the placement of a methyl (**7b**,**8b**), chloro (**7c**,**8c**), fluoro (**7e**,**8e**), or methoxy (**7f**,**8f**) group into the 4 position of the aryl rings led to compounds having similar potencies. However the introduction of a 4-dimethylamino substituent (**7i**,**8i**) into the aryl rings caused potency to plummet considerably. Second, the addition of a 3-chloro group to the 4-chloro analogs leading to the **7d** and **8d** cause a dramatic reduction in potency, e.g., there is a 668-fold difference between the IC_{50} values of **7c** and **7d** in the HCT116 screen.

The next part of the analysis of the biodata involved a comparison of the potencies of some of the compounds in series **7** and **8**. These comparisons are presented as $\Delta_{7/8}$ values which were obtained by dividing the IC_{50} value of a compound in **7a-h** by that of the analog in series **8** which possesses the same aryl substituent. In this way one may able to ascertain whether an oxalyl [-C(O)C(O)-] or malonyl [-C(O)CH_2C(O)-] linker between the piperidinyl nitrogen atoms is optimal. Taking standard deviations into account, in the HCT116 bioassay **7b-g** are more potent than **8b-g** while **8h** has a lower IC_{50} value than **7h**. The unsubstituted analogs **7a** and **8a** are equipotent. In the case of the HT29 screen, **7b,e,f** are more potent than **8b,e,f** while **8a,c,d,h** have lower IC_{50} figures than **7a,c,d,h** and **7g** and **8g** have equal potency. Thus the oxalyl and malonyl linkers are favoured in 56%and 31%, respectively, of the enones **7a-h** and **8a-h** while equipotency was observed in 13% of the comparisons made. A quantitative estimate of the differences in potencies between **7a-h** and **8a-h**, which are dependent on the nature of the spacer group, is presented as $\Delta_{8/7}$ values in Table 2. Thus the oxalyl group is marginally preferable to the malonyl linker although excellent cytotoxic potencies were observed in each series of compounds.

A question to be addressed is whether the substituted dimers **7b-i** and **8b-i** display greater cytotoxic potencies than the unsubtituted analogs **7a** and **8a**, respectively. For series **7**, the enone **7a** has greater potency than **7d,h,i** and was equipotent with **7b,c,e-g** in the HCT116 assay. For the HT29 screen, **7a** was more potent than **7b-d,f-i** and was equipotent with **7e**.

Table 2. Comparisons between some of the potencies of the compounds in series **7** and **8**

Series **7, 8**	Aryl substituent	$\Delta_{8/7}$[a]		$\Delta_{7b\text{-}i/7a}$[b]		$\Delta_{8b\text{-}i/8a}$[c]	
		HCT116	HT29	HCT116	HT29	HCT116	HT29
a	H	0.50	0.21	---	---	---	---
b	4-CH_3	4.56	1.55	2.25	9.21	20.5	66.7
c	4-Cl	1.67	0.12	0.75	55.7	2.50	30.0
d	3,4-Cl_2	1.33	0.12	501	353	1337	2017
e	4-F	30.0	6.00	0.50	2.14	30.0	60.0
f	4-OCH_3	3.00	4.53	0.50	12.1	3.00	257
g	3,4-$(OCH_3)_2$	6.00	0.54	0.75	40.7	9.00	103
h	3,4,5-$(OCH_3)_3$	0.07	0.15	6.75	19.3	1.00	13.3
i	4-$N(CH_3)_2$	>0.19	>1.35	684	1326	>1250	>8333

[a]The $\Delta_{8/7}$ values are the quotients of the IC_{50} values in series **8** divided by the analog in series **7** which has the same aryl substituent.

[b]The $\Delta_{7b\text{-}i/7a}$ values are the quotients of the IC_{50} values of **7b-i** divided by the figure for the unsubstituted compound **7a**.

[c]The $\Delta_{8b\text{-}i/8a}$ values are the quotients of the IC_{50} values of **8b-i** divided by the figure of the unsubstituted compound **8a**.

In the case of series **8**, the unsubstituted analog **8a** has lower IC_{50} values than **8b-g,i** and displays equal potency as **8h** in the HCT116 screen while against HT29 cells, **8a** is more potent than **8b-i**. Thus overall **7a** and **8a** are more potent than the substituted dimers **7b-i** and **8b-i** in 78% of the comparisons made while the same potency was noted in 22% of the cases. These comparisons are presented in quantitative form in Table 2 where, in general, the $\Delta_{7b\text{-}i/7a}$ figures are smaller than the $\Delta_{8b\text{-}i/8a}$ values. Hence substitution in the aryl rings in series **7** rather than **8** may lead to potency increases over the substituted components.

Further, the importance of the amidic groups and their contributions towards the cytotoxicity of the dimers were verified by comparing the IC_{50} values of **8a** and **9** against HCT116 and HT29 cells. Compound **9** was found to be a potent cytotoxin compared to 5-FU and curcumin towards both cell lines. However it displayed more than 30-fold and 700-fold lower cytotoxic potencies in comparison to **8a** against HCT116 and HT29 cells, respectively. These observations suggest that the amidic carbonyl groups contribute significantly to cytotoxic potencies. Therefore further analog development of these classes of compounds should retain the amidic groups.

In order to evaluate whether the magnitude of some of the physicochemical properties of the aryl substituents such as electronic (σ/σ*), hydrophobic(π), and steric properties (MR) influence the cytotoxic potencies in series **7** and **8**, linear and semilogarithmic plots were made between these constants and IC_{50} values of **7a-i** and **8a-j** in the HCT 116 and HT29 screens. Statistically significant negative correlations ($p<0.05$) were noted between σ/σ* and IC_{50} values of **7a-i** and **8a-j** in the HCT116 screen and a trend to significance ($p=0.06$) was noted between σ/σ* and IC_{50} values of **7a-j** in the HT29 bioassay. These results suggest that further development of this class of compounds should include stronger electron-withdrawing groups in the aryl rings. No correlations were noted between the π and MR constants of the aryl substituents and the IC_{50} values.

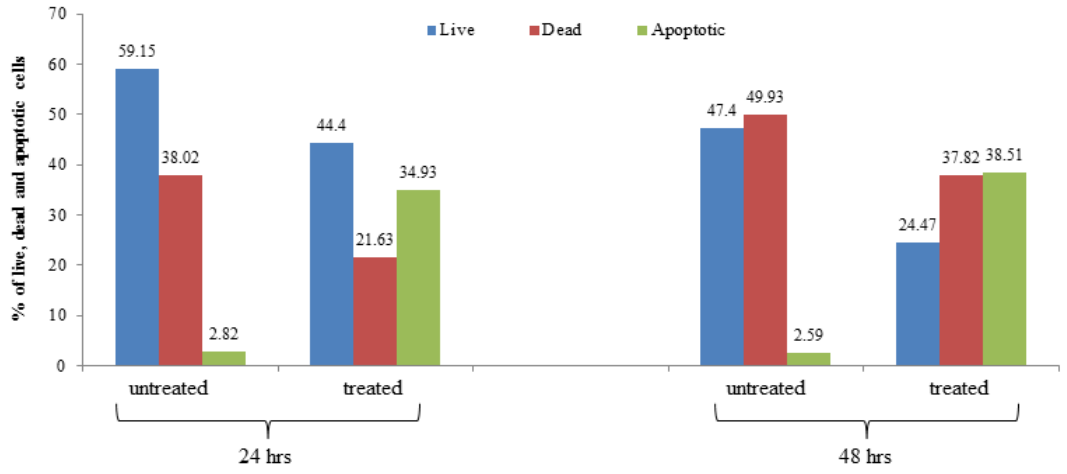


Figure 2. Analysis of the effect of **7f** on HCT116 cells after incubation for 24 hours and 48 hours by flow cytometry.

A final issue to be investigated is the way in which these classes of compounds exert their cytotoxicity. A number of anticancer agents exert their activity by inducing apoptotic cell death in cancer cells. Compound **7a** was shown to trigger apoptosis, not necrosis, in a number of T-cell and B-cell lymphomas [24]. In order to examine whether the substituted dimers also induce

apoptotic cell death in colon cancer cells, HCT116 cells was treated with a potent representative compound **7f** using a concentration of 1μM. The result is presented in Figure 2. After 24 hours, 34.9% apoptotic cell death was observed in the treated cells compared to 2.82% in untreated cells. After 48 hours, the percentage of apoptotic cells was increased slightly to 38.5%. This observation suggests that apoptosis is one of the ways by which **7f** displays cytotoxicity in HCT116 colon cells. Due to its structural similarity to other analogs in series **7** and **8**, it is possible that these compounds also follow the same mode of action.

5. Conclusions

This study reveals that in general the dimers in series **7**-**9** are potent cytotoxic agents. A number of these compounds possess submicromolar to low nanomolar IC_{50} values. Various potent cytotoxins possessing high selective toxicity towards a particular colon cancer cell type were identified. In particular, **7a**, **7e**, **8a, c** and **8h** emerged as potent lead molecules for further evaluations. The hypothesis that introduction of an aryl substituent enhance cytotoxic potencies was verified in less than half of the biodata in the HCT116 and HT29 screens. The benefit of retaining amidic groups in the molecules and their contribution towards cytotoxicity was observed. An investigation of the mode of action of a potent representative compound **7f** revealed that it induces apoptosis in HCT116 cells.

6. Experimental protocols

6.1. Chemistry

Melting points were determined on a Gallenkamp instrument and are uncorrected. ^{1}H NMR spectra were obtained using a Bruker Avance spectrometer 500 mHz equipped with a BBO probe. Chemical shifts (δ) are reported in ppm. Elemental analyses were undertaken using an Elementer CHNS analyzer.

*6.1.1. Synthesis of 3,5-bis(arylidene)-4-piperidones (**6a-j**)*

The synthesis of **6a-j** was reported previously [18,19].

*6.1.2. General procedure for the synthesis of 3,5-bis(arylidene)-4-piperidone dimers (**7**)*

Oxaloyl chloride (0.003 mol, 0.39 gm) in DCE (5 ml) was added dropwise to a stirred

suspension of a 3,5-bis(benzylidene)-4-piperidone **6** (0.006 mol) prepared by a literature method [19] in DCE (20 mL) containing triethylamine (0.006 mol, 0.61 gm) at ~20 ^{o}C for a period of 30 min. The reaction was stirred at room temperature 8-12 hrs. The solvent was removed under reduced pressure at 45 ^{o}C. An aqueous solution of potassium carbonate (25 mL, 5 % w/v) was added to the crude mass and stirred for 2h. The solid obtained was filtered, dried, and crystallized from a suitable solvent to yield pure products.

*6.1.2.1. 1,2-bis[3,5-bis(Benzylidene)-4-oxo-piperidin-1-yl]ethane-1,2-dione (**7a**)*

The synthesis of **7a** was reported previously [24].

*6.1.2.2. 1,2-bis[3,5-bis(4-Methylbenzylidene)-4-oxo-piperidin-1-yl]ethane-1,2-dione (**7b**)*

Yield: 53%; mp (chloroform/methanol) 275 ^{o}C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.79(s, 2H, 2×=CH), 7.65 (s, 2H, 2×=CH), 7.29 (d, J=7.70 Hz, 8H, Ar-H), 7.18(q, 8H, Ar-H), 4.53 (d, J=15.61 Hz, 8H, 4×NCH_2), 2.44 (s, 6H, 2×CH_3), 2.28 (s, 6H, 2×CH_3). Anal.calcd for $C_{44}H_{40}N_2O_4.1.5\ H_2O$: C 76.76; H 6.30; N 4.07 %, found: C 76.46; H 6.39; N 3.93%.

*6.1.2.3. 1,2-bis[3,5-bis(4-Chlorobenzylidene)-4-oxo-piperidin-1-yl]ethane-1,2-dione (**7c**)*

Yield: 61%; mp (methanol) 289 ^{o}C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.72(s, 2H, 2×=CH), 7.54 (s, 2H, 2×=CH), 7.46 (d, J=8.40 Hz, 2H, Ar-H), 7.37(d, J=8.37 Hz, 2H, Ar-H), 7.31(d, J=8.42 Hz, 4H, Ar-H), 7.20(d, J=8.38 Hz, 4H, Ar-H), 4.62 (s, 4H, 2×NCH_2), 4.52 (s, 4H, 2×NCH_2). Anal.calcd for $C_{40}H_{28}Cl_4N_2O_4.3\ H_2O$: C 60.26; H 3.54; N 3.51 %, found: C 60.35; H 3.70; N 3.15%.

*6.1.2.4. 1,2-bis[3,5-bis(3,4-Dichlorobenzylidene)-4-oxo-piperidin-1-yl]ethane-1,2-dione (**7d**)*

Yield: 64%; mp (chloroform/methanol) 271 ^{o}C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.68 (s, 2H, 2×=CH), 7.55 (s, 1H, =CH), 7.53 (s, 1H, =CH), 7.46 (d, J=8.15 Hz, 6H, Ar-H), 7.41(d, J=1.71Hz, 2H, Ar-H), 7.20 (dd, J=1.67Hz, J=1.71Hz, 2H, Ar-H), 7.05 (dd, J=1.71Hz, J=1.77Hz, 2H, Ar-H), 4.67 (s, 4H, 2×NCH_2), 4.56 (s, 4H, 2×NCH_2). Anal.calcd for $C_{40}H_{24}Cl_8N_2O_4$: C 54.58; H 2.75; N 3.18 %, found: C 54.52; H 2.90; N 2.95 %.

*6.1.2.5. 1,2-bis[3,5-bis(4-Fluorobenzylidene)-4-oxo-piperidin-1-yl]ethane-1,2-dione (**7e**)*

Yield: 67%; mp (chloroform/methanol) 253 ^{o}C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.73(s, 2H, 2×=CH), 7.57 (s, 2H, 2×=CH), 7.37(q, 4H, Ar-H), 7.25 (q, 4H, Ar-H), 7.18 (t, 4H, Ar-H), 7.098(t, 4H, Ar-H), 4.63 (s, 4H, 2×NCH_2), 4.51 (s, 4H, 2×NCH_2). Anal.calcd for $C_{41}H_{26}Cl_8N_2O_4.H_2O$: C 53.93; H 3.09; N 3.07 %, found: C 53.84; H 2.98; N 2.74%.

*6.1.2.6. 1,2-bis[3,5-bis(4-Methoxybenzylidene)-4-oxo-piperidin-1-yl]ethane-1,2-dione (**7f**)*

Yield: 71%; mp (chloroform/methanol) >300 ^{o}C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.74(s, 2H, 2×=CH), 7.58 (s, 2H, 2×=CH), 7.35 (d, J=8.65 Hz, 4H, Ar-H), 7.22 (d, J=8.63 Hz, 4H, Ar-H), 6.98 (d, J=8.71 Hz, 4H, Ar-H), 6.91 (d, J=8.67 Hz, 4H, Ar-H), 4.60 (s, 4H, 2×NCH_2), 4.53 (s, 4H, 2×NCH_2), 3.90 (s, 6H, 2×OCH_3), 3.76 (s, 6H, 2×OCH_3). Anal.calcd for $C_{44}H_{40}N_2O_8$: C 72.91; H 5.56; N 3.86 %, found: C 72.57; H 5.94; N 3.75%.

*6.1.2.7. 1,2-bis[3,5-bis(3,4-Dimethoxybenzylidene)-4-oxo-piperidin-1-yl]ethane-1,2-dione (**7g**)*

Yield: 60%; mp (chloroform/methanol) 282 ^{o}C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.71(s, 2H, 2×=CH), 7.42 (s, 2H, 2×=CH), 6.99 (d, J=8.47 Hz, 4H, Ar-H), 6.94(d, J=8.08 Hz, 2H, Ar-H), 6.80(m, 6H, Ar-H), 4.70 (s, 4H, 2×NCH_2), 4.64 (s, 4H, 2×NCH_2), 4.00 (s, 6H, 2×OCH_3), 3.96 (d, 12H, 4×OCH_3), 3.74 (s, 6H, 2×OCH_3). Anal.calcd for $C_{48}H_{48}N_2O_{12}.H_2O$: C 66.75; H 5.84; N 3.24 %, found: C 66.31; H 5.62; N 3.06%.

*6.1.2.8.1,2-bis[3,5-bis(3,4,5-Trimethoxybenzylidene)-4-oxo-piperidin-1-yl]ethane-1,2-dione (**7h**)*

Yield: 57%; mp (ethanol) 273 ^{o}C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.63(s, 2H, 2×=CH), 7.44 (s, 2H, 2×=CH), 6.66 (s, 4H, Ar-H), 6.49 (s, 4H, Ar-H), 4.75 (s, 4H, 4×NCH_2), 4.68 (s, 4H, 4×NCH_2), 3.96 (s, 12H, 4×OCH_3), 3.94 (s, 9H, 3×OCH_3), 3.88 (s, 9H, 3×OCH_3), 3.84 (s, 6H, 2×OCH_3). Anal.calcd for $C_{52}H_{56}N_2O_{16}$: C 64.72; H 5.85; N 2.90 %, found: C 64.95; H 6.16; N 2.78%.

*6.1.2.9.1,2-bis[3,5-bis{4-(N,N-Dimethylamino)benzylidene}-4-oxo-piperidin-1-yl]ethane-1,2-dione (**7i**)*

Yield: 46%; mp (chloroform/methanol) >300 ^{o}C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.75(s, 2H, 2×=CH), 7.65 (s, 2H, 2×=CH), 7.33 (d, J=8.75 Hz, 4H, Ar-H), 7.21(d, J=8.71 Hz, 4H, Ar-H), 6.73 (d, J=16.93 Hz, 8H, Ar-H), 4.58 (s, 4H, 2×NCH_2), 4.54 (s, 4H, 2×NCH_2), 3.08 (s, 12H,

4×NCH_3), 2.95 (s, 12H, 4×NCH_3). Anal.calcd for $C_{44}H_{40}N_2O_4$.1.5 H_2O: C 76.76; H 6.30; N 4.07 %, found: C 76.46; H 6.39; N 3.93%.

*6.1.3. General procedure for the synthesis of 3,5-bis(arylidene)-4-piperidone dimers (**8a-j**)*

Malonyl chloride (0.01 mol, 1.4 gm) in DCE (5 ml) was added dropwise to a stirred suspension of 3,5-bis(arylidene)-4-piperidone **6** (0.015 mol) in DCE (20 mL) containing triethylamine (0.01 mol, 1.4 gm) at ~20 ^oC for a period of 30 min. The reaction was stirred at room temperature 8-12 hrs. The solvent was removed under reduced pressure at 45 ^oC. An aqueous solution of potassium carbonate (25 mL, 5% w/v) was added to the crude mass and stirred for 2h. The solid obtained was filtered, dried, and crystallized from a suitable solvent to yield pure products.

*6.1.3.1. 1,3-bis-[3,5-bis(Benzylidene)-4-oxo-piperidin-1yl]propane-1,3-dione (**8a**)*

The synthesis of **8a** was reported previously[24].

*6.1.3.2. 1,3-bis-[3,5-bis(4-Methylbenzylidene)-4-oxo-piperidin-1yl]propane-1,3-dione (**8b**)*

Yield: 43%; mp (chloroform/methanol) 251 ^oC; ^1H NMR (500 MHz, $CDCl_3$): δ 7.81(s, 2H, 2×=CH), 7.74 (s, 2H, 2×=CH), 7.40 (d, J=7.90 Hz, 4H, Ar-H), 7.27 (d, J=7.60 Hz, 4H, Ar-H), 7.21(q, 8H, Ar-H), 4.89 (s, 4H, 2×NCH_2), 4.65 (s, 4H, 2×NCH_2), 3.15 (s, 2H, CH_2), 2.43 (s, 6H, 2×CH_3), 2.41(s, 6H, 2×CH_3). Anal.calcd for $C_{45}H_{42}N_2O_4.H_2O$: C 77.94; H 6.40; N 4.04 %, found: C 77.94; H 6.63; N 3.98%.

*6.1.3.3. 1,3-bis-[3,5-bis(4-Chlorobenzylidene)-4-oxo-piperidin-1yl]propane-1,3-dione (**8c**)*

Yield: 58%; mp (chloroform/methanol) 260 ^oC; ^1H NMR (500 MHz, DMSO-d_6): δ 7.64(s, 2H, 2×=CH), 7.60 (s, 2H, 2×=CH), 7.57 (d, J=11.46 Hz, 4H, Ar-H), 7.54 (d, J=7.60 Hz, 4H, Ar-H), 7.49 (q, 8H, Ar-H), 4.62 (d, 8H, J=10.15 Hz, 4×NCH_2), 3.51 (s, 2H, CH_2). Anal.calcd for $C_{41}H_{30}Cl_4N_2O_4$.2.5 H_2O: C 61.38; H 3.77; N 3.49 %, found: C 61.10; H 3.69; N 3.30%.

*6.1.3.4. 1,3-bis-[3,5-bis(3,4-Dichlorobenzylidene)-4-oxo-piperidin-1yl]propane-1,3-dione (**8d**)*

Yield: 65%; mp (chloroform/methanol) 236 ^oC; ^1H NMR (500 MHz, $CDCl_3$): δ 7.71(s, 2H, 2×=CH), 7.63 (s, 2H, 2×=CH), 7.56 (q, 8H, Ar-H), 7.51 (d, J=8.29 Hz, 2H, Ar-H), 7.30 (d,

J=1.45Hz, 2H, Ar-H), 7.31 (dd, J=1.45Hz, J=1.53Hz, Ar-H), 7.15 (dd, J=1.48Hz, 2H, Ar-H), 4.78 (d, 8H, J=6.72Hz, 4×NCH_2), 3.30 (s, 2H, CH_2). Anal.calcd for $C_{41}H_{26}Cl_8N_2O_4$. H_2O: C 53.93; H 3.09; N 3.07 %, found: C 53.84; H 2.98; N 2.74%.

*6.1.3.5. 1,3-bis-[3,5-bis(4-Fluorobenzylidene)-4-oxo-piperidin-1yl]propane-1,3-dione (**8e**)*

Yield: 56%; mp (chloroform/methanol) >300 ^oC; ^1H NMR (500 MHz, $CDCl_3$): δ 7.81(s, 2H, 2×=CH), 7.75 (s, 2H, 2×=CH), 7.49(q, 4H, Ar-H), 7.33 (q, 4H, Ar-H), 7.18 (t, 4H, Ar-H), 7.12 (t, 4H, Ar-H), 4.84 (s, 4H, 2×NCH_2), 4.79 (s, 4H, 2×NCH_2), 3.22 (s, 2H, CH_2 Anal.calcd for $C_{41}H_{30}F_4N_2O_4.H_2O$: C 69.42; H 4.27; N 3.95 %, found: C 69.58; H 4.30; N 3.71%.

*6.1.3.6. 1,3-bis-[3,5-bis(4-Methoxybenzylidene)-4-oxo-piperidin-1yl]propane-1,3-dione (**8f**)*

Yield: 61%; mp (chloroform/methanol) 245 ^oC; ^1H NMR (500 MHz, $CDCl_3$): δ 7.80(s, 2H, 2×=CH), 7.73 (s, 2H, 2×=CH), 7.48 (d, J=8.6 Hz, 4H, Ar-H), 7.29 (d, J=10.17 Hz, 2H, Ar-H), 6.98 (d, J=8.49 Hz, 4H, Ar-H), 4.85(s, 4H, 2×NCH_2), 4.72 (s, 4H, 2×NCH_2), 3.88 (d, J=7.72 Hz, 12H, 4×OCH_3), 3.22 (s, 2H, CH_2). Anal.calcd for $C_{45}H_{42}N_2O_8.4\ H_2O$: C 66.59; H 5.22; N 3.45 %, found: C 66.68; H 5.38; N 3.27%.

*6.1.3.7. 1,3-bis-[3,5-bis(3,4-Dimethoxybenzylidene)-4-oxo-piperidin-1yl]propane-1,3-dione (**8g**)*

Yield: 65 %; mp (chloroform/methanol) >300 ^oC; ^1H NMR (500 MHz, DMSO-d_6): δ 7.86(s, 4H, 4×=CH), 7.17 (s, 4H, Ar-H), 7.13 (s, 8H, Ar-H), 4.54 (s, 8H, 4×NCH_2), 3.84 (d, J=7.27 Hz, 24H, 8×OCH_3), 3.48 (s, 2H, CH_2). Anal.calcd for $C_{49}H_{50}N_2O_{12}.7\ H_2O$: C 59.69; H 6.54; N 2.84 %, found: C 59.82; H 6.18; N 2.71%.

*6.1.3.8. 1, 3-bis-[3,5-bis(3,4,5-Trimethoxybenzylidene)-4-oxo-piperidin-1yl]propane-1,3-dione (**8h**)*

Yield: 53%; mp (chloroform/methanol) 115 ^oC; ^1H NMR (500 MHz, $CDCl_3$): δ 7.78(s, 2H, 2×=CH), 7.74 (s, 2H, 2×=CH), 6.72 (s, 4H, Ar-H), 6.58 (s, 4H, Ar-H), 4.96 (s, 4H, 2×NCH_2), 4.90 (s, 4H, 2×NCH_2), 3.92 (d, J=15.37 Hz, 30H, 10×OCH_3), 3.86 (s, 6H, 2×OCH_3), 3.37 (s, 2H, CH_2). Anal.calcd for $C_{53}H_{58}N_2O_{16}.H_2O$: C 63.83; H 6.07; N 2.81 %, found: C 63.84; H 6.27; N 2.51%.

*6.1.3.9. 1, 3-bis-[3, 5-bis{4-(N,N-Dimethylamino)benzylidene}-4-oxo-piperidin-1yl]propane-1,3-dione (**8i**)*

Yield: 42%; mp (chloroform/methanol) >300 ^{o}C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.78(s, 4H, 4×=CH), 7.36 (d, J=8.64 Hz, 8H, Ar-H), 6.73 (d, J=8.70 Hz, 8H, Ar-H), 4.21(s, 8H, 4×NCH_2), 3.05 (s, 26H, 8×NCH_3 and CH_2). Anal.calcd for $C_{49}H_{54}N_6O_4$.1.5 H_2O: C 71.88; H 7.02; N 10.61 %, found: C 71.59; H 7.28; N 10.78 %.

*6.1.3.10. 1,3-bis-[3,5-bis(4-Hydroxybenzylidene)-4-oxo-piperidin-1yl]propane-1,3-dione (**8j**)*

Yield: 56%; mp (chloroform/ethanol) >300 ^{o}C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.64(s, 2H, 2×=CH), 7.60 (s, 2H, 2×=CH), 7.57 (d, J=11.46 Hz, 4H, Ar-H), 7.54 (d, J=7.60 Hz, 4H, Ar-H), 7.49 (q, 8H, Ar-H), 4.62 (d, 8H, J=10.15 Hz, 4×NCH_2), 3.51 (s, 2H, CH_2). Anal.calcd for $C_{41}H_{34}N_2O_8$.4.5 H_2O: C 64.46; H 5.68; N 3.67 %, found: C 64.59; H 5.31; N 3.59%.

*6.1.4. Synthesis of 1,3-bis-[3,5-bis(benzylidene)-4-oxo-piperidin-1yl]propane (**9**)*

A mixture of 3,5-bis(benzylidene)-4-piperidone **6a** (0.004 mol, 1 gm), 1,3-dibromopropane (0.002 mol, 0.41gm), potassium carbonate (0.002, 0.28gm) and a catalytic amount of potassium iodide (10mg) in acetonitrile (30 ml) was heated at reflux temperature for 6-7 hrs. A further quantities of 1,3-dibromopropane (0.001 mol, 0.2 gm) added and the reaction continued for another 3-4 hrs. The completion of the reaction was monitored by TLC (solvent: chloroform-methanol 98:2). After the reaction was complete, the solvent was removed under reduced pressure at 45 ^{o}C. An aqueous solution of potassium carbonate (25 mL, 10% w/v) was added to the crude mass and stirred for 2h. The solid obtained was filtered, dried, and crystallized from acetone to yield pure product. Yield: 63 %; mp (acetone) 135 ^{o}C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.81(s, 4H, 4×=CH), 7.39 (m, 20H, Ar-H), 3.78 (s, 8H, 4×NCH_2), 2.54 (t, 4H, 2×NCH_2), 1.60 (p, 2H, CH_2). Anal.calcd for $C_{41}H_{38}N_2O_2$: C 83.36; H 6.48; N 4.74 %, found: C 83.74; H 6.79; N 5.05%.

6.2. Biology

6.2.1. Cell culture

Phosphate buffered saline (PBS), pH 7.8, versene, and trypsin (2.5%) was obtained from Invitrogen Inc. (Burlington, ON, Canada). Fetal bovine serum was purchased from Fisher scientific (Toronto, ON, Canada). McCoy's 5A medium (ATCC cat No: 30-2007) was purchased

from the ATCC (American Type Culture Collection, Rockville, USA). The penicillin-streptomycin antibiotic solution and all other chemicals unless otherwise indicated were purchased from Sigma (Oakville, ON, Canada). MilliQ water was obtained from a MilliQ water purification system (Millipore, MA, USA). Dr. Keith Bonham, Saskatoon Cancer Center, Saskatoon kindly gifted the HCT-116 and HT-29 colon cancer cell lines. The HCT-116 and HT-29 cell lines were subcultured in McCoy's 5A medium (ATCC cat No: 30-2007) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin antibiotic solution. The cells were grown in an atmosphere of 95% O_2 and 5% CO_2 with 95% humidity.

6.2.2. Cytotoxicity assay

The cytotoxicity of the compounds **7-9,** 5-fluorouracil and curcumin were determined using the sulforhodamine B assay as reported previously [27,28]. The cells were harvested using 0.25 % trypsin in versene and the cell count was determined using the trypan blue exclusion method. In a 96-well plate, about 5×10^3 cells in 100 µL of complete media was plated per well and allowed to grow for 24 hours. An aliquot of 100 µL of media containing a range of different concentrations of the test compounds and 1% DMSO (control) were added and incubated for 48 hours. The cells were fixed by adding 50 µL of 50% w/v aqueous trichloroacetic acid to each well and incubated at 4°C for one hour. To obtain the cell numbers at zero time point (Tz), a plate was fixed at the time of treatment of the cells with a solution of the test compound. The plates were rinsed with tap water four times and air dried. The fixed cells were stained with 0.4% w/v sulforhodamine in 1% v/v acetic acid for 10 minutes and washed four times with 1% v/v acetic acid and air dried. The dye was re-dissolved in 200 µL of 10 mM Trizma base and the absorbance read at 515 nm. The percent growth was calculated as per the equation below. The IC_{50} values were derived by fitting a four-parameter curve into the percent cell growth versus log concentration data using GraphPad Prism 5.0 for windows (GraphPad Software, San Diego, California, USA).

$$\% \text{ cell growth} = \frac{OD_{sample} - ODTz}{OD_{control} - ODT_2}$$

6.2.3. Apoptosis assay

Apoptosis assay was conducted using Annexin V-FITC apoptosis detection kit (Biovision; Catalogue No: ALX-850-250-KI02) as per the suggested protocol. HCT116 cells were grown in

McCoys'5A Modified media (ATCC) supplemented with 10% fetal bovine serum (Fisher Scientific) and 1% antibiotic-antimycotic solution (Sigma) in a humidified incubator at 37°C with 5% CO_2. About 1.5×10^3 cells/well were seeded in 6 well plates and left for 24 hrs at 37^oC following which the cells were treated with a solution of **2f** in DMSO and further diluted to a final concentration of 1% in the well. Apoptotic cell death was examined at different time points 1, 5, 11, 24 and 48 hrs and compared against untreated cells. The cells were collected by trypsinization (0.2% Trypsin-EDTA) followed by centrifugation for 5 minutes at 1000g at 4°C. The pallet was washed with 1ml of 1X PBS, resuspended in 500 microlitres of 1X binding buffer and treated with 5 microlitres of Annexin V-FITC (fluorescein isothiocyanate labeled annexin V) and 5 microlitre of propidium iodide. After incubating the samples in the dark for 5 mins at room temperature, apoptotic cell death was detected by a fluorescence-activated cell sorter (FACS) and analyzed by CellQuest (Becton-Dickinson) software.

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CHAPTER 6

Chapter 6 consists of copies of three articles: one published in the European Journal of Medicinal Chemistry in 2009*†, another in Die Pharmazie 2008**$, and the last one in Bioorganic and Medicinal Chemistry Letters***#

Relation of Chapter 6 to the objectives of this project

The objectives are to investigate the cytotoxic properties of a number of 1,5-diaryl-3-oxo-1,4-pentadienes possessing piperidine and cyclohexane rings and to evaluate the effect of representative potent cytotoxic molecules on rat liver mitochondria. The question to be addressed is whether cytotoxicity shown by these molecules includes targeting mitochondria.

Description

A number of structurally divergent molecules were developed as cytotoxic agents that includes: Part A. 2,4-bis(Benzylidene)-8-methyl-8-azabicyclo[3.2.1]octan-3-ones (**2**) and 3,5 bis(benzylidene)-1-methyl-4-piperidones (**3**), Part B. 2-Benzylidene-6-(nitrobenzylidene)-cyclohexanones (**1**-**3)**, and Part C. 1-[4-(2-Diethylaminoethoxy)phenylcarbonyl]-3,5-bis-(benzylidene)-4-piperidones (**2**) and their methiodide analogs (**3**). A number of representative potent cytotoxic molecules from Part A (**2a** and **2d**) and Part B (**1d**, **2d** and **3d**) were evaluated for their effect on respiration and swelling in rat liver mitochondria. In Part C, the molecules are positively charged quaternized methiodide enones **3** and their corresponding free bases **2** which were evaluated for their effect on mitochondrial functions. Owing to the negative membrane potential of mitochondria, the positively charged cytotoxic compounds are of great interest in mitochondria targeted chemotherapy.

Author Contributions

My contributions to the *Eur. J. Med. Chem, Bioorg. Med. Chem.* and *Die Pharmazie* publications were the evaluation of the compounds for their effect on mitochondrial functions such as respiration and swelling. The coauthors on these papers are U. Das, who synthesized some the compounds, supervised the synthesis of other compounds and undertook the QSAR and molecular modeling studies, H. N. Pati and A. Doroudi undertook the synthesis of some of the compounds, E. De Clercq and J. Balzarini supervised the cytotoxic assays against human Molt 4/C8 and CEM T-lymphocytes and murine leukemic L1210 cells, H. Sakagami and M. Kawase supervised the selective toxicity studies against some malignant and non-malignant cell lines, B.

Bandy supervised the mitochondrial work, J. W. Quail elucidated the X-ray crystal structure of **2e** in Part A and J. P. Stables supervised the short term toxicity of compounds in rodents in the *Eur. J. Med. Chem.* publication , and this project was supervised and guided by J. R. Dimmock and D. K. J. Gorecki.

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$ Das et al. *Pharmazie* **2008**, 63, 827-829.

Das et al. Bioorg. Med. Chem. **2008**, 16, 6261-6268.

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Original article

The cytotoxic properties and preferential toxicity to tumour cells displayed by some 2,4-*bis*(benzylidene)-8-methyl-8-azabicyclo[3.2.1] octan-3-ones and 3,5-*bis*(benzylidene)-1-methyl-4-piperidones

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Abstract

This study demonstrated that replacement of the axial protons on the C2 and C6 atoms of various 1-methyl-3,5-*bis*(benzylidene)-4-piperidones **3** by a dimethylene bridge leading to series **2** lowered cytotoxic potencies. Four compounds **2a** and **3a–c** emerged as lead molecules based on their toxicity towards different neoplasms and their selective toxicity for malignant rather than normal cells. Some possible reasons for the disparity between the IC_{50} values in the two series of compounds are presented based on molecular modeling, log *P* values and respiration in rat liver mitochondria.

Keywords: Tropinones; 4-Piperidones; Cytotoxicity; Molecular modeling; X-ray crystallography; Mitochondria

1. Introduction

The major interest in these laboratories is the development of antineoplastic agents which are structurally divergent from contemporary anticancer drugs. These novel compounds are principally conjugated unsaturated ketones which are known to react with thiols [1] but have low or nonexistent affinities for amino and hydroxy groups [2,3]. Since thiols, in contrast to amino or hydroxy groups, are not found in nucleic acids, α,β-unsaturated ketones may be bereft of the carcinogenic and mutagenic properties displayed by various anticancer drugs [4]. There are a number of critical biochemical processes which involve thiols and the importance of compounds which interact with multiple molecular targets has been emphasized recently [5,6].

The 1,5-diaryl-3-oxo-1,4-pentadienyl group has been mounted on a variety of cyclic scaffolds leading to the discovery of a number of potent cytotoxins [7,8]. This group is considered to react at a primary binding site. However, the magnitude of the bioactivity observed will be influenced by the presence of other structural units in the molecule which align at an auxiliary site. These possibilities are illustrated in Fig. 1A. In order to probe as to the nature of the groups in the vicinity of the pharmacophore which affect cytotoxic potencies, various compounds possessing the general structure **1** were prepared (Fig. 1B). Several studies revealed that

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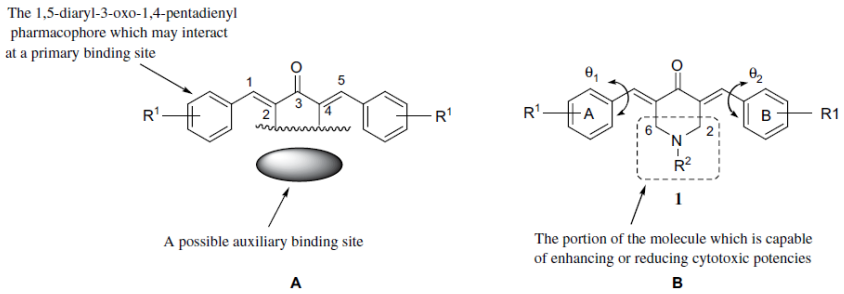


Fig. 1. A) The possible interactions of series **1**–**3** at binding sites. (B) The general structure **1**.

compounds in which R^2 is an acyl group have increased cytotoxic potencies compared to the analogs when R^2 is a hydrogen atom [9,10]. In fact a number of *N*-acyl compounds have submicromolar IC_{50} values and displayed selective toxicity to neoplasms than normal cells [11]. Thus by expanding the size of the molecules, there is the possibility of additional binding of the ligand at a receptor which results in the lowering of the IC_{50} values. The hypothesis formulated in this study is that by increasing the size of the heterocyclic scaffold, cytotoxic potencies will be elevated compared to the analogs lacking this additional structural unit. In the present case, a dimethylene bridge was placed between carbon atoms 2 and 6 of the piperidine ring to give series **2** with a view to comparing cytotoxic potencies with the analogs **3** which lack this structural feature.

Previous studies revealed that the lack of coplanarity of rings A and B with the adjacent unsaturated linkages in **1** was caused, inter alia, by nonbonded interactions between one of the *ortho* protons of each aryl ring with the equatorial hydrogen atoms at C2 and C6 [12]. The decision was made, therefore, to replace the axial and not equatorial protons on the C2 and C6 atoms by substituents. In this way, changes in the cytotoxic potencies between **1** and various analogs could be attributed to the topographical, physicochemical and chemical properties of the groups at C2 and C6 per se and the interpretation of the results would not be complicated by changes in the torsion angles θ_1 and θ_2. X-ray crystallography revealed that the displacement of the C2 and C6 axial hydrogen atoms of various piperidines by a dimethylene bridge afforded 8-azabicyclo[3.2.1]octanes [13,14]. Hence the aim of the present investigation was to prepare a small cluster of prototypic molecules related to **1** which bear C2 and C6 substituents, namely series **2**, and to compare their cytotoxic properties with the analogs having both axial protons intact on the C2 and C6 atoms viz series **3**. In particular, the information gained from this study may contribute to an understanding of those structural features which lead to marked cytotoxic properties.

2. Chemistry

The compounds in series **2** and **3** were prepared by the synthetic chemical route presented in Scheme 1. X-ray crystallography was undertaken on **2e** and an ORTEP diagram [15] of this compound is displayed in Fig. 4. Molecular models of **2a**–**e** and **3a**–**e** were built and the torsion angles θ_1 and θ_2 are recorded in Table 1.

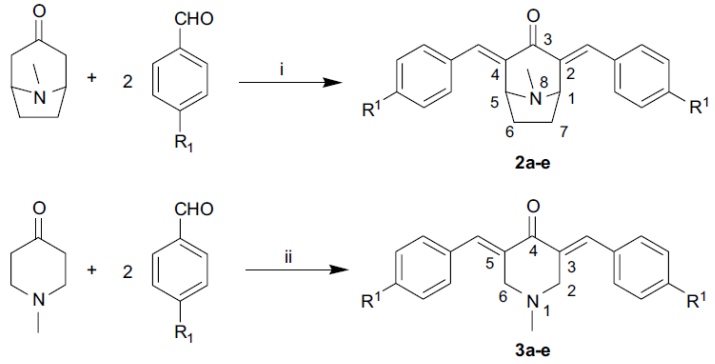
3. Bioevaluations

All of the compounds in series **2** and **3** were evaluated against human Molt 4/C8 and CEM T-lymphocytes and murine L1210 lymphoid leukemia cells. These results are portrayed in Table 1. In addition, these compounds were assayed for inhibitory effects towards human HSC-2, HSC-3 and HSC-4 oral squamous cell carcinomas and human HL-60 promyelocytic leukemia cells. Three normal human cell lines were also used, namely HGF gingival fibroblasts, HPC pulp cells and HPLF periodontal ligament fibroblasts. The data from these determinations are presented in Table 2. The effects of **2a** and **2d** on respiration and swelling of rat liver mitochondria are presented in Figs. 5 and 6, respectively. Doses of 30, 100 and 300 mg/kg of **2a**–**e** and **3a**–**e** were injected intraperitoneally into mice and the animals were observed after 0.5 and 4 h for any mortalities. In addition, using these doses and time intervals, the mice were examined for neurotoxicity by the rotorod test [16]. Two representative compounds, namely **2e** and **3c**, were administered orally to rats using a dose of 50 mg/kg and the animals were examined for mortalities and neurotoxicity after 0.25, 0.5, 1 and 2 h and in the case of **2e** after 4 h also.

4. Results and discussion

^{1}H NMR spectroscopy revealed that the compounds in series **2** and **3** are isomerically pure. X-ray crystallography of 8-methyl-2,4-*bis*(3-thienylmethylene)-8-azabicyclo[3.2.1]octan-3-one [17] as well as **2e** revealed that the olefinic double bonds adopted the *E* configuration. In addition, the same stereochemistry was noted with various 3,5-*bis*(benzylidene)-1-methyl-4-piperidones [18,19]. Hence the assumption was made that all of the compounds in series **2** and **3** are the *E*,*E* isomers.

All of the compounds in series **2** and **3** were evaluated against human Molt 4/C8 and CEM T-lymphocytes in order to ascertain the toxicity of these compounds towards human neoplastic cells. In addition, **2a**–**e** and **3a**–**e** were examined towards murine L1210 cells since a number of anticancer drugs are cytotoxic to this cell line [20] and hence it serves



Scheme 1. Synthetic routes employed in the synthesis of series **2** and **3** in which i = NaOH and ii = HCl/CH_3COOH. The R^1 substituents are as follows, namely **a**: $R^1 = H$; **b**: $R^1 = Cl$; **c**: $R^1 = NO_2$; **d**: $R^1 = CH_3$; **e**: $R^1 = OCH_3$.

as an indicator of compounds having potential clinical utility. These data are presented in Table 1.

The results indicate that among the tropinone derivatives, only **2a** displayed noteworthy cytotoxicity having an IC_{50} value of approximately 10 μM and on average possessing approximately one-quarter of the potency of melphalan. The 4-methoxy analog **2e** exhibited moderate potency towards L1210 cells but not to the T-lymphocytes while the remaining compounds in series **2** have IC_{50} values considerably in excess of 100 μM. On the other hand, in the 4-piperidone series, both **3a** and **3c** are potent cytotoxins especially towards the T-lymphocytes. Compound **3a** is 1.6 times more potent than melphalan towards Molt 4/C8 cells and is equipotent with this drug in the CEM assay. The 4-*nitro* analog **3c** is equipotent with melphalan in both the Molt 4/C8 and CEM tests. Clearly both **3a** and **3c** are useful lead molecules. Compound **3b** demonstrated modest potencies while the IC_{50} values of **3c** and **3d** are in excess of 100 μM. The unsubstituted compound in both series **2** and **3** possesses the lowest IC_{50} values which may indicate that an E_4 operating parameter is in effect [21]; i.e., the 4-substituent in **2b–e** and **3b–e** may cause an unfavourable steric impedance to the alignment of the molecules at critical binding sites. A second factor which may influence cytotoxic potencies in series **3** is the electronic contributions of the nuclear substituents. Thus in **3a–c**, the σ values of the R^1 group are 0.00–0.78 [22] while in the substantially less potent molecules **3d**,**e**, the σ constants are −0.17 to −0.27 [22]. Thus in the latter two compounds the methyl and methoxy aryl groups lower the fractional positive charge on the olefinic protons relative to **3a–c** thereby reducing electrophilicity towards cellular thiols.

The biodata presented in Table 1 reveal very clearly that the substitution of a dimethylene bridge for the axial protons attached to the C3 and C5 atoms of series **3** which generated **2a–e** leads to a reduction in cytotoxic potency. This conclusion may be drawn by noting that when the same substituents are present in the aryl rings, the compounds in series **3** are more potent than the analogs **2a–e** with the exception that **2e** is more cytotoxic than **3e** in the L1210 screen.

Table 1
Some cytotoxic and physicochemical properties of **2a–e** and **3a–e**

Compound	IC_{50} (μM)[a]				Torsion angles			
	Molt 4/C8	CEM	L1210	Average[b]	θ_1[c]	θ_2[c]	log P[d]	TPSA[d]
2a	8.51 ± 0.60	8.99 ± 0.54	11.8 ± 2.0	9.77	47.4	−47.4	4.33	20.3
2b	>500	>500	>500	>500	121.7	−45.3	5.68	20.3
2c	>500	247 ± 112	336 ± 3	>361	120.2	−120.5	4.25	112.0
2d	>500	>500	>500	>500	47.1	−47.1	5.22	20.3
2e	>500	–[e]	40.0 ± 18.4	–	46.2	−46.3	4.44	38.8
3a	1.98 ± 0.27	3.32 ± 2.30	8.77 ± 0.28	4.69	45.6	−46.0	3.95	20.3
3b	36.9 ± 8.0	33.9 ± 12.9	96.8 ± 3.5	55.9	45.5	−45.9	5.31	20.3
3c	2.42 ± 0.38	5.21 ± 3.06	14.0 ± 1.8	7.21	46.7	−47.3	3.87	112.0
3d	277 ± 6	233 ± 27	305 ± 10	171	45.5	−46.0	4.85	20.3
3e	230 ± 1	172 ± 6	281 ± 15	228	43.2	−43.7	4.06	38.8
Melphalan[f]	3.24 ± 0.56	2.47 ± 0.21	2.13 ± 0.02	2.61	–	–	–	–

[a] The IC_{50} values represent the concentrations of compounds required to inhibit the growth of the cells by 50%.
[b] These figures indicate the average of the IC_{50} figures towards the three cell lines.
[c] The θ values refer to the torsion angles between the aryl rings and the adjacent olefinic linkage.
[d] The letters log P and TPSA indicate the calculated log P and total polar surface area values, respectively, of the molecules.
[e] The percentage inhibition of CEM cells by **2e** was inconsistent viz 61 ± 7, 46 ± 3, 64 ± 4 and 12 ± 4 at concentrations of 500, 100, 20 and 4 μM, respectively.
[f] The data for melphalan was taken from Pharmazie 52 (1997) 182–186 with the permission of the copyright owner.

Table 2
Examination of **2a–e**, **3a–e** and melphalan against some human malignant and normal cells

Compound	Human tumour cells CC_{50} (μM)[a]					Human normal cells CC_{50} (μM)[a]			
	HSC-2	HSC-3	HSC-4	HL-60	Average[b]	HGF	HPC	HPLF	SI[c]
2a	21	44	23	8.3	24	298	>400	>400	>15
2b	>400	>400	>400	>400	>400	>400	>400	>400	~1.0
2c	308	>400	>400	>400	>377	>400	>400	>400	~1.1
2d	>400	>400	>400	>400	>400	>400	>400	>400	~1.0
2e	300	380	250	364	324	162	>400	366	~1.0
3a	4.2	7.9	7.4	2.0	5.4	45	64	40	9.2
3b	7.4	16	39	14	19	323	369	>400	>19
3c	16	47	22	20	26	170	>400	326	>11
3d	276	>400	338	>400	>354	>400	>400	>400	~1.1
3e	344	>400	>400	>400	>386	>400	>400	>400	~1.0
Melphalan[d]	35	115	81	6	59	>200	>200	>200	>3.4

[a] The CC_{50} values are the concentrations of the compounds required to kill 50% of the cells. Determinations were carried out in duplicate and the variation between experiments was less than 5%.
[b] The average values reflect the mean of the CC_{50} figures for the compounds generated using HSC-2, HSC-3, HSC-4 and HL-60 cells.
[c] The letters SI refer to the selectivity index (SI) values which are the quotients of the average CC_{50} figures of the compounds towards normal cells divided by the average CC_{50} data for the malignant cell lines.
[d] Solubility considerations precluded the use of concentrations higher than 200 μM. The data for melphalan are taken from Bioorganic and Medicinal Chemistry 2007; 15:3373–3380 and is reproduced with the permission of Elsevier.

Attempts were made to determine the reasons for the lowering of potencies when substituents were placed on the C2 and C6 atoms in series **3**. The information generated may assist in gleaning further knowledge of those structural features which influence cytotoxicity. First, molecular models of **2a–e** and **3a–e** were made and the torsion angles θ_1 and θ_2 are listed in Table 1. Apart from the anomolous behaviour of **2b** and **2c**, these angles are all in the range of 43–47°. Thus, in general, substitution of the C2 and C6 axial protons in both series of compounds does not affect the magnitude of the deviation from coplanarity between rings A and B and the adjacent olefinic linkages. The changes in bioactivity between series **2** and **3** are, therefore, likely due to factors in the loci of the carbon atoms attached to the nitrogen atom. The biodata in Table 1 reveal that in all three assays, **3a** possessed greater cytotoxicity potencies than **2a**. Models of both compounds are presented in Fig. 2 and the greater steric bulk in the vicinity of the basic centre and the adjacent carbon atoms in **2a** are apparent. Furthermore in order to provide some quantitative information pertaining to the steric bulk in the vicinity of the C1/C5 and C2/C6 atoms in **2a** and **3a**, respectively, the interatomic distances d_1 and d_2 as indicated in Fig. 3 were measured. The d_1 values of **2a** and **3a** are 2.21 and 1.11 Å, respectively, while the d_2 figures are 2.25 and 2.38 Å, respectively. Thus while both the C1/C5 and C2/C6 atoms in **2a** and **3a**, respectively, could align at the same portion of a binding site, the dimethylene bridge likely exerts a significant steric repulsion. The areas occupied by the axial substituents in **2a** and **3a**, i.e., $d_1 \times d_2$, are 4.97 and 2.64 sq Å, respectively. Thus, in future, the design of compounds occupying an area in between these two values may afford further information as to the effect of substituents on the C2 and C6 atoms in series **3**, e.g., a methyl or trifluoromethyl group could be placed on one of the C2 or C6 atoms in series **3**.

X-ray crystallography of a representative compound in series **2**, namely **2e**, was undertaken to confirm the *E* stereochemistry of the olefinic double bonds and to compare the θ_1 and θ_2 values with the published X-ray crystallographic data for **3e**. In the case of **2e** there are two molecules, designated **2e1** and **2e2**, in the asymmetric unit of the centrosymmetric space group $P2_1/c$. These molecules are very similar in shape and an ORTEP-3 diagram [15] of **2e1** is presented in Fig. 4. Both **2e1** and **2e2** are the *E*,*E* isomers. The C4-C9-C10-C11 (θ_1) and C2-C16-C17-C18 (θ_2) values for **2e1** are 22.1° and −14.0°, respectively, while the comparable θ_1 and θ_2 figures for **2e2** are 13.9° and −15.4°, respectively. The θ_1 and θ_2 values for **3e** are 18.4° and −26.8°, respectively [18]. In general, therefore, the X-ray crystallographic data support the concept that replacement of the axial protons in series **3** by a dimethylene bridge does not change the torsion angles θ_1 and θ_2 to an appreciable extent.

Since the hydrophobicity of molecules may influence the extent of bioactivity significantly [23], the log *P* values of **2a–e** and **3a–e** were computed. These data are presented in Table 1. The lower log *P* values of the compounds in series **3** than **2** when comparing pairs of compounds having identical aryl substituents may have contributed to the generally greater cytotoxic potencies of the 1-methyl-4-piperidones **3**. Compounds in series **2** and **3** which have the same R^1 substituents have identical total polar surface area (TPSA) values as indicated in Table 1; consequently TPSA values do not contribute to potency differences between the two series of compounds. In order to seek possible correlations between cytotoxic potencies in series **3** and both the log *P* and TPSA values, linear, semilogarithmic and logarithmic plots were made between these physicochemical parameters and the IC_{50} values of **3a–e** in each of the three assays. However, no correlations were observed ($p > 0.1$).

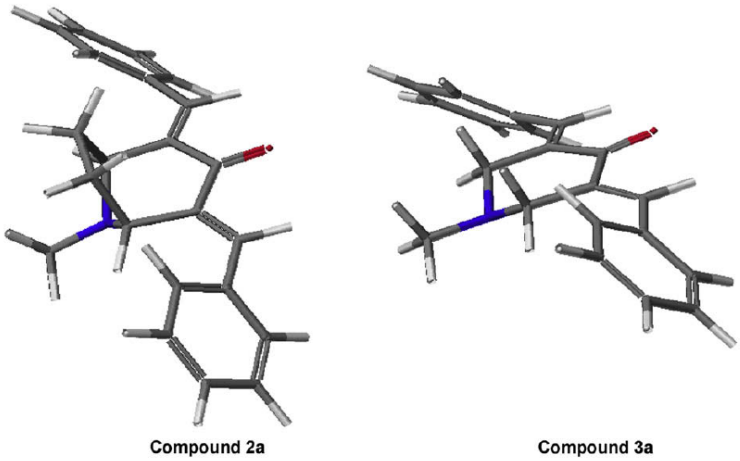


Fig. 2. Molecular models of **2a** and **3a**.

Various acyclic 3-aminoketones inhibit or stimulate respiration in mitochondria isolated from rat and mouse liver cells [24,25]. Since the compounds prepared in this study are cyclic 3-aminoketones, the question arises whether different effects on mitochondria may explain the variation in cytotoxic potencies. Accordingly two compounds displaying markedly divergent cytotoxic properties, namely **2a** and **2d**, were examined. Fig. 5 shows that after a delay both compounds exert a strong stimulating effect on mitochondrial respiration, with compound **2a** giving a significantly shorter latent period (1.96 ± 0.05 min versus 4.45 ± 0.13 min). Measurements shown in Fig. 6 reveal that **2a** produces rapid mitochondrial swelling, while **2d** does so only more slowly. Mitochondrial swelling involves opening of the mitochondrial permeability transition pore and collapse of the mitochondrial membrane potential [26]. This collapse of the membrane potential decreases the resistance to electron flow in the respiratory chain and increases mitochondrial respiration [27], which accounts for the increase in respiration observed in Fig. 5. The mitochondrial permeability transition is a critical trigger for apoptosis [28] and has been identified as a target for cancer therapy [29–31]. The greater ability of **2a** to induce mitochondrial swelling, therefore, may have contributed to its higher cytotoxic potency in the cancer cell lines.

A difference in electrophilicity may explain the difference in the ability of **2a** and **2d** to cause mitochondrial swelling. The opening of the mitochondrial permeability transition pore involves alkylation or cross-linking of a critical thiol on a protein of the permeability transition pore complex [32,33], and as noted earlier these conjugated unsaturated ketones are known to react with thiols [1]. In the less potent **2d**, the R^1 methoxy substituents are less electronegative (σ value $= -0.17$) than the R^1 protons (σ value $= 0.00$) of **2a** and would thereby decrease the electrophilicity of **2d** towards thiols compared to **2a**.

All of the compounds in series **2** and **3** were evaluated further using human HSC-2, HSC-3, HSC-4 and HL-60 neoplasms. These data are presented in Table 2. The results are similar to the biodata generated using Molt 4/C8, CEM and L1210 cells, namely in series **2** only **2a** displays noteworthy cytotoxicity while **3a–c** are substantially more potent than **3d,e**. Taking into consideration the average CC_{50} values towards these four cell lines, **2a** and **3a–c** possess 2.5, 10.9, 3.1 and 2.3 times the potency of melphalan and are clearly lead molecules. The CC_{50} values of **2a** and **3a–c** towards human HGF, HPC and HPLF normal cells reveal their excellent selectivity (SI) figures demonstrating the preferential toxicity of these compounds for neoplastic cells, which further confirms their importance as templates for further development.

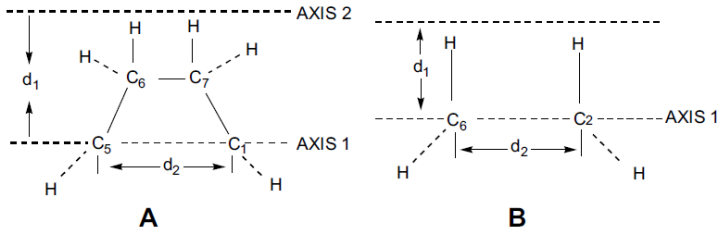


Fig. 3. A comparison of the steric bulk of portions of the structures of **2a** (A) and **3a** (B).

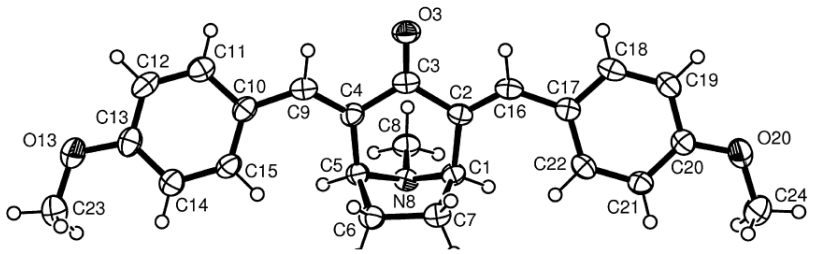


Fig. 4. An ORTEP-3 diagram of **2e1** determined by X-ray crystallography.

A number of acylic 3-aminoketones or Mannich bases are lethal to mice at low doses, e.g., 30 mg/kg, and also display neurotoxicity [34]. Since 4-piperidones may be considered cyclic 3-aminoketones, the evaluation of the compounds in series **2** and **3** with regard to mortality and neurological deficit was undertaken. Doses up to and including 300 mg/kg of **2a**–**e** and **3a**–**e** were administered intraperitoneally to mice and after 4 h, no deaths of the animals were noted. Minimal neurotoxicity was observed with **2e**, **3a**,**c**,**e** after 0.5 h and with **3a** after 4 h. A dose of 300 mg/kg of **2e** caused neurotoxicity in all of the animals. No neurological disturbances were caused by the other compounds. A dose of 50 mg/kg of two representative compounds **2e** and **3c** was administered orally to rats and the animals were examined at different time intervals up to 4 (**2e**) and 2 (**3c**) h. No mortalities or neurological deficit was noted. The conclusion drawn from this short-term toxicity evaluation is that the compounds in series **2** and **3** are well tolerated in rodents thereby enhancing their potential for future development.

5. Conclusions

This study has revealed clearly that in general replacement of the 2a and 6a protons in series **3** by a dimethylene bridge leading to **2a**–**e** is accompanied by a reduction in cytotoxic potencies. Thus development of the cytotoxic 3,5-*bis*(benzylidene)-4-piperidones in which two protons are present on the carbon atoms attached to the basic centre appears to be a prudent decision. However, limited molecular modifications whereby groups of varying sizes are placed on the 2 and 6 carbon atoms of series **3** may establish the generality or otherwise that such structural changes are disadvantageous in terms of cytotoxic potencies. The reasons for the disparity in cytotoxic potencies between series **2** and **3** may have been due to the dimethylene bridge in **2a**–**e** exerting a steric impedance to alignment at one or more binding sites as well as variation in hydrophobic properties and possibly differential effects on mitochondrial respiration.

In terms of cytotoxic potencies, the data in Table 1 reveal that **2a**, **3a** and **3c** are lead molecules while moderate potency was displayed by **3b**. The assessment of these four compounds against human tumour cell lines as revealed in Table 2 confirmed their cytotoxic properties which in these assays are on average more potent than a reference drug melphalan. The importance of these four compounds as templates for future development was enhanced by two additional observations. First, **2a** and **3a**–**c** display preferential cytotoxicity for malignant rather than normal cells as revealed by the SI

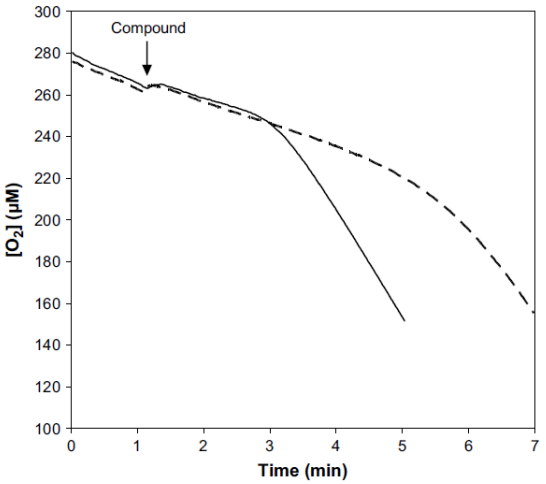


Fig. 5. Effects of **2a** and **2d** on respiration of rat liver mitochondria. Where indicated by the arrow, compounds were added to a respiring suspension of rat liver mitochondria to a concentration of 250 μM: — **2a**, – – – **2d**.

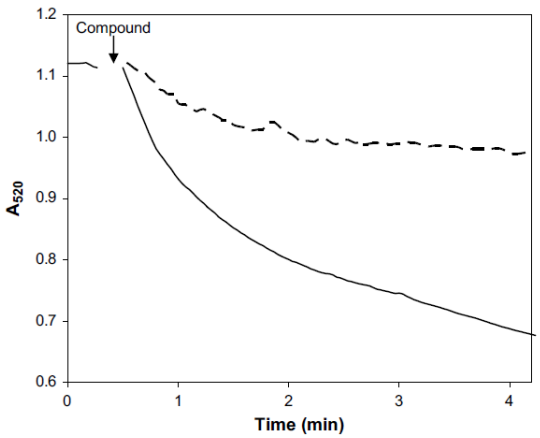


Fig. 6. Effects of **2a** and **2d** on swelling of rat liver mitochondria. Where indicated by the arrow, compounds were added to a respiring suspension of rat liver mitochondria to a concentration of 250 μM: — **2a**, – – – **2d**.

figures in Table 2. Second, these compounds and their analogs in series **2** and **3** are well tolerated in mice.

6. Experimental protocols

6.1. Chemistry

Melting points which are uncorrected were determined using a Gallenkamp instrument. ^{1}H NMR spectra were recorded using a Bruker AMX 500 FT machine while elemental analyses (C, H, N) were obtained using an Elementer analyzer and were within 0.4% of the calculated values. 4-Piperidones **3a**,**c**,**d** were crystallized with 0.25, 0.75 and 0.25 mol of water of crystallization, respectively. X-ray crystallography was undertaken using a Nonius instrument.

6.1.1. Synthesis of 2,4-bis(benzylidene)-8-methyl-8-aza-bicyclo[3.2.1]octan-3-ones (2a–e)

Sodium hydroxide solution (5N, 1 ml) was added dropwise to a solution of 8-methyl-8-aza-bicyclo[3.2.1]octan-3-one (0.5g, 0.0036 mol) and the appropriate aryl aldehyde (0.0072 mol) in ethanol (20 ml) at room temperature. The reaction mixture was stirred under nitrogen for 2 h at room temperature and then water (15 ml) was added. The precipitate was collected and recrystallized from ethanol.

*6.1.1.1. 2,4-Bis(benzylidene)-8-methyl-8-aza-bicyclo[3.2.1]octan-3-one (**2a**).* Yield: 70%; m.p. 148 °C, (lit. [35] m.p. 150–151 °C). ^{1}H NMR ($CDCl_3$) δ: 2.06 (q, 2H), 2.33 (s, 3H), 2.61 (p, 2H), 4.43 (dd, 2H), 7.40 (m, 10H), 7.86 (s, 2H). Anal. calcd. for $C_{22}H_{21}NO$: C, 83.78; H, 6.74; N, 4.44%. Found: C, 83.57; H, 6.54; N, 4.40%.

*6.1.1.2. 2,4-Bis(4-chlorobenzylidene)-8-methyl-8-aza-bicyclo[3.2.1]octan-3-one (**2b**).* Yield: 85%, m.p. 183 °C. ^{1}H NMR ($CDCl_3$) δ: 2.02 (q, 2H), 2.32 (s, 3H), 2.62 (p, 2H), 4.35 (p, 2H), 7.34 (d, 4H, $J = 8.38$ Hz), 7.43 (d, 4H, $J = 8.61$ Hz), 7.78 (s, 2H). Anal. calcd. for $C_{22}H_{19}Cl_2NO$: C, 68.76; H, 4.98; N, 3.64. Found: C, 68.71; H, 4.98; N, 3.77%.

*6.1.1.3. 8-Methyl-2,4-bis(4-nitrobenzylidene)-8-aza-bicyclo[3.2.1]octan-3-one (**2c**).* Yield: 73%, m.p. 247 °C. ^{1}H NMR ($CDCl_3$) δ: 2.06 (q, 2H), 2.34 (s, 3H), 2.68 (p, 2H), 4.34 (dd, 2H), 7.55(d, 4H, $J = 8.60$ Hz), 7.83 (s,2H), 8.33 (d, 4H, $J = 8.67$ Hz). Anal. calcd. for $C_{22}H_{19}N_3O_5$: C, 65.18; H, 4.72; N, 10.37. Found: C, 64.95; H, 4.67; N, 10.60%.

*6.1.1.4. 8-Methyl-2,4-bis(4-methylbenzylidene)-8-aza-bicyclo[3.2.1]octan-3-one(**2d**).* Yield: 80%, m.p. 172 °C. ^{1}H NMR ($CDCl_3$) δ: 2.05 (q, 2H), 2.33 (s, 3H), 2.42 (s, 6H), 2.63 (p, 2H), 4.43 (dd, 2H), 7.26 (d, 4H, $J = 7.92$ Hz), 7.33 (d, 4H, $J = 7.99$ Hz), 7.84 (s, 2H). Anal. calcd. for $C_{24}H_{25}NO$: C, 83.93; H, 7.34; N, 4.08. Found: C, 84.13; H, 7.02; N 4.20%.

*6.1.1.5. 2,4-Bis(4-methoxybenzylidene)-8-methyl-8-aza-bicyclo[3.2.1]octan-3-one (**2e**).* Yield: 82%, m.p. 160 °C (lit. [35] m.p. 162–163 °C). ^{1}H NMR ($CDCl_3$) δ: 2.03 (q, 2H), 2.34 (s, 3H), 2.62 (p, 2H), 3.87 (s, 6H), 4.41 (dd, 2H), 6.98 (d, 4H, $J = 8.64$ Hz), 7.40 (d, 4H, $J = 8.68$ Hz), 7.82 (s, 2H). Anal. calcd. for $C_{24}H_{25}NO_3$: C, 76.77; H, 6.71; N, 3.73. Found: C, 76.89; H, 6.54; N, 3.77%.

6.1.2. Synthesis of 3,5-bis(benzylidene)-1-methyl-4-piperidones (3a–e)

Dry hydrogen chloride was passed into a solution of 1-methyl-4-piperidone (0.05 mol) and the appropriate aryl aldehyde (0.10 mol) in acetic acid (25 ml) at room temperature. The mixture was stirred at room temperature for 6–8 h and the precipitate was collected, washed with acetone (20 ml) and added to a solution of aqueous potassium carbonate solution (5% w/v). The free base was collected, dried under vacuum at 45–50 °C and crystallized from ethanol (**3a**), chloroform–methanol (**3b**,**d**,**e**) or chloroform–ethanol (**3c**).

*6.1.2.1. 3,5-Bis(benzylidene)-1-methyl-4-piperidone (**3a**).* Yield: 71%, m.p. 110 °C (lit. [36] m.p. 115–117 °C). ^{1}H NMR ($CDCl_3$) δ: 2.49 (s, 3H), 3.80 (s, 4H), 7.42 (m, 10H), 7.85 (s, 2H). Anal. calcd. for $C_{20}H_{19}NO.0.25H_2O$: C, 81.66; H, 6.46; N, 4.76%. Found: C, 81.90; H, 6.36; N, 4.69%.

*6.1.2.2. 3,5-Bis(4-chlorobenzylidene)-1-methyl-4-piperidone (**3b**).* Yield: 88%, m.p. 183 °C (lit. [36] m.p. 174–176 °C). ^{1}H NMR ($CDCl_3$) δ: 2.49 (s, 3H), 3.75 (s, 4H), 7.35 (d, 4H, $J = 8.44$ Hz), 7.43 (d, 4H, $J = 8.46$ Hz), 7.77 (s, 2H). Anal. calcd. for $C_{20}H_{17}Cl_2NO$: C, 67.05; H, 4.78; N, 3.91. Found: C, 66.89; H, 4.67; N, 3.81%.

*6.1.2.3. 3,5-Bis(4-nitrobenzylidene)-1-methyl-4-piperidone (**3c**).* Yield: 81%, m.p. 223 °C (lit. [36] m.p. 229–231 °C). ^{1}H NMR ($CDCl_3$) δ: 2.89 (s, 3H), 3.56 (br s, 4H), 7.52 (d, 4H, $J = 8.62$ Hz), 7.76 (s, 2H), 8.23 (d, 4H, $J = 8.61$ Hz). Anal. calcd. for $C_{20}H_{17}N_3O_5$ 0.75 H_2O: C, 61.08; H, 4.32; N, 10.69. Found: C, 61.05; H, 4.42; N, 11.07%.

*6.1.2.4. 3,5-Bis(4-methylbenzylidene)-1-methyl-4-piperidone (**3d**).* Yield: 76%, m.p. 201 °C (lit. [36] m.p. 192–195 °C). ^{1}H NMR ($CDCl_3$) δ: 2.42 (s, 6H), 2.49 (s, 3H), 3.79 (s, 4H), 7.26 (d, 4H, $J = 7.95$ Hz), 7.33 (d, 4H, $J = 8.0$ Hz), 7.82 (s, 2H). Anal. calcd. for $C_{22}H_{23}NO$ 0.25 H_2O: C, 82.00; H, 7.14; N, 4.35. Found: C, 81.92; H, 7.19; N, 4.25%.

*6.1.2.5. 3,5-Bis(4-methoxybenzylidene)-1-methyl-4-piperidone (**3e**).* Yield: 83%, m.p. 186 °C (lit. [36] m.p. 199–201 °C). ^{1}H NMR ($CDCl_3$) δ: 2.51 (s, 3H), 3.79 (s, 4H), 3.88 (s, 6H), 6.98 (d, 4H, $J = 8.71$ Hz), 7.40 (d, 4H, $J = 8.67$ Hz), 7.80 (s, 2H). Anal. calcd. for $C_{22}H_{23}NO_3$: C, 75.62; H, 6.63; N, 4.01. Found: C, 75.72; H, 6.71; N, 4.22%.

6.1.3. X-ray crystallography of 2,4-bis(4-methoxybenzylidene)-8-methyl-8-aza-bicyclo[3.2.1]octan-3-one (2e)

With the exception of the structure factors, data pertaining to the X-ray crystallographic determination of **2e** have been deposited with the Cambridge Crystallographic Data Centre

as supplementary publication no. CCDC 656960. This information may be obtained without cost by applying to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK, (fax: +44 (0) 1223 336033 or e-mail deposit@ccdc.cam.ac.uk).

6.1.4. Molecular modeling

The molecular models of **2a**–**e** and **3a**–**e** were built using the BioMedCache program [37]. MOPAC optimized geometry calculations using AM1 parameters were employed in order to produce the lowest energy conformations.

6.1.5. Determination of the calculated log P and total polar surface area values of 2a–e and 3a–e

The log *P* and TPSA data were generated using the JME molecular editor [38].

6.1.6. Statistical calculations

The linear, semilogarithmic and logarithmic plots between the IC_{50} values of **3a**–**e** in different bioassays and the *c* log *P* and TPSA data were made using a software package [39].

6.2. Bioassays

6.2.1. Cytotoxicity evaluations

The assays using human Molt 4/C8 and CEM T-lymphocytes and murine L1210 cells have been described previously [40]. In brief, the cells are incubated with different concentrations of compounds in RPMI 1640 medium at 37 °C for 72 h (Molt 4/C8 and CEM cells) and 48 h (L1210 cells).

A literature procedure was utilized for the bioevaluation of **2a**–**e** and **3a**–**e** towards HSC-2, HSC-3, HSC-4, HL-60, HGF, HPC and HPLF cells [41]. In brief, with the exception of assays using HL-60 cells, different concentrations of compounds were incubated with the cell lines in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum. Cell viability was determined by the MTT method after 24 h incubation at 37 °C. A similar procedure was followed for the HL-60 cells except RPMI 1640 media containing 10% fetal bovine serum was used and cytotoxicity was assessed by the trypan blue exclusion procedure.

6.2.2. Evaluation of 2a and 2d on respiration in rat liver mitochondria

Rats were euthanized by isoflurane anesthesia and decapitation. The mitochondria from the liver were isolated by differential centrifugation using a literature procedure [42]. The effect of **2a** and **2d** on the consumption of oxygen in mitochondria was measured by polarography by a previously reported methodology [43]. In these measurements, freshly isolated mitochondria were incubated at 30 °C in a respiratory buffer of 125 mM sucrose, 65 mM KCl, 10 mM HEPES, 5 mM potassium phosphate, 1 mM $MgCl_2$, pH 7.2 containing 5 mM succinate as respiratory substrate. Under the same conditions, mitochondrial swelling was measured spectrophotometrically by the loss in light scattering at 520 nm as described previously [42].

6.2.3. Toxicity and neurotoxicity evaluations in rodents

The toxicity and neurotoxicity evaluations were undertaken by the National Institute of Neurological Disorders and Stroke, USA according to their protocols [44]. Doses of 30, 100 and 300 mg/kg of **2a**–**e** and **3a**–**e** were injected intraperitoneally into mice and observed for both mortalities and neurotoxicity at the end of 0.5 and 4 h. No deaths were observed. Neurotoxicity was observed in the following cases (dose in mg/kg, time of observation in hours, number of animals displaying neurological deficit/total number of animals in the test) viz **2d** (300, 4, 2/2), **2e** (100, 0.5, 1/8), **3a** (100, 0.5, 2/8; 100, 4, 1/4), **3c** (100, 0.5, 1/8) and **3e** (300, 0.5, 1/4). In addition, doses of 50 mg/kg of **2e** and **3c** were administered orally to rats. At the end of 0.25, 0.5, 1 and 2 h (also 4 h in the case of **2e**), no deaths or neurotoxicity was observed.

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E,*E*-2-Benzylidene-6-(nitrobenzylidene)cyclohexanones: Syntheses, cytotoxicity and an examination of some of their electronic, steric, and hydrophobic properties

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Abstract—Three series of structurally isomeric 2-benzylidene-6-(nitrobenzylidene) cyclohexanones **1**–**3** were prepared and evaluated against human Molt/C8 and CEM T-lymphocytes as well as murine L1210 cells. The IC_{50} values of the majority of compounds are less than 10 μM and in some assays, the figures for **1d** and **1e** are submicromolar. Correlations were discerned between cytotoxic potencies and the atomic charges on one of the olefinic carbon atoms, the torsion angles between an aryl ring, and the adjacent unsaturated group as well as log *P* values. Three representative compounds were examined for their effect on respiration in rat liver mitochondria.

1. Introduction

The principal interest in our laboratory is the syntheses of a variety of conjugated styryl ketones as candidate antineoplastic agents. These compounds are thiol alkylators having little or no capacity to interact with amino or hydroxy groups[1,2]; since these latter groups, but not thiols, are found in nucleic acids, enones may be devoid of the genotoxic problems displayed by a number of anticancer drugs.[3] Recently molecules have been designed to enable successive alkylation of thiols to occur since on occasion sequential reactions with cellular constituents have been claimed to be more detrimental to malignant cells than the corresponding normal tissues.[4] These considerations led to the decision to prepare a number of compounds which contain the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore (ArCH=CR–CO–CR=CHAr)[5,6] thereby allowing stepwise alkylation of cellular thiols. Recently a small number of 2,6-bis(benzylidene) cyclohexanones were prepared in which the substituents in each of the aryl rings differed in their electronic properties.[7,8] In these molecules, the charges on the olefinic carbon atoms are predicted to be divergent thereby enhancing sequential reactions.

The objectives of the present study were twofold. First, an evaluation was planned of the hypotheses that cytotoxic potencies were correlated with both the charge densities and the steric environment of the olefinic carbon atoms. Second, the original series consisted of a small group of compounds which possessed widely differing potencies in the Molt 4/C8, CEM, and L1210 bioassays.[8] Hence expansion of the cluster of compounds was indicated in order to draw meaningful conclusions pertaining to those structural features which contribute to cytotoxicity.

In ring A of series **1**, the strongly electron-attracting 2-nitro group was proposed which should cause the olefinic carbon atoms, designated C^A and C^B as indicated in Figure 1, to be electron deficient thereby enhancing thiol alkylation. Substituents with varying Hammett sigma (σ) values were considered for insertion onto ring B, including the 3,4,5-trimethoxy group due to our recent disclosure of the cytotoxicity of compounds containing the 3-(3,4,5-trimethoxyphenyl)-2-propenoyl substituent.[9] In addition, the rate of electrophilic attack with

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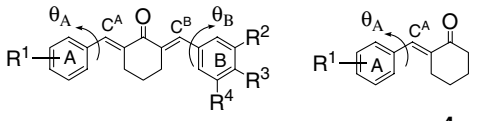


1a-g: R^1= 2-NO_2
2a-g: R^1= 3-NO_2
3a-g: R^1= 4-NO_2

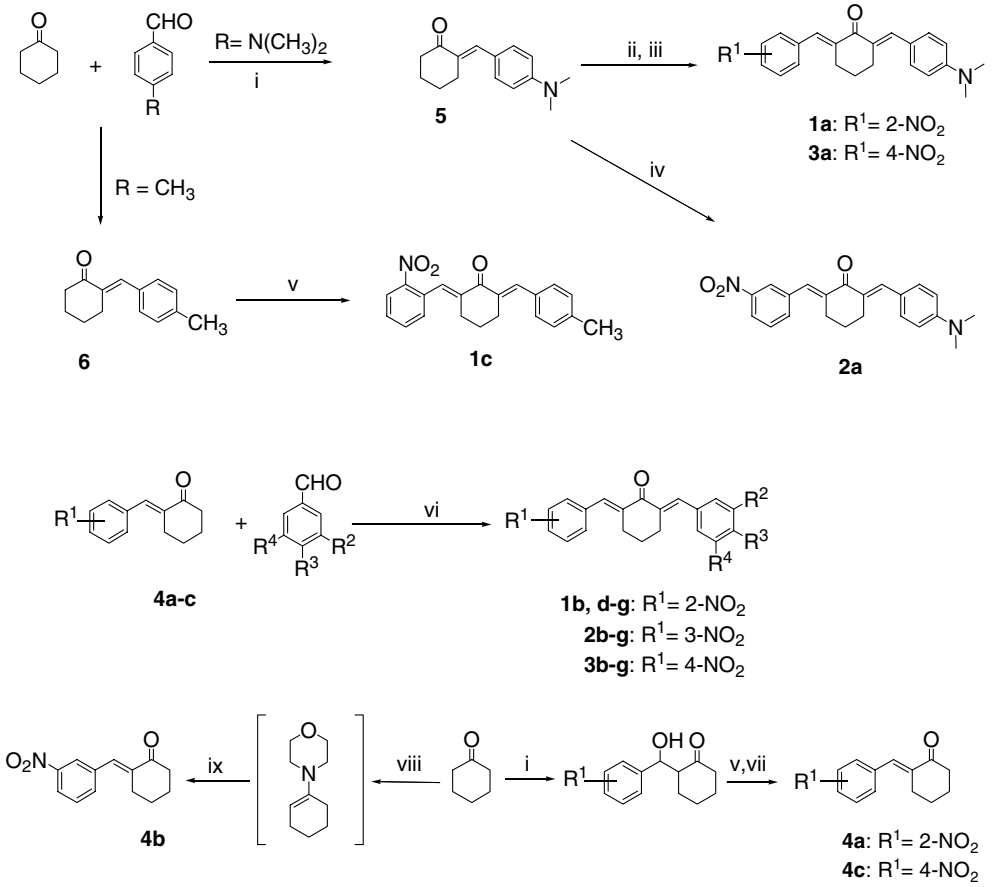
4a: R^1= 2-NO_2
4b: R^1= 3-NO_2
4c: R^1= 4-NO_2

Figure 1. General structures of series **1**–**4**. The R^2, R^3, and R^4 substituents in series **1**–**3** are as follows, namely **a**: $R^2 = R^4$ = H, R^3 = $N(CH_3)_2$; **b**: $R^2 = R^4$ = H, R^3 = OCH_3; **c**: $R^2 = R^4$ = H, R^3 = CH_3; **d**: $R^2 = R^3 = R^4$ = OCH_3; **e**: $R^2 = R^3 = R^4$ = H; **f**: $R^2 = R^4$ = H, R^3 = F; **g**: $R^2 = R^4$ = H, R^3 = Cl.

thiols will be influenced by the topography of the molecules in the environment of the C^A and C^B atoms. Hence the determination of the torsion angles θ_A and θ_B created between the aryl rings A and B with the adjacent olefinic carbon atoms was suggested. Such considerations led to the decision to prepare series **1**. In order for these hypotheses to be examined further, the placement of the nitro group in other locations of ring A was planned leading to series **2** and **3**. In addition, to assist in the understanding of those structural features in series **1**–**3** which contribute to cytotoxic potencies, the monobenzylidene analogs **4a–c** were also proposed. The general structures of these compounds are presented in Figure 1.

2. Results

The compounds in series **1**–**4** were prepared by the synthetic routes outlined in Scheme 1. The majority of the bis(benzylidene)cyclohexanones were prepared by condensation of **4a**, **4b**, or **4c** with various aryl aldehydes under acidic conditions. However, attempts to use this procedure in the preparation of **1a**, **1c**, **2a**, and **3a** led to the formation of dark polymeric material from which no products were obtained. Under basic conditions, 2-(4-dimethylaminobenzylidene) cyclohexanone **5** and the related 4-methyl analog **6** reacted with the appropriate nitrobenzaldehyde to afford **1c** and **2a**. However under these conditions, reaction of **5** with both 2-nitrobenzaldehyde and 4-nitroarylaldehydes led to the formation of multiple products but under acidic conditions, both **1a** and **3a** were formed. Initial attempts to prepare **4a–c** from cyclohexanone and the relevant nitrobenzaldehyde under acidic conditions led to isolation of the corresponding 2,6-bis(nitrobenzylidene)cyclohexanones. In the presence of sodium hydroxide solution, the 2-nitro and



Scheme 1. Synthetic chemical routes in the preparation of the compounds in series **1**–**4**. Reagents: (i) NaOH; (ii) HCl/2-$NO_2C_6H_4CHO$; (iii) HCl/4-$NO_2C_6H_4CHO$; (iv) NaOH/3-$NO_2C_6H_4CHO$; (v) NaOH/2–$NO_2C_6H_4CHO$; (vi) HCl; (vii) NaOH/4-$NO_2C_6H_4CHO$; (viii) morpholine/4-$CH_3C_6H_4SO_2OH$; (ix) 3-$NO_2C_6H_4CHO$.

4-nitro benzaldehydes condensed with cyclohexanone to produce the intermediate aldols which were dehydrated by acid to give **4a** and **4c**, respectively. Under basic conditions, the 3-nitroaldehyde gave only 2,6-bis(3-nitrobenzylidene)cyclohexanone. Hence **4b** was prepared via the enamine route as illustrated in Scheme 1. ^{1}H NMR spectroscopy revealed that each of the products in series **1**–**4** was isomerically pure. The absorptions of the olefinic protons were in the region of 7.47–7.97 ppm which is characteristic of *E* isomers, since compounds possessing the *Z* configuration absorb at higher fields.[10] Furthermore, X-ray crystallography revealed that the olefinic double bonds adopted the *E* configuration in **3c**[11] and **3f**[12] as well as a related 2,6-bis(benzylidene)cyclohexanone.[13] The assumption was made therefore that the olefinic bonds in series **1**–**4** adopted the *E* configuration. Models of the compounds in series **1**–**4** were built and the charge densities of the C^A and C^B atoms as well as the torsion angles θ_A and θ_B were determined and are presented in Table 2. In addition, the log *P* values of all of the compounds were obtained and are portrayed in Table 2.

All the compounds in series **1**–**4** were evaluated against human Molt 4/C8 and CEM T-lymphocytes and murine L1210 lymphoid leukemia cells. These data are summarized in Table 1. The effect of representative compounds on respiration in mitochondria isolated from rat liver cells is presented in Figure 2.

Table 1. Cytotoxic properties of compounds **1–4**

Compound	IC_{50}[a] (μM)		
	Molt 4/C8	CEM	L1210
1a	122 ± 6	168 ± 36	164± 27
1b	8.90±0.20	7.45±0.08	42.4±1.3
1c	7.52±0.45	6.09±2.12	7.77±0.45
1d	0.702±0.22	0.402±0.033	1.52±0.29
1e	1.48±0.11	0.925±0.056	4.84±0.40
1f	1.87±0.06	1.51±0.04	8.40±0.13
1g	3.86±1.00	1.75±0.14	9.38±0.47
2a	10.9±0.8	11.7±0.8	156±134
2b	7.98±0.54	8.22±0.12	29.5±9.8
2c	9.53±1.23	10.1±0.6	41.8±3.7
2d	44.0± 2.7	45.2±7.5	42.2±3.3
2e	1.70±0.42	2.29±0.75	9.44±1.07
2f	5.12±2.31	5.05±3.02	16.3±0.3
2g	2.02±0.28	1.75±0.00	9.16±0.87
3a	>500	>500	>500
3b[b]	300±54	250±6	240±8
3c	8.44±0.49	8.53±0.31	8.16±0.35
3d[b]	6.42±1.07	4.61±3.89	6.97±1.80
3e	8.35±0.95	9.32±0.20	9.80±0.18
3f	17.1±4.6	18.6±6.9	26.8±2.8
3g[b]	9.12±0.28	8.18±0.20	9.41±0.97
4a	33.3±3.1	36.4±1.3	23.8±12.4
4b	8.28±0.69	8.12±0.92	50.1±10.4
4c	13.9±1.0	19.3±1.5	46.5±9.1
Melphalan[b]	3.24 ±0.79	2.47 ± 0.30	2.13±0.03

[a] The IC_{50} values indicate the concentrations of compounds required to inhibit the growth of the cells by 50%.

[b] The data were previously reported in Ref. 8 [copyright (2006) by Elsevier].

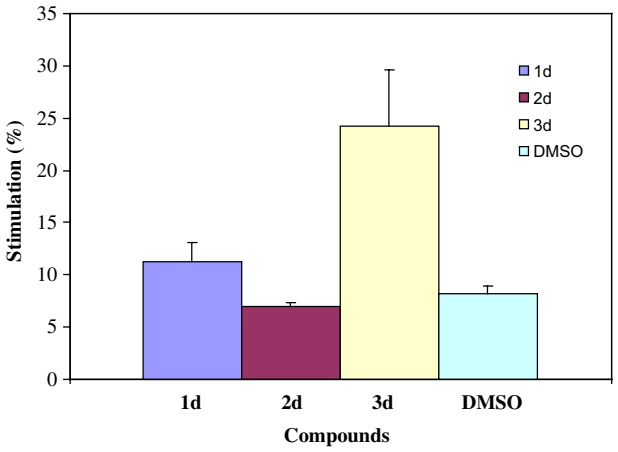


Figure 2. The effect of **1d**, **2d**, **3d**, and solvent control (dimethylsulfoxide 4 μL) on respiration in rat liver mitochondria. The figures for **1d**, **2d**, and **3d** are different from each other and the solvent control taking standard deviations into account.

3. Discussion

The bioevaluations of **1a–g**, **2a–g**, **3a–g**, and **4a–c** toward three cell lines are presented in Table 1. The IC_{50} values of **1d** and **1e** are submicromolar in some of the bioassays and 58% of the IC_{50} values were less than 10 μM. In view of these promising results, various studies were initiated to seek correlations between cytotoxic potencies and different physicochemical and biochemical parameters of these molecules with the aim of obtaining guidelines for expansion of this project.

Table 2. Some physicochemical properties of compounds **1–4**

Compound	Atomic charges[a]		Torsion angles[b]		log *P*
	q_A	q_B	θ_A	θ_B	
1a	−0.086	−0.032	76.79	−51.12	4.80
1b	−0.081	−0.041	76.76	−51.27	4.76
1c	−0.082	−0.047	−76.83	51.58	5.15
1d	−0.092	−0.053	76.90	−51.53	4.33
1e	−0.133	−0.055	76.59	−51.86	4.70
1f	−0.130	−0.058	−76.79	51.55	4.86
1g	−0.130	−0.061	−76.84	51.72	5.38
2a	−0.093	−0.028	−51.30	50.72	5.01
2b	−0.089	−0.042	−51.28	51.12	4.96
2c	−0.083	−0.045	−51.27	51.42	5.35
2d	−0.079	−0.053	−51.32	51.83	4.53
2e	−0.087	−0.053	−51.27	51.56	4.90
2f	−0.085	−0.056	−51.25	51.36	5.07
2g	−0.084	−0.059	−51.24	51.58	5.58
3a	−0.092	−0.022	−51.14	50.98	5.03
3b	−0.096	−0.039	−51.11	51.09	4.98
3c	−0.089	−0.043	−51.14	51.41	5.38
3d	−0.086	−0.053	51.17	−51.86	4.56
3e	−0.095	−0.050	−51.14	51.56	4.93
3f	−0.092	−0.053	−51.14	51.37	5.09
3g	−0.092	−0.056	−51.16	51.57	5.61
4a	−0.092	—	−69.54	—	2.92
4b	−0.083	—	−51.50	—	3.12
4c	−0.090	—	−50.68		3.14

[a] The atomic charges in esu are the electron densities on the carbon atoms designated C^A and C^B in Figure 1.

[b] θ_A and θ_B refer to the torsion angles which are illustrated in Figure 1.

Interactions with cellular thiols are believed to occur at the olefinic carbon atoms designated C^A and C^B. The atomic charges on these atoms in the compounds **1–4** are presented in Table 2. The nitro group in ring A is the most electron-attracting substituent having a Taft σ^* value of 0.97[14] (series **1** and **4a**) and Hammett σ values of 0.71 (series **2** and **4b**) and 0.78 (series **3** and **4c**).[15] The σ constants for the ring B substituents in **a–g** are −0.83, −0.27, −0.17, −0.03, 0.00, 0.06, and 0.23, respectively, [16] and are arranged in sequence with **a** bearing the most electron-repelling group and **g** the most electron-attracting substituent. For each compound in series **1–3** , the electron densities are lower on the C^B rather than the C^A atoms. Thus the polarization of the π electrons in the conjugated 1,5-diaryl-3-oxo-1,4-pentadienyl group is toward the nitro substituents, causing the C^B atom to have lower electron densities than C^A. Hence thiol alkylation is predicted to take place initially at C^B and subsequently at C^A. In order to examine whether cytotoxic potencies are correlated with the electron densities on the C^A and C^B atoms, linear plots were made between these values and the IC_{50} figures of **1a–g** in each of the three bioassays.This experiment was repeated with **2a–g** and finally with **3a–g**. Positive correlations ($p < 0.05$) were noted when considering the atomic charges on the C_B atoms except for the Molt 4/C8 and CEM biodata for series **2**. No correlations were noted between the IC_{50} figures and the charges on the C_A atom ($p > 0.05$). This evaluation was repeated except that the IC_{50} values were plotted against the σ constants in ring B. Negative correlations ($p < 0.01$) were obtained in all cases except for **2a–g** in the Molt 4/C8 and CEM tests ($p > 0.05$). Thus potency increases (IC_{50} values diminish) as the electron densities on the C^B atom are decreased (positive correlation) and the σ constants are elevated (negative correlation). This observation may be rationalized by considering that attack of cellular thiols at C^B will be enhanced by a reduction in the atomic charges on the C^B atoms. Thus in the future, compounds may be designed having substituents in ring B which have large positive sigma values.

Consideration was given to the possibility that the steric environment at the olefinic carbon atoms influences the extent of thiol alkylation and hence cytotoxic potencies. Thiolation is believed to occur initially at C^B and the θ_B values recorded in Table 2 reveal that they are virtually constant. Thus the average θ_B values for series **1**, **2**, and **3** are 51.5°, 51.4°, and 51.4°, respectively, and there are very small variations in these torsion angles within each series. Hence the differences in cytotoxic potencies are unlikely to be influenced by the torsion angles θ_B. The average θ_A angles in series **1**, **2**, and **3** are 76.8°, 51.3°, and 51.1°, respectively, and minimal variation of these torsion angles was noted within each series. Since the cytotoxic potencies of the compounds in series **1** are greater than the analogs in series **2** and **3** vide infra, these torsion angles may influence the magnitude of the cytostatic effect. Hence in the future, groups with larger molecular refractivity values than nitro group should be placed in the 2 position of ring A or two ortho substituents should be employed which may lead to more potent analogs. The insertion of a second arylidene ring onto **4a–c** leading to series **1–3**, respectively, caused only minimal changes in the C^A and θ_A values as the data in Table 2 indicate.

The biodata in Table 1 were examined further with a view to discerning those structural features which influence cytotoxic potencies. First, the optimal position of the nitro group in ring A was considered. A point system of 3 (highest potency), 2, and 1 (lowest potency) was used in comparing the IC_{50} values of compounds having the same substituents in ring B. Thus in the Molt 4/C8 assay, the IC_{50} figures of **1a**, **2a**, and **3a** were compared, then **1b**, **2b**, and **3b** and so forth. Standard deviations were taken into account and when the IC_{50} values were statistically indistinguishable, equal points were allocated bearing in mind that for each comparison of three compounds, a total of six points were invariably awarded. Use of this methodology indicated that the figures for series **1**, **2**, and **3** are 16.5, 16.5, and 9, respectively (Molt 4/C8 assay), 19.5, 13.5, and 9, respectively (CEM test) and 18, 13, and 11, respectively (L1210 screen). Hence the optimal position of the nitro group in ring A in terms of potency is the 2 position.

In order to assess whether the compounds in series **1**–**3**, which permit sequential alkylation to occur, have increased cytotoxic potencies vis-à-vis the analogs in which this process will not occur (series **4**), the IC_{50} values of **1a–g**, **2a–g**, and **3a–g** were compared with those generated for **4a**, **4b**, and **4c**, respectively. The results are summarized in Table 3. In general, structural modification of **4a**, **4b**, and **4c** into series **1**, **2**, and **3**, respectively, was accompanied by increases in potencies in all three bioassays except conversion of **4b** into **2a–g** did not lead to compounds with lower IC_{50} values toward CEM cells.

The rate and extent of the ability of compounds to penetrate the cell membranes of neoplastic and transformed cells is dependent on a number of structural features including the lipophilicity of the molecules. The log *P* values of the compounds were calculated and are presented in Table 1. The average log *P* values for **1a–g**, **2a–g**, and **3a–g** are 4.86, 5.06, and 5.08, respectively, and hence the lower lipophilicity of the compounds in series **1** may have contributed to the greater potencies than the analogs in series **2** and **3**. The generally lower IC_{50} potencies displayed by the compounds in series **1–3** than **4a–c** also reflect a negative correlation with the log *P* values.

Table 3. Comparison between the potencies of the bisalkylators **1a–g**, **2a–g**, and **3a–g** with the monobenzylidene analogs **4a**, **4b**, and **4c**, respectively

Bioassay	Comparison of potencies[a]								
	1a–g	**4a**	Equal	**2a–g**	**4b**	Equal	**3a–g**	**4c**	Equal
Molt 4/C8	86	14	0	43	29	29	57	29	14
CEM	86	14	0	29	43	29	57	29	14
L1210	71	29	0	57	0	43	71	29	0
Total	81	19	0	43	24	33	62	29	9

[a] The figures represent the percentage of compounds displaying greater potency or were equipotent. The standard deviations of the IC_{50} figures were taken into account when making these comparisons.

Various compounds which are thiol reagents such as *N*-ethylmaleimide and mersalyl interact with different mercapto groups in mitochondria.[17] Furthermore, a Mannich base of a conjugated styryl ketone inhibited respiration in rat liver mitochondria and the mode of action, at least in part, was based on competition with the conjugated unsaturated ketone coenzyme Q_{10}.[18] Thus the decision was made to determine whether representative compounds interfered with respiration in mitochondria isolated from rat liver cells, and if so whether the magnitude of this effect correlated with cytotoxic potencies. Three related compounds, namely, **1d**, **2d**, and **3d**, were chosen since they possessed markedly different potencies having average IC_{50} figures of 0.88, 43.8, and 6.00 μM, respectively, in the three cytotoxicity screens. A concentration of 10 μM of each compound was chosen which is in excess of the IC_{50} values of **1d** and **3d** and substantially below that of **2d**. The data in Figure 2 reveal that **1d** and **3d** stimulated respiration. However, the magnitude of the stimulatory effect was negatively correlated with cytotoxic potencies. The least potent of these three compounds, namely **2d**, had virtually no effect on respiration. Increasing the concentration of **2d** to 100 μM revealed no statistically significant difference in stimulation of respiration from the solvent control (data not shown). Nevertheless if the causes for the relative cytotoxic potencies observed in this study are multifactorial, the differences in the effects on mitochondrial function may have exerted some contributions to the disparity of IC_{50} values.

4. Conclusions

A number of novel cytotoxic agents have been prepared, many of which display good potencies toward Molt 4/C8, CEM, and L1210 cells. The highest potencies were displayed by the compounds in series **1** and in particular **1d** and **1e** are lead molecules having submicromolar IC_{50} values in some of the assays. Factors which influence cytotoxic potencies in series **1**–**3** include the atomic charges on the C^B atoms, the torsion angle θ_A, and log *P* values. Another factor which may have contributed to the variation in IC_{50} values is the differences in the effects on respiration in rat liver mitochondria. A number of guidelines for amplifying this project have been proposed.

5. Experimental

5.1. Synthesis of compounds

Melting points in Celsius degrees were determined on a Gallenkamp apparatus and are uncorrected. ^{1}H NMR spectra were recorded using a Bruker AMX 500 FT machine while elemental analyses were obtained using an Elementer analyzer.

5.1.1. Syntheses of 1a, 2a, and 3a. 2-(4-Dimethylaminobenzylidene)cyclohexanone **5** was prepared by a reported procedure[19] and crystallized from ethanol at 5–6 °C to give the desired product in 45% yield, mp 130 °C [lit.[19]] 127–127.5 °C]. ^{1}H NMR ($CDCl_3$): 1.79 (p, 2H), 1.91 (p, 2H), 2.52 (t, 2H), 2.88 (t, 2H), 3.03 (s, 6H, 2× NCH_3), 6.71 (d, 2H, Ar–H, J = 8.85 Hz), 7.41 (d, 2H, J = 8.83 Hz), 7.55 (s, 1H, =CH).

Dry hydrogen chloride was passed into a solution of **5** (0.005 mol) and 2-nitrobenzaldehyde (0.005 mol) in acetic acid (15 mL) and the mixture stirred at room temperature overnight. Acetic acid was removed in vacuo and the residue triturated with potassium carbonate solution (10% w/v, 20 mL) and extracted with chloroform. The organic extract was washed with water and dried. Evaporation of the solvent gave a semisolid which was purified by chromatography using a column of silica gel 60 (70–230 mesh) and an eluting solvent of 10–30% ethyl acetate in hexane to produce **1a**, mp 152 °C in 41% yield. ^{1}H NMR ($CDCl_3$): δ 1.79 (p, 2H), 2.60 (t, 2H), 2.96 (t, 2H), 3.05 (s, 6H, 2× $N(CH_3)_2$), 6.73 (d, 2H, Ar–H, J = 8.80 Hz), 7.38 (d, 1H, Ar–H, J = 7.60 Hz), 7.49 (m, 3H, Ar–H), 7.64 (t, 1H, Ar–H), 7.84 (s, 1H, =CH), 7.95 (s, 1H, =CH), 8.13 (d, 1H, Ar–H, J = 8.20 Hz). Anal. Calcd for $C_{22}H_{22}N_2O_3$: C, 72.91; H, 6.12; N, 7.73. Found: C, 72.62; H, 5.98; N, 7.53%.

Aqueous sodium hydroxide solution (20% w/v, 1 mL) was added to a solution of **5** (0.005 mol) and 3-nitrobenzaldehyde (0.005 mol) in ethanol (15 mL) at 8–10 °C. The solution was stirred at room temperature for 0.5 h. The resultant precipitate was collected, washed with water (3× 15 mL), dried and crystallized from chloroform/ethanol (1:9) to give **2a**, mp 169 °C in 68% yield. ^{1}H NMR ($CDCl_3$): δ 1.86 (p, 2H), 2.93 (t, 2H), 3.01 (t, 2H), 3.07 (s, 6H, 2× $N(CH_3)_2$), 6.75 (d, 2H, Ar–H, J = 8.84 Hz), 7.50 (d, 2H, Ar–H, J = 8.82Hz), 7.60 (t, 1H, Ar–H), 7.76 (d, 1H, Ar–H, J = 7.7 Hz), 7.79 (s, 1H, =CH), 7.84 (s, 1H, =CH), 8.20 (d, 1H, Ar–H, J = 8.18 Hz), 8.32 (s, 1H, Ar–H). Anal. Calcd for $C_{22}H_{22}N_2O_3$: C, 72.91; H, 6.12; N, 7.73. Found: C, 72.87; H, 6.0; N, 7.46%.

Dry hydrogen chloride was passed into a solution of **5** (0.005 mol) and 4-nitrobenzaldehyde (0.005 mol) in acetic acid (15 mL) and the mixture was stirred overnight at room temperature. The precipitate was collected, washed with diethyl ether (2× 10 mL), and potassium carbonate solution (10% w/v, 30 mL). The solid obtained was washed with water (3× 10 mL), dried and crystallized from chloroform/ethanol (1:9) to give **3a**, mp 91–92 °C in 66% yield. ^{1}H NMR ($CDCl_3$): δ 1.86 (p, 2H), 2.91 (t, 2H), 3.0 (t, 2H), 3.07 (s, 6H, 2× $N(CH_3)_2$), 6.75 (d, 2H, Ar–H, J = 8.84 Hz), 7.50 (d, 2H, Ar–H, J = 8.84 Hz), 7.60 (d, 2H, Ar–H, J = 8.63 Hz), 7.79 (s, 1H, =CH), 7.84 (s, 1H, =CH), 8.27 (d, 2H, Ar–H, J = 8.65 Hz). Anal. Calcd for $C_{22}H_{22}N_2O_3$: C, 72.91; H, 6.12; N, 7.73. Found: C, 72.94; H, 6.13; N, 7.52%.

5.1.2. Synthesis of 1c. 2-(4-Methylbenzylidene)cyclohexanone **6** was prepared by a literature procedure[20] and crystallized from methanol to give **6**, mp 71 °C [lit.[20] mp 60 °C] in 40% yield. ^{1}H NMR ($CDCl_3$): 1.78 (p, 2H), 1.92 (m, 2H), 2.38 (s, 3H, CH_3), 2.53 (t, 2H), 2.86 (t, 2H), 7.21 (d, 2H, Ar–H, J = 7.90 Hz), 7.32 (d, 2H, Ar–H, J = 7.96 Hz), 7.50 (s, 1H, =CH).

Aqueous sodium hydroxide solution (20% w/v, 1 mL) was added to a solution of **6** (0.005 mol) and 2-nitrobenzaldehyde (0.005 mol) in ethanol (15 mL) at 8–10 °C. The solution was stirred at room temperature for 0.5 h. The reaction mixture was acidified with dilute hydrochloric acid and extracted with chloroform. Evaporation of the organic solvent gave a viscous oil which was purified by chromatography using a column of silica gel 60 (70–230 mesh) and an eluting solvent of ethyl acetate/hexane (1:9) to give **1c**, mp 120 °C in 30% yield. ^{1}H NMR($CDCl_3$): δ 1.78 (p, 2H), 2.40 (s, 3H, CH_3), 2.62 (p, 2H), 2.94 (t, 2H), 7.24 (d, 2H, Ar–H, J = 7.94 Hz), 7.39 (t, 3H, Ar–H), 7.52 (t, 1H, Ar–H), 7.65 (t, 1H, Ar–H), 7.83 (s, 1H, =CH), 7.96 (s, 1H, =CH), 8.14 (d, 1H, Ar–H). Anal. Calcd for $C_{21}H_{19}NO_3$: C, 75.66; H, 5.74; N, 4.20. Found: C, 75.42; H, 5.71; N, 4.27%.

5.1.3. Synthesis of 1b, d–g, 2b–g, and 3b–g. The enones **1b**, **d–g**, **2b–g**, and **3b–g** were prepared by the following general procedure. Dry hydrogen chloride was passed into a solution of **4a** , **4b**, or **4c** vide infra (0.005 mol) and the appropriate aryl aldehyde (0.006 mol) in ether (40 mL) and methanol (4 mL). The reaction mixture was stirred at room temperature for 24 h and the resultant solid was collected and crystallized from chloroform/methanol (1:3).

5.1.3.1. *E*,*E*-2-(4-Methoxybenzylidene)-6-(2-nitrobenzylidene)cyclohexanone (1b). Mp 157 °C; yield 80%. ^{1}H NMR ($CDCl_3$): δ 1.79 (p, 2H), 2.61 (t, 2H), 2.94 (t, 2H), 3.86 (s, 3H, OCH_3), 6.98 (d, 2H, Ar–H, J = 8.3 Hz), 7.38 (d, 1H, Ar–H, J = 7.65 Hz), 7.48 (d, 2H, Ar–H, J = 8.45 Hz), 7.52 (t, 1H, Ar–H), 7.65 (t, 1H, Ar–H), 7.82 (s, 1H, =CH), 7.96 (s, 1H, =CH), 8.14 (d, 1H, Ar–H, J = 8.20 Hz). Anal. Calcd for $C_{21}H_{19}NO_4$: C, 72.19; H, 5.48; N, 4.01. Found: C, 71.89; H, 5.53; N, 3.90%.

5.1.3.2. *E*,*E*-2-(3,4,5-Trimethoxybenzylidene)-6-(2-nitrobenzylidene)cyclohexanone (1d). Mp 159 °C; yield 94%. ^{1}H NMR ($CDCl_3$): δ 1.80 (p, 2H), 2.62 (t, 2H), 2.97 (t, 2H), 3.91 (s, 9H, 3× OCH_3), 6.73 (s, 2H, Ar–H), 7.39 (d, 1H, Ar–H, J = 7.60 Hz), 7.52 (t, 1H, Ar–H), 7.66 (t, 1H, Ar–H), 7.77 (s, 1H, =CH), 7.96 (s, 1H, =CH), 8.14 (d, 1H, Ar–H, J = 8.15 Hz). Anal. Calcd for $C_{23}H_{23}NO_6$: C, 67.47; H, 5.66; N, 3.42. Found: C, 67.60; H, 5.61; N, 3.58%.

5.1.3.3. *E*,*E*-2-(Benzylidene)-6-(2-nitrobenzylidene)-cyclohexanone (1e). Mp 116 °C; yield 47%. ^{1}H NMR ($CDCl_3$): δ 1.78 (p, 2H), 2.63 (t, 2H), 2.95 (t, 2H), 7.40 (m, 5H, Ar–H), 6.79 (d, 2H, Ar–H, J = 7.6 Hz), 7.52 (t, 1H, Ar–H), 7.65 (t, 1H, Ar–H), 7.85 (s, 1H, =CH), 7.97 (s, 1H, =CH), 8.15 (d, 1H, Ar–H, J = 8.20 Hz). Anal. Calcd for $C_{20}H_{17}NO_3$: C, 75.22, H, 5.37; N 4.39. Found: C, 74.82; H, 5.26; N, 4.04%.

5.1.3.4. *E*,*E*-2-(4-Fluorobenzylidene)-6-(2-nitrobenzylidene)cyclohexanone (1f). Mp 136 °C; yield 52%. ^{1}H NMR ($CDCl_3$): δ 1.79 (p, 2H), 2.63 (t, 2H), 2.91 (t, 2H), 7.12 (t, 2H, Ar–H), 7.39 (d, 1H, Ar–H, J = 7.65 Hz), 7.47 (q, 2H, Ar–H), 7.52 (t, 1H, Ar–H), 7.65 (t, 1H, Ar–H), 7.80 (s, 1H, =CH), 7.97 (s, 1H, =CH), 8.15 (d, 1H, Ar–H, J = 8.20 Hz). Anal. Calcd for $C_{20}H_{16}FNO_3$: C, 71.21; H, 4.78; N 4.15. Found: C, 70.93; H, 4.79; N 3.88%.

5.1.3.5. *E*,*E*-2-(4-Chlorobenzylidene)-6-(2-nitrobenzylidene)cyclohexanone (1g). Mp 149 °C; yield 41%. ^{1}H NMR ($CDCl_3$): δ 1.79 (p, 2H), 2.63 (t, 2H), 2.84 (t, 2H), 7.40 (m, 5H, Ar–H), 7.53 (t, 1H, Ar–H), 7.66 (t, 1H, Ar–H), 7.77 (s, 1H, =CH), 7.97 (s, 1H, =CH), 8.15 (d, 1H, Ar–H, J = 8.2 Hz). Anal. Calcd for $C_{20}H_{16}ClNO_3$: C, 67.90; H, 4.56; N, 3.96. Found: C, 67.70; H 4.61; N 3.78%.

5.1.3.6. *E*,*E*-2-(4-Methoxybenzylidene)-6-(3-nitrobenzylidene)cyclohexanone (2b). Mp 114 °C; yield 68%. ^{1}H NMR ($CDCl_3$): δ 1.85 (p, 2H), 2.94 (t, 2H), 2.98 (t, 2H), 6.96 (d, 2H, Ar–H, J = 8.50 Hz), 7.49 (d, 2H, Ar–H, J = 8.45 Hz), 7.60 (t, 1H, Ar–H), 7.75 (d, 1H, Ar–H, J = 7.7 Hz), 7.79 (s, 1H, =CH), 7.81 (s, 1H, =CH), 8.19 (d, 1H, Ar–H, J = 8.10 Hz), 8.32 (s, 1H, Ar–H). Anal. Calcd for $C_{21}H_{19}NO_4$: C, 72.19; H, 5.48; N 4.01. Found: C, 71.91; H, 5.46; N, 3.90%.

5.1.3.7. *E*,*E*-2-(4-Methylbenzylidene)-6-(3-nitrobenzylidene)cyclohexanone (2c). Mp 156 °C; yield 60%. ^{1}H NMR ($CDCl_3$): δ 1.84 (p, 2H), 2.94 (t, 2H), 2.98 (t, 2H), 7.25 (t, 2H, Ar–H), 7.41 (d, 2H, Ar–H, J = 7.85 Hz), 7.60 (t, 1H, Ar–H), 7.76 (d, 2H, Ar–H, J = 7.65 Hz), 7.81 (s, 1H, =CH), 7.82 (s, 1H, =CH), 8.20 (d, 1H, Ar–H, J = 8.20 Hz), 8.32 (s, 1H, Ar–H). Anal. Calcd for $C_{21}H_{19}NO_3$: C, 75.66; H, 5.74; N, 4.20. Found: C, 75.58; H, 5.88; N, 3.92%.

5.1.3.8. *E*,*E*-2-(3,4,5-Trimethoxybenzylidene)-6-(3-nitrobenzylidene)cyclohexanone (2d). Mp 172 °C; yield 82%. ^{1}H NMR ($CDCl_3$): δ 1.86 (p, 2H), 2.95 (t, 2H), 3.00 (t, 2H), 3.91 (s, 9H, 3× OCH_3), 6.74 (s, 1H, Ar–H), 7.61 (t, 1H), 7.76 (d, 2H, Ar–H, =CH, J = 7.70 Hz), 7.80 (s, 1H, =CH), 8.21 (d, 1H, Ar–H, J = 8.15 Hz), 8.29 (s, 1H, Ar–H). Anal. Calcd for $C_{23}H_{23}NO_6$: C, 67.47; H, 5.66; N, 3.42. Found: C, 67.29; H, 5.57; N, 3.30%.

5.1.3.9. *E*,*E*-2-(Benzylidene)-6-(3-nitrobenzylidene)-cyclohexanone (2e). Mp 120 °C; yield 22%. ^{1}H NMR ($CDCl_3$): δ 1.85 (p, 2H), 2.97 (m, 4H), 7.38 (t, 1H, Ar–H), 7.44 (m, 2H, Ar–H), 7.49 (d, 2H, Ar–H, J = 7.65 Hz), 7.61 (t, 1H, Ar–H), 7.76 (d, 1H, Ar–H, J = 7.65 Hz), 7.80 (s, 1H, =CH), 7.84 (s, 1H, =CH), 8.21 (d, 1H, Ar–H, J = 8.25 Hz), 8.33 (s, 1H, Ar–H). Anal. Calcd for $C_{20}H_{17}NO_3$: C, 75.22, H 5.37; N, 4.39. Found: C, 75.28; H, 5.44; N, 4.28%.

5.1.3.10. *E*,*E*-2-(4-Fluorobenzylidene)-6-(3-nitrobenzylidene)cyclohexanone (2f). Mp 126 °C; yield 63%. ^{1}H NMR ($CDCl_3$): δ 1.85 (p, 2H), 2.81 (t, 2H), 7.13 (t, 2H, Ar–H), 7.48 (dd, 2H, Ar–H), 7.61 (t, 1H, Ar–H), 7.76 (d, 1H, Ar–H, J = 7.65 Hz), 7.80 (s, 2H, =CH), 8.21 (d, 1H, Ar–H, J = 8.15 Hz), 8.32 (s, 1H, Ar–H). Anal. Calcd for $C_{20}H_{16}FNO_3$: C, 71.21; H, 4.78; N, 4.15. Found: C, 70.97; H, 4.70; N, 4.08%.

5.1.3.11. *E*,*E*-2-(4-Chlorobenzylidene)-6-(3-nitrobenzylidene)cyclohexanone (2g). Mp 138 °C; yield 68%. ^{1}H NMR ($CDCl_3$): δ 1.99 (p, 2H), 2.96 (q, 4H), 7.42 (m, 4H), 7.62 (t, 1H), 7.77 (d, 2H, Ar–H & =CH, J = 8.80 Hz), 7.81 (s, 1H, =CH), 7.84 (s, 1H, =CH), 8.16 (d, 1H, Ar–H, J = 8.16 Hz), 8.34 (s, 1H, Ar–H). Anal. Calcd for $C_{22}H_{16}ClNO_3$: C, 67.90; H, 4.56; N, 3.96. Found: C, 67.33; H, 4.58; N, 3.84%.

5.1.4. Compounds 3b–g. The synthesis of **3b**, **d**, and **g** has been reported previously.[8]

5.1.4.1. *E*,*E*-2-(4-Methylbenzylidene)-6-(4-nitrobenzylidene)cyclohexanone (3c). Mp 136 °C; yield 54%. ^{1}H NMR ($CDCl_3$): δ 1.84 (p, 2H), 2.41 (s, 3H, CH_3), 2.92 (t, 2H), 2.98 (t, 2H), 7.25 (d, 2H, Ar–H, J = 7.8Hz), 7.41 (d, 2H, Ar–H, J = 7.80 Hz), 7.60 (d, 2H, Ar–H, J = 8.35 Hz), 7.79 (s, 1H, =CH), 7.82 (s, 1H, =CH), 8.27 (d, 2H, Ar–H, J = 8.40 Hz). Anal. Calcd for $C_{21}H_{19}NO_3$: C, 75.66; H, 5.74; N, 4.20. Found: C, 75.45; H, 5.75; N, 4.13%.

5.1.4.2. *E*,*E*-2-(Benzylidene)-6-(4-nitrobenzylidene)cyclohexanone (3e). Mp 141 °C; yield 40%. ^{1}H NMR ($CDCl_3$): δ 1.84 (p, 2H), 2.93 (t, 2H), 2.98 (t, 2H), 7.38 (t, 1H, Ar–H), 7.44 (t, 1H, Ar–H), 7.49 (d, 2H, Ar–H, J = 7.75 Hz), 7.60 (d, 2H, Ar–H, J = 8.30 Hz), 7.80 (s, 1H, =CH), 7.84 (s, 1H, =CH), 8.27 (d, 2H, Ar–H, J = 8.40 Hz). Anal. Calcd for $C_{20}H_{17}NO_3$: C, 75.22; H, 5.37; N, 4.39. Found: C, 75.02; H, 5.29; N, 4.19%.

5.1.4.3. *E*,*E*-2-(4-Fluorobenzylidene)-6-(4-nitrobenzylidene)cyclohexanone (3f). Mp 167 °C; yield 33%. ^{1}H NMR ($CDCl_3$): δ 1.85 (p, 2H), 2.94 (t, 4H), 7.13 (t, 2H), 7.48 (t, 2H), 7.60 (d, 2H), 7.79 (s, 2H, =CH), 8.27 (d, 2H, Ar–H, J = 8.5 Hz) Anal. Calcd for $C_{20}H_{16}FNO_3$: C, 71.21; H, 4.78; N, 4.18. Found: C, 70.88; H, 4.70; N, 4.10%.

5.1.5. Synthesis of 4a. A solution of sodium hydroxide (0.6 g, 0.015 mol) in water (5 mL) was added dropwise to a mixture of cyclohexanone (3.0 g, 0.02 mol) and 2-nitrobenzaldehyde (5.85 g, 0.056 mol) at room temperature for 0.25 h and the stirring was continued for 4 h. The solid was collected, dried, and recrystallized from chloroform/methanol to yield 2-(α-hydroxy-2-nitrobenzyl) cyclohexanone (**7**), mp 126 °C in 28% yield. ^{1}H NMR ($CDCl_3$): δ 1.71 (m, 4H), 2.13 (m, 1H), 2.45 (m, 2H), 2.82 (m, 1H), 4.18 (d, 1H), 5.46 (t, 1H), 7.44 (t, 1H), 7.65 (t, 1H), 7.78 (d, 1H, J = 7.9 Hz), 7.86 (d, 1H, J = 8.15 Hz).

Hydrochloric acid (2 mL) was added to a solution of **7** (10.5 g, 0.045 mol) in ethanol (30 mL) and the reaction mixture was heated at 40–45 °C for 4 h. The solvent was removed in vacuo at 40–45 °C and water (100 mL) was added to the residue. The solid was collected and dried to give **4a**, mp 92 °C in a yield of 64%. ^{1}H NMR ($CDCl_3$): δ 1.75 (p, 2H), 1.95 (p, 2H), 2.54 (t, 2H), 2.58 (t, 2H), 2.58 (t, 2H), 7.33 (d, 1H, Ar–H, J = 7.63 Hz), 7.51 (t, 1H, Ar–H), 7.60 (s, 1H, =CH), 7.64 (t, 2H, Ar–H), 8.12 (d, 1H, Ar–H, J = 8.21Hz). Anal. Calcd for $C_{13}H_{13}NO_3$: C, 67.52; H, 5.67; N, 6.06. Found: C, 67.40; H, 5.49; N, 6.29%.

5.1.6. Synthesis of 4b. A solution of cyclohexanone (4.9 g, 0.05 mol), morpholine (4.75 g, 0.055 mol), 4-toluenesulfonic acid (0.02 g) in toluene (50 mL) was heated under reflux using a Dean-Stark apparatus until the stoichiometric amount of water separated (~8 h). 3-Nitrobenzaldehyde (6.8 g, 0.045 mol) was added to the reaction mixture and heating under reflux was continued for 12 h. Water (25 mL) was added to the reaction mixture which was heated at 50–55 °C for ~1 h. The organic phase was separated, washed with hydrochloric acid (5%, 20 mL) and water (3× 50 mL), and dried. Toluene was removed in vacuo at 50–55 °C to give a viscous oil which was purified by chromatography using a column of silica gel 60 (70–230 mesh) and an eluting solvent of ethyl acetate/hexane (1:9) to give **4b**, mp 51–52 °C in 45% yield. ^{1}H NMR($CDCl_3$): 1.82 (p, 2H), 1.98 (p, 2H), 2.59 (t, 2H), 2.86 (t, 2H), 7.52 (s, 1H, =CH), 7.58 (t, 1H, Ar–H), 7.69 (d, 1H, Ar–H, J = 7.6 Hz), 8.19 (d, 1H, Ar–H, J = 8.15 Hz), 8.25 (s, 1H, Ar–H). Anal. Calcd for $C_{13}H_{13}NO_3$: C, 67.52; H, 5.67; N, 6.06. Found: C, 67.30; H, 5.48; N, 6.41%.

5.1.7. Synthesis of 4c. This compound was prepared by a literature procedure[21] to give **4c**, mp 119 °C [lit.[21]] mp 118–120 °C] in 72% yield with respect to 4-nitrobenzaldehyde. ^{1}H NMR($CDCl_3$): 1.82 (p, 2H), 1.98 (p, 2H), 2.59 (t, 2H), 2.83 (m, 2H), 7.47 (s, 1H), 7.53 (d, 2H), 8.25 (d, 2H).

5.2. Molecular modeling

Models of the compounds in series **1**–**4** were built using a BioMedCache program.[22] The lowest energy conformers were generated using the CONFLEX program and optimized by mechanics using augmented MM2 parameters.

5.3. Determination of log *P* values

The log *P* values for enones **1**–**4** were generated with the JME molecular editor.[23]

5.4. Cytotoxicity assays

A literature procedure was employed to examine the cytotoxicity of **1a–g** , **2a–g** , **3a–g** , and **4a–c** toward human Molt 4/C8 and CEM T-lymphocytes as well as murine L1210 cells.[24] In brief, different concentrations of compounds were incubated with the cells in RPMI 1640 medium at 37 °C for 72 h (Molt 4/C8 and CEM T-lymphocytes) or 48 h (L1210 cells). The correct IC_{50} values for **4c** are presented in Table 1 which replaces the figures quoted previously.[25]

5.5. Evaluation of 1d, 2d, and 3d on respiration in rat liver mitochondria

Rats were anesthetized with isoflurane and decapitated. A previously reported procedure was employed to isolate mitochondria from the liver.[26] The consumption

of oxygen by mitochondria was determined by polarography using a literature methodology.[27]

5.6. Statistical analyses

The linear, semilogarithmic and logarithmic plots were constructed using a statistical software package.[28]

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ORIGINAL ARTICLES

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The effect of some 1-[4-(2-diethylaminoethoxy)phenylcarbonyl]-3,5-bis (benzylidene)-4-piperidone methiodides and related compounds on respiration and swelling of rat liver mitochondria

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The effect of a number of *N*-aroyl-3,5-bis(benzylidene)-4-piperidones **2** and related quaternary ammonium compounds **3** on the rates of respiration in rat liver mitochondria were determined. All of the compounds stimulated respiration and the greatest effect was displayed by the compounds in series **3** which caused swelling of mitochondria.

1. Introduction

3,5-bis(Benzylidene)-4-piperidone (**1**) displays potent cytotoxicity to a number of transformed and neoplastic cell lines (Dimmock et al. 2001; Das et al. 2007). In addition, it has excellent antimycobacterial properties (Das et al. 2008). Thus **1** was used as the lead molecule in the simultaneous development of further candidate cytotoxins and antimycobacterials, including the formation of the corresponding *N*-aroyl derivatives **2a**–**d** and related quaternary ammonium salts (**3a**–**d**) (Das et al. 2007). Most of the compounds in series **2** and **3** also possess excellent cytotoxic and antimycobacterial properties (Das et al. 2007; Das et al. 2008).

2. Investigations, results and discussion

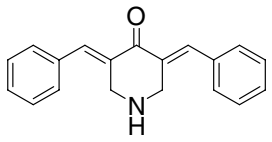
The aim of the present study was to investigate how these compounds exert their bioactivities. A possible target organelle is the mitochondrion for a number of reasons including the following considerations. First, respiration in rat liver mitochondria was stimulated and then inhibited as the concentrations of an acyclic Mannich base of a conjugated arylidene ketone increased (Hamon et al. 1982). The compounds in series **1**–**3** are cyclic Mannich bases (3-aminoketones) of arylidene ketones and hence may affect mitochondrial respiration in a similar fashion. Second, some compounds which are structurally related to **1** and **2** stimulate respiration in mitochondria at a low concentration (10 μM) (Das et al. 2008). Third, some antineoplastic agents alter the rates of respiration in mitochondria (Marín-Hernández et al. 2003; Lemeshko and Kugler 2007).

The data in the Table indicate that the compounds **1**–**3** stimulate respiration in rat liver mitochondria. At the concentrations employed in this study, no inhibition of respiration was noted although on occasions reduction of stimulation was observed as the concentrations of the compounds increased. This observation may have been due to the coexistence of the induction of both inhibitory and stimulatory effects on respiration in mitochondria. An attempt was made to ascertain whether the extent of respiration was controlled by the electronic, hydrophobic and steric properties of the atom or group in the arylidene aryl rings in series **2** and **3**. Thus linear and semilogarithmic plots were constructed between the Hammett σ, Hansch π and molar refractivity constants of the R group and the percentage increase in respiration when 50 μM of the compounds were employed. A trend to a positive correlation was noted only in series **2** with the σ values when linear ($p = 0.081$) and semilogarithmic ($p = 0.100$) plots were made. In the remaining cases, no correlations were observed ($p > 0.1$). Thus the insertion of strongly electron-attracting substituents in the arylidene aryl rings of compounds related to **2a**–**d** such as the 3,5-dinitro group ($\Sigma\sigma = 1.42$) (Hansch and Leo 1979) would be predicted to increase respiration. Conversely the placement of one or more electron-donating groups such as the 4-methylamino substituent ($\sigma = -0.84$) (Hansch and Leo 1979) would likely reduce respiration compared to the biodata generated for **2a**–**d**.

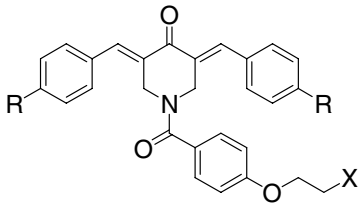
Stimulation of respiration in mitochondria can be caused by a number of biochemical mechanisms including the induction of swelling of these organelles. In order to explore this possibility, **1**, **2a** and **3a**–**d** were evaluated for this property. The results which are portrayed in the Figure reveal that swelling in mitochondria was caused by the quaternary ammonium compounds in series **3** while this effect was absent in **1** and **2a**. Mitochondrial swelling has been observed previously, such as with the anticancer quaternary ammonium compound erucylphosphohomocholine (Lemeshko and Kugler 2007).

An important consideration in deciding to develop compounds as candidate drugs is their mammalian toxicity. A previous study revealed that when a dose of 300 mg/kg of **1**, **2a**–**d** and **3a**–**d** was administered to mice and the animals observed at the end of 4 h, there were no mortalities

Table: Stimulation of respiration in rat liver mitochondria by 1, 2a-d and 3a-d



1



2a-d: X = $N(C_2H_5)_2$ HCl
+
3a-d: X = $N(C_2H_5)_2$ I^-
CH_3

Compd.	R	Percentage increase in respiration[a]							
		10 µM	Δ[b]	20 µM	Δ[b]	50 µM	Δ[b]	100 µM	Δ[b]
1	–	20.2 ± 1.22	–	22.8 ± 0.61	–	43.3 ± 4.51	–	48.3 ± 4.80	–
2a	H	27.7 ± 2.80		28.4 ± 2.17		61.2 ± 5.14		87.2 ± 11.6	
3a	H	32.4 ± 5.68	1.2[c]	53.6 ± 3.86	1.9	134 ± 2.49	2.2	168 ± 10.5	1.9
2b	Cl	25.4 ± 3.81		29.7 ± 6.97		52.5 ± 9.35		62.4 ± 11.3	
3b	Cl	136 ± 3.67	5.4	119 ± 3.45	4.0	100 ± 2.78	1.9	80.0 ± 4.13	1.3
2c	CH_3	23.6 ± 2.60		36.0 ± 6.71		41.2 ± 7.60		67.8 ± 8.58	
3c	CH_3	60.0 ± 3.03	2.5	83.7 ± 2.20	2.3	254 ± 10.6	6.2	83.9 ± 1.79	1.2
2d	NO_2	32.3 ± 2.11		63.5 ± 2.76		88.9 ± 8.22		155 ± 4.46	
3d	NO_2	66.8 ± 2.91	2.1	54.7 ± 3.50	0.9	94.9 ± 2.52	1.1[c]	60.3 ± 3.56	0.4

[a] The rates of oxygen consumption by mitochondria (1 mg protein/mL) respiring on succinate were calculated for the period 1 min prior to, and 1 min after, addition of the compound
[b] The Δ figures are the quotients of the percentage increases in respiratory stimulation of the compound in series **3** with the analog in series **2** which has the same aryl substituent
[c] The differences between the percentage increases in respiration are not statistically significantly different

caused by the compounds in series **1** and **2** but all of the mice receiving **3a, c, d** were dead. Reducing the dose to 100 mg/kg led to the deaths of most of the mice receiving **3a, d** (Das et al. 2008). It is conceivable that these differences in tolerability in mice may be due, at least in part, to variations in the effects on mitochondria. Thus, in general, stimulation of respiration is greater in series **3** than **2** as the Δ values in the Table indicate. This observation may be due to the fact that quaternary ammonium salts being completely ionized can interact with anionic binding sites in the mitochondria. On the other hand, amines such as **2a–d** exist in solution as a mixture of protonated and unprotonated molecules (Albert 1985) and hence the extent of their interacting with anionic sites will be lower than is capable with **3a–d**. Furthermore, the compounds in series **3** cause swelling of mitochondria. These two observations are important, since the toxicity of quaternary ammonium compounds is often attributed to neurological deficit (Pandeya and Dimmock 1997) and hence future toxicity studies of these compounds should take into consideration their effect on mitochondria.

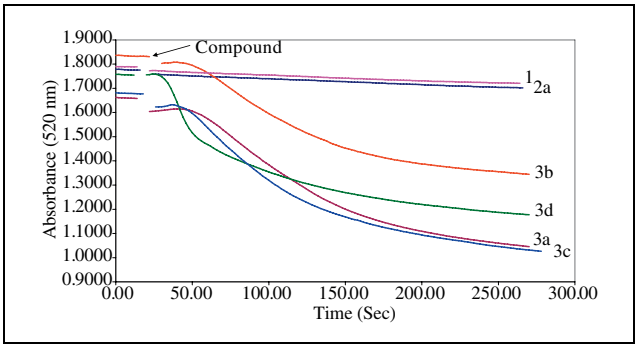


Fig.: Evaluation of 50 µM of **1**, **2a** and **3a-d** for causing swelling of rat liver mitochondria

In conclusion, the mode of action of the promising bioactive molecules **1–3** includes stimulation of respiration in mitochondria and in the case of the quaternary ammonium compounds swelling of these organelles takes place.

3. Experimental

The preparation of **1**, **2a–d** and **3a–d** has been described previously (Dimmock et al. 2001; Das et al. 2007). The Hammett σ, Hansch π and molar refractivity constants were taken from the literature (Hansch and Leo 1979) and the linear and semilogarithmic plots between these values and the percentage increases in mitochondrial respiration were made using a commercial software package (SPSS 2005).

A literature method was used to isolate the mitochondria (Kowaltowski et al. 1996) and the increase in mitochondrial oxygen consumptions was determined polarographically by a previously reported procedure (Estabrook 1967). A reference compound carbonyl cyanide 3-chlorophenylhydrazone caused an increase in respiration of 606 ± 5.34% when 10 µM was utilized. Mitochondrial swelling was determined spectrophotometrically at 520 nm as described previously (Kowaltowski et al. 1996). For obtaining the data in the Table and the Fig., the mitochondria at 1 mg protein/mL were incubated at 30 °C in an aqueous buffer pH 7.2 containing sucrose (125 mM), HEPES (10 mM), potassium phosphate (5 mM), magnesium chloride (1 mM) and succinate (5 mM).

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CHAPTER 7

GENERAL DISCUSSION

For a number of years, α, β-unsaturated ketones have generated enormous interest among researchers owing to their potential to display an array of potent pharmacological properties [1-3]. One of the important ways the α,β-unsaturated ketones display their cytotoxic properties is by selectively alkylating cellular thiols rather than hydroxyl and amino groups present in nucleic acids [4,5]. This property of α,β-unsaturated ketones has created special interests in designing cytotoxic agents possessing this pharmacophore with the aspiration that these molecules may be free of the mutagenic properties that are inherent with many chemotherapeutic drugs [6]. Since the discovery of the natural product curcumin, which contains enone groups and displays potent cytotoxic activities *in vitro* and *in vivo* [7, 8], a major interest has been directed toward developing novel curcuminoids as future anticancer drug candidates.

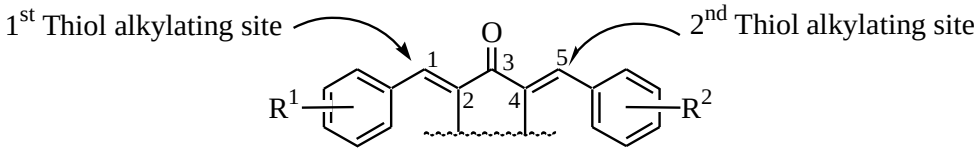
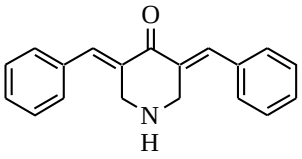


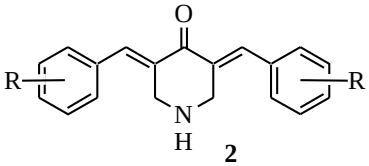
Figure 1: 1, 5-Diaryl-3-oxo-1, 4-pentadienyl (dienone) group. The sequential alkylation of thiol groups occurs at the β-carbon of the α,β-unsaturated carbonyl group.

To improve the metabolic stability of curcumin, the 1, 5-bis (arylidene)-pentan-2,4-dione group of curcumin was replaced by a 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore. Incorporation of this group onto a number of alicyclic and heterocyclic rings led to the development of potent cytotoxic agents [9, 10]. In particular, novel cytotoxic agents based on the 3,5-bis (arylidene)-4-piperidone core structure has been undertaken [11-13]. In the current study, a number of hypotheses were formulated to design novel cytotoxic molecules. It was hypothesized that inclusion of one or more α,β-unsaturated groups would enhance the thiol alkylating potential of the molecules, which may ultimately enhance the cytotoxic potencies of the molecules substantially.

One of the major problems in chemotherapy is the damaging effect of anticancer drugs on normal cells. A hypothesis of sequential cytotoxicity formulated previously by Dimmock et al [13] was utilized to design tumour selective cytotoxins. This hypothesis states that "sequential attack of one or more cytotoxic agents on cellular thiols in cancer cells could be more detrimental to malignant cells compared to the normal cells." In other words, the initial thiol alkylation sensitizes the cancer cells and further alkylation would cause a preferential deleterious effect on cancer cells. Therefore the molecules carrying a 1,5-diaryl-3-oxo-1,4-pentadieny group may prove to be tumour-selective cytotoxins while causing minimal or sparing effects on normal cells.

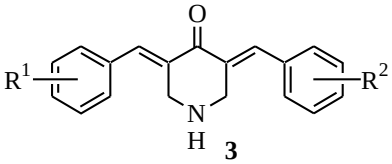


1



2

R = Cl, NH_2, OH, Br, CF_3, COOH,
$COOC_2H_5$, $^{+}N(CH_3)_3I^{-}$

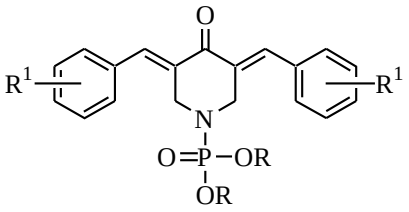


3

R^1= $O(PO)(OR)_2$; R^2= O(PO)(OR)(OH)
R= C_2H_5, CH_3

In-line with the first objective of this thesis project, a series of curcuminoids **2** and **3** based on a lead cytotoxin **1** were synthesized and evaluated against two human T-lymphocytes Molt4/C8 and CEM and a murine leukemia L1210 cancer cell line [13a]. Various substituents with diverse physicochemical properties were placed at the ortho, meta and para positions of the aryl rings and their effects on cytotoxic potencies were examined. In series **2** the molecules possessing ortho and meta substituents displayed greater cytotoxic potencies than their para analogs. To improve water solubility, the 4-O-phosphoryl derivatives **3** were prepared which displayed significantly lower cytotoxic potencies compared to their 4-hydroxy precursor. A number of molecules demonstrated greater cytotoxic potencies than melphalan which is an alkylating anticancer agent used in the clinic. The excellent cytotoxic potencies of a number of

lead molecules from the initial screen were also confirmed by evaluating these compounds against a panel of cancer cell lines that includes neoplasms of leukemic, lung, colon, CNS, breast and prostate origins. In general, the leukemic and colon cancer cells were more sensitive than other malignant cell lines to the compounds. Many of the compounds displayed remarkable cytotoxic potencies (GI_{50}: <0.5μM) and high selective toxicities towards some cancer cell lines compared to others. Another important property of these enones is that they can safely be administered to rodents up to and including 300 mg/kg. The biodata generated from this study provides useful information for further designing of analogs of this class of compounds.



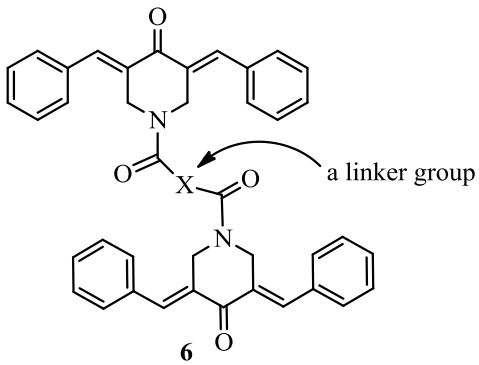
4: R= C_2H_5; **5**: R=H

R^1= H, 4-CH_3, 4-Cl, 4-OCH_3, 3,4-$(OCH_3)_2$, 3,4,5-$(OCH_3)_3$, 2-NO_2, 3-NO_2, 4-NO_2

A further goal was to develop some water soluble prodrugs of 3,5-bis(arylidene)-4-piperidones. In view of this aspiration, two series of N-phosphonate derivatives **4** and **5** were prepared [14]. These compounds display potent cytotoxicity toward human Molt 4/C8 and CEM T-lymphocytes as well as murine leukemia L1210 cells. The compounds in series **5** were in general more potent than the ethyl ester analogues **4**. 3,5-bis-(2-Nitrobenzylidene)-1-phosphono-4-piperidone, a member of the phosphonic acid series **5** displayed remarkable cytotoxic potency with an average IC_{50} value of 34 nM towards the human T-lymphocyte cell lines. Many of the compounds were more potent than the anticancer drug melphalan.

Various physicochemical properties influence the cytotoxic potencies observed. In the NCI screen, the majority of the compounds demonstrated submicromolar to double digit nanomolar range IC_{50} values towards several colon cancer cell lines and leukemic cells. The ability of the compounds to reverse MDR in a mouse lymphoma cancer cell line transfected with the human mdr1 gene was evaluated. Most of the compounds demonstrated potent MDR modulating properties. Thus, these two series of compounds are found to be not only potent cytotoxins but to display potent MDR reversal activity. The study was further expanded to prove the concept of sequential cytotoxicity using these two series of compounds and this investigation was carried out using a panel of malignant (HL-60, HSC-2, HSC-3, HSC-4) and normal (HGF,

HPC, HPLF) cell lines [15]. The biodata obtained from this study revealed that over 70 % of the CC_{50} figures are submicromolar and they also display greater toxicities to the neoplastic cells compared to normal cells. In other words, these compounds were potent tumour-selective cytotoxins. QSAR studies show that cytotoxic potencies and selective toxicity were influenced by the nature of the aryl substituents. The modes of action of representative compounds include induction of apoptosis, interference with cellular respiration, activation of caspase-3 and internucleosomal DNA fragmentation. Very interestingly, some of these mechanisms are only noted in some cancer cells, but not in others. For example, one of the potent compounds in series **5** induces caspase-3 in HL-60 cells, but this effect was not observed in HSC-2 and HSC-4 cells. This pleiotropy shown by these compounds may account for their selective toxicities towards cancer cells.



6

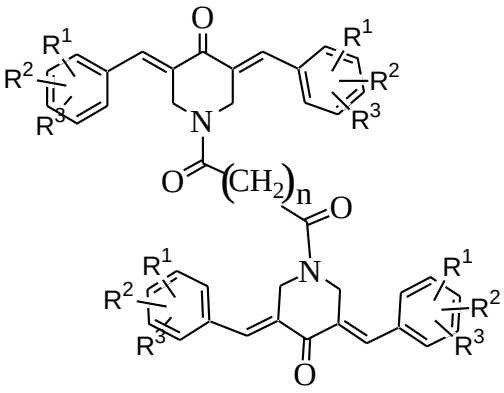
X= $(CH_2)_n$, CH=CH, $(CH=CH)_2$, C≡C

o,m,p-Ar

n=0, 1, 2,3,6

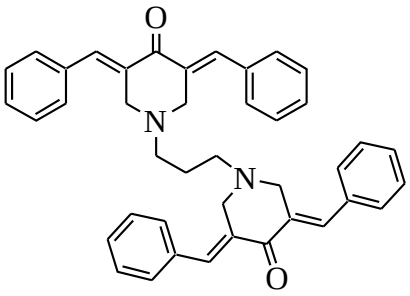
It was hypothesized that doubling the number of sites available for thiol alkylation in a series of candidate cytotoxins would lead to increases in potency by more than two-fold. To prove this concept of cytotoxic synergism, a series of dimers **6** was synthesized [16]. These compounds possess at least double the number of thiol alkylating sites compared to **1**. A linker group X with a varying carbon chain length was introduced between the two amidic carbonyl groups with the consideration that the relative location of one of the 1,5-diaryl-3-oxo-1,4-pentadienyl groups with respect to other will influence cytotoxic potencies substantially. The hypothesis of cytotoxic synergism was verified in one-third of our comparisons using human Molt 4/C8 and CEM T-lymphocytes and murine L1210 cancer cells. Some correlations between the relative locations of the amidic groups with cytotoxicity was established using molecular modeling. Two potent compounds **6a** (X= --) and **6b** ($X=CH_2$) were found to be the most potent

compounds. This observation was also further supported by the biodata generated from a panel of cancer cell lines in the NCI screen. The average GI_{50} values of **6a** and **6b** were 0.31 and 0.24 μM, respectively [17]. These compounds displayed the following important properties. First, greater toxicity was demonstrated towards certain tumours than various non-malignant cells [17]. Second, various compounds in series **6** are toxic to a number of human colon cancer and leukemic cells i.e., the GI_{50} values were in the submicromolar to low nanomolar range against a number of colon and leukemic cell lines. Third, these compounds reverse P-gp mediated multidrug resistance. The mode of action of **6a** includes induction of apoptosis and necrosis while **6b** induces apoptosis via internucleosomal DNA fragmentation and PARP cleavage in HSC-2 and HL-60 cells. Flow cytometry analysis revealed that **6b** arrests the G2/M and S phases in the cell cycle of human colon cancer HCT-116 cells.



7: n=0**; 8**: n=1

R^1,R^2,R^3= H, 4-CH_3, 4-Cl, 3,4-Cl_2, 4-F,
4-OCH_3, 3,4-$(OCH_3)_2$, 3,4,5$(OCH_3)_3$



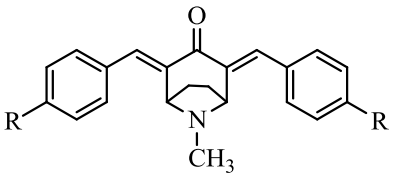
9

The next endeavor was directed to developing further analogs of the lead cytotoxins **6a** and **6b** which emerged from the previous study in a quest to find more potent cytotoxic compounds. The major interest was to find novel compounds that are effective against colon cancer cells. This persuasion was based on the observation that **6a** and **6b** demonstrate potent growth-inhibiting properties against a panel of colon cancer cell lines in the NCI screen. Two series of dimers **7** and **8** were prepared by placing a number of substituents onto the aryl rings of **6a** and **6b**, respectively, and were screened against HCT116 and HT29 colon cancer cell lines [18]. In these series, statistical correlations between their electronic, hydrophobic and steric

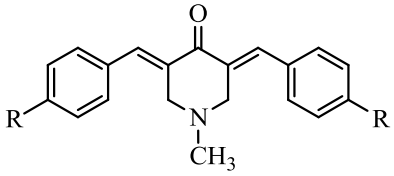
properties with cytotoxic potencies were sought. The data reveals that cytotoxic potency increases with the increase in the electron-withdrawing properties of the aryl substituents in both the series in HCT116 cells. A similar observation is also noted in series **7** against HT29 cells. Therefore, further analog development should include stronger electron-withdrawing groups in the aryl rings. A number of hypotheses were verified and the following observations were obtained from the biodata generated for these two series of compounds. First, compounds in the series **7- 9** are potent cytotoxins which display remarkable IC_{50} values that are in the submicromolar to low nanomolar range. Most of the compounds show greater cytotoxic potencies than melphalan and 5-fluorouracil. Second, the compounds in series **7** display greater cytotoxic potencies than the compounds in series **8**. Third, in both series, the compounds demonstrate greater selective toxicities towards HCT116 cells compared to HT29 cells. Fourth, in a number of cases, the introduction of an aryl substituent enhanced cytotoxic potencies compared to the unsubstituted analog in both series. Fifth, excision of both the amidic carbonyl groups of **6b** as represented by the compound **9** leads to a reduction in cytotoxic potencies against both the colon cancer cell lines which proved the importance of the amidic carbonyl groups in these dimeric molecules. An investigation was made to establish the mode of action a representative potent cytotoxin by which the cytotoxicity of this class of compounds is mediated in colon cancer cells. A potent cytotoxin in series **7** (R=4-OCH_3) induced apoptosis in HCT116 cells.

The last part of the study was to evaluate an important question whether mitochondria are also a target for cytotoxic 1,5-diaryl-3-oxo-1,4-pentadienes and whether the variation in cytotoxic potencies of these compounds can be accounted for by their effects on mitochondrial functions. Three structurally different group of compounds, bis(arylidene)-4-piperidones, N-aroyl-bis(arylidene)-4-piperidones and bis(arylidene)cyclohexanones were evaluated for their effect on two important mitochondrial functions namely respiration and swelling. As observed earlier, alkylation or cross-linking of a number of conjugated unsaturated ketones with a critical thiol of a protein [4] leads to the opening of the mitochondrial permeability transition pore [19, 20] which causes mitochondrial swelling and collapse of the mitochondrial membrane potential [21]. The collapse of the membrane potential decreases the resistance to electron flow in the respiratory chain and stimulates mitochondrial respiration [22]. The mitochondrial permeability

transition is a critical trigger for apoptosis [23] and has been identified as a target for cancer therapy [24-26].



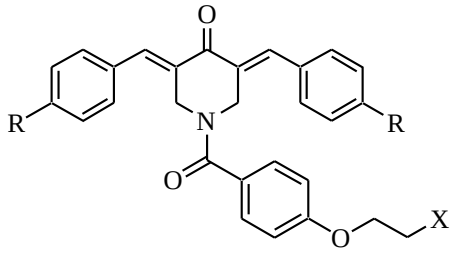
10



11

R= H, Cl, NO_2, CH_3, OCH_3

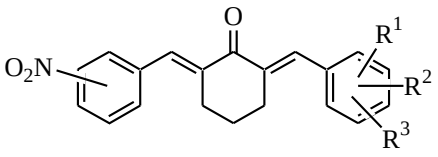
Two series of molecules with divergent topography were chosen. In general, the replacement of the protons at C-2 and C-6 position in series **11** by a dimethylene bridge as represented by series **10** led to a considerable reduction in cytotoxic potencies [27]. This disparity in cytotoxic potencies between both series was thought to be due to the dimethylene bridge in **10** exerting a steric impedance to aligning at a binding site. In addition, the variation in the hydrophobic properties of the molecules might be a contributing factor. In terms of cytotoxic potencies, the best compounds in both the series were the unsubstituted analogs **10a** (R=H) and **11a** (R=H). **11a** displays greater cytotoxic potencies toward malignant cells than normal cells as compared to **10a**. Two compounds **10a** and **10d** (R=OCH_3) which possessing markedly divergent cytotoxic potencies towards malignant cells were examined for their effect on mitochondrial respiration and swelling. Both the compounds exert a strong stimulating effect on mitochondrial respiration, with compound **10a** having a significantly shorter latent period. A significant difference in mitochondrial swelling was noted with these two compounds. A rapid mitochondrial swelling was observed with **10a**, while this phenomenon progressed very slowly in the case of **10d**. The greater ability of **10a** to induce mitochondrial swelling, therefore, may have contributed to its higher cytotoxic potency in malignant cell lines. A difference in the ability of **10a** and **10d** to cause mitochondrial swelling was also explained in the context of the electrophilicity of the aryl substituents. In the less potent **10d**, the R^1 methoxy substituents are electron repelling compared to the R^1 protons of **10a** and would thereby decrease the electrophilicity of **10d** towards thiols compared to **10a**.



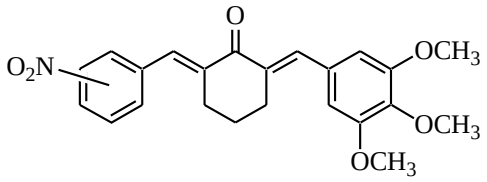
R= H, Cl, CH_3, NO_2

12 X= $N(C_2H_5)_2$; **13** X= $^{+}N(C_2H_5)_2CH_3\ I^{-}$

In addition, a series of cytotoxic N-aroyl-3,5-bis(arylidene)-4-piperidones **12** and their methiodide analogs **13** were evaluated for their effect on mitochondrial respiration [28]. All the compounds in both series demonstrated a stimulating effect on mitochondria. The compounds in series **13** showed considerably greater stimulating effect than the series **12**. In a dose-response study, an increase in mitochondrial respiration was noted with the increase in concentration in both series. Evaluation of the effect of aryl substituents possessing diverse physicochemical properties on mitochondrial respiration in series **12** revealed that mitochondrial respiration increases with the increase in electron-withdrawing properties of the aryl group. The quaternized salts in series **13** caused rapid swelling of mitochondria while this effect was absent in **12a** (R=H) and **11a** (R=H). This observation partly accounted for the murine toxicity shown by the compounds in series **13**.



14



15 R=2-NO_2; **16** R=3-NO_2; **17** R=4-NO_2

Finally, a number 1,5-diaryl-3-oxo-1,4-pentadienes **14** possessing a cyclohexane nucleus were evaluated against Molt4/C8, CEM and L1210 malignant cell lines [29]. The IC_{50} values of the majority of 2-benzylidene-6-(nitrobenzylidene)cyclohexanones were less than 10 µM. The order of cytotoxic potencies of the compounds in series **15-17** were in order of **15**(ortho)>**17**(para)>**16**(meta). A general observation is that bis(arylidene)cyclohexanones were less toxic to malignant cells compared to 3,5-bis(arylidene)-4-piperidones. The most potent compounds emerged from this study were **15** (R= 2-NO_2, ave IC_{50}: 0.88 µM) and **17** (4-NO_2, ave

IC_{50}: 6 μM) whereas a corresponding meta analog **16** (3-NO_2, ave IC_{50}: 43.8 μM) was found to be less potent. To explain this disparity in cytotoxic potencies, the effect of these three related compounds were evaluated on mitochondrial respiration. Using a concentration of 10 μM, **15** and **17** stimulated respiration whereas **16** had virtually no effect on respiration. Increasing the concentration of **16** as high as 100 μM also revealed no statistically significant difference in the stimulation of respiration. The causes for the relative cytotoxic potencies observed in this study may be multifactorial; the differences in the effects on mitochondrial function may have exerted some contributions to the disparity of IC_{50} values.

In conclusion, some novel series of potent cytotoxins were developed. A number of lead cytotoxic molecules with submicromolar to low nanomolar range were identified and warrants further development of these molecules as candidate anticancer agents. Multidrug resistance reversal properties displayed by a number of cytotoxic molecules were noteworthy. A number of potent cytotoxic molecules identified in this study did not cause any significant toxicity in rodents up to and including a dose of 300 mg/kg. The modes of action of these cytotoxic molecules include apoptosis, cell cycle arrest, DNA fragmentation, caspase-3 activation, PARP cleavage and interference with mitochondrial functions.

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FUTURE WORKS AND CHALLENGES

This thesis project work was my first step in drug discovery in the College of Pharmacy and Nutrition, University of Saskatchewan which was aimed at the development of novel cytotoxic agents based on various medicinal chemistry approaches. This study resulted in a number of novel curcuminoids possessing remarkable cytotoxic potencies *in vitro* against human cancer cell lines. Some of these compounds also demonstrate potent multidrug resistance reversal properties. These interesting results warrant further examination of these molecules as candidate cytotoxins and the next phase of investigation should be pursued.

The first phase of the investigation in drug discovery is the identification of leads for preclinical evaluations. In this step, a systematic investigation is proposed which is presented in Figure 1. A number of other important questions that are relevant to this thesis work need to be addressed in the future:

- Though the majority of the compounds designed in this project comply to drug likeliness properties, there are some compounds, for example the dimers, which are exceptions. Although drug likeness characteristics of molecules are considered important physicochemical parameters for prediction of oral bioavailability, a number of clinically used drugs do not meet these criteria. From the *in vitro* studies, the dimers are found to be very interesting molecules. Very special interests have been expended in developing a number of these molecules. Considering the dimers are large molecules, one may expect that solubility could be one of the problems with these molecules which may limit their further development. Therefore, to address this issue, one or more hydrophilic groups such as hydroxyl and phosphate should be introduced onto the aryl rings of the lead dimeric compounds to improve their aqueous solubility.
- To examine whether these cytotoxic molecules target one or more sulfhydryl macromolecules such as glutathione S-transferase, thioredoxin reductase, and h-NMT which are known as potential targets for anticancer agents.
- To evaluate whether these molecules are effective against drug-resistant cancer cell lines.
- To examine whether the phosphate prodrugs are hydrolyzed to their active precursor 3,5-bis(arylidene)-4-piperidones and the cytotoxicity observed is due to the parent compound or its precursor.

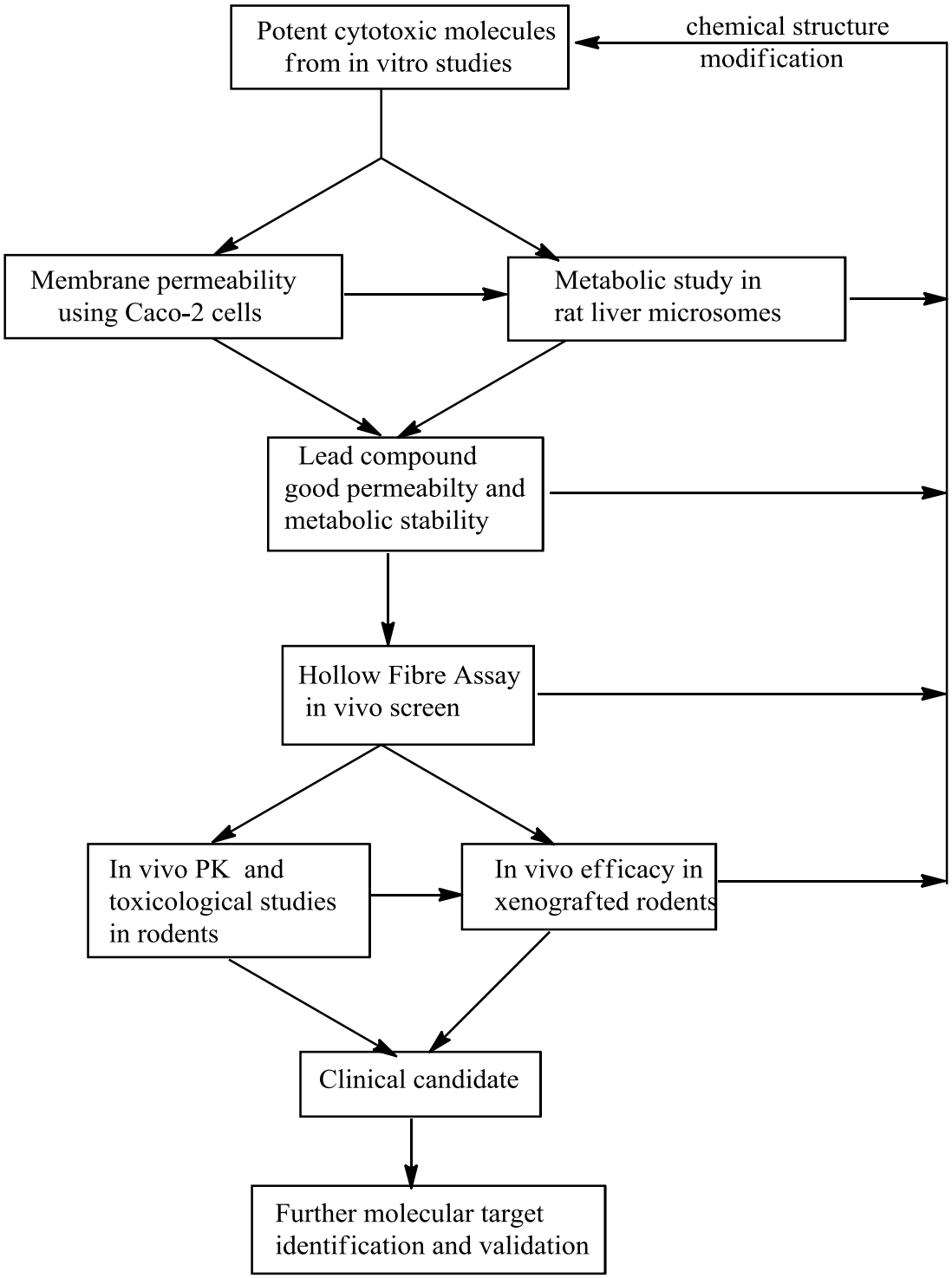

Figure1. Systematic screening strategies for obtaining cytotoxic drug candidates for clinical evaluations.

Drug discovery is an expensive and very challenging job which takes a number of years to develop a suitable molecule for clinical evaluations. A molecule may fail to display the desired properties at any stage of the investigation and thereafter it goes back again to the first stage of the development process. In this scenario, structural modifications of the molecule are undertaken to improve its physicochemical characteristics. A common problem like solubility can be addressed by introducing hydrophilic groups, or deploying a suitable formulation strategy. One of the biggest challenges in drug discovery research is to reproduce *in vitro* cytotoxic potencies of a molecule *in vivo*. Hence, suitable strategies and dynamic approaches are required to overcome potential hurdles in this endeavor.

APPENDIX

Mitochondrial Study-Experimental protocol

Mitochondria isolation[1**]:**

Rats (250-300 g body weight) were euthanized by isoflurane and decapitation. Mitochondria were isolated from the liver following rapid removal of the organ. Briefly, freshly isolated liver was homogenized using a glass Teflon homogenizer using an isolation medium containing 250 mM sucrose, 10mM HEPES and 1 mM EGTA (pH 7.2). The homogenate was centrifuged at 1000g for 8 min at 4°C. The supernatant was collected and centrifuged at 10,000g for 10 min at 4°C. The pallet was collected and added to a medium containing 250 mM sucrose, 10mM HEPES and 0.1 mM EGTA (pH 7.2) and centrifuged at 10000g for 10 min at 4°C. Again the final pallet was washed twice and suspended in 2 ml of a same medium except EGTA was omitted.

Oxygen uptake studies:

The mitochondrial protein was measured at a wavelength of 520 nm in a spectrophotometer using 30/40 µl of freshly prepared mitochondria in 1 ml of respiratory buffer containing 62 mM KCl, 125 mM sucrose, 10 mM Hepes, 2 mM diabasic potassium phosphate and 1 mM $MgCl_2$ (pH 7.2). Approximately 0.5 mg of protein/ml was used in each oxygen consumption experiment. Respiration rates were measured with a Clark-type electrode (Hansatech Instruments Ltd, Norfolk, England) in a water-jacketed glass chamber with a magnetic stirrer in it. An oxygen electrode system, which consists of two electrodes immersed in an electrolyte solution of 50% saturated solution of KCl. Application of a polarizing voltage of 700 mV ionizes the electrolyte and initiates the current flow. This current is directly proportional to the amount of dissolved oxygen within the sample held in the reaction vessel. The current produced by the electrode disc is converted to a voltage signal, which is digitized into a reproducible unit (nmol/ml) by the electronics within the control unit. The temperature in the respiration chamber was kept constant at 30°C. Before testing the compounds one initial experiment was carried out by using 0.25mM succinate as a respiratory substrate with 1 mg protein of the mitochondrial suspension with transitions from state 4 to 3^*. After being confirmed that the mitochondria are coupled, further experiments were conducted with the compounds where succinate was used as a substrate. The compounds were dissolved in DMSO having a final

concentration in the chamber as 1%, whereas in some special cases it is >1% due to the solubility problem of the compounds. Respiration was monitored for 10 minutes. Assays were performed in triplicate using fresh mitochondria. Rates were calculated from Windows® software package, Oxygraph Plus. The % of inhibition /stimulation was calculated by using the following formula:
% of inhibition = [(rate before treatment– rate after treatment) / rate before treatment] x 100.
The difference between control and treated rates gives positive results when there is inhibition of respiration and a negative result indicates stimulation of respiration. Antimycin A was used as the standard inhibitor.

*[Mitochondrial respiratory state 4 is the resting state, which is differentiated by relatively slow oxygen uptake without ADP. On the other hand, mitochondrial respiratory state 3 is the active state with a high rate of oxygen uptake and with a sufficient ADP supply [2]. State 3 is initiated by adding ADP (0.2 micromoles). State 4 must be produced before producing state 3. In state 3 the increase in respiration rate is because of the binding of the ADP to the ATP synthase in the presence of inorganic phosphate, which results in the opening of a channel that permits the flow of protons into the matrix from outside the inner membrane. Due to the flow of protons across the membrane, the energy released is used to produce ATP. As soon as the energy in the gradient is removed the electron chain accelerates. When all of the ADP is used, the respiration rate goes back to state 4. Mitochondrial function was assessed by the respiratory control ratio (RCR) and ADP:O ratios. These two parameters are well accepted as indicators of electron transport chain coupling to ATP synthesis and the efficiency of oxidative phosphorylation in the presence of different substrates. The ADP:O ratio was calculated by taking the ratio of total amount of ADP added to the medium to the percentage of total oxygen used up in order to phosphorylate ADP (i.e. from the beginning to the end of state 3 respiration). RCR was calculated by taking the ratio of respiratory rate in state 3 to the respiratory rate in state 4[3]. The RCR value was higher than 4 and ADP:O was higher than 2. These values are generally accepted as normal for carefully prepared mitochondria from liver.]

Mitochondrial swelling study:

Using the fresh mitochondria isolated from rat liver (procedure explained earlier), a swelling study was performed. Mitochondrial swelling was determined by light absorbance measured at 520 nm wavelength using a spectrophotometer following the literature method [4].

Briefly, 1 mg protein was used in the incubation medium (62 mM KCl, 125 mM sucrose, 10 mM hepes, 2 mM potassium phosphate diabasic and 1mM $MgCl_2$) with 0.25 mM of succinate each time with or without the sample. All used reagents were purchased from Sigma Chemical Co. Each experiment was performed twice. The temperature in the spectrophotometer was maintained at 30 °C.

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