PHARMACOKINETICS AND PHARMACODYNAMICS

OF

ANTICANCER CURCUMIN ANALOGUES

IN

AN ACADEMIC DRUG DISCOVERY UNIT

A Thesis Submitted to the
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in Partial Fulfillment of the Requirements
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By

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ABSTRACT

Recently, several universities including the University of Saskatchewan initiated drug design and discovery programs to meet the shift in emphasis to translational research by the major funding organizations. The nascent Drug Design and Discovery Research Group at the University of Saskatchewan initiated an anticancer drug discovery program where one of the active projects focused on the development of curcumin analogues containing the 1,5-diaryl-3-oxo-1,4-pentadienyl group as anticancer agents. This became my Ph. D. dissertation research. My current work identifies the inadequacy in the current drug design and discovery model and provides initial direction for the future design of the curcumin analogues with better pharmacokinetic and pharmacodynamic properties. A battery of pharmacokinetic tests on NC 2083, a curcumin analogue that exhibits high *in vitro* efficacy but does not show any effect *in vivo*, suggested low permeability, high plasma protein binding and rapid partitioning into RBC as the probable reasons for its inability to control tumour growth in xenografted nude mice. Solubility and permeability assays on different curcumin analogues suggest that the majority of curcumin analogues exhibit low water solubility and low permeability. Our permeability assessment tests on the curcumin analogues identified the differential effect of these curcumin analogues on Caco-2 and MDCK cell monolayer integrity where MDCK cell monolayer was adversely affected while Caco-2 was not. The comparison of physicochemical properties of our analogues with analogues from laboratories reporting preclinical success highlighted that the majority of molecules from successful laboratories conform to the Lipinski's rule of five while most of our compounds violated it. In order to identify the mechanism of action, we tested the effect of curcumin analogues on the mRNA and protein expression of essential proteins of tumour angiogenesis. The tested curcumin analogues exhibited differential modulation of the angiogenesis mediating proteins. All these studies laid the fundamental building blocks for the anticancer drug discovery process at the College of Pharmacy and Nutrition, University of Saskatchewan and a new model of drug design and discovery was proposed. However, the efficiency of the proposed model remains to be seen.

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Dedicated
to
My Parents, Wife and Son

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

μM	Micromolar
5-FU	5-Fluorouracil
A-B	Apical to Basal transport
ADDU	Academic Drug Discovery Unit
ADME	Absorption Distribution Metabolism Excretion
ADMET	Absorption Distribution Metabolism Excretion Toxicology
Ahr	Aryl Hydrocarbon Receptor
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	Analysis of Variance
APC	Adenomatous Polyposis Coli
ASBT	Apical Sodium Dependent Bile Acid Transporter
ATCC	American Type Culture Collection
AUC	Area under the Plasma Concentration Versus Time Curve
B2MG	Beta-2-Microglobulin
B-A	Basal to Apical Transport
BBB	Blood Brain Barrier
BCRP	Breast Cancer Resistance Protein
BM	Biomimetic
Caco-2	Colon Adenocarcinoma Cell Lines
cDNA	Complementary DNA
CIHR	Canadian Institutes of Health Research
cLogP	Calculated LogP
CL_S	Systemic Clearance
C_{max}	Maximum Concentration in the Blood
COX-2	Cyclooxygenase 2
C_T	Threshold Cycle
CYP P450	Cytochrome P 450
Da	Dalton
DDDRG	Drug Design and Discovery Research Unit

DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DOPC	Dioleyoylphosphatidylcholine
DS	Double Sink
EDTA	Ethylendiaminetetraacetic Acid
Efr	Efflux Ratio
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme Linked Immunosorbent Assay
EMT	Epithelial Mesenchymal Transition
Et	Ethyl
F	Bioavailability
FAP	Familial Adenomatous Polyposis
FaSSGF	Fasted State Simulated Gastric Fluid
FaSSIF	Fasted State Simulated Intestinal Fluid
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FIPNet	Fully Integrated Pharmaceutical Network
GIT	Gastrointestinal Tract
GLO	Glyoxalase
GPCR	G-Protein Coupled Receptor
GSH	Glutathione
GST	Glutathione S Transferase
HBA	Hydrogen Bond Acceptors
HBD	Hydrogen Bond Donors
HBSS	Hanks Balanced Salt Solution
HDM	n-Hexadecane
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic Acid
HPLC	High Pressure Liquid Chromatography
HTS	High Throughput Screening
IC_{50}	Concentration that Inhibits the Growth by 50 Percent

IL	Interleukin
IS	Internal Standard
IV	Intravenous
IVIVC	In Vitro In Vivo Correlation
JAK	Janus Kinase
k	Elimination Rate Constant
LLOQ	Lowest Limit of Quantitation
LogP	Logarithm of Permeability Coefficient
LRH	Liver Receptor Homologue
LY	Lucifer Yellow
MAPK	Mitogen Activated Protein Kinase
MCT	Monocarboxylic Acid Transporter
MDCK	Madin-Darby Canine Kidney Cell Line
MDR	Multi Drug Resistance
miLogP	Calculated Logp by Cheminformatics Tool by Molinspiration
MMP	Matrix Metalloproteinase
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
MRP	Multidrug Resistance Associated Protein
MS	Mass Spectrometry
MW	Molecular Weight
NCE	New Chemical Entity
NCI	National Cancer Institute
NDA	New Drug Application
nHBA	Number of Hydrogen Bond Acceptors
nHBD	Number of Hydrogen Bond Donors
NICE	National Institute for Health and Clinical Excellence
NIH	National Institutes of Health
NMR	Nuclear Magnetic Resonance
nOHNH	Number of Hydrogen Donors
nON	Number of Hydrogen Bond Acceptors

Nrotb	Number of Rotatable Bonds
NSAID	Non Steroidal Anti Inflammatory Drug
OATP	Organic Anion-Transporting Polypeptide
OCTN	Organic Cation/Carnitine Transporter
OD	Optical Density
OST	Organic Solute Transporter
PAINS	Pan Assay Interference Compounds
PAMPA	Parallel Artificial Membrane Assay
Papp	Apparent Permeability
PBS	Phosphate Buffered Saline
PD	Pharmacodynamics
PEG	Polyethyleneglycol
PEPT	H+/Di-Tripeptide Transporter
PET	Positron Emission Tomography
Pgp	P-Glycoprotein
pH	-Log $[H^+]$
PI3	Phosphoinositide 3 Kinase
PK	Pharmacokinetics
PPAR	Peroxisome Proliferator Activated Receptor
R&D	Research and Development
RAS	Rat Sarcoma
RBC	Red Blood Cells
RNA	Ribonucleic Acid
RO5	Lipinski's Rule of Five
RT	Reverse Transcription
SD	Standard Deviation
SHP	Small Heterodimer Partner
SiRNA	Small Interfering Ribonucleic Acid
SMC	Scottish Medicines Consortium
SMILES	Simplified Molecular Input Line Entry Specification
STAT	Signal Transducer and Activator of Transcription

SULT	Sulfotransferase
T/C	Treated/Control
$T_{1/2}$	Elimination Half-Life
TBME	Tert-butyl methyl ether
TCA	Trichloroacetic Acid
TEER	Trans-Epithelial Electrical Resistance
TGF	Transforming Growth Factor
T_{max}	Time Required to Achieve C_{max}
TNF	Tumour Necrosis Factor
TPSA	Total Polar Surface Area
TRUS	Transrectal Ultrasonography
Tz	Cells Fixed at Time Zero
UGT	UDP-Glucuronosyl Transferase
USD	United States Dollar
USFDA	United States Food And Drug Administration
UV	Ultraviolet
V_d	Volume of Distribution
VEGF	Vascular Endothelial Growth Factor
VIDO	Vaccine and Infectious Disease Organization
WHO	World Health Organization

CHAPTER 1
LITERATURE REVIEW

1.1 Introduction

For decades, pharmaceutical industries guided the drug discovery and development while the academic institutions provided logical support. Traditionally, academic researchers explore the basic aspects of disease, target identification (1), elucidation of pathways of pathogenesis and development of tools rather than the drug discovery. The current emphasis of funding agencies to fund projects with direct application and marketing ability has redirected the course of academic research such that academia has become further engaged in the drug discovery and development process. At the University of Saskatchewan a drug design and discovery research group was formalized to bring together different researchers to coordinate different aspects of drug design and discovery. This newly formed research team required a drug discovery strategy for efficient and rapid identication of lead molecules to support its drug design efforts (2).

A major focus of the Drug Design and Discovery Research Group at the University of Saskatchewan is the development of anticancer agents. Despite the progress made in anticancer treatment strategies to date, cancer still remains a devastating diagnosis and is associated with significant morbidity and mortality. According to Canadian statistics, cancer is the most fatal disease in Canada and caused 8.3% more deaths than cardiovascular disease in 2008 (3). Worldwide, cancer mortality has increased in the past few years (4) and about 27 million new cases of cancer and 17.5 million more deaths due to cancer are expected by 2050 (5). These rising statistics suggests a need for the discovery of new anticancer agents with high efficacy and low side effects. Despite this need, anticancer compounds have one of the highest attrition rates compared to other classes of compounds in the drug discovery and development pipeline (6, 7). The implementation of several strategies including a paradigm shift in anticancer drug discovery from non-targeted cytotoxic drugs to targeted antibodies resulted in only minor improvements in success rate (8). The high attrition rate is due to several challenges ranging from drug design to clinical trials (9, 10). Such drug discovery and development challenges increase several fold in academic drug discovery settings, challenges that include lack of discovery experience and financial and labor constraints (11).

At the University of Saskatchewan, Dimmock and coworkers have identified the potential efficacy of the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore (12), a pharmacophore similar

to curcumin in structure (13), in a variety of disease conditions including cancer. Like curcumin, these compounds demonstrated anticancer activity against colon cancer at very low concentrations (12-14). However, the major focus remained on the development of quantitative structure activity relationships to improve the efficacy of compounds with little emphasis on their optimum pharmacokinetic characteristics. The pharmacokinetic studies on NC 2083, a compound containing 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore, suggested that the poor absorption likely explained its lack of in vivo efficacy. Although most of anticancer drugs are administered intravascularly, the development of orally active anticancer drug is always desirable. To establish a general screening process for drug discovery and using the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore as model compounds, I developed a screening strategy for the rapid characterization of pharmacokinetic properties, with a focus on absorption characteristics. This screening strategy will help support drug design efforts of future pharmacophores developed by the Drug Design and Discovery Resarch Group at the University of Saskatchewan.

1.2 Background

1.2.1 Colon Cancer: Current Status and Future Challenges

Cancer is caused by an alteration in normal cellular mechanisms that leads to the perturbation of a delicate balance between cell division and cell death to favor cell proliferation (15). Tumour suppressing genes encode proteins responsible for suppressing uncontrolled growth while proto-oncogenes promote cell growth (16, 17). Gene mutations or chromosomal aberrations lead to expression of defective proteins that are unable to perform the same functions as their normal counterparts and consequently lead to uncontrolled cell growth (18).

Colon cancer is the second most fatal cancer in developed countries including Canada. Worldwide, more than 940,000 new cases of colorectal cancers are recorded and about 500,000 die each year (5). In 2011, 22,200 new cases and 8,900 deaths were estimated in Canada (19). The high mortality rates with colon cancer result from its lack of symptomology early stages of disease progression. Early diagnosis with effective treatment can significantly decrease the incidence of mortality associated with colon cancer (20).

Diet, lifestyle and environmental factors influence colon cancer prevalence (21). A high incidence of colorectal cancer in affluent societies is associated with diets rich in fat, refined

carbohydrates and processed meats (22). The susceptibility of immigrants from developing nations, who are at relatively low-risk in their homeland, increases within a few years of their immigration. Rapid increase in risk level of carcinogenesis suggests the role of environmental factors and lifestyle (23, 24). The relatively lower incidence rates of colon cancer in Asian countries may be due to the consumption of turmeric and onion in curry. Turmeric and onions contain curcumin and quercetin, respectively, which exhibit chemopreventive activity against colorectal neoplasia (25, 26). The combination dosing of curcumin and quercetin in five patients with familial adenomatous polyposis, an autosomal dominant disorder, which eventually leads to colorectal cancer, decreased the size and number of ileal and rectal adenomas (25)

1.2.1.1 Aetiology of Colon Cancer:

The development of colon cancer involves stepwise progression of normal epithelium to adenomatous polyp and eventually into carcinoma (27, 28). This progression towards carcinoma involves a series of mutations in proto-oncogenes, tumour suppression genes and DNA repair genes. Tumour suppressing genes and proto-oncogenes code proteins, which suppress and promote cell growth, respectively. DNA repair genes code for proteins which identify and correct the damage caused by endogenous or exogenous factors and the errors during replication (16). Accumulation of such mutations in cells eventually leads to carcinoma (27). As per the clonal selection theory, the cells having genetic mutations are more labile to subsequent genetic changes and, therefore, favour the accumulation of genetic abnormalities (29, 30).

Normal colorectal epithelium undergoes a series of genetic changes to form colon carcinoma (Figure 1.1). The loss of tumor suppressor gene, APC (31), leads to early adenoma. Subsequently, KRAS mutation (32, 33), loss of SMAD 2 or SMAD 4 (34-36) and alteration in DNA methylation (37) transform early adenoma into late adenoma. Subsequent loss of p53 gene (38) (a tumour suppressing gene), transforms late adenoma into carcinoma. This sequence of events is usually consistent among most patients but alteration in the sequence of genetic mutations can still lead to carcinoma. The accumulation of genetic mutations governs the transformation of normal epithelium into carcinoma (27, 39).

1.2.1.2 Invasion and Metastasis

Invasion and metastasis are characteristics of malignant tumours (40). Direct migration and

penetration of cancerous cells to the surrounding tissues is termed invasion, whereas metastasis refers to the entry of cancerous cells into the blood stream or lymphatic system to reach distant sites (15). Metastasis involves a series of events. First, cancerous cells invade the surrounding tissue and penetrate the basal lamina and endothelial cells of blood vessels to gain access to the blood stream. In the second step, blood carries these cancerous cells to distant favourable organ sites. In step three, the cancer cells adhere to capillaries, invade the capillaries to move out of the blood stream and reinvade the neighbouring tissue and grow again (41).

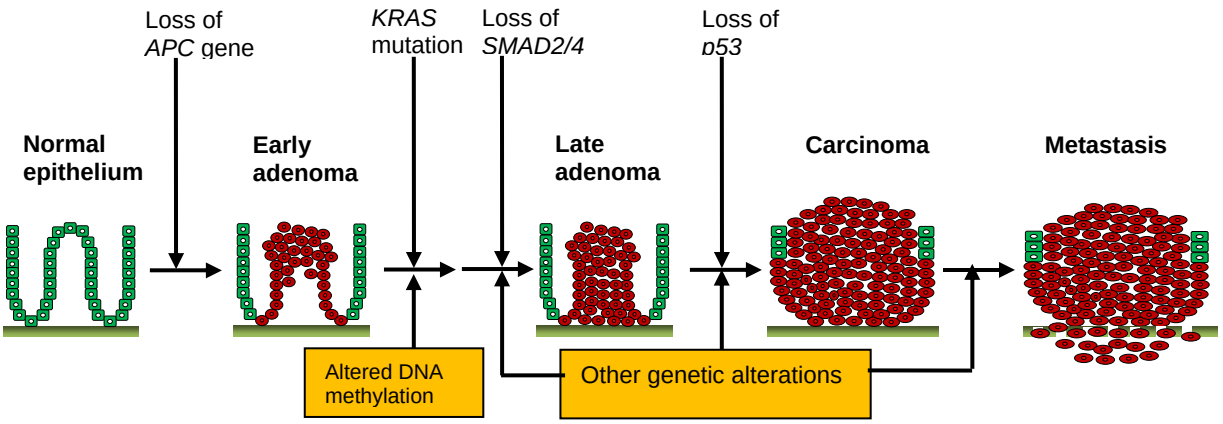

Figure 1.1: The sequence of events in development of colorectal carcinoma. See text for explanation. (Adapted from Davies *et al* (42))

Unlike normal cells, cancer cells are easily dislodged from their original location and penetrate other tissues via different mechanisms (43, 44). The first mechanism is a decrease in cell-cell adhesiveness resulting primarly from the loss of cell adhesion proteins such as integrins, cadherins, selectins, some immunoglobulins, and hyaluronate binding proteins (45). In particular, integrins are critical in mediating cellular adhesion, interaction with extracellular matrix, and angiogenetic processes (46). The second mechanism is increased cell motility, which permits cells to detach and migrate. To gain cellular motility cells must attain mesenchymal cell characteristics, which is referred as epithelial-mesenchymal transition (EMT) (47). Cancer cells or surrounding tissues produce signaling molecules that increase cellular motility and act as chemoattractants. The third mechanism is activation of proteases, an enzyme responsible for protein degradation. One such protease produced by cancer cells is plasminogen activator (48,

49), which converts plasminogen into its active form plasmin, a protease. Plasminogen is present in high concentrations in all tissues and plasminogen activator can generate large quantities of plasmin. The plasmin degrades components of the basal lamina and extracellular matrix and converts the matrix metalloproteinase enzyme (MMP) (50) precursor into active protease MMPs to degrade the extracellular matrix.

1.2.1.3 Angiogenesis

Once access to tissues at distant sites is gained, cancer cells can form secondary tumours. Angiogenesis, or the formation of capillaries, is vital for tumor growth beyond a few millimeters (51). Angiogenesis provides a continuous supply of nutrients and oxygen to dividing tumour cells (52). Tumours may become necrotic or apopotic in the absence of a vascular blood supply. Angiogenesis is a four step process (15). The first step is local injury of basement membranes of the tissues causing destruction and hypoxia. In the second step, angiogenic factors activate endothelial cells to migrate to form a new tubular structure. In the third step, endothelial cells are stimulated to proliferate and in fourth step, these angiogenic factors keep influencing the angiogenic process (53).

A balance of angiogenic activators and inhibitors controls tumour angiogenesis. Several angiogenic activators and inhibitors have been identified (54). Some of the major endogenous angiogenic activators are vascular endothelial growth factor (VEGF), basic fibroblast growth factor, angiogenin, transforming growth factor (TGF)-alpha and beta, tumour necrosis factor (TNF)-alpha, and interleukin 8, whereas the endogenous inhibitors are angiostatin, caveolin, and interferon alpha (55, 56).

1.2.1.3.1 Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor (VEGF) is a very powerful angiogenic agent produced by most human tumours and normal cells (57). VEGF binds with VEGF receptors to initiate signal transduction, which leads to increased matrix metalloproteinase (MMPs) production. The MMPs break down extracellular components and allow endothelial cells to move. Five different VEGF have been identified, VEGF-A to VEGF-E. VEGF-A, VEGF-B, VEGF-C and VEGF-E cause proliferation of blood vessels whereas VEGF-C and VEGF-D cause proliferation of lymph vessels (58). VEGF-A is the most widely studied member of VEGF family. VEGF-A is required

for the normal development of vasculogenesis and angiogenesis, as even one missing allele can be lethal to animals (59). VEGF-A is over expressed in most human solid tumours and, thus, a very good target for antitumourigenic agents (60, 61).

1.2.1.4 Inflammation and Cancer

As early as 1863, Rudolf Virchow, a pathologist, identified the link between inflammation and cancer (62). He noted the presence of leucocytes in neoplastic tissue and hypothesized that the origin of cancer is at the site of chronic inflammation. The use of NSAIDs in chemoprevention in people with familial adenomatous polyposis gene further corroborated the association between inflammation and cancer (63). Chronic infection causing inflammatory conditions results in development of carcinoma (64, 64, 65). Chronic infection by human papilloma virus and hepatitis B virus causes cervical and hepatocellular carcinoma, respectively (66, 67). Chronic irritants such as cigarette smoke, asbestos and silica exposure cause inflammation and consequently lead to cancer (64). Thus, cancer is related with inflammation, histopathologically and epidemiologically, and corroborated by prophylactic effect of anti-inflammatory drugs in cancer (68).

1.2.1.4.1 Glyoxalase System

The glyoxalase system is possibly a common link between inflammation and cancer (69). The glyoxalase enzyme system is responsible for detoxification of reactive aldehydes such as methylglyoxal, which are produced as normal byproducts of the metabolic processes (70). This system includes glyoxalase I and II, which catalyzes the conversion of cytotoxic methylglyoxal into non toxic d-lactate (71). Although an increase in glyoxalase activity is a biomarker of prostate cancer (72), glyoxalase activity was also significantly higher in human colon tumours (73) compared with normal human colon tissue. Curcumin's ability to inhibit glyoxalase implicates this enzyme system as a possible link between its anti-inflammatory and anti-colon cancer effects (69).

1.2.1.4.2 Interleukin 8

Interleukin 8 (IL-8), a member of the proinflammatory chemokine family, is an activator and chemoattractant for neutrophils (74, 75). The role of IL-8 varies in different cancer types.

Most solid tumours constitutively express high levels of IL-8, which is necessary for tumour growth, but endothelial IL-8 causes apoptosis of leukemic cells. In solid tumours, expression of IL-8 is principally regulated by inflammatory signals (TNF-α, IL-1ß) in the tumour microenvironment, hypoxia, acidosis, and nitric oxide (74). The activity of IL-8 is mediated through the G-protein coupled receptor (GPCR), CXCR1/2 (76). Binding of IL-8 to these receptors causes conformational changes in the receptor, which then interacts with various different molecules to initiate a signaling cascade. Some of the pathways influenced by the activation of CXCR1/2 receptors are PI3-K-Akt pathway, ras-raf-MAPK pathway and JAK-Stat pathway. Increased Akt modulates cell survival, migration and angiogenesis in solid tumours. In leukemic cells, the NH2-terminal pentapeptide of endothelial IL-8 causes apoptosis and antitumour effect in vivo (75, 77, 78).

1.2.1.4.3 Cyclooxygenase 2

Cyclooxygenase 2 (COX-2), an enzyme catalyzing the first step in the conversion of arachidonic acid to prostaglandins, prostacyclins and thromboxanes, plays an important role in angiogenesis (79, 80). COX-2 is not usually present in cells but can be induced by inflammatory or mitogenic stimuli, growth factors and cytokines. COX-2 also plays an important role in different stages of cancer including carcinogen activation, tumourigenesis, prevention of apoptosis, and metastasis (81). These roles are believed to be mediated through prostaglandins. Prostaglandins activate secondary messenger in signal transduction pathways to promote the tumour growth. The versatile role played by COX-2 during different stages of cancer makes it a suitable target for anticancer therapy (82).

1.2.1.5 Diagnosis, Prevention and Treatment of Colon Cancer:

Early diagnosis of colorectal cancer is the key to successful treatment. The initial symptoms such as symptomatic anemia, bright red blood in the feces, change in bowel habits, weight loss, anorexia, nausea, vomiting or fatigue, are not specific to colorectal cancer; however, clustering of these symptoms suggests presence of tumour. Patients may present these cancers as a palpable mass but the staging of tumour is done by different imaging techniques such as magnetic resonance imaging (MRI), transrectal ultrasonography (TRUS), positron emission tomography (PET) (83). Family history and genetic screening also helps to identify patients with

high risk of hereditary colorectal cancer such as familial adenomatous polyposis (FAP), hereditary nonnpolyposis colorectal cancer, and MYH associated polyposis (84). Regular screening using sigmoidoscopy and colonoscopy also helps to detect polyps in early stage of cancer. Colonoscopic surveillance is recommended in persons with familial risk, persons undergoing polypectomy, and persons with inflammatory bowel disease and in the (> 50 years) who do not have any family history of colorectal cancer (42, 85, 86). The surveillance and early detection of colon cancer can decrease its high mortality rate. With an increase in our understanding of the molecular mechanisms of colon cancer, genetic screening can help to confirm diagnosis, modify screening strategies, and inform prognosis and the likelihood of an appropriate chemotherapeutic response in patients.

Apart from genetic causes, dietary, lifestyle, disease and environmental factors have been correlated with the prevalence of colon cancer (87). The World Health Organization (WHO) suggests a correlation between red meat intake and prevalence of colon cancer (88, 89). Heterocyclic amines and polycyclic aromatic hydrocarbons formed during high heat cooking are believed to be responsible for this relationship. On the other hand consumption of vegetables and multivitamins are suggested to reduce risk of colon cancer (90). One of the nutrients responsible for reduction of risk is folic acid. Folate is involved in methyl group metabolism and thus may influence methylation of DNA (91-93). The lifestyle factors which influence the occurrence of the disease are physical inactivity and consumption of alcohol and tobacco (88, 94, 95). Exposure to environmental toxicants, radiation, and other occupational hazards also can increase the risk of colorectal cancer (87). Medical conditions such as inflammatory bowel disease (96), cholescystectomy and diabetes mellitus (97) also put the patients at higher risk of colon cancer. Consumption of laxatives increases the risk of colon cancer whereas consumption of non-steroidal anti inflammatory drugs (NSAIDs) reduces the risk of colon cancer (63).

The main treatments of colon cancer are surgery, chemotherapy and radiation therapy (98). Non-metastasized tumours are removed surgically using a variety of techniques (99). Radiation therapy is also useful for colorectal cancers, which are locally advanced or locally recurrent. Radiation therapy is used during surgery (intra operative radiation therapy) as well as adjuvant radiation therapy (100). Chemotherapy, which is a systemic treatment of colon cancer with chemotherapeutic agents, is used either alone or with radiation therapy and surgery (101).

Systemic chemotherapy involves several different drugs ranging from non-targeted

cytotoxic compounds to targeted antibodies to treat metastatic as well as non-metastatic colorectal tumours. The oldest chemotherapeutic agent 5-fluourouracil (5-FU), a fluropyrimidine analog, is the backbone of the chemotherapy of advanced colorectal cancer (102). 5-FU in conjunction with Leucovorin is used as first line therapy to metastatic colorectal cancer (103). Irinotecan, a synthetic analogue of the natural alkaloid camptothecin, is the earliest drug to be added to 5-FU-Leucovorin combination therapy (104). Oxaliplatin, a platinum containing compound, is added to the first and second line therapy of metastatic colorectal cancer along with 5-FU (105). A prodrug of 5-FU, capacitabine, is given orally and simulates the infusion profile of 5-FU and has a favourable toxicity profile (106). Another new class of compounds, epidermal growth factor receptor antibodies, target specific proteins important for tumour cell survival. Epidermal growth factor receptors (EGFR) dimerize and subsequently phosphorylate to activate different signalling pathways including PI3-AKT pathway, Ras-Raf-MapKinase Pathway, and JAK-STAT pathway. The EGFR is a good target for chemotherapy because of its very high expression in colon carcinoma (107). Cetuximab, a monoclonal antibody specific to EGFR, blocks the ligand dependent phosphorylation of these receptors to inhibit the growth of tumours. Several clinical trials suggest the advantage of cetuximab either alone or in combination with fluoropyrimidines or irinotecan in patients with EGFR expression; however, this was not effective against patients with K-ras mutation. Cetuximab exhibited its effect either alone or in combination in patients with wild type K-ras gene (108). Bevacizumab is a monoclonal antibody against VEGF and inhibits angiogenesis in colon cancer (109). Bevacizumab is added in the first line therapy (5-FU/Lecovorin) and second line therapy (5-FU/Irinotecan/Lecovorin) to improve response to therapy. All these chemotherapeutic drugs approved as first or second line treatments have helped to improve the overall survival rate of patients; however, many of these therapies include long-term treatment and exhibit severe adverse effects such as alopecia, fatigue and infertility. While these drugs serve to alleviate the condition of cancer patients, new, efficacious and safe anticancer drugs are required to effectively treat colon cancer.

1.2.2 Anticancer Drug Discovery and Development

The drug discovery process is a high-risk high-reward business. Consequently, it was traditionally the domain of the pharmaceutical industry. Despite its less than 1 percent success

rate, drug discovery and development attracts significant investment due to the enormous financial reward associated with success (7). Drug developers who discover a drug obtain market exclusivity until the patent expires. Usually, a patent expiry time of 20 years provides 8-12 years of market exclusivity to the drug developer, which helps to recover their investment, make profit, and assure brand image (110). Thus, most pharmaceutical companies are actively involved in drug discovery in order to garner the benefits of market exclusivity.

Traditionally, academic institutions and government-funded institutions provided the basic research necessary for drug discovery. However, with the recent emphasis on translational research by major funding agencies such as the National Institutes of Health (NIH) (111), academic institutions have delved into drug discovery processes (11, 112). Academic drug discovery programs usually focus on the unmet human health needs but face numerous challenges in the process (113). The first challenge is the limited financial resources and staff. Academic institutions, which are financially dependent on funding agencies to support their research, have limited funds to manage the enormous cost of drug discovery and development. So far, those academic institutions who have ventured into drug discovery research are involved in pre-clinical candidate identification and selection. After this pre-clinical stages, either they collaborate with industries or out-license their product (114).

The second challenge is to create a balance between academic achievement and business prospects. Academic researchers must vie for rapid publication to assure their academic growth, but business prospects require delayed disclosure to ensure appropriate patent positioning. These conflicting interests need unification if universities wish to consider the potential financial benefits of drug discovery (113, 115). The third challenge is the naïve understanding of the academic community of the drug discovery process. Historically, academic research is hypothesis driven and the non-hypothesis driven drug discovery process often invites disapproving remarks (113, 115). Such attitude reflects the belief that the drug discovery is the realm of pharmaceutical industries and not for academic research. Incorporation of the teaching of drug discovery processes in graduate courses, organization of symposia and external speaker invitations highlighting various aspects of drug discovery processes has helped some major universities (113). Despite these challenges, academia has an important role to play in the resurrection of a slumping drug discovery process by innovative thinking, collaboration with industry and nonprofit organizations, and preparing a new generation of researchers for

translational research (116).

1.2.2.1 Drug Discovery Process

Drug discovery and development is a complex, costly, low throughput and time consuming process. Development of a new drug takes about 8-10 years and more than 800 million USD (117, 118). The compounds have to undergo different stages of assessment ranging from pre-clinical efficacy and safety to clinical efficacy and safety studies. At every stage of the drug discovery process only approximately 1% success rates are observed. In 2001, the year with lowest success rate, a research and development cost of $26.4 billion resulted in the approval of only 9 NCE's as drugs (119). Various novel approaches in rational drug design have helped to expedite the drug discovery process but efficiency is still a major concern for pharmaceutical industry.

The drug discovery and development process involves different stages beginning with target identification and ending with regulatory approval (Figure 1.2) (120, 121). In the first stage, medical necessity and suitable targets for the medical conditions are identified and validated (122). Alternatively, a validated target is selected and chemicals are designed against those targets. These chemicals undergo a variety of *in vitro* and *in vivo* efficacy and safety screening assays during the pre-clinical stage (123, 124). The promising candidates are tested for formulation, scale-up synthesis and long term safety in animals. Subsequently, an investigational new drug application (INDA) is filed to the Food and Drug Administration (FDA). Upon approval, clinical testing of the candidate is performed in Phase I, Phase II and Phase III studies to assess safety in healthy volunteers, efficacy in small number of patients, and efficacy in large number of patients, respectively. The company then files a new drug application (NDA) to the FDA for approval. The FDA reviews the application with all the clinical and pre-clinical test data and either rejects or approves the application. The successful application allows the company to sell the drug in the market.

The drug discovery process has moved from random compound synthesis to the era of rational drug design (125). The first step in rational drug design is target identification and validation. Completion of the human genome project has provided numerous potential gene targets (126) but many have unknown functions and are not useful as drug targets unless validated. The various strategies employed in validating a target are simulation of interactions on

a computer-generated model, sense reversal in the gene of interest to understand the physiological role, manipulation of proteins, and assessment of its role in animal models by knockout animals or by introducing an inhibitor in humans in case a suitable animal model is not available (127).

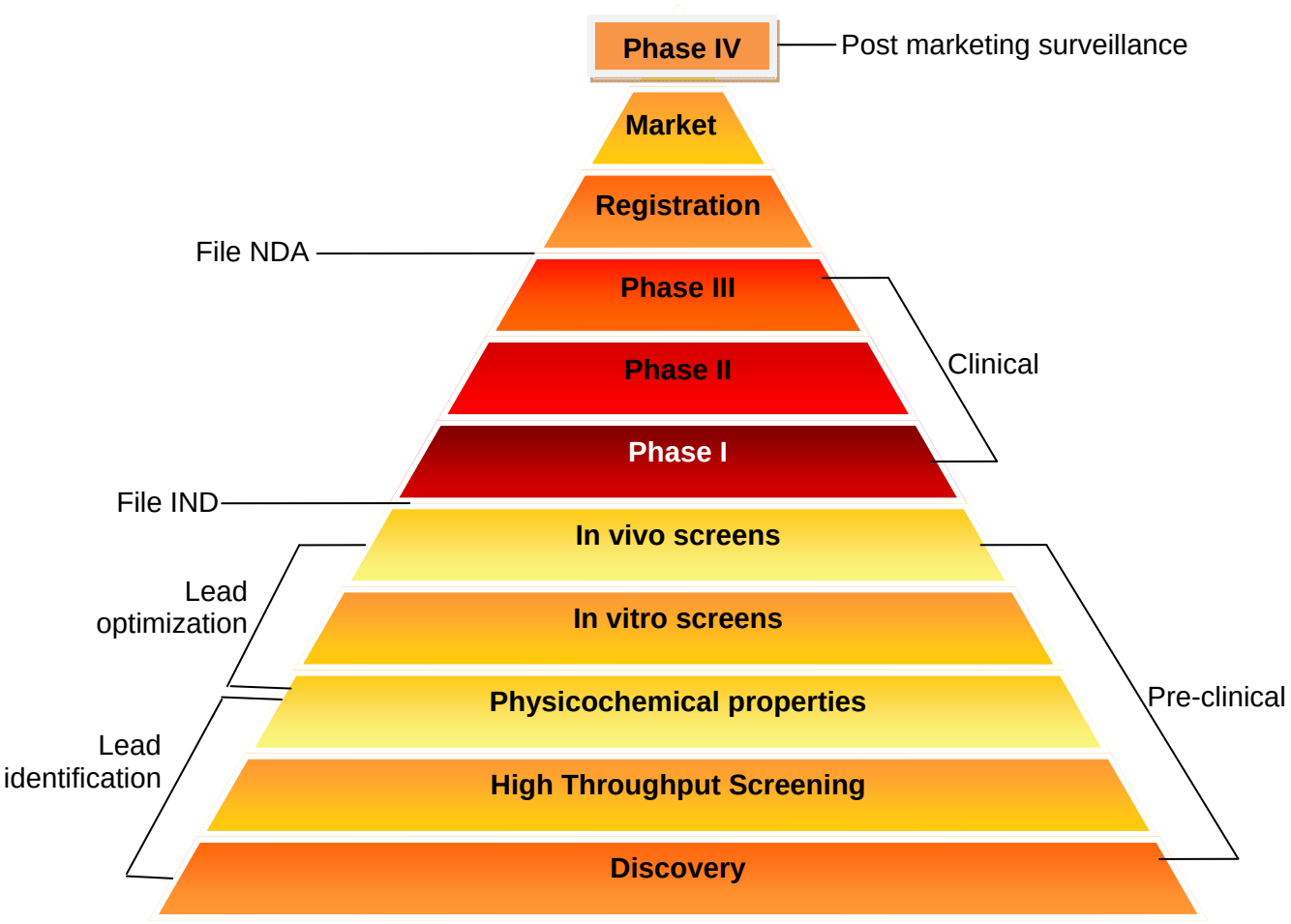

Figure 1.2: Drug discovery and development processes (adapted from C. Gunaratna, *Current Separations*, 19:1, p17, 2000). See text for explanation.

The second step is "hit" identification, which is defined as selection of compounds with suboptimal activity. Hits are identified by screening a large number of compounds through high throughput screening (HTS), nuclear magnetic resonance (NMR) fragment screening (fragment based drug design). The subsequent step to hit identification is "hit to lead" where hits are

modified to obtain optimum PK and PD properties (128). During this phase, “hits” are refined to obtain “leads”, which are again optimized before regulatory preclinical studies are conducted. This “hit” identification and “lead” optimization processes uses a variety of high throughput techniques including solution phase parallel synthesis, combinatorial chemistry, microwave assisted organic synthesis, mass directed preparative HPLC, high throughput biological assays, high-throughput pharmacokinetic and metabolic assays (129). Results of these high throughput biological assays help in the design of a new library of compounds. This closed loop work flow of lead optimization continues during the drug discovery and development processes. During this step only initial investigations on the modes of action are carried out.

After lead optimization, the regulatory pre-clinical and clinical studies are conducted. Regulatory pre-clinical and clinical studies assess the safety, efficacy and pharmacokinetics of the compound in animals and humans, respectively (119). General toxicity, genotoxicity, carcinogenicity are determined in animals along with pharmacokinetics during pre-clinical studies. Regulatory pre-clinical studies are followed by Phase I clinical study, which evaluates the safety of compound in healthy human volunteers. Subsequently, Phase II study evaluates the efficacy of compound in patients for the first time. Phase III study, which follows Phase II, evaluates the efficacy and safety in large number of patients in randomized multi-centric trials. After Phase III, the company files new drug application (NDA) to FDA for approval to launch into the market. In final phase of drug development, Phase IV, the company collects the safety and efficacy data from the market.

The drug discovery and development process is becoming increasingly more costly and time-consuming. In 1979, the total capitalized cost of development per new drug was 138 million, which increased from 318 to 802 million US dollars in 1991 and 2002, respectively (117, 118). The current cost of development ranges from 800-1200 million dollars per drug (117). The enormous cost of R&D in pharmaceutical company laid the foundation for strategies to eliminate unpromising compounds early in the drug discovery pipeline. The cost of failure adds to the cost of new drugs. Collaboration with other companies, academics and clinical research organizations are among various strategies to decrease the cost of research (130).

Identification of causes of failure in different stages helps pharmaceutical industries to develop strategies for improvement. In 1991 (131), poor PK characteristics were the major reason for compound attrition, but rational approaches in drug design have overcome this. By the

year 2000, the major reason for failure was poor efficacy and significant toxicity (7). Consequently, toxicological screening has become a major part of the drug screening processes today (132).

1.2.2.2 Challenges in the Development of New Cancer Chemotherapeutic Agents

1.2.2.2.1 Selection of Targeted Vs Non-Targeted Approach

Targeted versus non-targeted chemotherapy is still heavily debated but in recent times chemotherapeutic drug discovery has moved from a non-targeted to targeted approach (10). Until recently, cytotoxic drugs dominated chemotherapy in cancer patients. Cytotoxic drugs tend to have a low therapeutic index and exhibit severe adverse reactions. These cytotoxic drugs inhibit the growth of rapidly dividing cells, showing selectivity to cancerous cells over normal cells. The advent of imatinib, a drug specific to activated Abelson kinase in chronic myeloid leukemia, transformed drug discovery from non-targeted cytotoxic drugs to the development of targeted chemotherapeutics (133). Abelson kinase has limited requirement in normal cells while leukemic cells are dependent on Abelson kinase, and thus this drug is well tolerated (134). These targeted chemotherapeutics have high effectiveness, high therapeutic windows, and less severe adverse reactions as compared with cytotoxic drugs (135); however, development of tumor resistance against various targeted molecules has sparked the debate about long-term therapeutic uses (136, 137). Solid tumours are much tougher to treat than leukemia, which is now curable with imatinib (138). Solid tumours carry several mutations and are genetically unstable; they quickly adopt alternate pathways for survival upon inhibition of an important cellular pathway. Most targets are an intermediate protein in the signaling cascade. Modulation of upstream or downstream proteins may render targeted therapies ineffective; however, combination therapy with non-targeted cytotoxic drugs improves the efficacy of targeted chemotherapeutics (139, 140).

Selection of appropriate target for targeted new chemothereaputic discovery and development is a major challenge due to variety of reasons. First, most of the targets are non-validated and require rigorous target validation. Poor target validation could a major cause of drug failure in drug discovery and development (141). Consequently, drug discovery researchers focus on a very small number of targets leaving many possible viable targets untouched. For example, the human kinome, i.e. the complete set of human kinases (142), is composed of 518 enzymes, but more than 300 enzymes have received no attention (143, 144). Second, over

expression of targets in cancer cells do not necessarily correlate with treatment outcomes. This lack of correlation may be due to changes in upstream or downstream proteins in the signaling cascade, dependence of cancer cells to alternate pathways for survival, and selection of the appropriate patient population (145). Third, fresh targets for cancer chemotherapeutic drug development are increasing due to technological advances (1). The companies investing in cancer research must make prudent decisions as to which targets to choose for drug design projects. Companies want to be first to launch a drug against a fresh target for financial benefit but run a high risk of failure. Fourth, the discovery of biomarkers to allow selection of the appropriate patient population is very vital. Often it is not possible to identify a correct biomarker. Cetuximab, an EGFR antibody, did not benefit many patients with high EGFR expression. Later it was identified that cetuximab is effective in only patients with wild type K-ras (108).

1.2.2.2.2 Models of Preclinical Screening

The models used in pre-clinical efficacy studies such as cultured human tumour cell lines and subcutaneously xenografted tumours in immune-compromised mice often fail to predict treatment outcomes of chemotherapy in patients (10, 146). Several reasons may explain this lack of correlation. First, the characteristics of cultured tumour cell lines change with continued passaging and thus may not represent the true nature of the tumour. Second, cell culture systems lack the architectural and cellular complexity of a tumour, such as inflammatory cells, vascular and stromal components. As well, the murine stroma of xenografted tumours behaves differently than human stroma (147). Finally, tumours take years to grow at their original site. During these years of growth they accumulate genetic and epigenetic changes and exhibit invasion and metastasis characteristics. In contrast, subcutaneously xenografted tumuors grow very quickly and possibly carry only a fraction of entire genetic and epigenetic heterogeneity of tumours in the human patient (148).

The failure of pre-clinical models to predict clinical outcome also depends upon the interpretation of results (146). Often, incongruity exists in comparison of pre-clinical models and the target patient population. Pre-clinical models that mimic the target patient population often improve the prediction of clinical outcome (147). For example, melphalan demonstrated significant activity against rhabdosarcoma pre-clinical models but exhibited only marginal

activity in patients that failed standard chemotherapy. However, when dosed to untreated patients with very advanced disease at diagnosis, melphalan exhibited significant activity. This suggests that comparison of pre-clinical models which mimic patients will improve the prediction. Another concern is the metrics chosen for comparisons. The criteria used to consider a drug as active is less stringent in pre-clinical assessments than clinical trials. For example, 58% inhibition of tumour growth in a xenograft model is considered an effective treatment whereas in human clinical patients this is not considered as effective (147). Understanding the outcome without taking the comparative drug systemic exposure into account is misleading. Many compounds are inactive in the clinic because their comparative systemic exposure at the maximum tolerated dose is much lower than levels found in mice. Sometimes, comparative systemic exposure of a compound is not attainable in the clinic and can be easily predicted to fail in clinical trials. So, integration of pharmacokinetic knowledge in interpretation of pre-clinical results is very important to predict outcomes in human clinical trials.

Despite the change in course of anticancer drug discovery from non-targeted cytotoxic drugs to targeted drug molecules, we have continued to use the same models for cytotoxic drug screening (127, 149, 150). In some cases, such as imatinib against chronic myeloid leukemia, these screening models have been very successful; however, the use of such models adds to the poor predictability of uncharacterised tumour xenograft models. Recent advancements in knock-out gene models, which are biochemically more characterized, have witnessed an increase in their use; however, the predictability of these models still requires validation (147).

1.2.2.2.3 Regulatory Approval

All drugs need approval by regulatory agencies before being marketed in the respective countries. Regulatory agencies have unclear efficacy and safety criteria for anticancer drugs, which pose a major challenge for drug developers (9). Unlike other drug classes, regulatory agencies have no strict defined criteria for benefit over risk ratio for the drug developers to follow. More so, regulatory agencies lack harmonization over benefit to risk ratio assessment. Understandably, safety and efficacy hurdles are the greatest when the drug is designed to target early stages of cancer where the chance of successful chemotherapy is the highest. On the other hand, the safety and efficacy concerns are lowest for drugs used in heavily treated, refractory patients, but the chance of success with chemotherapy is the lowest with these patients. To gain

easy regulatory approvals, drug developers target late stage, heavily treated recurrent cancer first and then work towards early stage cancers. Targeting recurrent cancers significantly decreases the chance of successful therapy.

1.2.2.2.4 Stratified/Personalized Medicine

The "one drug fits all" is not a suitable approach for use in a heterogeneous cancer population; however, individualized therapy requires biochemical mapping of patients to identify the correct therapeutic regimen (151, 152). The use of biomarkers, "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacological responses to a therapeutic intervention (153)", is a promising prospect in biochemical mapping of patients for individualized therapeutic regimen design. Identification of the link between tamoxifen with estrogen receptor status in breast cancer patients improved chemotherapy outcomes. Subsequently, trastuzumab's link to ERBB2 (also called HER2) receptors and imatinib's link to BCR-ABL revolutionized the idea of individualized therapy using biomarkers (154). Additionally, biomarker studies may help in selecting patient-specific dose and dosing schedules. For example, biomarker studies in patients on bortezomib treatment helped to correctly identify dose and dosing schedule for sustained target inhibition, which otherwise had recovered between doses in early clinical studies.

The use of biomarkers in early clinical trials is expected to decrease drug attrition rates, decrease costs and improve clinical outcomes (8, 155). Patients who are not likely to benefit from treatments can be eliminated from trials to prevent unwanted toxicity by decreasing heterogeneity in the patient population (139). Decrease in unwanted toxicity will subsequently lead to a decrease in cost and time of approval. Recent paradigm shift in anticancer drug discovery towards development of targeted anticancer drug development needs new methods to identify optimal dosing schedules in trials because the toxicity profile of targeted drugs are different from cytotoxic drugs. The study on modulation of biomarkers can help in identification of dosing schedules. Additionally, biomarker-based clinical trials may help to identify drug effects on different organ tumours (156).

Despite promising prospects and initial successes with biomarkers, critics still see a limited utility due to variety of reasons (140). The main reason for such apprehension is based on the very few instances that have identified a clear link between clinical outcome and the biomarker.

Many targeted chemotherapeutics have been approved in the market but biomarkers are available only for a few of these as the clinical outcome of most of the targeted chemotherapeutics failed to exhibit a significant link with biomarkers. Cetuximab's failure to link with EGFR expression level is an example of the failure of association with biomarkers; however, it was later identified that the KRAS mutation correlated better than EGFR expression in tumours. Thus, a rigourous validation of the target is required to avoid inappropriate patient treatment, erroneous re-imbursement decisions and setting an erroneous direction to innovation (122, 141). An analysis of phase I clinical trials over a 12 year period suggests a rare impact of biomarkers on decision making, selection of dose and dosing schedule. Another major factor in failure of biomarkers is the lack of validated, accurate and reproducible analytical methods.

1.2.2.2.5 Price of the Drugs

The exorbitantly high increase in anticancer drug prices is a concern to patients, clinicians and medical care providers. The enormous cost raises debate over the rationalization of cost over efficacy in the literature and the media. In an analysis of drugs approved in Europe during 1995-2000, the newer chemotherapeutics do not offer substantial clinical advantage but cost is several times higher than the existing drugs (157). A new anticancer drug, Fotolyn (Pralatrexate) costs about 30,000 USD a month, which has only a modest increase in patient survival rates (158). Even clinicians feel that it might be unethical to treat a patient with a drug, which gives only modest benefit with exorbitant expense.

Exorbitant prices with only modest advantage force the health reimbursement policy-making agencies to be selective in their re-imbursement policies (159). The National Institute for Health and Clinical Excellence (NICE) and Scottish Medicines Consortium (SMC) approve 39% and 43% of drugs for medical coverage. These decisions deter clinicians and patients from usage of drugs due to low benefit over cost ratio. Drug developers strategize to target recurrent and heavily treated patients because the regulatory hurdles of safety and efficacy is low. Once approved they work towards the early stage conditions where safety and efficacy hurdles are high. However, targeting recurrent condition leaves the drug with modest advantage in treatment, which is taken as reason by these reimbursement policy-making agencies to not reimburse the prescription cost (9).

The reasons behind high cost with modest benefit of new anticancer agents are the

enormous investment in drug development, high attrition rates and stringent requirements of regulatory agencies (9). Firstly, anticancer drug development programs have the highest attrition rates than any other class of compounds. High attrition comes as an economical burden to successful drug development, which eventually increases cost to allow for some benefit to the drug developers (7). Secondly, the slow regulatory audit and approval process increase the time of drug development processes (160). Longer drug development processes decrease the market exclusivity period to the drug developers. This forces drug developers to recover expenses within a shorter period of time, which again adds to the increase in price. Lastly, approved and marketed drugs are still at risk of post-marketing withdrawal, which adds to the cost to drug developers. All these reasons force the drug development companies to charge high prices for the drugs.

Pharmaceutical companies continuously need to change their drug development strategies to counter these challenges (161). The possible solution to these issues are networking with academia and smaller industries to cut costs, working together with regulatory agencies during drug discovery, and signing agreements with public health service providers (130, 161, 162). Emphasis on the identification of biomarkers early in drug discovery and rigorous validation would help to decrease cost and expedite the approval processes. Validation of target for its druggability should be ascertained before venturing into drug development.

1.2.2.3 Possible Solutions to the Challenges in Anticancer Drug Discovery

Networking and collaboration is a proposed solution to increase the efficiency of drug discovery processes. Kiatin proposes a new fully integrated pharmaceutical network (FIPNet) model for R&D where a consortium of large pharmaceutical companies, small pharmaceutical companies, clinical research organizations and academia deconstruct the drug discovery processes for improved outcomes (161). The collaboration brings together the core competencies of all the major role players to improve efficiency in the drug discovery and development process. In this model, academia provides basic research, which lays the foundation of research, and translational medicine, which translates basic research into applied research and commercialization. Small pharmaceutical companies and clinical research organizations are highly motivated, have higher functional efficiency and low overhead costs to alleviate the cost of drug discovery. In this model, small pharmaceutical companies have specialized focus and

deliver the specialized objectives of innovative therapeutics or emerging technologies whereas clinical research organizations execute, monitor and analyze clinical trial results. Large scale pharmaceutical companies co-ordinate and manage overall processes in this model. The decentralization of R&D process eliminates the bias in the selection of appropriate candidates for the development to meet the productivity requirements of the companies. Companies should collaborate with experts from outside the company, maybe from academia or small scale industry, to select the medical needs or targets. Innovation should be imbibed from all sources (162).

Selection of validated biomarkers may decrease the risk of failure in clinical trials as validated biomarkers help identify the appropriate target population before initiation of clinical trials (122, 141). Furthermore, since single agents against a single target usually results in suboptimal treatment and development of resistance, collaboration with companies to facilitate combination therapies would also help to reduce the cost of clinical trials (130). Collaboration with diagnostic companies and regulatory agencies (maybe different approval agencies for diagnostics) should be made to develop companion diagnostic tests in parallel to facilitate the use of drugs in the clinic. Collaboration between federally funded institutes such as NIH and regulatory agencies to provide the knowledge needed to regulatory agencies and expedite the approval process. Lastly, collaboration between health service providers with drug developers ensures the coverage of prescription drug costs, which increases the clinical use of the drug. An example of such collaboration is an agreement between NICE and Janssen Cilag's agreement on response-rebate scheme (163). In this scheme, patients showing partial or full response to the bortezomib after first relapse will be funded by the national health service provider whereas the company will reimburse patients with no or minimal response after the treatment. Such incentives for the companies will motivate them to discover new biomarkers to identify correctly the target population.

1.2.3 ADMET in Drug Discovery and Development

Drugs cross several physiological barriers to the site of action to elicit their effect. These physiological barriers evolved to protect the body from external toxic substances (164). An understanding of physiological processes at these barriers can help in selection of the dosing regimen to maintain the drug concentration within the therapeutic window for an effective

therapy (165). Pharmacokinetics also helps to correlate dose with pharmacological response, to evaluate clinical drug interactions, to understand the effects of pathological conditions on these pharmacokinetic processes and dosage adjustments in pathological conditions for individual patients, if necessary.

1.2.3.1 Role in Drug Discovery and Development

Until two decades ago, pharmacokinetics received limited attention in drug discovery. An analysis of reasons of failure of molecules in the drug discovery and development pipeline showed that most compounds failed in the clinic due to pharmacokinetic problems (131). Since then, pharmaceutical companies have incorporated pharmacokinetic screening early in the drug discovery pipeline. Early identification and elimination of compounds with pharmacokinetic issues reduces the cost of drug discovery. As a result, the number of failures due to pharmacokinetic issues decreased in ensuing years (7).

Today, pharmacokinetic studies form an integral part in drug discovery (166). Pharmacokinetic studies help to understand the pharmacological response and select optimum dosing regimens for therapeutic efficacy screening. Similarly, pharmacokinetic studies correlate toxicological outcomes and clinical outcomes with systemic drug concentrations and systemic exposure.

1.2.3.2 Pharmacokinetic Screening in Pre-Clinical Study

Drug developers employ a cassette of pre-clinical pharmacokinetic assays at different stages of drug discovery (167). Because large numbers of compounds are screened during early stages of drug discovery, the assays employed should be high throughput and require small quantity of test compounds (124, 128). These assays aim to categorize the compounds from high to low priority compounds. As compounds move into later stages of drug discovery throughput decreases but PK studies at this stage provide more information and confidence than earlier high throughput screening techniques. Although the set of screening assays employed varies among companies the assays can be generalized as follows: During the “Hits” stage, high throughput screening assays are employed to generate pharmacokinetic data on a large number of compounds (168). Such pharmacokinetic data helps to correlate molecular structure with predicted pharmacokinetic properties of the compounds. Some of the properties calculated from

molecular structures *in silico* include lipophilicity, ionization, solubility, ADME profile and oral bioavailability using different predictive techniques such as QSAR, 3D-QSAR, linear and non-linear regression techniques etc (169, 170). Many software packages are available commercially for ADME prediction such as TIMES, METEOR, META, Metabol Expert, ADMET predictor, SimCYP, and M-CASE. Predicted ADME properties help in prioritization of compounds for the next stage of drug optimization.

In the drug optimization stage, compounds with a high score in predictive software are screened against various *in vitro* pharmacokinetic screening tools. Solubility, permeability across cell Caco-2 and MDCK monolayers or PAMPA, metabolic stability using the S9 fraction, microsomes and hepatocytes, P450 enzyme induction and inhibition studies, and rapid *in vivo* screening are conducted at this stage (171). These screening assays are more costly and time consuming than *in silico* prediction; however, several of these assays are automated to increase assay throughput. Fewer numbers of compounds are screened at this stage of discovery than the "Hits" stage. In the final selection stage, protein binding, tissue distribution, metabolite profiling and in vivo PK and PD are conducted (172, 173).

1.2.3.3 Tools to Assess In Vitro Pharmacokinetic Studies

Drug developers use several *in vitro* pharmacokinetic studies to select drugs with optimum pharmacokinetic characteristics and to understand efficacy studies in animals or humans. Several *in vitro* tools are available to assess different pharmacokinetic processes such as absorption (174), distribution and elimination (metabolism and excretion) (124). Some of the commonly used tools used in pharmacokinetic assessment are discussed.

1.2.3.3.1 Absorption

Most designed drugs are intended for oral administration due to ease of administration and patient compliance; however, orally administered drugs must cross the intestinal barrier before reaching their site of action. The intestinal epithelium is a polarized epithelium with structurally and functionally distinct apical and basal surfaces due to the presence of tight junctions (175). Tight junctions formed by the membrane proteins block the free movement of proteins from apical and basal surfaces.

Compounds cross the polarized epithelium via the paracellular or transcellular route.

Paracellular transport refers to the movement of compound across the polarized epithelium through tight junctions whereas transcellular transport refers to the movement of compound across the polarized epithelium through the membranes (176). Paracellular transport is slower than the transcellular route due to much lower available surface area than the area available for transcellular transport. High molecular weight compounds are less likely to undergo paracellular transport due to the small size of the pores (177, 178). Compounds undergoing transcellular transport should possess the characteristics to dissolve in the gastrointestinal media (aqueous in nature) and traverse through the lipid bilayer (lipid in nature). Thus, compounds must have balanced hydrophilic and lipophilic characteristics. Permeation via the transcellular route may involve passive diffusion or carrier-mediated processes. Carrier-mediated transport may be classified into facilitated diffusion or active transport depending upon the direction of transport, concentration gradient and energy requirement. Facilitated diffusion is the movement of drug in the direction of concentration gradient and does not require energy; however, active transport may happen against a concentration gradient and requires energy. A compound may be transported by any of these mechanisms or by more than one mechanism.

1.2.3.3.1.1 Caco-2 and MDCK Cell Monolayers

The most commonly used tools to assess the *in vitro* permeability of compounds are Caco-2 and MDCK cell lines (174, 179-181). Colon adenocarcinoma cell (Caco-2) line is derived from human colon whereas Madin Darby Canine Kidney (MDCK) cell line is derived from the dog. These cell lines are widely used to assess the permeability of compounds across polarized epithelium barriers to understand human intestinal absorption. Similar to the small intestine, these cell lines develop microvilli structure on the apical side of the monolayer upon differentiation in a suitable media. The Caco-2 is the most extensively characterized model and considered a “gold standard” assay for permeability assessment due to its structural and functional similarity with small intestine. Not only is Caco-2 structurally similar to the small intestine due to the presence of brush border on its apical side, Caco-2 expresses a number of metabolic enzymes, uptake and efflux transporters present in enterocytes. This model often provides excellent correlation with human absorption data (182-184).

Caco-2 cells express several transporters, which facilitate transport of various drugs and nutrients at its basolateral and apical membranes (185). The transporters responsible for uptake

of drugs or nutrients into the cell present at the apical membrane in Caco-2 cells include organic cation/carnitine transporter (OCTN2) (186), organic anion-transporting polypeptide 2B1 (OATP-B) (187){{}}, H+/di-tripeptide transporter (PEPT1) (188), monocarboxylic acid transporter (MCT-1) (189) and apical sodium dependent bile acid transporter (ASBT) (190, 191). Efflux transporters at the apical membrane, which are responsible for the removal of drug or nutrients into the intestine, include P-glycoprotein (P-gp) (192), multidrug resistance associated protein 2 (MRP2) (193) and the breast cancer resistance protein (BCRP) (194). Efflux transporters at the basolateral membrane of Caco-2 cells, which transport/remove drug or nutrients into the blood stream, are not completely characterized; however, mRNA expression of multidrug resistance associated proteins (MRP2, MRP4 and MRP6) and organic solute transporters (OSTα-OSTβ) has been reported (195-198). Additionally, Caco-2 expresses nuclear receptors, which bind with DNA and regulate the expression of other genes upon interaction with ligands, including farnesoid X receptor, peroxisome proliferator activated receptor (PPAR), vitamin D receptor, aryl hydrocarbon receptor (Ahr), small heterodimer partner (SHP) and liver receptor homologue-1(LRH-1) (199-204).

Caco-2 expresses phase I and phase II metabolic enzymes. Some of the major metabolic enzymes include cytochrome CYP1A, UDP-glucuronosyl transferase (UGTs) (184), sulfotransferases (SULTs) (183) and glutathione S-transferase (GSTs) (205). Unfortunately, the most important P450 isoform, CYP3A4, is not expressed in Caco-2; however, cell lines transfected with CYP3A4 have been developed to study the interplay between metabolic enzymes and transporters.

Despite the expression of transporters and metabolic enzymes in Caco-2 that mimic the small intestine, its extensive time period for differentiation (21 days) is a disadvantage. MDCK is used for preliminary high throughput screening of compounds due to its shorter culture period (3 days) and good correlation with human absorption values for passively absorbed drugs (181). A shorter culture period decreases the cost and potential for contamination but lack of transporter expression limits its utility for compounds that undergo carrier-mediated transport. Additionally, the MDCK cell line does not constitutively express the complement of metabolic enzymes expressed in the intestine. However, the cell line has been transfected with genes encoding for various transporters and metabolic enzymes (206-208). These transfected MDCK cell lines are widely used as a tool to study transport mechanisms and metabolic pathways due to their ability

to differentiate in a shorter time period.

Among various models to assess intestinal absorption, cell monolayers are more widely used due to their ease of experimentation, cost effectiveness, high degree of *in vitro-in vivo* correlation (IVIVC), and presence of all mechanisms of transport.

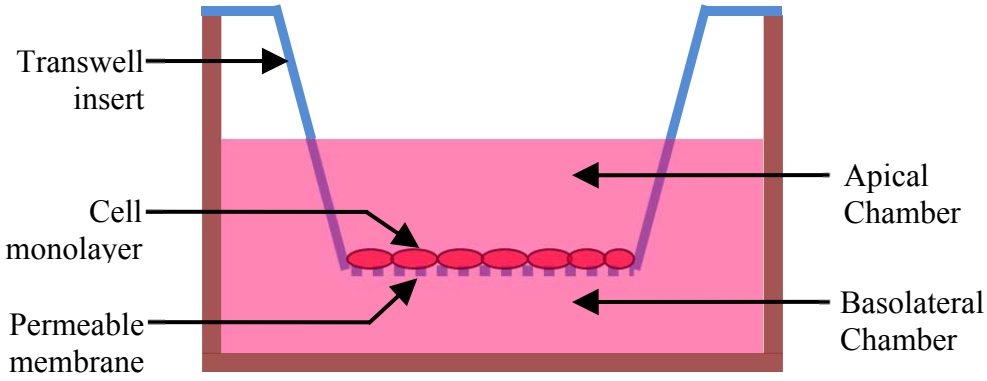

Figure 1.3: Schematic diagram of Transwell® used in the permeability assessment of compounds using Caco-2 and MDCK cell monolayer

In these models, cells seeded on the porous membrane in a Transwell system grow to form a polarized epithelial monolayer (Figure 1.3) (174, 209,210). Cells growing on the membrane physically separate into apical and basal compartments. Compounds are added to the donor compartment (either apical or basal depending upon the purpose of the experiment) and the concentration of compound in the acceptor compartment is determined to calculate an apparent permeability co-efficient using equation 2.1.

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{C_0} \times \frac{1}{A_f} \qquad \text{Equation 2.1}$$

Where dQ/dt is the permeability rate, C_0 is the initial concentration of the donor compartment, and A_f is the surface area of the monolayer (filter).

Maintenance of tight junctions in these cell lines is an important consideration for correct estimation of the apparent permeability coefficient. The formation of tight junctions is measured using transepithelial electrical resistance ($\Omega.cm^2$). Alternatively, the integrity of the tight junction

is assessed using paracellular markers such as mannitol, inulin, dextran, PEG 4000 and Lucifer yellow (185). These markers permeate only via paracellular transport and low permeability suggests integrity of cell monolayers.

1.2.3.3.1.2 PAMPA

Since most drugs passively permeate across membranes, the parallel artificial membrane assay (PAMPA) allows for the rapid evaluation of passive permeability and has received much attention by pharmaceutical companies as a primary permeability screening tool (174, 211). As the name suggests, this technique uses ingredients present in biological membranes to prepare an artificial membrane to mimic the biological membrane. Since its inception in 1998 several modifications of this technique (212-214) have been developed to improve quality and prediction potential including dioleyoylphosphatidylcholine (DOPC-PAMPA), n-hexadecane (HDM-PAMPA), biomimetic (BM-PAMPA), blood-brain barrier (BBB-PAMPA) and double sink (DS-PAMPA). Double sink PAMPA first creates sink conditions by ion entrapment due to a differential pH between donor and acceptor compartments and a second sink condition by the addition of chemical scavengers such as surfactants in the acceptor compartment. Double sink PAMPA has the best permeability prediction among the different variants of the technique and is recommended for the most applications (215). Due to the spectacular rise in the popularity of PAMPA within a decade, Lipinski speculated that this technique might replace cell monolayer based assays; however, the use of cell monolayers and PAMPA in a mutually supportive role is the best use for screening in drug discovery (212).

1.2.3.3.2 Distribution

The extent and ability of a drug to distribute to its site of action is a key determinant of drug efficacy. Consequently, an evaluation of the distribution characteristics of a compound becomes important during drug discovery and development. Previously, drug distribution was determined only in pre-clinical animals and *in vitro* assays. Recent advancements in microdialysis techniques, however, have improved our understanding of drug distribution in humans.

In the biological system, an equilibrium exists between the unbound drug and drug bound reversibly to plasma and tissue components (e.g. proteins). Only the unbound form of a drug is

capable of crossing polarized epithelial and membrane barriers and exhibit pharmacological action. In vitro assessments of plasma protein binding using equilibrium dialysis or ultrafiltration (216) provide important information regarding the fraction of a dose freely available to cross membranes and interact with the compound's site of action. Microdialysis is a technique that allows determination *in vivo* of unbound concentration of drug in plasma, tissue and other biological fluids (217). Equilibrium dialysis uses a semipermeable membrane to dialyse plasma.

The determination of plasma protein binding of drug is important during the drug discovery. Theoretically, drugs with high plasma protein binding require higher dose to achieve the same level of unbound systemic concentration. Since only unbound fraction of drug is responsible for the pharmacological effect, generally, the compounds with high plasma protein binding are not desirable during the drug discovery. However, in some cases the high plasma protein binding may have no clinical significance or may be valuable. (218-220). Rifapentine (>97 % plasma protein bound) (221) and acenocoumarol (>98% plasma protein bound) (222), despite being highly bound, are effective at relatively low doses. The high plasma protein binding of warfarin confers advantage over other anticoagulants. . The plasma protein bound warfarin (> 99 % plasma protein bound) acts as a depot and increases the half-life (20-60 h) (223). The longer half-life is an advantage over other anticoagulant such as heparin, which has half life of 1.5 h (220, 224).

The magnitude of apparent volume of distribution provides a measure of the extent of tissue distribution. However, this pharmacokinetic parameter does not provide organ specific drug distribution (165). Drug concentration in the biophase governs the pharmacodynamic effect; however, the measurement of drug concentration in biophase is often impractical (225). An understanding of organ specific distribution of drugs and their correlation with efficacy or toxicity could help decide the fate of a compound in drug discovery and development. Such information can be arrived at indirectly by determination of the ratio of fraction unbound in plasma and tissue, the, physicochemical characteristics of the drug, organ architechture, and the interplay with transporters (226). For example, lipophilicity is directly correlated with brain penetration (227); however Pgp expression at the blood brain barrier is responsible for poor permeation of some highly lipophilic drugs (228). Poor brain penetration of the Merck compound, L-365260, is attributed to the presence of Pgp at blood brain barrier. Inhibition of Pgp with quinine or verapamil increased the BBB penetration by 3- to 5-fold in rats (171, 229).

1.2.3.3.3 Metabolism

Most drugs undergo enzymatic transformation into more polar metabolites in the liver or other organs and subsequently excreted in the urine or bile. The knowledge of metabolic susceptibility of a compound and its role as substrate, inducer or inhibitor of metabolic enzymes helps to predict its potential for poor oral bioavailability, high clearance, toxicity, drug-drug interactions, size of dose and dosing frequency (230).

Compounds with low bioavailability and high clearance are usually not desired during the drug development, and therefore, the compounds are usually structurally modified for high bioavailability and low clearance during drug discovery (231). Given the strategic location of liver between intestine and systemic circulation, most of the orally absorbed drugs are exposed to liver enzymes. Metabolic lability of compound in liver fractions (S9 fraction, microsomes, cytosol) or hepatocyte (232) provides information on the rate and extent of metabolism and predicts the in vivo hepatic clearance and oral bioavailability. Metabolic lability data are used as a filter in the selection processes and in the prospective design of molecules in early lead identification or hit finding stages (233). An understanding of metabolically labile groups helps chemists to manipulate the structure to meet optimum clearance and oral bioavailability. Currently, high-throughput is employed to determine the metabolic lability of compounds in the very early stages of drug discovery (234, 235).

The microsomal system is the most widely used model to determine metabolic lability because of its ease of preparation, long-term storage, and high reproducibility. Microsomes also contain P450 and UGT enzymes, which are metabolic enzymes responsible for the metabolism of most drugs, but lack most of the Phase II metabolic enzymes. Hepatocytes contain all the phase I and phase II metabolic enzymes and provide better prediction of in vivo hepatic clearance over microsomes (236); however, the prediction based on microsomal data is usually reasonable and does not impact the decision significantly (237). Based on the microsomal prediction, about 64% of the 1163 compounds from GlaxoSmithKline were correctly classified as high or low in vivo clearance (237). Due to the ease in experimentation and reasonably good prediction, the microsomes are favoured in the preliminary screening of metabolic lability.

The metabolic fate of the drugs may influence their efficacy and toxicity. The drug may get converted into active, inactive, toxic and reversible metabolites, which may influence the

efficacy and toxicity of the drug (120). While formation of inactive metabolite leads to the loss of activity, active metabolite formation may lead to altered activity and toxicity. Formation of toxic metabolites mediates toxic effects and formation of reversible metabolites is likely to prolong the activity (120).

The knowledge about the nature of metabolites helps to design the various pharmacokinetic, efficacy and safety studies. Selection of appropriate species is an important criterion in the design of toxicity studies. The extents and pathways of metabolism vary widely among different pre-clinical species for many drugs, which may influence the efficacy and toxicity of drugs. For example losartan, a nonpeptide angiotensin II receptor antagonist shows species dependent metabolism. While the oxidative metabolism is the primary route of metabolism in rats, glucuronidation predominates in monkeys; however, both metabolic pathways have equal contribution in human (238). An oxytocin receptor antagonist from Merck, L-368899, is metabolized very rapidly in monkey than rat or dog (239). These differences in metabolism among different preclinical animals make inter-species extrapolation of toxicity more difficult. Acetaminophen exhibits hepatotoxicity in mice but does not show significant hepatotoxicity in rats. These species difference is attributed to formation of reactive toxic metabolite formed and GSH content in the liver. (240-243). Therefore, the in vitro metabolic profile of drugs in different species should be determined and the selected species for toxicity and efficacy studies should have similar in vitro metabolite profile with human (244). Additionally, some of the metabolites exhibit organ specific toxicity. The N-hydroxy metabolite of 2-naphthylamine causes bladder cancer but does not show significant toxicity in any other organ. The glucuronidation of N-hydroxy metabolite of 2-naphthylamine, an aromatic hydrocarbon present in cigarette smoke, forms N-hydroxy-N-glucuronide and excreted in the urine. At acidic pH of urine, N-hydroxy-N-glucuronide decomposes to form protonated nitrenioum ion that binds with DNA and initiates bladder cancer (245, 246).

Given the fact that most of the known clinically significant drug-drug interactions are mediated by induction or inhibition of metabolic enzymes, the determination of metabolic pathways is important to predict possible interactions during development of the compounds. Phase I metabolic enzymes, which catalyze oxidation, reduction and hydrolysis of drugs, are high affinity low capacity enzymes (247). Therefore, the systemic concentrations of compounds that are primarily metabolized by a single Phase I enzymes are likely to change upon inhibition

or induction of the metabolic enzyme (248). Various endogenous or exogenous factors can influence the activity of the metabolic enzyme and thus likely to affect efficacy and toxicity of the drugs. A prior understanding of metabolic pathways may help in predicting clinically significant interactions (249).

Given the important role of drug metabolism, the metabolic studies are integral to the drug discovery and development persuit and help in the selection of appropriate molecules for other safety and efficacy studies.

1.2.3.3.4 Optimum pharmacokinetic characteristic of molecules

Theoretically, a compound should have good bioavailability, optimum half-life and favourable metabolism to be successful in the drug development (229); however, these pharmacokinetic (PK) reasons alone do not dictate the go/no-go decision. PK parameters in conjunction with mechanisms of efficacy and toxicity determine whether the PK will have significant impact on the therapeutic outcome. Various strategies are adopted accordingly to overcome suboptimal PK characteristics. A drug administered via the extravascular route should have good oral bioavailability but some drugs, despite having poor oral bioavailability, are clinically successful as oral drugs. For example, poorly orally bioavailable (<1%) alendronate is used successfully in the clinic for the treatment of osteoporosis because most of the systemically available alendronate is taken up in bones and very slowly cleared (half-life >10 years) (250-252). Alternatively, a drug with low oral bioavailability may undergo chemical modification, reformulation or be administered via another route. Poorly orally bioavailable enalaprilat (<10 %) was chemically modified to an ethyl ester prodrug (enalapril) to improve oral bioavailability to 60% (253, 254). A formulation change increased the oral bioavailability of cholecystokinin (CCK_B) receptor antagonist at Merck (L-365,260). An oral administration of L-365,260 with PEG 600 exhibited increased bioavailability (4-fold in rats and 8-9 fold in dogs) than its administration as suspension in 0.5% methylcellulose (255). Ceftriaxone, a third generation cephalosporin, is unstable at gastric pH and exhibits poor oral bioavailability; however, administration via the intravenous or intramuscular route increased its clinical efficacy (256, 257).

Similarly, a drug with very short or very long half-life is not desirable. While very short half-life drugs require frequent administrations or large doses to maintain systemic drug

concentrations above the minimum effective concentration, drugs with long half-lives may accumulate and cause toxicity. However, several drugs with very short and very long half-life are clinically successful. Given the short half-life of ~2h, nifedipine requires t.i.d administration, which may result in decrease of patient compliance in chronic treatment (258). Therefore, a chemical modification of nifedipine yielded another drug, amplodipine, which exhibited a longer half-life allowing its administration once a day (259-261).

Other strategies such as formulation modifications and co-administration of an inhibitor of drug metabolizing enzyme have been successfully employed to decrease the frequency of administration of very short half-life drugs. A sustained release formulation of metoprolol (half-life <3 h) is given once a day and produces uniform and prolonged effects on systemic blood pressure and heart rate (262). Co-administration of amoxicillin with clavulanic acid resulted in a decrease in dosing frequency. Clavulanic acid is a suicide inhibitor of microbial beta-lactamase, the enzyme responsible for the metabolism of amoxicillin, thereby decreasing the clearance of amoxicillin (263). Omeprazole, despite having very short half-life (~1h), is used clinically once a day because of its mechanism of action. Omeprazole is ultimately converted into a cyclic sulfonamide via spiro intermediate formation in the parietal cells, where cyclic sulfenamide irreversibly binds with proton pump to inhibit acid secretion in the stomach. Since the spiro intermediate formation occurs only at acidic pH, omeprazole undergoes pH dependent accumulation in parietal cells and inhibits the proton pump for a long duration (264).

While the extensive first pass metabolism of an orally administered drug is not a desirable characteristic, it proves advantageous for some drugs. Tioconazole, one of the early antifungal azole, could not be developed as an oral antifungal agent due to its extensive first pass metabolism (265). Recently discovered antifungal azoles, ketoconazole, fluconazole and itraconazole, do not undergo extensive first pass metabolism (266). The systemic concentration of nifedipine exhibits high degree of intra-individual variability due to substantial first pass metabolism (267). On the other hand ezetimibe, a cholesterol absorption inhibitor, undergoes significant (80-90%) glucuronidation and excretion into the bile. The ezetimibe glucuronide and ezetimibe acts at the Niemann pick C1 like 1 protein (NPC1L1) to inhibit cholesterol absorption. Since ezetimibe glucuronide is as active as ezetimibe, the extensive first metabolism does not have any effect on the therapeutic outcome (268).

In addition to abovementioned examples and approaches employed to overcome various

pharmacokinetic issues, other innovative approaches may be used depending upon the merit of individual compounds. These examples emphasize that optimum pharmacokinetic cannot be defined without consideration of other aspects including efficacy and toxicity. Therefore, the go/no-go decision on the molecule should be taken after consideration of all different factors that can contribute to the effectiveness of the compound.

1.2.4 Physicochemical properties that can affect ADMET

In the early 1980's the major focus of drug design was to improve potency. In 1988, a report to determine reasons for attrition of compounds found inappropriate pharmacokinetics as the major reasons of failure. In order to reduce the cost of attrition and meet timelines, early drug discovery evolved to incorporate pharmacokinetic screening, which resulted in a significant decrease in failures due to inappropriate pharmacokinetics by 2000 (7, 131).

Although integration of pharmacokinetics into the drug discovery process decreased the attrition rate, this did not help in the design of molecules until Lipinski proposed the Rule-of-Five (RO5), which identifies compounds that may have potential oral bioavailability issues prior to experimental evaluations (269). Since then, several different physicochemical parameters and models to predict pharmacokinetic behaviour have been proposed (170, 270-272). Based on these theories and models several ADME prediction sotwares are now available. The Lipinski's RO5 states that if a compound violates more than one of the following rules: molecular weight of <500D, <5 hydrogen bond donors, <10 hydrogen bond acceptors and cLogP of <5, the compound is likely to have an oral bioavailability problem (269, 273-275). These physicochemical properties are interdependent and thus very difficult to segregate one from the other. Oral bioavailability (F) is dependent on permeation through the intestine (Fa), metabolism by the intestine (Fg) and metabolism by the liver (Fh). The number of violations of RO5 affects only permeation not metabolism by intestinal or liver enzymes (276). In 2002, Veber identified structural properties, such as the number of rotatable bonds and polar surface area that had a remarkable effect on oral absorption in rats and proposed that compounds should have ≤ 10 rotatable bonds and total polar surface area (TPSA) should be ≤140 Å2.(277)

Distribution of a drug in the body also depends upon the physicochemical space of the drug. In general, lipophilic compounds distribute widely in the extravascular tissues and exhibit high volumes of distribution, unless these compounds exhibit extensive plasma protein binding.

Compounds with a basic centre that ionize at physiological pH exhibit high volumes of distribution due to increased tissue binding affinity by virtue of an ion pair interaction with charged acidic groups of phospholipid membranes (278). Thus, incorporation of basic centre in the molecule can increase the volume of distribution of the drug and vice-versa. Despite having low lipophilicity Amlodipine, a long acting calcium channel blocker, exhibits high volume of distribution due to presence of basic centre. Increase in hydrogen bonding potential decreases the blood brain permeation as well.

The role of transporters in pharmacokinetic processes receives increasing attention. Some physicochemical parameters associated with substrate characteristics of transporters include charge, molecular weight and hydrogen bonding capability. Similar to Lipinski's rule, Didziapetris et al proposed the rule of four for the determination of P-glycoprotein (Pgp) substrates (279). The rule of four states that compounds with ≥ 8 nitrogen and oxygen atoms, > 400 D molecular weight, pKa < 4 (weak organic acid) are likely to be substrates, and compounds with ≤4 nitrogen and oxygen atoms, <400 D molecular weight, and pKa <8 (weak organic base) are likely to be nonsubstrates.

1.2.4.1 Lipophilicity

Lipophilicity is often correlated in various models of *in silico* prediction with permeability, absorption, distribution, metabolism, excretion and toxicity. The measures of lipophilicity are logP and logD, which are log of partition coefficient and distribution coefficient, respectively. The partition coefficient is the ratio of concentration of drug in the octanol (oil) and water at equilibrium, whereas logD is the ratio of concentration of drug in the octanol and buffer at specific pH (164).

During intestinal absorption, drugs must first dissolve in gastrointestinal media (aqueous) and then traverse through bilayer lipid membrane; theoretically, log P should demonstrate a bell shaped curve with the oral bioavailability of compounds. However, the relationship between permeability and lipophilicity depends upon several factors including type of transport across the membrane (paracellular and transcellular), molecular size, and pH of the donor and acceptor compartment in permeability assays (178, 280). The ideal logP for good ADME properties is suggested to be 1 to 3. Further, the median logP values of molecules in different stages of drug discover shows decreasing trend (bioactive molecules>clinical candidates> drugs). The median

log P values of drugs, clinical candidates and bioactive compounds were 2.83, 3.47 and 4.09, respectively; however, dispersion around the median values (standard deviation) remained constant (281, 282).

Theoretically an increase in lipophilicity should increase potency of the molecule. To interact at the binding site, complete removal of water from binding site of the molecule is necessary. The removal of water from lipophilic groups is energetically favourable and has positive solvation energy (270, 278). Thus, higher potency is likely to correlate with increased lipophilicity. However, the increase in lipophilicity increases the promiscuity, which is the ability to interact with off target proteins. Increase in promiscuity is likely to result in toxicity.

1.2.4.2 Number of Hydrogen Bond Donors and Acceptors

Compounds form hydrogen bonds to increase solubility in water. These bonds are necessary to break before permeation across a lipid bilayer. An increase in the number of hydrogen bonds decreases the passive diffusion of compound (164). Thus, the number of hydrogen bond donors and acceptors should be limited in order to minimize the stability of compound in aqueous media. Lipinski identified the acceptable number of hydrogen bond donors and acceptors as 5 and 10, respectively (269, 275).

1.2.4.3 Molecular Weight

Molecular weight is related with size of the molecule which should be smaller than the pore size of tight junctions. Permeation of compounds through the paracellular route is often limited by the size of the molecule (177). Fat soluble compounds can cross the intestinal membrane even when the molecular weight is high, mainly through transcellular transport, whereas water soluble compounds' permeability is largely dependent upon molecular weight (275). High molecular weight compounds are likely to show high toxicity as promiscuity of compounds is also likely to increase (282). Additionally, the systemic clearance of a compound is inversely proportional to the molecular weight (278). Ideally, the molecular weight of the compound for good ADME should be below 500 Da (283).

1.2.4.4 Molecular Flexibility

Veber *et al* (277) identified that molecular flexibility affects oral bioavailability. The

number of rotatable bonds present in the molecule is an indicator of molecular flexibility. Studies have shown that with an increase in the number of rotatable bonds, oral bioavailability in rats can be significantly decreased. The maximum number of rotatable bonds should be ≤10 (277). However, similar studies on the compounds from Pharmacia did not show a similar trend (284). The physicochemical properties of compounds from different chemical classes clustered around different values of the number of rotatable bonds and polar surface area. Thus, the rule does not apply uniformly across all class of compounds.

1.2.4.5 Total Polar Surface Area (TPSA)

Total polar surface area is the total sum of area over the polar atoms in the molecule. Polar surface is associated with hydrogen bond formation, which must be broken before permeation through biological membranes. TPSA is a very useful parameter in medicinal chemistry to predict the permeability and toxicity of compounds. Polar surface area of more than 140 $Å^2$ are poorly permeable at the intestinal membrane (277). Compounds with TPSA of more than 60 $Å^2$ are poorly permeable to the brain. Compounds with cLogP >3 and TPSA<75 have higher chances to exhibit toxicity (285, 286).

Combinatorial chemistry approaches in drug discovery have led to a great number of compounds with poor oral bioavailability and toxicity profiles. Currently, in rational drug design, compounds are optimized for their predicted physicochemical properties to improve a priori oral bioavailability and toxicological characteristics. These criteria of physiological properties were employed in the drug design process and subsequently reduced the attrition rates due to pharmacokinetic issues; however, these criteria are purposefully liberal as the goal of RO5 was to bring the medicinal chemistry process on the right path before 1995 (273). Passing RO5 criteria does not necessarily mean that a compound possesses drug-like properties; more stringent criteria should be defined by different drug developers for different projects. Toxicity is also found to correlate with the physicochemical properties of a compound. Compounds with clogP values greater than 3 and TPSA of less than 75 $Å^2$ have 2.5-fold greater risk for toxicity (285, 286). Software is available to predict the physicochemical properties of compounds based on their structures. Since these physicochemical properties are correlated with pharmacokinetic and toxicological outcomes, it helps to eliminate compounds in the drug design stage.

1.2.5 Curcumin and its Analogues in Cancer

Natural products have always been a venue for the search of new drugs or leads. Plants produce an array of chemically diverse compounds to safeguard them from predator animals. These chemicals are used directly as medicines or their synthetic analogues are designed to improve efficacy and safety profile. Natural products such as digoxin, digitoxin, quinine, morphine and taxol are still used today. The advantage of searching natural products is that they provide complex molecules such as bryostatin and camptothecin, which are difficult to envisage while designing (287). The challenges in the synthesis of complex natural molecules are evident from the fact that almost 40% of natural compounds have not been synthesized chemically (288).

Turmeric, a rhizome of *Curcuma longa* Linn., is widely used as a spice due to its color and taste and as a preservative in India (289, 290). The turmeric is named as "Indian saffron" in Europe due to its color and taste; however, the medicinal uses of the turmeric remained unrecognized except in India where it was used in the traditional medicine system, Ayruveda (290). This spice, an ingredient of Indian curry powder, has been used as anti-inflammatory agent, as an anticholic, against toothaches, against chest pains, against liver problems, to heal wounds and lighten scars and as a cosmetic (291, 292).

Turmeric contains several phytochemicals including curcumin, demethoxycurcumin, bisdemethoxycurcumin, turmerin, essential oils such as turmerones, atlantones and zingiberene. Curcumin, a yellow colored compound, constitutes 2-5% in turmeric preparations and is considered to be responsible for most of tumeric's pharmacological activities. Curcumin is hydrophobic in nature and has very poor aqueous solubility. Of the three curcumin analogues present in turmeric (curcumin, demethoxycurcumin, bis-demethoxycurcumin), the most active form is not clear as different forms show varying potency in different efficacy studies. However, curcumin exhibits high potency in most of the cases (293).

Curcumin exhibits a gamut of pharmacological activities. Some of the pharmacological activities of curcumin include anti-cancer, anti-inflammatory, antioxidant, antimalarial, antiseptic and wound healing. Since inflammation is central to many chronic diseases, presumably, the anti-inflammatory action of curcumin may explain its positive effects in a variety of pathological conditions (290).

Curcumin exhibits chemopreventive action against oral, gastrointestinal and colon cancers

by various mechanisms. First, curcumin blocks the metabolic activation of carcinogens or stimulates detoxification metabolism (294). Second, curcumin has capability to induce apoptosis in precancerous and metastatic cells or inhibit the cell cycle (295). Lastly, curcumin also inhibits angiogenesis, metastasis and invasion, which are vital for cancer progression (296).

The pharmacokinetic characteristics of curcumin are not favourable and are highly variable (297). Curcumin has poor oral bioavailability in rats and humans. An oral dose of 400 mg/kg of curcumin to rats did not result in detectable concentrations in cardiac blood whereas trace amounts of curcumin were found in portal blood. However, the level of curcumin in rat plasma was detectable upon the use of radioactive detection technique. In another study, an oral dose of 2 g/Kg to rats resulted in a plasma concentration of 1.35 ± 0.23 μg/mL whereas the same dose in humans resulted in undetectable or a very low concentration (0.006±0.005 μg/mL). The concomitant administration of piperine, an inhibitor of UGT, CYP3A4 and P-gp, increased the bioavailability of curcumin by 154 and 2000 % in rats and humans, respectively. Piperine also decreases endogenous UDP-glucuronic acid to inhibit glucuronidation in the enterocyte (298).

Curcumin undergoes extensive first pass metabolism in the liver and intestine (299). About 30-80% of curcumin disappeared from the mucosal side of the intestinal barrier but the concentration of curcumin in serosal fluid was below the limit of detection in an everted rat intestinal sac preparation suggesting that curcumin undergoes extensive metabolism in the intestine. The major metabolites formed are sulphate and glucuronide conjugates of curcumin. Pan *et. al.* identified that 99% of curcumin is present in the plasma as glucuronide conjugates (300). Curcumin also undergoes extensive phase I reduction, likely through alcohol dehydrogenase, to form dihydrocurcumin, tetrahydrocurcumin, hexahydrocurcumin and octahydrocurcumin, which are subsequently glucuronidated. Small traces of ferulic acid and dihydroferulic acid are also formed in the liver. A consensus on pharmacological activity of the metabolites of curcumin does not exist. Some of the studies found metabolites of curcumin more active whereas others found curcumin more active over their metabolites (301, 302).

Curcumin and its metabolites are excreted in feces, bile and urine. Unchanged curcumin is mostly excreted in feces and conjugated metabolites are excreted through bile or urine. Upon oral administration at the dose of 1 mg/kg, 75% of unchanged curcumin was excreted in the feces; however, intravenous and intraperitoneal injection resulted in biliary excretion. Urinary excretion of the curcumin is negligible (297, 299).

1.2.5.1 Development of Piperidone Based Curcumin Analogues

The bioavailability of curcumin may be increased using several strategies such as use of adjuvants, nanoparticle technology, liposomal formulation and the synthesis of new analogues (303, 304). As discussed earlier, piperine, which inhibits glucuronidation by inhibiting UGT and decreasing the endogenous UDP-glucuronic acid, increases the bioavailability of curcumin in animals and humans (298). Other adjuvants to improve the bioavailability of curcumin are eugenol, terpinol and quercetin. The second approach is the use of nanoparticle-based and liposome-based deliveries (305, 306). Solid-lipid nanoparticles and liposomal formulations of curcumin increased efficiency.The third approach is the preparation of new curcumin analogues with better efficacy and pharmacokinetic profile.

To improve the low oral bioavailability of curcumin several attempts have been made to modify curcumin into more potent and pharmacokinetically viable molecules. Curcumin contains two major substructures including two benzylidene keto substituent and a β-diketone moiety (Figure 1.4). Modifications have been made at both the substructures to develop structure activity relationships (12, 307-310). However, the alpha, beta unsaturated ketone, being a Michael acceptor, has been retained in most of the cytotoxic compounds to improve cytotoxicity.

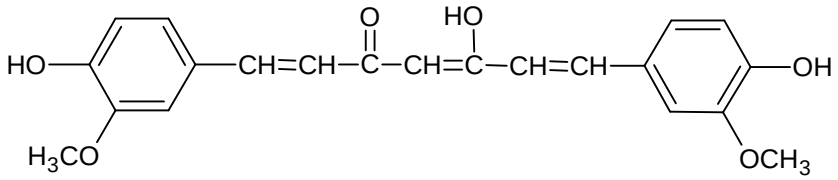

Figure 1.4: Chemical structure of curcumin

Michael acceptors react with nucleophiles such as thiols (311). In the case of compounds containing an α, β-unsaturated keto group, the olefinic double bond is polarized, which enables nucleophilic attack as illustrated in Figure 1.5. In general, α, β-unsaturated ketones react preferentially with thiols rather than amines or hydroxyl groups found in nucleic acids, which reduces their genotoxic potential.

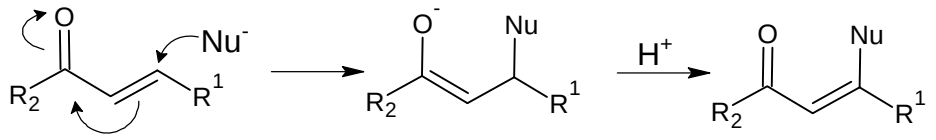

Figure 1.5: A schematic diagram representing the reaction between α, β-unsaturated ketone (Michael acceptor) with a nucleophile (Nu) (Adapted from Mosley *et. al* (311)).

Due to the presence of β-diketone moiety, curcumin undergoes keto-enol tautomerism. The curcumin degradation at neutral and basic pH is believed to be due to the presence of β-diketone moiety (312, 313). Secondly, the enolisation of β-diketone moiety create a potential site for glucuronidation. Both these factors may contribute to the low oral bioavailability of curcumin. Thus, to improve the oral bioavailability β-diketone moiety was replaced with a ketone moiety to form 1,5-diaryl-3-oxo-pentadienyl pharmacophore.

In the case of the 1,5-diaryl-3-oxo-1,4-pentadienes, there is no β-diketone moiety hence, enolisation does not occur. The two α, β-unsaturated keto group in 1,5-diaryl-3-oxo-1,4-pentadiene allows sequential interactions with thiols (12, 314). Since on a number of occasions, a chemical attack is more harmful to neoplasms than non-malignant cells, the second reaction could lead to substantial selective toxicity to tumours. Subsequently, a number of compounds containing 1,5-diaryl-3-oxo-1,4-pentadiene pharmacophore were reported to have good cytotoxic properties and improved oral bioavailability (311, 315).

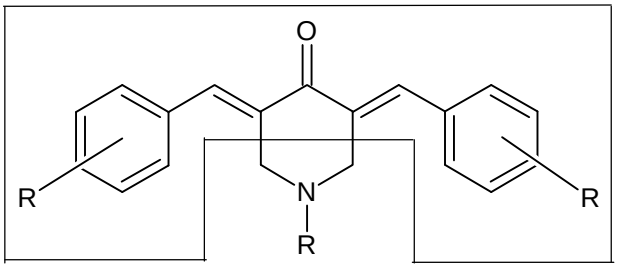

Figure 1.6: The 3,5-bis(benzylidene)-piperidone compounds containing 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore (indicated in box)

In 1990, Dimmock and coworkers reported on the cytotoxic properties of 3,5-bis(benzylidene)-4-piperidones against P388 leukemia cells (316). Subsequently, other labs also reported the cytotoxic activities of 4-piperidone analogues (315, 317). The inspiration for

designing this class of compounds differs between labs. Some researchers believe it to be chalcones whereas some of the researchers refer to them as curcumin analogues (313, 315, 318, 319), due to its structural similarity. Most of the recent literature refers to 3,5-bis(benzylidiene)-4-piperidones as curcumin analogues (12, 310, 320).

This 3,5-bis(benzylidiene)-4-piperidones exhibit efficacy against different types of pathological conditions including cancer (308, 316, 318, 319, 321-323). This class of compounds was also active as antimycobacterial (324, 325) agents, antioxidants (326, 327), analgesic and local anesthetic agents, antifungal agents, and antiviral agents. Its multiple targeted mechanism of action makes compounds from this series less likely to exhibit multidrug resistance (310).

EF24, a compound from the 3,5-bis(benzylidene) cluster of cytotoxins, is active against colon cancer in vivo and exhibits improved oral bioavailability with respect to curcumin (315). An intraperitoneal dose (0.2 mg/kg) of EF24 to HCT-116 colon cancer xenografted tumour demonstrated tumour regression and increased survival rates without any significant toxicity. The oral and intraperitoneal bioavailability in male CD2F1 mice was 60% and 35%, respectively. The absorption is very rapid with a T_{max} of 3 minutes. It exhibits high plasma protein binding (>98%) and binds mostly with albumin. About 40-50% of the compound is eliminated via metabolism; the systemic clearance was 0.482 L/min/kg, and the half-life was 73.6 min (328).

Like curcumin, these piperidone based curcumin analogues act via a variety of different mechanisms. EF24 acts via caspase induced apoptosis during mitosis in HCT-116 xenografted tumour in nude mice. These curcumin analogues containing 1,5-diaryl-3-oxo-1,4-pentadienyl group act via TNF-α induced NF-κB phosphorylation and degradation. The potency to deactivate NF-κB is ten times higher than curcumin. Other mechanisms include inhibition of VEGF, activation of the p38 signaling pathway, inhibition of fanconi anemia pathway, inhibition of STAT3 phosphorylation (315, 329-335), disruption of the microtubule skeleton, inhibition of angiogenesis and increase in PTEN expression.

1.2.5.1.1 Development of NC 2083

Das and coworkers developed a novel series of compounds containing 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore, which showed excellent cytotoxic activity against various tumor cell lines. Among these analogues, NC 2083 (3,5-bis(4-chlorophenylmethylene)-1-[4-{2-(4-

morpholinyl)ethoxy}phenylcarbonyl-4-piperidone) hydrochloride demonstrated strong cytotoxic activities in vitro against colon cancer cell lines. Its IC50 value was < 0.005 μM against HCC-2998 cells and 1.45 μM against SW-620 cell lines with a selectivity index of >2188 when considering all cell lines (336). Highly positive outcomes in the pharmacological screening of NC 2083 led to its in vivo evaluation in Ncr-nu/nu mice with the SW-620 tumor. Surprisingly, NC 2083 failed to reduce tumor growth rates up to a dose of 600 mg/kg administered intraperitoneally (i.p.) (Report from Southern Cancer Institute, USA-Unpublished). PK or PD issues may explain the lack of activity of NC 2083.

For many compounds, a relationship exists between the concentration at the site of action and pharmacological effect. The concentration of a compound at the site of action depends on the size of the dose and upon the processes of absorption, distribution and clearance. Poor absorption and distribution and/or rapid elimination characteristics may result in insufficient concentrations at the site of action. Alternatively, the compound may not elicit a pharmacodynamic response even when it reaches the site of action. A review of NC 2083's drug like properties (logP = 5.8, Molecular weight = 577.5 D) indicates that it violates more than one rule of Lipinski's RO5. If a molecule violates more than one rule it is likely to have gastrointestinal absorption issues (269). Given its high logP value, NC 2083 is expected to have poor aqueous solubility and likely poor absorption characteristics. These characteristics warranted both an in vitro and in vivo pharmacokinetic evaluation of NC 2083, the outcomes of which may offer supportive data to explain NC 2083's lack of in vivo efficacy.

1.2.5.1.2 Development of N-phosphono Derivatives

In order to decrease lipophilicity to an optimum value (i.e. log P values of 0 to 3), Dimmock et al developed a new series of 3,5-bis(benzylidene)-piperidone based curcumin analogues that involved modification at the nitrogen with a phosphono group (Figure 2.7) (310). Furthermore, a prodrug approach of this series was attempted by synthesizing the diethyl ester analogues of these N-phosphono curcumin compounds. The ubiquitous nature of esterases in the body will likely result in the rapid release of the active parent compound *in vivo*. This group of compounds exhibited very good antineoplastic properties against various cell lines (310). After successful in vitro evaluations, the next step in the drug discovery is to evaluate their preclinical PK and PD behavior.

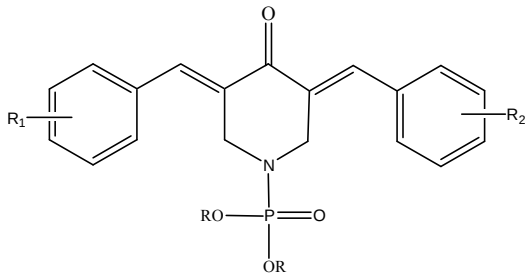

Figure 1.7. Chemical structure of N-phosphono derivatives of 3,5-bis(benzylidene)-4-piperidone based curcumin analogues(R=H or Et)

1.3 Perspective of the Current Study

As our lab is still in the lead identification stage of the drug discovery, our primary purpose is to identify the lead molecules for lead optimization. Feedback to the drug design group helps in the optimization of pharmacokinetic and pharmacodynamic properties through structural modifications of the compound. Usually, compounds with nonoptimum properties help to identify the pitfalls in the design and subsequently eliminate them. Despite having high efficacy against colon cancer cell lines *in vitro*, NC 2083 did not demonstrate activity *in vivo* in a xenograft tumour mouse model. We suspected a pharmacokinetic problem and investigated the physicochemical and pharmacokinetic causes for its failure to provide feedback to improve the drug design.

The second purpose of our current work is to identify a process for rapid evaluation of oral absorption properties. Drug discovery in academia faces various challenges such as high cost, lack of devoted specialized units and manpower, and lack of hypothesis driven research. To overcome these challenges we require an efficient absorption screening strategy for drug discovery. Currently, Dimmock and coworkers have synthesized several analogues with varying substitution groups, but the lead molecule possessing the optimum pharmacokinetic and pharmacodynamic characteristics remains unidentified. We selected these curcumin analogues as candidate molecules for the optimization of permeability screening strategy and identified highly permeable molecules which are likely to be orally bioavailable.

1.4 Hypotheses

1. Lack of *in vivo* efficacy of NC 2083 is due to poor absorption and/or high first pass metabolism and/or high plasma protein binding
2. Several of 3,5-bis(benzylidene)-4-piperidone containing curcumin analogues have good permeability characteristics.
3. Several 3,5-bis(benzylidene)-4-piperidone containing curcumin analogues have favourable pharmacokinetic behavior.
4. The 3,5-bis(benzylidene)-4-piperidone inhibits angiogenesis and inflammatory biomarkers such as interleukin 8 (IL8), vascular endothelial growth factor (VEGF), Glyoxalase I, Glyoxalase II and cyclooxygenase-2 (COX-2).

1.5 Objectives

1. To develop and validate a bioanalytical method for the quantitative analysis of NC 2083 and other curcumin anaolgues
2. To conduct *in vitro* and *in vivo* pharmacokinetic studies on NC 2083
3. To establish the suitability of MDCK and Caco-2 cell line for permeability screening of 3,5-bis(benzylidene)-4-piperidone curcumin analoguesTo determine intravenous and oral pharmacokinetics of curcumin analogues with better permeability characteristics
4. To evaluate the effect of permeable analogues on the mRNA expression profile and protein expression/activity of interleukin 8 (IL8), vascular endothelial growth factor (VEGF), Glyoxalase I, Glyoxalase II and cyclooxygenase-2 (COX-2).

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CHAPTER 2

A GENERAL HPLC-UV METHOD FOR THE QUANTITATIVE DETERMINATION OF CURCUMIN ANALOGUES CONTAINING THE 1,5-DIARYL-3-OXO-1,4-PENTADIENYL PHARMACOPHORE IN RAT BIOMATRICES

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A general HPLC-UV method for the quantitative determination of curcumin analogs containing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore in rat biomatrices

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Abstract

Curcumin and its derivatives generally display favorable cytotoxic activities against a number of cancer cell types. We focus our rational antineoplastic drug design program on curcumin analogues containing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore. Favorable outcomes from pharmacological screens of this series demanded further pharmacokinetic evaluations to determine their suitability as effective compounds *in vivo*. To allow such evaluations and to provide a general, sensitive, rapid and simple method for the analysis of compounds containing the 1,5-diaryl-3-oxo-pentadienyl scaffold, we developed an HPLC method with ultraviolet detection for their detection in various biological matrices of a relevant preclinical species, i.e. the rat. Our HPLC method is specific for the analysis of many members in this series in rat blood, plasma, serum and hepatic microsomes following liquid-liquid extraction with TBME (1:30 v/v). The assay procedure involves chromatographic separation on a Zorbax-Eclipse C-18 column under isocratic conditions with the mobile phase consisting of acetonitrile and ammonium acetate buffer (pH 5.0, 10 mM) in different ratios depending upon the compound. The method was validated for NC 2083 in rat serum and rat liver microsomes, a potential lead compound, to demonstrate its applicability. The standard curve was linear ($r^2 \geq 0.997$) from 50-5000 ng/mL. Intra- and inter-day precision and accuracy of the method were within USFDA specified limits. The stability of NC 2083 was established in an auto-injector, on bench-top, during freeze-thaw cycles and long term stability at -80°C for 40 days. The method is suitable for a number of compounds containing the 1,5-diaryl-3-oxo-pentadienyl scaffold with divergent logP values with only minor adjustments in the buffer to acetonitrile ratio of the mobile phase.

KEY WORDS: Curcumin analogues, HPLC; Microsomal stability; Piperidones; Rat biomatrices; Validation

1. Introduction

Many naturally occurring chalcones and flavones contain an aryl ring conjugated with an alpha, beta unsaturated keto group (Ar-CH=CH-CO)[1,2]. Such molecules display various important therapeutic actions [3]and, therefore, these compounds have received considerable interest. Recently, Dimmock and coworkers extended this 3-aryl-2-propenoyl group to a 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore (Figure 2.1)[4]. The literature refers to this pharmacophore as a curcumin analogue[5], and a substantial number of these compounds have excellent antineoplastic properties[2,6-10].Other laboratories are also examining the biological potential of this pharmacophore[5,11-13]. In order to initiate some *in vitro* and *in vivo* studies on a rational basis, a robust analytical method is mandatory. In particular, pharmacokinetic evaluations have become necessary to advance promising compounds further in the discovery process and to provide feedback to the rational drug design process. Quantitative determination of this series of compounds will require the availability of a versatile bioanalytical method.

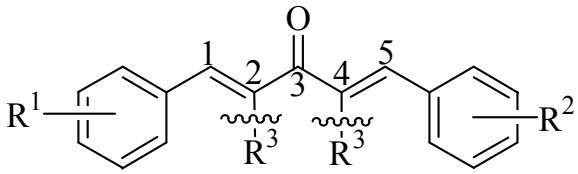
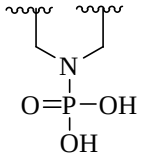
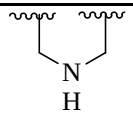
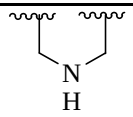
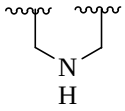
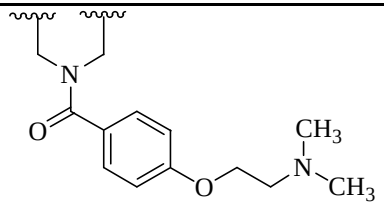
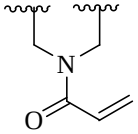
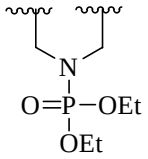
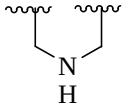
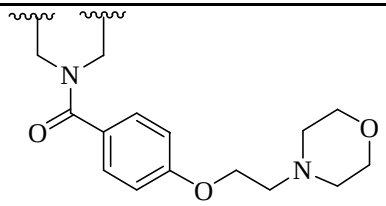
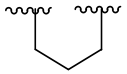


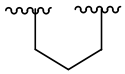
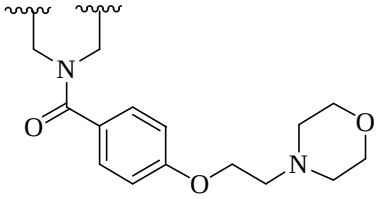
Figure 2.1 Basic structure of the 1,5-diaryl-3-oxo-1,4-pentadienyl group containing compounds (See Table 2.1)

To the authors' best knowledge, no bioanalytical method has been reported for curcumin analogues containing the 1,5-diaryl-3-oxo-1,4-pentadienyl group. Therefore, we developed a general HPLC method for the compounds containing this pharmacophore.

Table 2.1. HPLC mobile phase conditions and analyte retention times for the analysis of 1,5-diaryl-3-oxo-pentadiene containing cytotoxic compounds.

Code	R^1	R^2	R^3	miLogP value	Retention Time	ACN:AAB
NC 2324	3,4,5-$(OCH_3)_3$	3,4,5-$(OCH_3)_3$		1.93	6.72	40:60
NC 2443†	2-F	2-F		3.23	14.95	40:60
NC 1831	H	H		3.36	9.53	40:60
NC 1834	4-OCH_3	4-OCH_3		3.47	7.49	40:60
NC 2067*	H	H		4.60	7.12	40:60

Code	R^1	R^2	R^3	miLogP value	Retention Time	ACN:AAB
NC 2288	H	H	H	4.18	9.72	60:40
NC 1845	4-OCH_3	4-OCH_3		4.03	5.75	60:40
NC 2313	4-OCH_3	4-OCH_3		4.09	6.59	60:40
NC 1876	4-Cl	4-Cl		4.71	9.71	60:40
NC 2081*	H	H		4.45	5.38	60:40
NC 2095	4-NO_2	3,4,5-$(OCH_3)_3$		4.56	14.00	60:40

Code	R^1	R^2	R^3	miLogP value	Retention Time	ACN:AAB
NC 2164	3,4,5-$(OCH_3)_3$	3,4,5-$(OCH_3)_3$		4.23	9.52	60:40
NC 2083*	4-Cl	4-Cl		6.40	11.52	60:40

ACN-Acetonitrile; AAB-Ammonium acetate buffer (10 mM, pH 5.0); miLogP-molinspiration logP

*Obtained as hydrochloride salt; †Referred as EF24 in the literature [5,12,22]

Due to its widespread accessibility, HPLC with UV detection was selected as the instrument of choice.

We selected thirteen structurally diverse compounds containing the 1,5-diaryl-3-oxo-1,4-pentadienyl group with varying logP values (1.93 to 6.40), different substituents (with varying sigma and pi values) in both aryl rings, and different linker groups between C-2 and C-4 (Figure 2.1;Table 2.1). We tested the specificity of the method in different rat biomatrices including plasma, serum, blood and hepatic microsomes. Further, we selected a novel promising cytotoxin in this series, NC 2083 [3,5-bis(4-chlorophenylmethylene)-1-[4-{2-(4-morpholinyl)ethoxy}phenylcarbonyl]-4-piperidone hydrochloride], to validate this method. NC 2083 is under development at the University of Saskatchewan as an antineoplastic agent. This compound has an IC_{50} values of <0.005 and 1.45 μM toward the HCC-2998 and SW620 human colon cancer cells, respectively, *in vitro* [14,15]. The present communication reports a versatile HPLC method for the analysis of compounds that contain the 1,5-diaryl-3-oxo-1,4-pentadienyl substructure, such as NC 2083, in biological fluids. This method can be directly adopted after partial validation for pharmacokinetic screening of the compounds containing the 1,5-diaryl-3-oxo-1,4-pentadienyl group.

2. Materials and Methods

2.1. Chemicals and Reagents

Compounds containing the 1,5-diaryl-3-oxo-1,4-pentadienyl group were synthesized at the College of Pharmacy and Nutrition, University of Saskatchewan using reported procedures [2,6-9,14]. Tris (tris(hydroxymethyl)aminomethane) and HPLC grade acetonitrile were purchased from Fisher Scientific (Ottawa, ON, Canada). Tris

hydrochloride, tert-butyl methyl ether (TBME) and EDTA (ethylenediaminetetraacetic acid) were purchased from EM Sciences (Merck, Darmstadt, Germany). All other chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada). Milli-Q water with a resistance value of 18.2 MΩ was obtained from a Milli-Q Synthesis Water System (Millipore, MA). Drug free serum, blood and rat liver microsomes (RLM) samples were collected from adult, healthy male Wistar rats. Rat liver microsomes were prepared as described previously [16, 17], and the protein content was estimated with Lowry's method using bovine serum albumin as standard [18]. All protocols employed in the collection of serum and hepatic microsomal preparations were in accordance with the Canadian Council of Animal Care (CCAC) guidelines and approved by the University Committee on Animal Care and Supply.

2.2 Method Development and Validation

2.2.1 Instrumentation and HPLC conditions

The HPLC system consisted of an Agilent series 1200 quaternary pump with an online degasser, auto sampler and diode array detector (Agilent Technologies, Mississauga, ON, Canada). Sample aliquots (50 µL) were injected onto an Eclipse XDB-C_{18} column (4.6 × 150 mm, 5 µm) (Agilent Technologies, Mississauga, ON, Canada) and absorbance was monitored at 330 nm. The system was run in an isocratic mode with a mobile phase consisting of acetonitrile:ammonium acetate buffer (pH 5.0, 10 mM) in the ratio of 60:40 (v/v) (for NC 2083) and was delivered at a flow rate of 1 mL/min. An ammonium acetate stock solution (50 mM) was prepared and diluted with MilliQ water as necessary to achieve a final concentration of 10 mM. The mobile phase was filtered through a 0.22 µm Nylon filter (Pall Scientific, Ville St. Laurent, QC, Canada) and

degassed in an ultrasonic bath for 30 min prior to use. The column was maintained at room temperature during the run and subsequently washed with water followed by acetonitrile after every use. The carry over into the autosampler was negated by the injection of blank mobile phase following injection of the highest standard curve concentration. No carryover was detected.

2.2.2 Preparation of stock and working solutions

All stock and working solutions were prepared under low light conditions. Primary stock solutions (1 mg/mL) of analytes and the internal standard (NC 2288) were made by dissolving them first in DMSO and subsequently diluting with acetonitrile such that DMSO in the stock solution was ≤ 25% v/v. The stock solutions were diluted with acetonitrile to prepare secondary working stock solutions of 0.5, 1, 2, 5, 10, 20 and 50 µg/mL for NC 2083 and 40 µg/mL for the internal standard. The primary stock solution for the internal standard was also diluted with acetonitrile to prepare a working stock solution (40 µg/mL). Quality control (QC) samples were prepared similarly with stock solutions at 0.5, 1.5, 25 and 40 µg/mL, which were diluted 10-fold with rat blank serum to give the LLOQ (lowest limit of quantitation), low QC (LQC; 0.15 µg/mL), Middle QC (MQC; 2.5 µg/mL) and high QC (HQC; 4.0 µg/mL). All stock and working solutions were stored at -20±5°C and used to prepare the standard curve on the day of analysis.

2.2.3 Preparation of calibration standards and quality control (QC) samples

Calibration standards were prepared with each analysis run by spiking 10 µL of the individual working stock solutions to 90 µL pooled blank rat serum or heat inactivated rat liver microsomes (55°C for 5 min; 0.4 mg/mL) [16] with vortex mixing for 30 seconds. A

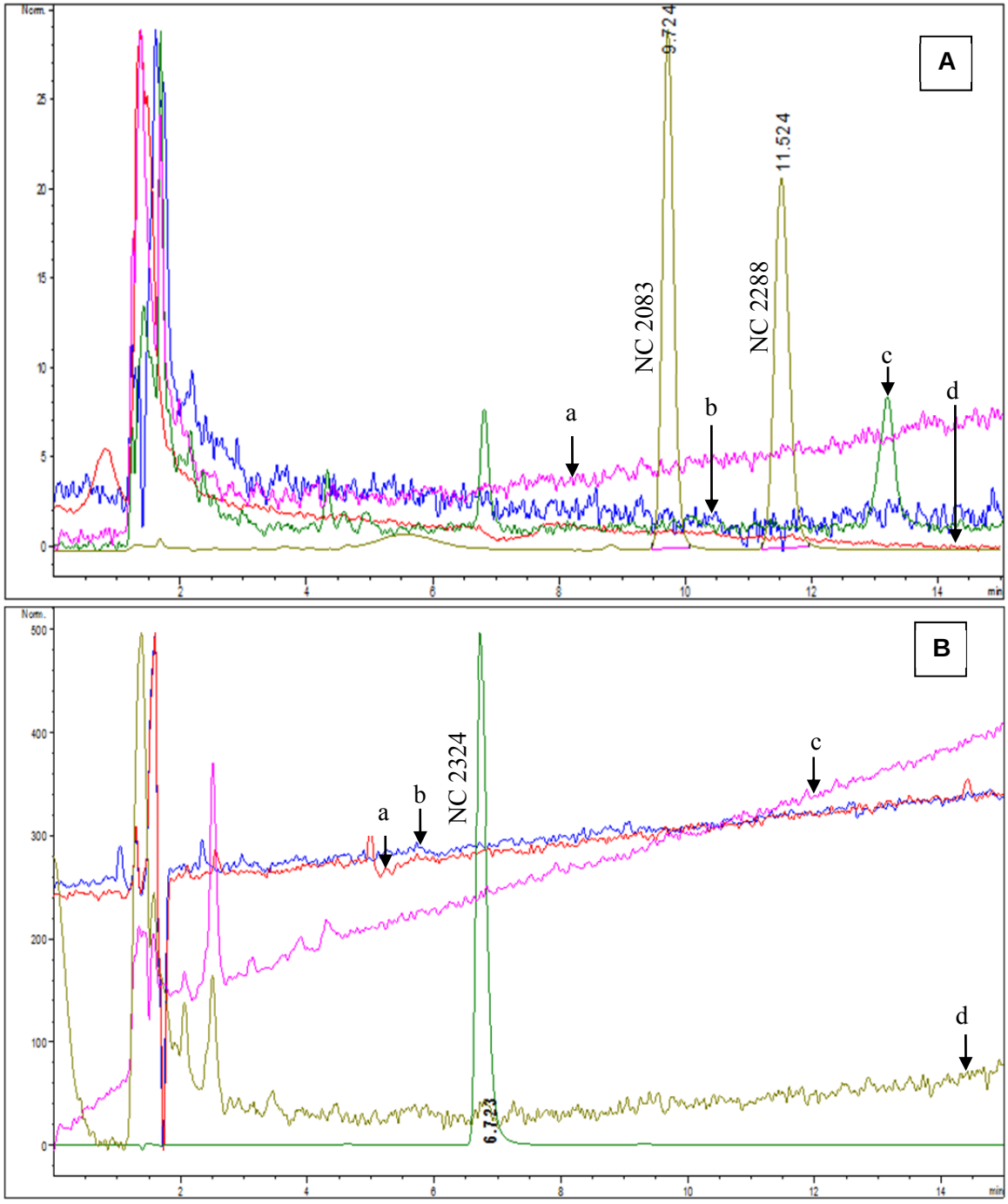


Figure 2.2. Chromatogram of A) NC 2083 and B) NC2324 overlaid with chromatograms of blank rat serum (a), plasma (b), blood (c), and liver microsomes (d). (A) 60:40 acetonitrile:ammonium acetate buffer mobile phase composition; B) 40:60 acetonitrile:ammonium acetate buffer mobile phase composition.

separate analyst prepared quality control samples, which were spiked in bulk at four concentration levels (LLOQ, LQC, MQC and HQC) on the first day of validation. These quality control samples were aliquoted into polypropylene microcentrifuge tubes and stored at -80±5°C in the dark until analysis. All calibration standards and samples were prepared under low light conditions.

2.2.4 Recovery

The recovery of NC 2083 and NC 2288 was determined following liquid-liquid extraction with tert-butyl methyl ether (TBME). For NC 2083, the recovery was determined at LQC, MQC and HQC, whereas recovery of NC 2288 was determined at 4 µg/mL. The recovery was assessed by comparison of post-extracted serum samples with un-extracted samples (in the reconstituting solvent) at the same concentrations. Post-extracted samples were prepared by the addition of 10 µL of appropriate QC working solutions into 90 µL of rat blank serum or rat liver microsomes and processed as described in section 2.2.5. Unextracted samples were prepared by the addition of 10 µL of working stock solutions of QC samples into 140 µL of the mobile phase.

2.2.5 Sample preparation

Ten µL of the internal standard solution (40 µg/mL) was added into the calibration standards, QC samples, and samples in other rat biomatrices (blood, plasma and liver microsomal suspensions). To each sample, 3 mL of TBME was added and vortex-mixed for 5 minutes and centrifuged at 778×*g* for 5 minutes at 4°C (5804 R, Eppendorf, Mississauga, ON, Canada). The aqueous layer containing serum was snap frozen using liquid nitrogen and the organic layer was transferred into a glass tube and evaporated to dryness under vacuum at 40°C in a CentriVap concentrator (LABCONCO, Kansas City,

Missouri, USA). The residue was reconstituted in 150 µL of mobile phase and vortex-mixed for 90 seconds. The reconstituted samples were transferred to amber colored HPLC vials and placed in the autosampler.

2.2.6 Method validation

The full method validation of NC 2083 was performed in accordance with USFDA guidelines for the assay of NC 2083 in rat serum and partial validation (precision, accuracy, autosampler stability and bench top stability) was conducted in heat inactivated rat liver microsomes (0.4 mg/mL). The specificity of the method was also established for all analytes in rat serum, plasma, blood and rat liver microsomes.

Specificity was evaluated by analysis of six different rat biomatrix samples to detect any potential interference with co-eluting endogenous substances. Linearity was determined by plotting the ratio of the peak area associated with the analyte concentration of seven calibration standards that ranged from 0.5-5 µg/mL to the peak area associated with the internal standard. A linear least square analysis was conducted with $1/X^2$ as weighting factor, and the slope, intercept and coefficient of determination (r^2) were determined to establish linearity. The limit of detection (LOD) was defined as the lowest detectable concentration with a signal to noise ratio of 3. The lowest limit of quantification was defined at the lowest concentration that gives precision and accuracy within ±20 % of the nominal value.

The intra- and inter-day precision and accuracy of the method was established by analysis of six replicates of samples at four different concentrations (LLOQ, LQC, MQC, HQC) on three different days. Single assay runs were accepted only when the relative standard deviation (RSD) was found to be less than ±15 % at all other concentrations

except at LLOQ, which allowed ±20 %. The criteria for accuracy was set at ±15 % of the nominal concentration of the QC samples except at LLOQ, where it was set at ±20%. In no case did more than one third of the QC samples violate these criteria.

Stability studies involving freeze-thaw stability, bench top stability and long-term stability were undertaken at LQC, MQC, and HQC, whereas autosampler stability was tested at LLOQ also. Freeze-thaw stability was tested after three freeze-thaw cycles spaced at least 24 hours apart with sample storage at -80±5°C between sample thawing. Bench top stability was established at room temperature and in an ice-bath for 6 hours. For autosampler stability, processed and reconstituted samples were kept in an autosampler for 24 hours before injection. Predicted concentrations were calculated using newly prepared calibration standards. Samples were stored at -80±5°C for 30 days before analysis to establish long-term stability. Samples were considered stable when the criteria for precision and accuracy were met.

Dilution integrity was assessed by dilution of 100 µg/mL spiked serum samples to 1 and 0.5 µg/mL with blank serum. These diluted samples were processed and analyzed with criteria for accuracy and precision as described previously.

2.3 Applications of the methods

2.3.1 Serum protein binding

Blood was collected from healthy male adult Wistar rats, (average weight 300±25 g) via cardiac puncture under isoflurane anaesthesia. After a thirty minute coagulation period at room temperature, serum was collected by centrifugation at 3200 rpm for 5 minutes. NC 2083 in DMSO (0.5 mg/mL) was diluted ten times with serum to yield serum samples (N=3) at a concentration of 50 µg/mL. Serum was equilibrated at 37°C for

20 min. One mL of serum samples were transferred into a micropartition kit (Millipore, MA, USA) and spun using a fixed angle (25°) rotor (TA-14-50) in a Beckman Coulter Allegra 25R centrifuge (Beckman Coulter, Mississauga, ON, Canada) for eight minutes at 1000×*g*. The filtrate was quantified for the presence of NC 2083 using reverse phase HPLC-UV method.

2.3.2 Hepatic microsomal stability study

Application of the method involved assessment of the metabolic stability of NC 2083 in rat liver microsomes. The rate of metabolism of NC 2083 was assessed in triplicate using the substrate depletion approach [19]with the following conditions: NC 2083 (5 µg/mL), protein concentration (0.4 mg/mL), phosphate buffer pH 7.4 (50 mM), and $MgCl_2$ (2 mM). The mixture was pre-incubated at 37.0±0.2°C for 15 minutes before initiation of the reaction by addition of NADPH (1 mM). The reaction was continued for up to 2 h at 37.0±0.2°C with orbital shaking at 80 rpm. The reaction was stopped at 0 (pre-incubation), 5, 10, 15, 30, 60, 90 and 120 min by addition of 50 µL of diluted (1:1) phosphoric acid. The samples were analyzed for NC 2083 using the method described above. The amount remaining to be metabolized was calculated and plotted on a log-normal scale to calculate the slope of the initial part of the curve [20]. The metabolic half-life was calculated by dividing 0.693 by the slope.

3. Results

3.1 Recovery

Analyte recovery was consistent at different concentration levels. The mean±SD recoveries of NC 2083 from rat serum were 53.0±6.5, 55.8±3.9 and 55.3±3.2 percent at the LQC, MQC and HQC, respectively, whereas mean±SD recoveries from rat liver

Table 2.2. Intra-day assay precision and accuracy for NC 2083 determination by HPLC-UV detection in rat serum (N = 6)

Quality Control (QC)	Analysis Day	Observed concentration (mean ± S.D., ng/mL)	Precision[a]	Accuracy[b]
LLOQ (50 ng/mL)	1	48.7±2.3	4.8	97.4
	2	46.4±2.8	6.0	92.8
	3	50.6±2.3	4.5	101.1
LQC (150 ng/mL)	1	148.4±7.0	4.7	98.9
	2	136.7±3.2	2.3	91.1
	3	135.6±5.0	3.7	90.4
MQC (2500 ng/mL)	1	2564.8±68.3	2.7	102.6
	2	2611.5±68.2	2.6	104.5
	3	2504.1±29.0	1.2	100.2
HQC (4000 ng/mL)	1	4058.6±206.2	5.1	101.5
	2	4166.8±103.8	2.5	104.2
	3	4109.7±95.0	2.3	102.7

[a]Expressed as % R.S.D. ((S.D./mean) × 100%).

[b]Calculated as (mean determined concentration/nominal concentration) × 100%.

Table 2.3. Intra-day assay precision and accuracy for NC 2083 determination by HPLC-UV detection in rat liver microsomes (N = 6)

Quality Control (QC)	Analysis Day	Observed concentration (mean ± S.D., ng/mL)	Precision[a]	Accuracy[b]
LLOQ (50 ng/mL)	1	52.8±3.8	7.1	105.6
	2	49.8±3.4	6.9	99.7
	3	51.1±4.5	8.8	102.4
LQC (150 ng/mL)	1	148.2±9.5	6.4	98.8
	2	156.7±10.4	6.6	104.4
	3	147.6±6.3	4.3	98.4
MQC (2500 ng/mL)	1	2784.2±33.2	1.2	111.4
	2	2717.5±44.1	1.6	108.7
	3	2600.5±67.1	2.6	104.0
HQC (4000 ng/mL)	1	4386.4±84.8	1.9	109.7
	2	4241.1±91.1	2.2	104.1
	3	4441.0±75.1	1.7	110.1

[a]Expressed as % R.S.D. ((S.D./mean) × 100%).

[b]Calculated as (mean determined concentration/nominal concentration) × 100%.

Table 2.4. Inter-day assay precision and accuracy for NC 2083 determination by HPLC-UV detection in rat serum and rat liver microsomes (RLM) (N=18)

	Nominal Concentration (ng/mL)	Observed Concentration (Mean ± S.D., ng/mL)	Precision [a]	Accuracy [b]
Serum	50	48.7±2.9	5.8	97.4
	150	140.5±7.8	5.6	93.7
	2500	2560.1±70.9	2.8	102.4
	4000	4111.7±142.8	3.5	102.8
RLM	50	51.3±3.9	7.6	102.5
	150	150.8±9.4	6.2	100.6
	2500	2700.7±91.2	3.4	108.0
	4000	4322.5±135.4	3.1	108.1

[a]Expressed as % R.S.D. ((S.D./mean) × 100%).

[b]Calculated as (mean determined concentration/nominal concentration) × 100%.

microsomes were 50.9±2.7, 47.1±3.2 and 50.2±4.4, respectively. The recovery of internal standard at 4 μg/mL concentration from rat serum and rat liver microsomes was found to be 56.8±4.1 percent and 58.6±4.7 percent, respectively.

3.2 Method Validation

The method is specific for the determination of all the compounds in Table 2.1. Figures 2A and 2B show representative chromatograms from blank serum, plasma, blood and rat liver microsomes spiked with NC 2083 (with NC 2288 as internal standard) and NC 2324, respectively, as illustrations of specificity at two diverse mobile phase conditions. Adjustment in the ratio of acetonitrile and ammonium acetate buffer allowed chromatographic separation of other compounds containing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore with varying logP values (log P =1.93-6.40) (Table 2.1), and specificity was established for these compounds in all rat biomatrices evaluated (Figure 2.2). For all analogues, the chromatographic run time was less than 15 minutes.

Table 2.5. Stability of NC 2083 in rat serum and rat liver microsomes (RLM) under various storage conditions (N=6).

Quality Control (QC)	Storage Condition	Concentration[a] (mean±SD, ng/mL)	Precision[b]	Accuracy[c]
LQC (150 ng/mL)	0 hr	148.4 ± 7.0	4.7	98.9
	24 hr (AIS)	147.7 ± 6.3	4.3	98.5
	6 hr (BT)	119.5 ± 14.1	17.7	79.6
	6 hr (BT in dark)	117.3 ± 22.6	19.3	78.2
	6 hr (BT on ice in dark)	131.2 ± 4.5	3.4	88.7
	F/T-3rd Cycle	136.7 ± 1.8	1.3	91.1
	40 Day at -80ºC	133.0 ± 2.9	2.2	88.7
	0 hr-RLM	148.2±9.5	6.4	98.8
	24 hr (AIS)-RLM	162.1±8.1	5.2	104.0
	6 hr (BT on ice in dark)-RLM	150.4±11.7	7.8	100.3
MQC (2500 ng/mL)	0 hr	2564.8 ± 68.3	2.7	102.6
	24 hr (AIS)	2550.8 ± 52.9	2.1	102.0
	6 hr (BT)	1975.1 ± 41.1	2.1	79.0
	6 hr (BT in dark)	1999.5 ± 91.5	4.6	80.0
	6 hr (BT on ice in dark)	2408.0 ± 52.5	2.2	96.3
	F/T-3rd Cycle	2493.7 ± 34.3	1.4	99.8
	40 Day at -80ºC	2369.3 ± 40.6	1.7	94.8
	0 hr-RLM	2784.2±33.2	1.2	111.4
	24 hr (AIS)-RLM	2572.6±141.7	5.5	102.9
	6 hr (BT on ice in dark)-RLM	2608.1±129.2	5.1	101.5
HQC (4000 ng/mL)	0 hr	4058.6 ± 206.2	5.1	101.5
	24 hr (AIS)	4044.7 ± 198.2	4.9	101.1
	6 hr (BT)	3211.3 ± 90.9	2.8	80.3
	6 hr (BT in dark)	3276.2 ± 168.4	5.1	81.9
	6 hr (BT on ice in dark)	3826.6 ± 97.9	2.6	95.7
	F/T-3rd Cycle	3914.7 ± 67.5	1.7	97.9
	40 Day at -80ºC	3551.4 ± 80.6	2.3	88.8
	0 hr-RLM	4386.4±84.8	1.9	109.7
	24 hr (AIS)-RLM	4620.4±90.4	2.1	110.2
	6 hr (BT on ice in dark)-RLM	4187.4±128.2	3.1	104.7

[a]Back calculated plasma concentrations

[b]Expressed as % R.S.D. ((S.D./mean) × 100%)

[c]Calculated as (mean determined concentration/nominal concentration) × 100%

AIS-Auto-injector stability; BT- Bench-top stability; F/T-Freeze-thaw stability

microsomes were 50.9±2.7, 47.1±3.2 and 50.2±4.4, respectively. The recovery of internal standard at 4 µg/mL concentration from rat serum and rat liver microsomes was found to be 56.8±4.1 percent and 58.6±4.7 percent, respectively.

3.2 Method Validation

The method is specific for the determination of all the compounds in Table 2.1. Figures 2A and 2B show representative chromatograms from blank serum, plasma, blood and rat liver microsomes spiked with NC 2083 (with NC 2288 as internal standard) and NC 2324, respectively, as illustrations of specificity at two diverse mobile phase conditions. Adjustment in the ratio of acetonitrile and ammonium acetate buffer allowed chromatographic separation of other compounds containing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore with varying logP values (log P =1.93-6.40) (Table 2.1), and specificity was established for these compounds in all rat biomatrices evaluated (Figure 2.2). For all analogues, the chromatographic run time was less than 15 minutes.

The validation results of this method for the quantitative determinations of NC 2083 in rat serum and rat liver microsomes satisfied the criteria specified by USFDA. The validation of other analogues was not carried out at this point in time; however, the laboratories adopting this method need to perform a partial validation for individual compounds.

The limit of detection and limit of quantification based on signal to noise ratios of 3 and 10 were 18.4 and 61.5 ng/mL, respectively. The method was linear in the concentration range of 50 to 5000 ng/mL with average coefficient of determination values of ≥0.997. The accuracy across different concentration levels of the calibration curve in rat serum and inactivated rat liver microsomes, varied from 90.5-108.4 percent

while the average percent CV varied from 0.1-5.5 (data not shown). Calibration curves gave reliable reproducibility at different concentration levels on different occasions. Intra-day and inter-day precision and accuracy in rat serum and liver microsomes is shown in Tables 2.2, 2.3 and 2.4. Intra-day accuracy in rat serum and liver microsomes ranged from 90.4 to 104.5 percent and 98.4 to 111.4, respectively, while precision ranged from 1.2 to 6.0 percent and 1.2 to 8.8, respectively. Inter-day accuracy in rat serum and liver microsomes ranged from 93.7 to 102.8 percent and 100.6 to 108.1, respectively, while precision ranged from 2.8 to 5.8 percent and 3.1 to 7.6, respectively. Dilution integrity of the method in rat serum was assessed following 100 to 200-fold sample dilutions. The average percent accuracy values were 102.1±2.5 and 95.6±2.8, while precision values were within 2.5 and 2.9 percent at 100-fold and 200-fold dilution, respectively, indicating good dilution integrity. Dilution integrity in rat liver microsomes was not established because the microsomal stability was conducted at 5 μg/mL, which was within the linearity range.

Given NC 2083's sensitivity to light, autosampler stability (24 h), bench-top stability (6 h) under light and dark conditions and on ice under dark, freeze-thaw stability (three cycles), and long term storage stability at -80±5°C (40 days) of NC 2083 in serum samples were determined. Autosampler stability (24 h) and bench-top stability (6 h; on ice in dark) of NC 2083 in rat liver microsomes were established; however, the freeze-thaw stability and long term stability studies were not carried out because the microsomal stability study samples were processed immediately after the experiment. NC 2083 was stable under all conditions except on bench top under light and dark conditions for 6 h

(Table 2.5). These results indicated a need to use an ice bath under low light conditions during sample preparation.

3.3 Application of the HPLC-UV method to assess

3.3.1 Serum protein binding

More than 99.9 % of NC 2083 exists as bound to rat serum proteins. Only free form of the drug exhibits its pharmacological effect, and thus only 0.1 % of the compound in systemic circulation will be responsible for its action. Since serum protein-drug complexes are usually reversible, the high protein binding characteristics may contribute to a longer duration of action.

3.3.2 Metabolic stability of NC 2083 in rat liver microsomes

Figure 2.3 illustrates the metabolic stability of NC 2083 in rat liver microsomes (0.4 mg/mL). Substantial depletion of NC 2083 was observed within two hours of initiation of the incubation with more than half of the compound metabolized by this time. Substrate depletion was linear up to 10 minutes on log-normal scale with a coefficient of determination of 0.9985 and a slope of -0.0219 (data not shown). The calculated *in vitro* half-life of NC 2083 in rat liver microsomes was 31.6 min. Based on the classification of McNaney *et al*.[20], this compound has intermediate systemic clearance suggesting its suitability for further pharmacokinetic and pharmacodynamic evaluations.

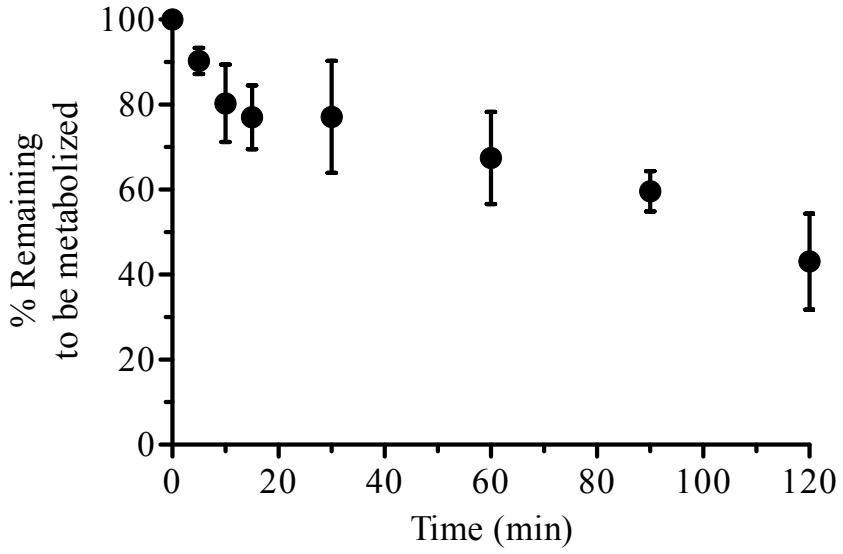


Figure 2.3 Mean ± SD of percent remaining to be metabolized of NC 2083 in rat liver microsomes (N = 3) using 0.4 mg/mL of protein concentration at different time intervals up to 2 h with incubation at 37±0.2°C.

4. Discussion

Currently, curcumin analogues containing the 1,5-diaryl-3-oxo-1,4-pentadienyl group are highly promising compounds in antineoplastic drug research. Curcumin itself is compound is attributed to poor absorption characteristics and/or first pass metabolism (mostly by the liver). Liver microsomal stability screening may identify the potential for extensive first pass metabolism, and such information is critical in the early phases of drug discovery to inform the rational drug design process. Early identification of curcumin analogues with similar pharmacokinetic problems is necessary to guide the rational drug design process. Rapid pharmacokinetic screening (*in vitro* and *in vivo*), though, requires a simple and versatile analytical method for the quantification of

different curcumin analogues in an appropriate preclinical species. This experimentation necessitates the development of a general HPLC-UV method that could quantify a large number of compounds containing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore within reasonably short chromatographic run times and with minimal alterations to chromatographic conditions.

Dimmock and coworkers synthesized a wide range of curcumin analogues possessing highly variable clogP values (range 1.93 to 6.40). We chose 13 compounds within the series that demonstrated good cytotoxicity against colon cancer cell lines and widely different logP values to develop a simple and versatile HPLC-UV method of analysis. The compound set included 4-piperidone analogues (Figure 2.1; R^3=-CH_2-NH-CH_2-) and cyclohexanone analogues (Figure 2.1; R^3=-CH_2-CH_2-CH_2-). This method requires minor modifications in the ratio of acetonitrile to ammonium acetate buffer to elute all compounds in a chromatographic run time of less than 15 minutes.

During the method development, a 60:40 acetonitrile:ammonium acetate buffer mobile phase resulted in analyte retention times that were directly proportional to the polarity of compounds, whereas this trend was reversed when the acetonitrile composition was reduced to 40%. This outcome is difficult to explain as the decrease in elution strength of the mobile phase should increase the retention times of non-polar compounds more than the polar ones; however, the observed trend was reversed. Nevertheless, we could achieve suitable and specific chromatographic separation of all chosen compounds in all rat biomatrices evaluated.

We subsequently validated this method for a promising compound, NC 2083, in rat serum to allow for further pharmacokinetic evaluations. The method was accurate,

precise and specific. NC 2083 was stable under most sample preparation, analytical and storage conditions but was unstable at bench top for 6 hours. Sample preparation under low light conditions with use of an ice bath provided assurance of NC 2083 stability. Given its putative affinity for thiol-containing proteins, we investigated its ability to bind covalently to albumin to explain the apparent loss of NC 2083 in rat serum during storage at room temperature. Our studies indicated minimal covalent binding with serum albumin (unpublished data) and the cause for this apparent loss of NC 2083 remains unknown.

5. Conclusion

Given the growing interest in 1,5-diaryl-3-oxo-1,4-pentadienyl containing curcumin analogues as a potential class of highly cytotoxic and selective antineoplastic compounds, we describe a versatile, simple, rapid and specific HPLC-UV method for the quantification of different compounds containing this pharmacophore in various bio-matrices. With minor modification in the mobile phase composition, this method accommodates compounds with a very wide range of clogP values (1.93 to 6.40) without losing specificity. Therefore, this method can be directly adopted for preclinical pharmacokinetic studies of this class of compounds; however, a partial validation for suitability of this method to individual compounds in laboratories adopting this method will be necessary.

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CHAPTER 3

PHARMACOKINETIC DIRECTED DISCOVERY OF A NOVEL ANTIMALARIAL AGENT

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Pharmacokinetic Directed Discovery of a Novel Antimalarial Agent

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Abstract

3,5-bis(4-Chlorobenzylidene)-1-[4-2-(4-morpholinyl)ethoxyphenylcarbonyl]-4-piperidone hydrochloride, 2, a potent cytotoxin in the series of 1-acyl-3,5-bis(benzylidene)-4-piperidone hydrochlorides 1, was inactive against SW620 colon tumor xenograft in athymic nude mice. Retrospective pharmacokinetic evaluations identified poor permeability characteristics in the Caco-2 model, intermediate metabolic lability, unfavourable physicochemical properties (e.g., large log P and high molecular weight), and sequestering of the compound to erythrocytes, which likely contributed to the lack of in vivo efficacy. Given its positive tropism to the erythrocyte, NC 2083 was evaluated for its growth-inhibiting properties towards Plasmodium falciparum, the malarial parasite whose life cycle includes the erythrocyte. This compound demonstrated significant toxicity to both a sensitive and a drug resistant strain of P. falciparum. This discovery reveals NC 2083 is a prototypic molecule for the development of candidate antimalarial drugs.
Keywords: Pharmacokinetics; Caco-2 cells; thiol concentrations; antimalarial

1. Introduction

A considerable amount of research has been described recently in regard to the antineoplastic properties of compounds containing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore [1]. There are several reasons for this interest. First, a number of conjugated unsaturated ketones react with thiols but not amino or hydroxyl groups [2,3] which are found in nucleic acids. Hence, these enones may not have the genotoxic properties displayed by a number of contemporary anticancer drugs [4]. Second, the initial lowering of thiol concentrations in malignant cells followed by a subsequent chemical insult has been shown on occasions to be more detrimental to tumours than normal cells [5,6]. In the case of the 1,5-diaryl-3-oxo-1,4-pentadienes, an initial interaction with thiols may take place followed by alkylation of cellular constituents at the remaining olefinic atom.

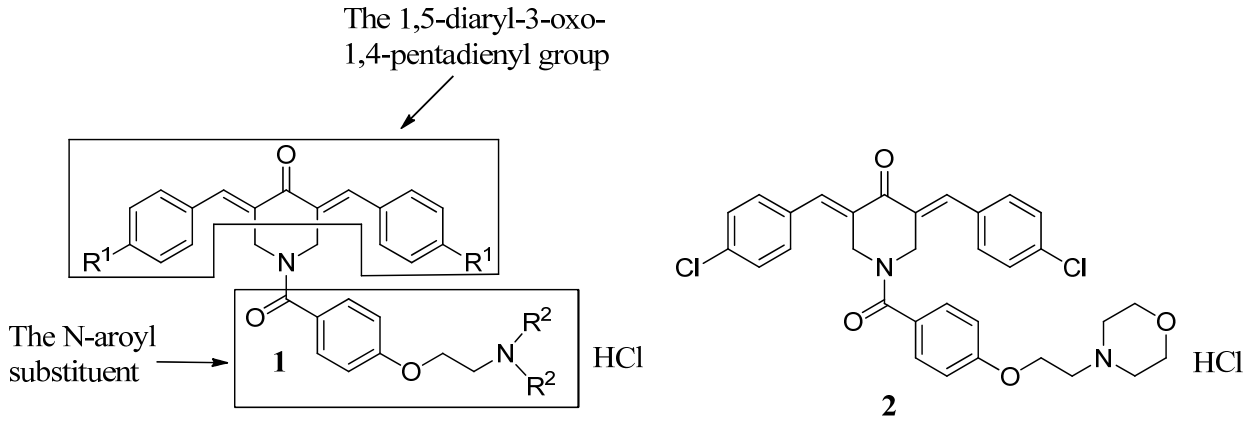

Figure 3.1: The structures of series **1** and compound **2** (**NC 2083)**.

Recently the potent cytotoxicity of a series of 1-acyl-3,5-bis(benzylidene)4-piperidone hydrochlorides 1 have been described [7]. These compounds possess the 1,5-diaryl-3-oxo-1,4-pentadienyl group, which is capable of interaction with cellular thiols. In addition, a 1-acyl group is present, which may allow further ligand binding to occur. In particular a member of this series of compounds namely NC 2083 exhibited IC50 values in the submicromolar and very low micromolar ranges towards a variety of human cancer cell lines. For example, the IC50 values of NC 2083 towards human HCC-2998 and SW-620 colon cancer cells are <0.005 and 1.45 μM, respectively. Subsequent studies revealed that NC 2083 had greater lethal effects towards a number of malignant cell lines than normal cells [8] and it possessed MDR-revertant properties [9]. In addition, this compound was well tolerated in mice [10].

Due to the promising in vitro efficacy of NC 2083 against colon cancer cell lines, this compound was administered to athymic nude mice with subcutaneously implanted SW-620 cancer cells; however, as reported herein, NC 2083 failed to control the growth of this tumour. The lack of in vivo efficacy and toxicity prompted a number of pharmacokinetic (PK) investigations. The identification of unfavorable PK characteristics may explain the in vivo observations and also may enable the design of further series of analogs having improved PK properties coupled with anticancer activity in vivo. These retrospective PK evaluations of NC 2083 led to the discovery of its novel antimalarial activity against sensitive and drug resistant strains of Plasmodium falciparum, a parasite responsible for the devastating global disease, malaria. In our current work, we describe the discovery process from a putative anticancer compound to a promising antimalarial agent.

2. Results and Discussion

Intraperitoneal injection of doses of 200, 400 and 600 mg/kg of NC 2083 to nude mice bearing the SW-620 tumour revealed that this compound had no inhibitory effect on tumour growth. A PK problem was suspected namely that NC 2083 has low bioavailability. In order to address this issue, a dose of 100 mg/kg of NC 2083 was administered to rats per os and 1 mg/kg by the intravenous route. Serial blood samples were collected over a 24 hour period. Examinations of the serum obtained from aliquots of blood revealed that any of the dienone NC 2083 that might be present was below the limit of quantification which was shown previously to be 50 ng/mL [11].

Although a number of factors may explain the absence of NC 2083 in serum, low bioavailability of NC 2083 due to poor permeation across the gut wall and/or extensive first-pass metabolism were initially suspected. An in vitro test which affords information on intestinal permeation uses the Caco-2 monolayer [12,13]. The transport rate of NC 2083 across the Caco-2 cells was linear up to two hours and neither metabolite peaks (upon HPLC analysis) nor loss of NC 2083 was noted during this time frame. The apparent permeability (Papp) of NC 2083 across the colon adenocarcinoma cell monolayer (Caco-2) was 1.20±0.07×10-6 cm/s. When compared to metoprolol (Papp = 29.88±3.17 ×10-6 cm/s), a marker compound used to segregate compounds into low and high permeability classes of the Biopharmaceutics Classification

System, NC 2083 can be considered a low permeability compound. In vitro hepatic microsomal studies can provide information regarding the metabolic stability of a compound. Compound NC 2083 exhibited an in vitro half life of ~32 minutes in rat liver microsomes suggesting that it is an intermediate clearance compound. The dienone NC 2083 also exhibited extensive binding to serum protein (> 99.9 percent) [11], which is consistent with its hydrophobic characteristics. In summary, these data suggest that additional factors are needed to explain the absence of NC 2083 in serum samples following intravenous administration. However, the low intestinal permeability, high plasma protein binding and intermediate clearance are likely to contribute to the lack of in vivo efficacy following extravascular administration on NC 2083 to tumour-bearing athymic nude mice.

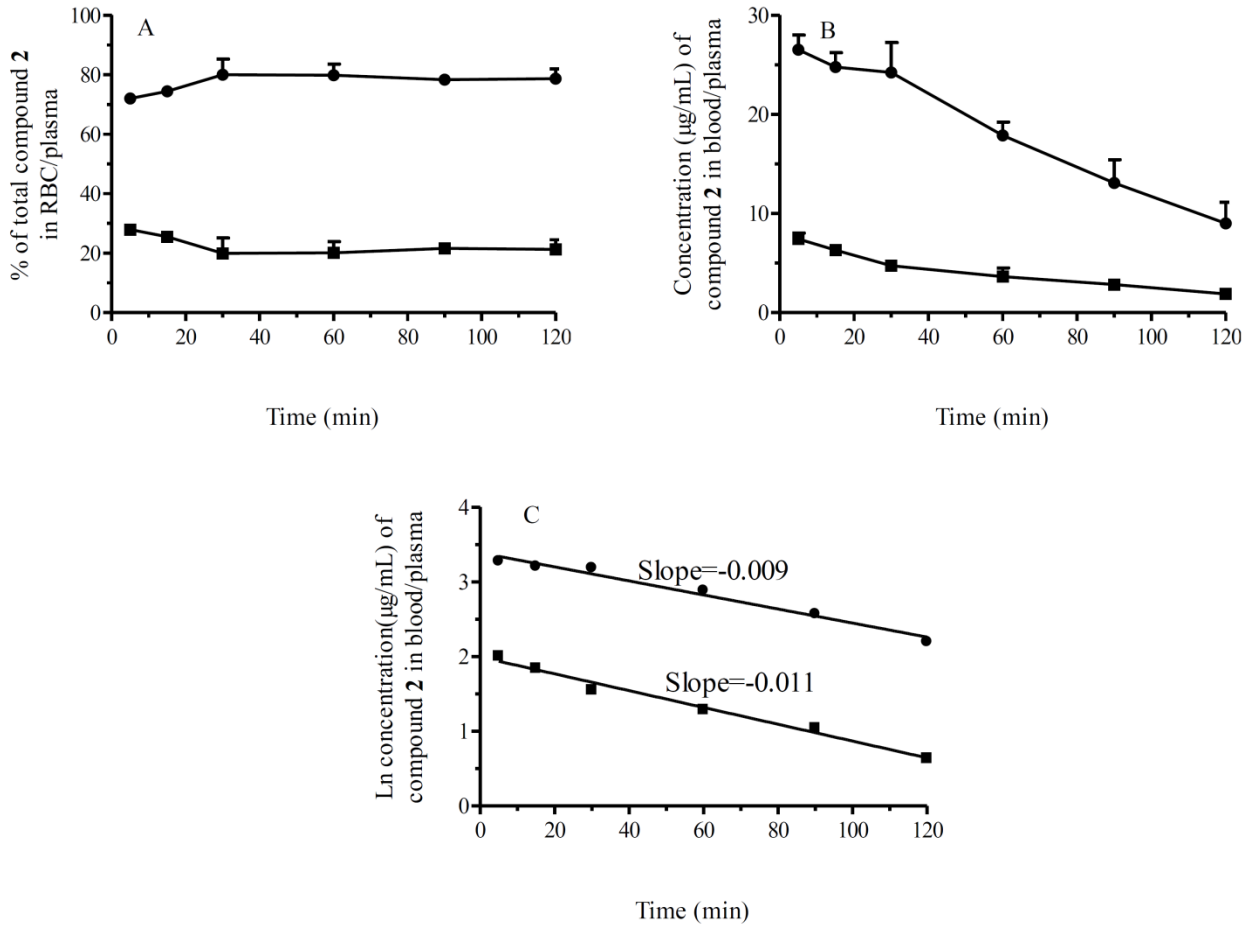

Figure 3.2. The concentrations of compound **2** (**NC 2083)** in blood (●) and plasma (■) expressed as the percentage (A) and also in μg/mL in both linear (B) and logarithmic (C) relationships.

An additional reason for the absence of NC 2083 in serum includes its passage into erythrocytes or very rapid peripheral distribution. In order to evaluate this hypothesis, a solution of NC 2083 was added to rat blood and the mixture was incubated at 37°C for two hours to assess partitioning into erythrocytes (Figure 3.2). Within 5 minutes of incubation, 75% of NC 2083 partitions into erythrocytes and reaches a maximum of 80 % within 30 minutes and stays at 80% up to 2 h of incubation (Figure 3.2A). The data in Figure 3.2B reveal the concentration of NC 2083 in blood and plasma at different time intervals. The higher concentration of NC 2083 in erythrocytes suggests that NC 2083 has a tendency to accumulate in red blood cells. Upon log transformation of the concentration versus time profile (Figure 3.2C), the terminal slopes were similar for erythrocytes and plasma, which indicates a rapid equilibration of NC 2083 between plasma and erythrocytes. The gradual decrease in the concentration in RBCs and plasma suggests the possibility that the compound reacts with various endogenous constituents in the erythrocytes and the plasma. Compound NC 2083 and related analogs were designed as thiol alkylators [7] and therefore interaction with cellular thiols, especially glutathione (GSH), was a possibility. Since NC 2083 concentrates preferentially into red blood cells (Figure 3.2A), this compound was incubated with erythrocytes and the concentration of GSH measured over two hours. The results are presented in Table 3.1 which reveal a nonsignificant reduction in GSH concentration. Hence the disappearance of NC 2083 from blood and plasma (Figures 3.2B and 3.2C) suggests that the compound is reversibly binding with one or more cellular components other than GSH.

Table 3.1. Effect of **NC 2083** on the concentration of total glutathione in erythrocytes[a]

	Total glutathione concentration (μM)	
Time (min)	Mean ± SD	% Decrease
0	185.78±3.64	0
5	162.40±5.62	12.58
15	162.84±8.69	12.34
30	161.87±6.97	12.87
60	169.16±8.23	8.95
90	171.82±1.89	7.51
120	165.60±10.74	10.86

[a]Compound **NC 2083** (25 μg/mL) was incubated with red blood cells at 37°C and aliquots were analyzed at different time intervals.

Two important observations were made from these studies which have valuable lessons in directing further drug research. First, the lack of in vivo anticancer properties of NC 2083

towards the SW-620 tumour is likely due to poor bioavailability and extensive serum protein binding characteristics. This conclusion was drawn from the in vivo experimentation and the use of Caco-2 cells, rat hepatic microsomes, and in vitro serum protein binding analyses. In addition, Lipinski's rules state that for good oral absorption, a compound with drug-like properties should have a molecular weight of less than 500, a logP value of less than five, the number of hydrogen bond donors should be less than five and the number of hydrogen bond acceptor atoms is less than ten [14]. The free base of NC 2083 violates two of these criteria having a molecular weight of 577.49 and a logP value of 6.4. Hence synthetic endeavours in the future should be directed towards reducing both the size and hydrophobicity of the molecules. For example, elision of the aryl ring in the N-acyl substituent and changing the 4-morpholinyl group to a primary amino function reduces the molecular weight to 431.31. In addition, the introduction of hydrophilic substituents into the arylidene aryl rings with hydroxy and carboxy groups which have π values of - 0.67 and - 0.32 [15] will reduce the logP value.

Second, an intriguing feature of the current investigation is the positive tropism of NC 2083 for red blood cells. One of the stages in the life cycle of the malarial parasite is located in the erythrocytes. Hence if the parasite is sensitive to NC 2083, the compound may have promise as an antimalarial drug. In fact, when one considers some of the characteristics of an ideal antimalarial, it is surprising that NC 2083 fulfills the following criteria.(1) The compound should accumulate preferentially in red blood cells. This stratagem is based on the observation that the sporozoites from a mosquito bite multiply in the liver forming merozoites which, following the rupture of their host cells, pass into the blood stream and invade red blood cells. The antimalarial drugs such as chloroquine, mefloquine and artemisinin have been used successfully to treat malaria due to their having a positive tropism for and accumulation within the red blood cells. However, the development of drug resistance by the malaria parasite has created an exigency for new antimalarial drugs with potent activity against resistant parasite strains. (2) A corollary of the required affinity for erythrocytes is that there is only limited distribution of the candidate drug to other tissues. Extensive serum protein binding suggests that extensive distribution of NC 2083 to various tissues is likely minimal which reduces the likelihood of off-target toxicity. (3) In regard to creating molecules which are effective against multidrug resistant (MDR) strains of Plasmodium species, the compounds should be structurally unrelated to current medication. In this way both sensitive and resistant strains of the malarial parasite may be susceptible to such

compounds. (4) An ideal compound would have MDR-revertant properties per se. This attribute could, for example, antagonize the development of Pgp-dependent drug resistance. As mentioned earlier, NC 2083 and related analogs have potent Pgp-revertant properties [9]. Finally the recent practice of employing antineoplastic agents in the treatment of malaria [16-18] confirmed the decision to evaluate NC 2083 against Plasmodium falciparum.

Compound NC 2083 was assessed against the drug sensitive D6 strain and drug resistant C235 strain of Plasmodium falciparum. The IC50 of NC 2083 towards the D6 strain is 0.37 μg/mL (0.60 μM) compared to the IC50 of chloroquine and mefloquine at 0.02 μM. This result suggests that NC 2083 is a lead in the evolution of novel antimalarials. When evaluated against the C235 strain of the parasite, which contains multiple copies of the pfmdr1 transporter gene and is resistant to many antimalarial drugs, compound NC 2083 inhibited the growth of the C235 protozoan displaying an IC50 figure of 1.21 μg/mL or 1.97 μM. This promising biodata indicates that analogs of NC 2083 should be assessed in a similar manner for the following reasons namely (1) to determine if this series of compounds display antimalarial properties and thus one can claim the discovery of a novel class of antimalarials (2) to find compounds with increased potencies towards malarial parasites i.e. analogs with the IC50 values of 0.1 μg/mL or less and (3) to find those molecules in which the IC50 figures between sensitive and resistant strains of Plasmodium species are the same revealing compounds which have the potential to deal with a major clinical problem of drug-resistant strains of malarial parasites [19,20].

3. Conclusions

This study has provided some indication why the promising cytotoxic properties of NC 2083 did not lead to in vivo activity. The important discovery of the affinity of NC 2083 for erythrocytes was unexpected but fortuitous insofar as it led to the discovery of the antimalarial properties of NC 2083. Thus this study revealed that NC 2083 is a prototypic molecule for developing further candidate anticancer and antimalarial drugs. Some directions for the synthesis of analogs of NC 2083 which may have more favourable PK characteristics were made. This discovery indicates the need to develop suitable formulations or to undertake structural modification of NC 2083 in the light of improving its pharmacokinetic characteristics.

4. Experimental

4.1 In Vivo Evaluation of NC 2083 Against the SW-620 Tumour

The SW-620 tumour was passsaged in female athymic NCr-nu/nu mice. Doses of 200, 400 and 600 mg/kg in a vehicle of 25% dimethylsulfoxide and 75% polyethyleneglycol 400 were injected intraperitoneally into mice. A control group received the vehicle alone. Each treatment group and mice injected with vehicle consisted of six animals. The positive control, irinotecan (60 mg/kg), in saline was administered intravenously to ten mice. Doses were administered every fourth day for a total of three doses. Tumor weights were recorded. The median doubling times of the vehicle and irinotecan treated groups were 9.8 and 24.1 days, respectively. No delay in tumour growth was observed in those animals which received varying doses of NC 2083.

4.2 Pharmacokinetic Studies

All procedures were conducted in accordance with the Canadian Council of Animal Care guidelines for the care and use of laboratory animals and were approved by the Animal Research Ethics Board of the University of Saskatchewan. One day prior to the study, the right jugular vein of each rat was surgically cannulated (silastic tubing, 0.64-mm i.d.1.19-mm o.d.,Dow

Corning) under isoflurane anesthesia. The next day 100 mg/kg of NC 2083 in a vehicle of 25% dimethylsulfoxide and 75% polyethyleneglycol 300 were administered orally to each of the rats. Blood samples were taken just before dosing and after 0.25, 0.5, 0.75, 1, 2, 4, 8, 12 and 24 h. The blood was left to coagulate in the dark and centrifuged at 10,000 rpm for five minutes. The serum was removed and stored at -80°C until analyzed by a literature method [11]. The methodology was repeated except that 1 mg/Kg of NC 2083 in a vehicle of equal proportions of dimethylsulfoxide, polyethyleneglycol 300, tween 80 and ethanol was administered via a femoral cannula and blood was collected from the jugular cannula just prior to dosing and after 5, 10, 20, 30 and 45 minutes as well as 1, 2, 4, 8, 12 and 24 hours.

4.3 Erythrocyte Partitioning of **NC 2083**

Blood, collected by cardiac puncture from a male Wistar rat which had been anaesthesised by isoflurane, was placed in tubes containing ethylenediaminetetracetic acid. An aliquot of a stock solution of NC 2083 in dimethylsulfoxide (2.5 mg/mL) was added to blood so that a concentration of 25 μg/mL was attained. The mixture was incubated at 37°C with shaking at 80

rpm. Two aliquots of the blood samples were collected after 5, 10, 15, 30, 60, 90 and 120 minutes. One aliquot was centrifuged at 2000×g for 10 minutes and stored at -80°C while the other aliquot was stored in a similar way. Subsequently, the concentrations of NC 2083 in whole blood and in the plasma from the centrifuged sample were determined by a literature method [11].

4.4 Glutathione Estimation

Red blood cells which were obtained by centrifugation as described in the erythrocyte partitioning experiment vide supra were lysed with the addition of ice cold water and centrifuged at 10,000×g for 15 minutes at 4°C. The supernatant was removed and deproteinated by the addition of an equal volume of metaphosphoric acid (10% w/w). Protein was separated by centrifugation at 5,000×g for five minutes and the supernatant was stored at -20°C until analyzed. After the samples were thawed, 50 μL of a solution of triethanolamine in water (53.1 %) was added for each milliliter of the supernatant. The glutathione content was determined using a Cayman glutathione binding assay kit in kinetic mode using a Biotek Synergy HT microplate reader [21].

4.5 Permeability Experiment using Caco-2 Cells

The Caco-2/TC-7 cells were obtained from Dr. W. Koester, Vaccine and Infectious Disease Organization, University of Saskatchewan and grown in Dulbecco Modified Eagle's Medium containing fetal bovine serum (10%) and non essential amino acids at 37°C in an atmosphere of oxygen (95%) and carbon dioxide (5%). Monolayers were grown in Transwells (Corning Life Sciences) and the media was replaced in the apical and basal compartments three times every week. After 21 days, the cells were washed three times with Hank's Balanced Salt Solution (HBSS) containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 10 mM), pH 7.4. The monolayers were equilibrated at 37°C for 0.5 h and then the transepithelial electrical resistance (TEER) values (500-700 mΩ) were obtained using a Millicell ERS system [Millipore (Canada) Ltd]. A mixture of NC 2083 (50 μM) in HBSS containing human serum albumin (10%) and dimethylsulfoxide (1%) was added to the apical compartment of the Transwells. After incubation at 37°C for 2 h in an atmosphere of oxygen (95%) and carbon dioxide (5%), aliquots were collected from both the apical and basal compartments and stored at

-20°C. Analysis was performed by extraction of a 100 µL sample with tert-butyl methyl ether as described previously [11]. The cells were washed three times with HBSS containing HEPES (10 mM), pH 7.4 and equilibrated for 0.5 h before measuring the post-assay TEER values. Lucifer yellow in HBSS was added to the apical compartment of the Transwell and incubated under the same conditions for 1 hour. Lucifer yellow rejection was determined by the assessment of fluorescence in the basal compartment at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a Biotek Synergy HT microplate reader (Fisher Scientific, Canada). Lucifer yellow rejection rates and post-assay TEER values were used to assess monolayer integrity during the permeability studies.

4.6 Evaluation of NC 2083 against D6 and C235 Strains of Plasmodium falciparum

Compound NC 2083 was screened against D6 and C235 strains of Plasmodium falciparum using the SYBR Green-I based fluorescence assay [22]. In brief, different concentrations of the compound were added to the parasites in a supplemented RPMI 1640 medium at a starting parasitemia of 0.3% and a haematocrit of 2% for a dose response determination. The cultures were incubated at 37°C in a humidified atmosphere of carbon dioxide (5%), oxygen (5%) and nitrogen (90%) for 72 hours. Lysis buffer containing SYBR Green-I dye was added to each well. After 24 hour incubation in the dark at room temperature, the plates were examined for relative fluorescence. The IC50 value of NC 2083 was generated with GraphPad Prism (GraphPad Software Inc., San Diego, USA) using the nonlinear regression (sigmoidal dose-response/variable slope) equation.

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Army Institute of Research are solely those of the authors and do not reflect the views of the US Department of Defense. R.S.P. Singh was supported by a Dean's scholarship, College of Graduate Studies and Research, University of Saskatchewan.

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CHAPTER 4

COMPARISON OF CACO-2 AND MDCK PERMEABILITY OF THE CYTOTOXIC CURCUMIN ANALOGUES AND A RELATED CHALCONES

Ravi Shankar Prasad Singh, Deborah Michel, Umashankar Das, Jonathan R. Dimmock, Jane Alcorn

Comparison of Caco-2 and MDCK Permeability of Cytotoxic Curcumin Analogues and a Related Chalcone

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Abstract

Curcumin demonstrates good in vitro antineoplastic activity but poor oral bioavailability. To improve pharmacokinetic and pharmacodynamic characteristics of curcumin, we synthesized analogues with enhanced cytotoxicity against various cancer cell lines. We then wished to evaluate promising curcumin analogs for their in vitro permeability characteristics. Although the Caco-2 model system is the 'gold standard', MDCK model often correlates well with Caco-2. No study has compared these two intestinal permeability screening assays for cytotoxic compounds that have potential to exert differential effects on monolayer integrity and permeability characteristics by virtue of their cytotoxic potential. Our objective was to determine the suitability of and correlation between Caco-2 and MDCK models for permeability assay of curcumin analogues and a related chalcone. We selected compounds with at least one conjugated α,β-unsaturated ketone exhibiting high cytotoxicity in NCI screening. Aliquots from acceptor compartments in transwell systems were analyzed using reverse phase HPLC-UV to calculate the apparent permeability (Papp) and efflux ratio. Both assays show similar apical-to-basal transport and good Spearman's rank correlation of apparent permeability values, but curcumin analogues increased Lucifer Yellow (LY) permeability, a marker of paracellular transport, in MDCK. Furthermore, these compounds caused up to 8% cell loss when incubated with MDCK for 2 h, which correlated with the Papp of LY in MDCK; however, no loss in cell viability was observed in Caco-2. Our data suggests the use of Caco-2 for permeability assessments of curcumin analogues. However, MDCK may provide a suitable ranking of permeabilities for rapid screening during lead optimization in drug discovery.

Keywords: curcumin analogues; Caco-2; MDCK; correlation; chalcone

Introduction

Curcumin, a widely used spice and coloring agent in Asian countries (1, 2), exhibits antineoplastic (3), antimutagenic (4, 5) and anti-inflammatory (6) activities. However, curcumin has poor oral bioavailability due to high first pass gastrointestinal and hepatic metabolism, and requires a very high dose to achieve minimum effective concentrations in the systemic circulation (7). Thus, many researchers have modified curcumin in hopes to improve antineoplastic activity and to enhance oral bioavailability.

In order to estimate oral bioavailability of compounds in early phases of drug discovery, permeability of compounds across biological membranes is established. In permeability screening, various assays which mimic in vivo intestinal absorption are employed such as the PAMPA (parallel artificial membrane assay), the Caco-2 (colon adenocarcinoma cells) assay, the MDCK (Madin-Darby canine kidney) assay, and the rat everted gut sac assay (8). PAMPA and Caco-2 are the most widely used models for this purpose. PAMPA is preferred for high-throughput screening but only assesses passive permeability characteristics (9-11). Permeability across monolayers such as Caco-2 and MDCK mimics better the intestinal epithelium, morphologically and functionally. Other methods of permeability screening are rare and require animal sacrifice.

The Caco-2 monolayer, which is derived from the human colon adenocarcinoma, is the only in vitro system for human permeability screening accepted by regulatory bodies. The apparent permeability across Caco-2 monolayer is considered “gold standard” due to its very good correlation with human in vivo intestinal absorption and widely used in drug discovery processes (12, 13). Caco-2 grown on Transwell® inserts in 12- or 24- well plates requires 21-28 days to polarize, which limits high throughput screening and increases the potential for contaminations. Recently, a 3-day culture and automation of this assay has been employed to increase the throughput {{572 Chong,S. 1997}}. Transporter expression is inadequate in a 3-day culture and automation of the assay in sterile conditions requires an enormous investment, which is not always possible especially in academic situations.

Another widely used assay, MDCK, which also exhibits morphological and functional similarity with human enterocytes, correlates with in vivo human intestinal absorption and is suitable for high throughput screening due its very short culture period (3 days) (14). Rank ordering of compounds based on their apparent permeability coefficient in MDCK and Caco-2

correlates well but permeability coefficients in Caco-2 appears to be more relevant with BCS classification (14). This correlation study suggested MDCK as a viable alternative to Caco-2 screening. However, the correlation studies failed to include cytotoxic compounds, which is a significant limitation as cytotoxic compounds have potential to be disruptive to polarized monolayer integrity (15-18). The integrity of the polarized cell monolayer is the indispensible requirement in these types of permeability assessments as the polarized epithelium and tight junctions create a barrier for the movement of drug from one compartment to other, a barrier that mimics epithelia in vivo.

Curcumin analogues tend to exhibit greater cytotoxicity than existing anticancer agents such as melphalan and 5-fluorouracil. Such high cytotoxicity against many cancer cell lines causes concern as these compounds have potential to cause loss of cell viability and, in turn, disruption of the polarized monolayer. Since these cytotoxic agents have differential effects on different cancer cell lines, we have concern regarding their influence on the commonly used cancer cell lines exploited for permeability assessments. Thus, in our current work, we selected highly cytotoxic compounds from a series of curcumin analogues and a related chalcone and tested their effect on the Caco-2 and MDCK cell lines. In addition, to advance our own drug discovery efforts, we evaluated the permeability characteristics of the selected analogues and a related chalcone in these two systems and correlated the apparent permeability values of MDCK and Caco-2 assays for cytotoxic compounds in order to draw conclusions regarding the most appropriate model system and the influence of cytotoxic compounds on these commonly employed permeability screening assays.

Materials and Methods

Chemicals and Reagents

The compounds were synthesized in the College of Pharmacy, University of Saskatchewan by previously reported methods for NC 158, 823, 236, 2242 (19) NC 710, 2209, 2165, 2166 (20) NC 2314, 2315, 2423 (21) while preparation of other compounds will be reported in due course. Phosphate buffered saline (PBS), versene, trypsin (2.5%), heat inactivated fetal bovine serum (FBS) and non-essential amino acids were obtained from Invitrogen Corporation (Burlington, ON, Canada). Dulbecco Modified Eagle's Medium (DMEM) and Transwell® plates and polyethylene inserts (6.5 mm diameter, 0.4 μm pore size) were procured from Fisher Scientific

(Toronto, ON, Canada). Minimal Essential Medium and all other chemicals unless otherwise indicated were obtained from Sigma-Aldrich (Oakville, ON, Canada). MilliQ water at 18.2 MΩ resistance was obtained from a MilliQ water purification system (Millipore, MA, USA). Millicell ERS system for the measurement of transepithelial electrical resistance (TEER) was purchased from Millipore (Billerica, MA, USA). Caco-2/TC-7 was gifted by Dr. Wolfgang Koester, Vaccine and Infectious Disease Organization (VIDO), University of Saskatchewan (Saskatoon, Saskatchewan, Canada) and MDCK-II cell line was purchased from ATCC (American Type Culture Collection, Rockville, USA).

Cell Culture

The Caco-2/TC-7 cell line was subcultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum and 1% non essential amino acids. Cells were grown in an atmosphere of 95% O2 and 5% CO2 with 95% humidity at 37°C. At 80-90 % confluence, cells were passaged using 0.25% trypsin in versene and seeded onto Transwell® inserts (1×105 cells per insert; insert area=0.33 cm2) preequilibrated with complete culture medium. Media was replaced with fresh complete media three times in a week for 21 days. Permeability assay was conducted on the 21st day.

MDCK cells were subcultured in Minimal Essential Medium (MEM) supplemented with 10 % FBS and gentamicin (50 µg/mL). Cells were passaged as above for Caco-2, but the cell seeding density for MDCK was 2.2×105 cells per insert (insert area=0.33 cm2). Media was replaced with fresh media one hour and 24 hour post seeding. The permeability assay was conducted the third day.

Cytotoxicity Assay

Cytotoxicity was determined using the sulforhodamine B assay (22). . Cells in the exponential phase of growth were harvested using 0.25% trypsin in PBS. Harvested cells were re-suspended in complete growth media and 1.5 ×104 and 3.0×103 cells/cm2 for Caco-2 and MDCK, respectively, were seeded in 96-well flat bottom plates. The cells were incubated with 50 µM of test compounds and a control (1% DMSO) for 2 h (duration of permeability assay). After incubation, the cells were fixed by the addition of 50 µL of 50 % w/v trichloroacetic acid (TCA) in water into each well. Subsequently, the plates were incubated at 4°C for an hour and washed with tap water and subsequently air dried. The fixed cells in each well were stained by

the addition of 100 µL of 0.4% w/v sulforhodamine B solution in 1% acetic acid and incubated for 10 minutes at room temperature. The plates were quickly washed with 1% acetic acid and air dried. The stains of the cells were dissolved in 200 µL of 10 mM Trizma base (unadjusted pH) and absorbance was measured at 515 nm.

Monolayer Integrity Testing

On the day of the permeability assessments, cells were washed three times with transport medium (Hank's balanced saline solution with 10 mM HEPES, pH 7.4) and equilibrated for 30 min at 37°C, in 95% humidity with 5% CO_2 and 95% O_2 before the measurement of pre-assay transepithelial electrical resistance (TEER). Electrodes of the Millicell ERS system were also equilibrated in transport medium at 37°C for 15 minutes before resistance measurements. The TEER (Ω.cm2) values were measured using Millicell ERS system. After the permeability assay, post assay TEER was measured in the same manner as the pre-assay TEER measurements.

Post-assay Lucifer yellow (LY) rejection rate was also determined to ensure the integrity of the monolayer throughout the assay period. At the end of the permeability assay, cell monolayers were washed three times with the transport medium and equilibrated for 30 min. Transport media on the apical side was replaced with 0.2 mL of Lucifer yellow solution (100 µg/mL) and incubated at 37°C in an atmosphere containing 5% O_2, 95% CO_2 and 95% humidity for 1 h. The fluorescence emission of LY (excitation wavelength = 485 nm, emission wavelength = 535 nm) in basal compartment was read using a Biotek Synergy HT microplate reader (Fisher Scientific, Nepean, ON, Canada). The concentration of LY in basal compartment was determined by comparing fluorescence emission of basal compartment against standards after background subtraction.

Permeability Assay Conditions

The transport media was prepared by addition of 10 mM HEPES buffer to Hanks balanced salt solution. The donor solution of test compounds (50 µM) were prepared in transport media containing 1% dimethyl sulphoxide (DMSO). All test compounds were evaluated for their solubility (>50µM) in transport medium containing 1% DMSO and stability for the duration of the permeability assay (2 h). Compounds failing these criteria were not selected for the assay. To maintain sink conditions, 1% DMSO was added to transport media to prepare the acceptor solution. To study the apical-to-basal transport of each compound, a 0.2 mL aliquot of the donor

solution containing a test compound was added to the apical compartment and 0.6 mL of acceptor solution was added to the basal compartment. In order to study the basal-to-apical transport, 0.2 mL of acceptor solution was added to the apical compartment and 0.6 mL of donor solution containing test compounds was added to basal compartment. All permeability assays were conducted in triplicate and Transwell plates were incubated at 37°C for 2 h in an atmosphere of 95% humidity, 5% O2 and 95% CO2. At the end of assay period (2 h), samples were collected from acceptor compartment and analyzed using HPLC-UV. The permeability of representative compounds of the series was linear up to 2 h (data not shown).

Quantitative Analysis of NC Compounds

Samples were analyzed by modification of an isocratic reverse phase HPLC method reported earlier (23) An aliquot (50 µL) from the acceptor compartment was injected onto a Zorbax XDB-C18 (150 mm × 4.6 mm, 5 µm) column (Agilent Technologies, Mississuaga, ON Canada) under gradient elution using acetonitrile and ammonium acetate buffer (5 mM, pH 5.0) with detection wavelength set at 330 nm. The column was equilibrated with 20% ammonium acetate buffer and 80% acetonitrile for 3 min, and subsequently the percent of ammonium acetate buffer changed linearly to 40% by 8 min, to 50% by 12 min, to 80 % by 16 min , and maintained until 24 min before re-equlibrating with the initial mobile phase condition before then next run. The flow rate was 1.0 mL/min and run time was 30 min. A standard curve was constructed for each compound separately using a weighted linear or quadratic regression (with $1/X^2$ weighting factor) using GraphPad Prism version 5.0 for Windows, GraphPad Software (San Diego, California, USA).

Prediction of Molecular Properties

Molecular properties (logP, molecular weight, total polar surface area, number of rotatable bonds, number of hydrogen bond donors, number of hydrogen bond acceptors) of compounds were calculated using Molinspiration online cheminformatics services (www.molinspiration.com). The apparent permeability (Papp) of compounds was calculated using Equation 4.1:

$$P_{app} = \left(\frac{dQ}{dt}\right) \times \frac{1}{C_0} \times \frac{1}{A_f} \qquad \text{Equation 4.1}$$

Where dQ/dt is permeability rate, C_0 is the initial concentration of the donor compartment,

and A_f is the surface area of the monolayer (filter). The total amount of compound that permeated during the experiment was calculated by multiplying the concentration and the volume of the acceptor compartment (0.6 mL for apical-to-basal transport and 0.2 mL for basal-to-apical transport). The permeability rate was calculated by dividing total amount permeated by 2 h. When compound levels in the acceptor compartment fell below the limit of quantification of the analytical assay, the apparent permeability was not calculated and excluded from subsequent correlation analysis. More sensitive assays, such as LC-MS/MS based assays, may be used in the future to quantify these excluded compounds in acceptor compartment. Apparent permeability of LY across Caco-2 and MDCK were determined similarly using Equation 4.1.

The Pearson's correlation coefficient between apparent permeabilities (apical to basal and basal to apical) across Caco-2 and MDCK were calculated using Microsoft Excel 2007 for windows (Microsoft Corporation, Redmond, WA, USA). Based on the apparent permeability of compound across Caco-2 and MDCK, compounds were ranked. A Spearman's rank correlation coefficient, which is a measure of association between two rankings, was calculated by equation 4.2 and its significance (two tailed p value) was tested using GraphPad Prism 5.0.

$$r_s = 1 - \frac{6\sum d^2}{n^3 - n} \qquad \text{Equation 4.2}$$

Where d is the difference between two separate rankings of individual test compounds based on the Papp values and n is the number of compounds undertaken in correlation studies. Depending upon the rs value the degree of association between two rankings was determined. Higher rs value indicates high degree of association and lower r_s value suggests low degree of association. The Spearman's correlation coefficient was calculated using GraphPad Prism 5.0 and p (two tailed) value was calculated.

Statistical Analysis

Results were expressed as mean±SD which were calculated using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). Two tailed unpaired t-test was applied to pre- and postassay TEER values using GraphPad Prism version 5.0 (GraphPad Software, San Diego, California, USA) to determine monolayer integrity during the entire experiment. One way ANOVA with Dunnett's post hoc test was applied to the Lucifer Yellow permeability for all compounds to compare against control (transport media containing 1% DMSO).

Results

Selection of Test Compounds

Selection of NC compounds was done based on their cytotoxic properties. A set of 60 compounds with diverse structures containing at least one conjugated alpha, beta-unsaturated ketone were analyzed for their physicochemical properties. The NC compounds that did not violate more than one rule of "rule of five" (RO5) (24, 25) and Veber's rule (26) were selected for aqueous solubility determination which was conducted in transport medium containing 1% DMSO. The screening based on the physicochemical properties was necessary to eliminate compounds with predicted low oral bioavailability and toxicity. Compounds having solubility of more than 50 μM in transport medium containing 1% DMSO were assessed for their stability at 37°C for 2 h. The NC compounds that demonstrated solubility equal to or exceeding 50 μM solubility and that were stable (>95%) under the permeability assay conditions were selected for permeability assessments (Table 4.1 and Figure 4.1).

Monolayer Integrity and Apparent Permeability

The monolayer formation of MDCK and Caco-2/TC-7 cell lines were tested using pre-assay TEER value. Caco-2/TC-7 exhibited higher pre-assay TEER values (595±24 Ω.cm2) than MDCK (228±31 Ω.cm2) and these were very similar to values reported in the literature (14, 27). Post-assay TEER values were 570±37 Ω.cm2 and 235±11 for Caco-2/TC-7 and MDCK, respectively, which were not significantly different from pre-assay TEER values ($p = 0.10$ for Caco-2/TC-7 and $p = 0.05$ for MDCK). The post assay permeability of Lucifer Yellow across Caco-2/TC-7 was 5.4±0.3 nm/sec whereas that of MDCK was 54.4±6.8 nm/sec in control wells (transport media with 1% DMSO), respectively. None of the tested compounds affected the Lucifer yellow permeability across Caco-2 significantly with comparison to control wells, whereas all eight curcumin analogues significantly increased the Lucifer Yellow permeability in MDCK. The non-piperidone based curcumin analogue and chalcone did not increase the LY permeability in MDCK.

The Papp values of test compounds, calculated according to Equation 4.1 (Table 4.2) exhibited higher permeability from apical -to-basal compartment than basal-to-apical transport. Thus, efflux ratios of compounds were ≤ 0.8. NC 2314 and NC 2315 showed higher permeability across Caco-2 and MDCK than other compounds. The Papp (basal-to-apical) was

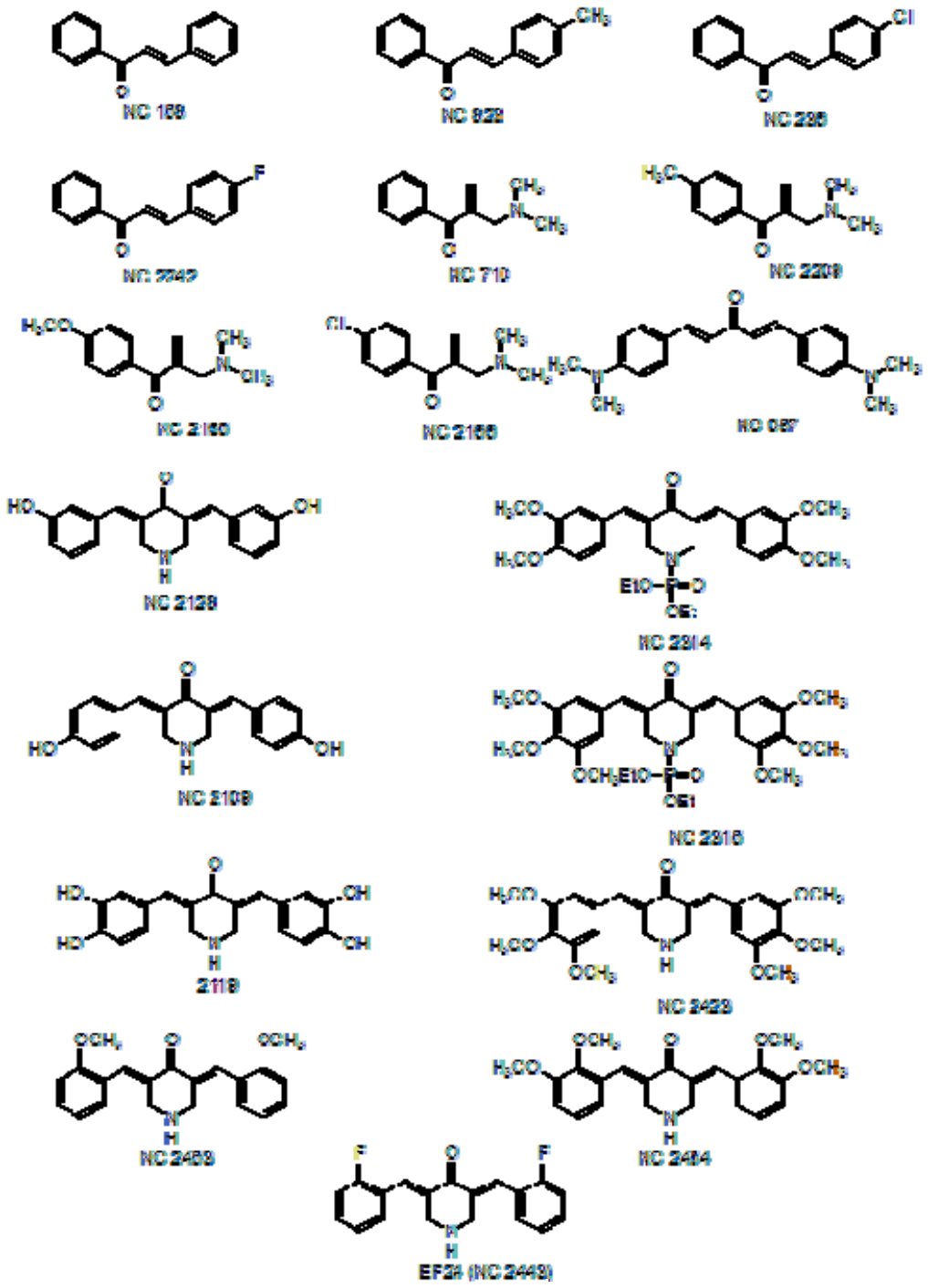

Figure 4.1. Structures of curcumin analogues containing 1,5-diaryl-3-oxo-pentadienyl group and related chalcones undertaken for permeability assessment using Caco-2 and MDCK model systems.

Table 4.1. Physicochemical properties (cLogP, Total Polar Surface Area (TPSA), Molecular Weight (MW), Volume) of curcumin analogs and a related chalcone (calculated from online cheminformatics services from www.molinspiration.com) and their anticancer activities against different cell lines.

Compound code	Physicochemical Parameter*				Anticancer Activity of Compounds (IC_{50} (μM) or Dose)†	References
	cLogP	TPSA	MW	Volume		
NC 158	3.81	17.07	208.26	201.85	7.94±0.32 (Molt4/C8) 11.1±3.1 (CEM)	(19)
NC 823	4.26	17.07	222.29	218.41	7.80±0.47 (Molt4/C8) 12.9±1.1 (CEM)	(19)
NC 236	4.49	17.07	242.71	215.39	9.00±0.53 (Molt4/C8) 8.66±0.29 (CEM)	(19)
NC 2242	3.98	17.07	226.25	206.78	8.23±0.11 (Molt4/C8) 5.80±3.46 (CEM)	(19)
NC 710	2.07	20.31	189.26	193.47	12 (HL-60); 4.9±1.0 (WiDr); 7.2±0.5 (CRL-2522)	(20, 37)
NC 2209	2.52	20.31	203.29	210.03	0.45 (HL-60); 2.0±0.1 (WiDr); 2.9±0.2 (CRL-2522)	(20, 37)
NC 2165	2.13	29.54	219.28	219.01	<3.4 (HL-60); 2.0±0.1 (WiDr); 2.5±0.6 (CRL-2522)	(20, 37)
NC 2166	2.75	20.31	223.70	207.00	<3.1 (HL-60); 4.1±0.6 (WiDr); 4.0±0.4 (CRL-2522)	(20, 37)
NC 957	4.39	23.55	320.44	321.08	Withheld	Unpublished
NC 2314	3.27	92.78	531.54	480.04	0.27±0.03 (Molt4/C8), 0.85±0.01 (CEM), 2.00±0.20 (L1210)	(21)
NC 2315	3.24	111.25	591.59	531.13	0.45±0.02 (Molt4/C8), 0.51±0.05 (CEM), 3.00±1.01 (L1210)	(21)
NC 2109	2.40	69.55	307.35	280.47	Withheld	Unpublished
NC 2110	1.42	110.01	339.35	296.51	Withheld	Unpublished
NC 2128	2.35	69.55	307.35	280.47	Withheld	Unpublished
NC 2423	2.65	66.03	395.46	366.62	Withheld	Unpublished
NC 2443	3.23	29.10	311.33	274.30	0.2 mg/kg in HCT-116 grafted athymic nude mice	(32)
5-FU[a]					>29.5 (All cell lines)	(38)
Melphalan					26.9 (All cell lines)	(38)

*cLogP=calculated log (partition coefficient between octanol and water); TPSA=total polar surface area; MW=molecular weight; IC_{50} = concentration at which 50% of cells are killed

† Name of cell line against which the compound exhibits maximum cytotoxicity is given in parentheses

[a]5-Fluorouracil

divided by Papp (apical-to-basal) to calculate efflux ratio which gives a preliminary idea concerning the mechanism of transport and involvement of uptake transporters (Table 4.2). An efflux ratio of >1.2 suggests involvement of efflux transporters, whereas a efflux ratio of <0.8 suggests involvement of active uptake processes; however, further confirmatory studies are required to conclude involvement of active uptake efflux processes (28).

Cytotoxicity Assessment

In order to assess whether curcumin analogues and a related chalcone show any detrimental effect to the Caco-2 and MDCK cells during the experiment, we incubated these compounds for 2 h with each of these cell lines. These compounds exhibited no significant decrease in the number of cells in comparison with the control (1% DMSO); however, these compounds reduced MDCK cell viability by 4-8%. The correlation between percent loss in number of MDCK cells and Papp of LY were significant (p=0043) but not in Caco-2 (p=0.8199).

Correlation between MDCK and Caco-2

Table 4.2 presents the apical-to-basal and basal-to-apical Papp values (mean±SD) and efflux ratios (Efr) of test compounds across MDCK and Caco-2. The mean values were used to calculate Pearson's correlation coefficients. The apical-to-basal Papp values of test compounds across Caco-2 correlated well with MDCK (r = 0.94) (Figure 4.2A). Most of the Papp values of compounds are near to the line of unity (slope = 1), suggesting that both assays resulted in similar Papp values. The permeability of NC 2314 across MDCK was much higher than that of Caco-2 (Figure 4.2A).

The basal-to-apical Papp values of compounds across MDCK and Caco-2 showed a relatively poor correlation (r = 0.76) (NC 2314 was removed as outlier). MDCK demonstrates higher basal-to-apical transport than Caco-2 (Figure 4.2B). No correlation (r = 0.24) was observed for efflux ratios of compounds between Caco-2 and MDCK (Figure 4.2C).

Rank Ordering of Compounds

In drug discovery, compounds are ranked to select the promising compounds based on their permeability assessment. To understand whether MDCK ranks the compound similar to Caco-2, Spearman's rank correlation was calculated. Spearman's rank correlation coefficient for apical-to-basal transport was 0.87 (p=0.0022) and for basal-to-apical transport was 0.83

Table 4.2. Apparent permeability (P_{app}) values and efflux ratios (Efr) of curcumin analogs and a related chalcone in MDCK and Caco-2 model systems at a substrate concentration of 50 µM (incubation time = 2h).

Compound Code*	Caco-2/TC-7			MDCK-II		
	P_{app} ($\times 10^{-6}$cm/s)			P_{app} ($\times 10^{-6}$cm/s)		
	A-B	B-A	Efr	A-B	B-A	Efr
	Mean±SD	Mean±SD		Mean±SD	Mean±SD	
NC 158	2.89±0.63	1.05±0.33	0.36	3.84±0.18	1.26±0.19	0.33
NC 957	8.42±0.66	7.02±0.79	0.83	19.70±0.51	15.90±0.49	0.81
NC 2315	28.50±0.32	8.82±0.27	0.31	105.00±7.63	50.50±10.90	0.48
NC 2314	54.70±1.43	8.17±1.09	0.15	62.30±7.39	27.70±3.24	0.44
NC 2128	15.40±.32	5.83±1.06	0.38	21.90±0.49	6.53±1.44	0.30
NC 2443	3.53±0.65	0.60±0.01	0.17	3.54±0.49	0.36±0.02	0.10
NC 2260	2.82±0.01	0.99±0.04	0.35	2.70±0.03	0.99±0.09	0.37
NC 2423	14.40±0.97	4.12±0.28	0.29	22.20±1.94	8.70±1.15	0.39
NC 2453	32.50±0.37	10.40±0.67	0.32	28.70±1.60	13.90±0.86	0.48
NC 2454	23.40±0.99	7.22±0.75	0.31	14.70±1.11	6.28±1.42	0.43

A-B: apical-to-basal transport, B-A: Basal-to-apical transport; Efr= Efflux ratio
*Compounds from Table 4.1 whose permeability coefficiencts could not be calculated has not been included

Table 4.3: Spearman's rank correlation coefficients (r_s) between cLogP and apparent permeabilites of curcumin analogs and a related chalcone using Caco-2 and MDCK model systems

Comparison	Spearman's rank correlation Coefficients (r_s)
Caco-2 Vs MDCK (A-B)	0.87 (p=0.0022)
Caco-2 Vs MDCK (B-A)	0.83 (p=0.0047)
cLogP vs MDCK (A-B)	-0.21 (p=0.56)
cLogP vs MDCK (B-A)	0.09 (p=0.81)
cLogP vs Caco-2 (A-B)	-0.39 (p=0.56)
cLogP vs Caco-2 (B-A)	-0.16 (p=0.66)

A-B: apical-to-basal transport, B-A: Basal-to-apical transport; cLogP was calculated using cheminformatics services of www.molinspiration.com

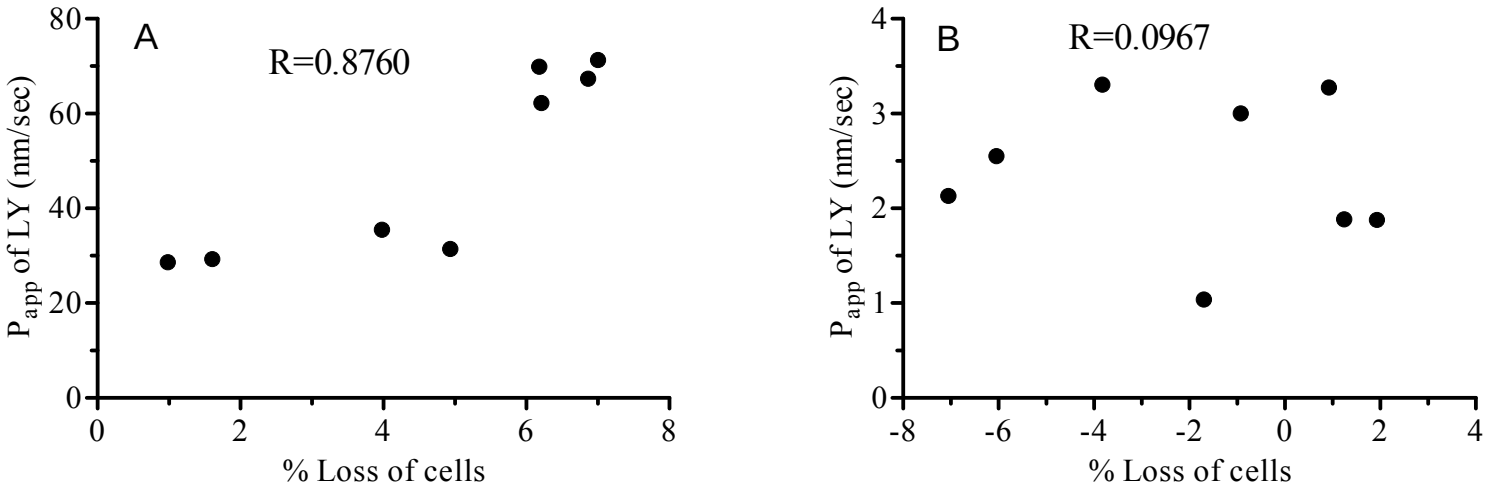

Figure 4.2. Correlation between apparent permeability of Lucifer Yellow (LY) and loss in cell viability after the incubation of MDCK (A) and Caco-2 (B) with curcumin analogs at 50 μM concentration in the environment containing 95% O_2 and 5%CO_2 at 37°C for 2h. The correlation was significant in MDCK (p=0.0043) while no significant correlation was obtained in Caco-2 (p=0.8199).

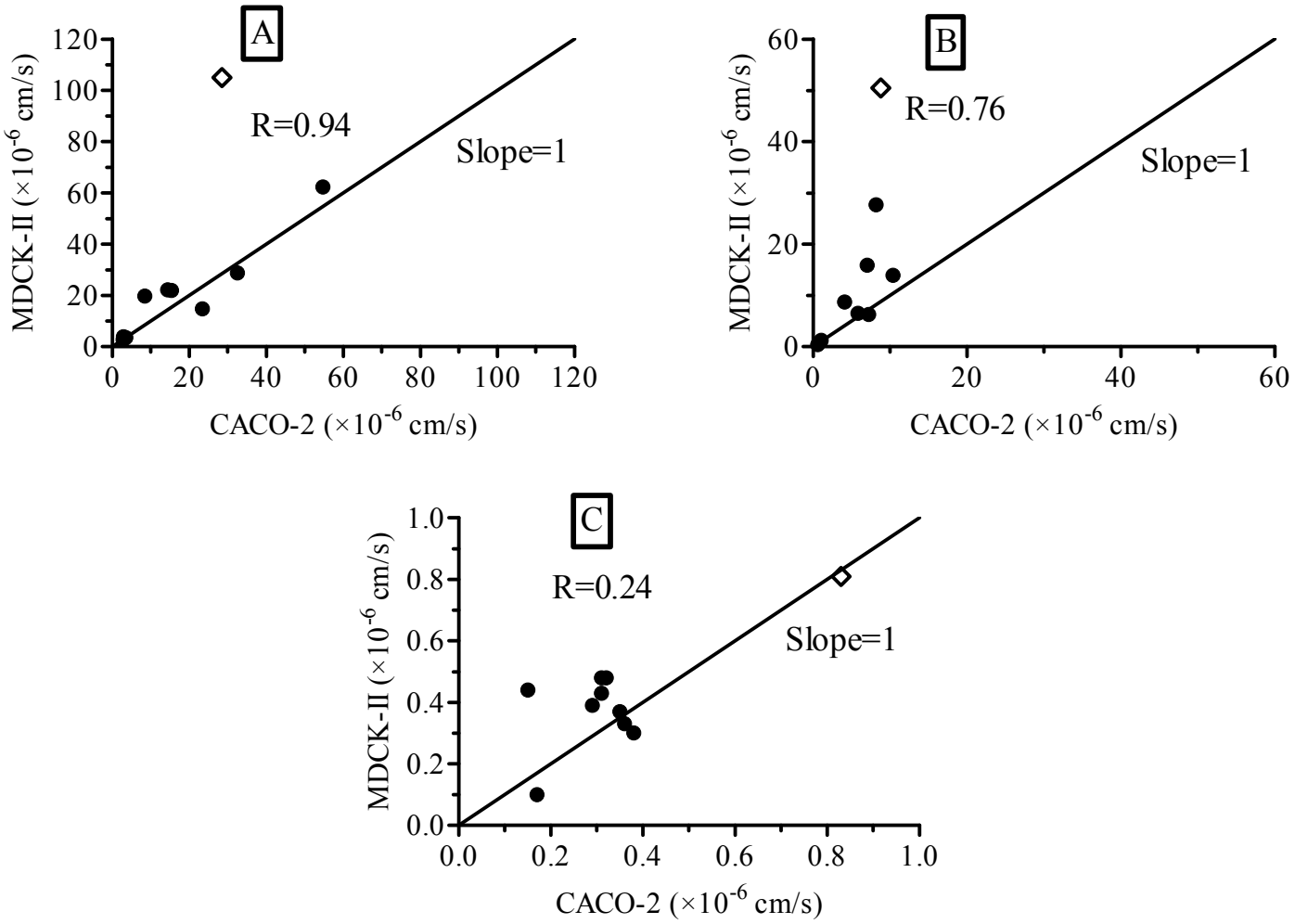

Figure 4.3. Correlation between apparent permeabilities of curcumin analogs and a related chalcone (Table 4.2) in the MDCK and Caco-2 model systems (substrate concentration = 50 μM, incubation time = 2h) and their deviation from line of unity (slope =1); A: apical-to-transport; B: Basal-to-apical transport; C: efflux ratio; unfilled symbol (◊) denotes outlier (NC 2314 was considered outlier for correlation analysis).

(p=0.0047), which indicates a good correlation between the rankings by these two cell lines. The rank correlation of between cLogP values of compounds and Papp values were not significant (Table 4.3).

Discussion

Caco-2 and MDCK are widely used cell lines for permeability assessments of compounds in the lead optimization stage of drug discovery. Maintenance of the integrity of the polarized epithelium during the experiment is required to ensure validity of the apparent permeability values. Tight junctions between cells in the monolayer maintain the polarized epithelium. Cytotoxic drugs may either influence tight junction function or cell viability to compromise the integrity of the polarized epithelial monolayer of the Caco-2 and/or MDCK cell lines (15, 17, 29). We evaluated both these models against a series of highly cytotoxic compounds to determine whether both cell lines may be a suitable model for routine permeability assessment of these curcumin analogues (15, 29, 30). Although these cell lines have been used to assess permeability characteristics of diverse compounds, to our knowledge no study has conducted a comparative analysis between the two models for highly cytotoxic compounds with potential to disrupt monolayer.

The cytotoxic curcumin analogues adversely affected the monolayer integrity of MDCK but not Caco-2, while the chalcone and non-piperidone based curcumin analogue, which lack the secondary amine group, had no effect on monolayer integrity of either cell line. In our study the formation of Caco-2 and MDCK monolayers was complete as pre-assay TEER values of Caco-2 and MDCK cell monolayers were similar to the earlier published works (14, 27) and Lucifer Yellow rejection rates approached 100%. Although the post-assay TEER was lower than pre assay TEER in both model systems, the difference between them were not significant. Since TEER is sensitive to several external factors including temperature and ion concentrations, the apparent permeability of Lucifer Yellow, a paracellular marker, was used as a reliable measurement of integrity of the polarized epithelium. The apparent permeability of Lucifer Yellow after exposure of Caco-2 with curcumin analogues was less than < 30 nm/sec, which was similar to control; however, the apparent permeability of Lucifer Yellow in MDCK after exposure with curcumin analogues was significantly higher than the control.

The cytotoxicity assessments showed that MDCK cells are more sensitive to curcumin

analogues than Caco-2 cells. A two hour incubation with curcumin analogues resulted in 4-8% loss of MDCK cells, which correlated significantly with the increase in the apparent permeability of Lucifer Yellow, while no loss in cell viability was observed in Caco-2. Although the sulphorhodamine B assay determines the percent cell attachment, loss of cell attachment may be due to loss of tight junction function or loss of cell viability. Hence, the elucidation of exact mechanism of the effects of curcumin analogues on MDCK cell line is a matter of further investigation. This loss of MDCK monolayer integrity does suggest an important limitation to the use of the MDCK cell line for the permeability screening of these cytotoxic compounds.

Most compounds of this series exhibit low permeability in the Caco-2 model. However, NC 2314 and NC 2315 demonstrated higher permeability characteristics than metoprolol (29.88±3.17 ×10-6 cm/s)(31), which is a compound that segregates high and low permeability compoundsin the biopharmaceutics classification system (BCS). Higher permeability characteristics than metoprolol suggests that these compounds may demonstrate favourable oral bioavailability characteristics than other compounds in the series. These two NC compounds also exhibit greater Papp values in Caco-2 than EF24 (NC 2443), an NC compound with reported activity against HCT-116 colon cancer implanted male athymic nude mice at the dose level of 0.2 mg/kg (i.p.)(32-34). EF24 did not show high permeability characteristics across both cell lines but its very low efflux ratio (0.1) suggests involvement of active uptake mechanisms. The permeability characteristics of NC 2314 and NC 2315 suggest that these compounds may show more favourable oral bioavailability characteristics than the other NC compounds evaluated in the series.

An evaluation of the efflux ratio is often used to provide suggestive evidence for the involvement of active transport processes (28). Conventionally, an Efr = 0.8-1.2 suggests passive diffusion whereas Efr < 0.8 suggests active uptake while Efr >1.2 suggests active efflux. Except NC 957, all other compounds exhibited low Efr values, which may suggest that permeation across the polarized monolayers involves both passive diffusion and active transport mechanisms, either active uptake transport at the apical membrane or active efflux transport at the basalateral membrane. Further studies on elucidation of the mechanism of transport are required.

Although both cell lines gave similar apical-to-basal Papp values (except NC2314 for which MDCK predicted much higher permeability characteristics than Caco-2), higher Papp

values in the basal-to-apical direction was observed with MDCK cells and Efr values did not correlate well between Caco-2 and MDCK. This discordance in Papp and Efr values and the generally low Efr suggest that MDCK and Caco-2 cell lines show significant qualitative and quantitative differences in the expression of active uptake and efflux transporters at the apical and/or basolateral membranes of the polarized monolayers. MDCK cell lines are reported to have low expression levels of transporters and low metabolic activity (35), while Caco-2 cells express a number of active transporters at higher or lower levels than endogenous transporter expression in the human intestine (36). MDCK is purported as a good model for the screening of compounds undergoing passive permeation, but our data suggests Caco-2 may provide more reliable assessment of NC compound permeation characteristics given the potential involvement of active transport mechanisms.

Interestingly, both the MDCK and Caco-2 polarized monolayers gave similar rank ordering of the apparent permeability for the evaluated NC compounds for both apical-to-basal or basal-to-apical directions. Given low expression levels of transporter proteins in MDCK, the compounds are expected to permeate predominantly by passive diffusion and therefore should correlate with clogP value. However, the clogP did not correlate well with the permeability values of NC compounds across either of the cell lines. This suggests clogP can not be used as surrogate predictor of the permeability of this series of compounds but the reasons for the lack of correlation are not apparent. Despite having a good correlation between apical-to-basal Papp values and a similar rank ordering of permeability of NC compounds with the Caco-2 system, our data indicates that MDCK is not suitable for apparent permeability determinations due to its effect on MDCK monolayer integrity. Nonetheless, MDCK may be used for rank ordering of compounds in drug discovery situations, where it is required for prioritizing compounds for further studies.

Conclusion

Curcumin analogs and related chalcone exhibit poor permeability characteristics and low efflux ratios, the latter of which may indicate the involvement of active transport mechanisms. Further studies are needed to confirm the contribution of transporters in intestinal permeation of these compounds. MDCK is not suitable for the determination of permeability characteristics of these series of compounds; however, MDCK assay can still be used for rank ordering of

compounds in drug discovery situations. The cLogP which is often correlated with passive permeability characteristics does not show any correlation, and therefore, can not be used as a surrogate marker for the permeability of this series of compounds. We are first to report that the highly cytotoxic compounds can adversely affect the monolayer integrity, and therefore the permeability assessment must be preceded by the evaluation of test compounds' ability to affect the monolayer integrity in the permeability assessment model.

Acknowledgements

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CHAPTER 5
PHARMACOKINETIC STUDIES OF SELECTED CURCUMIN ANALOGUES IN MALE WISTAR RATS

Ravi Shankar Prasad Singh, Umashankar Das, Jonathan R. Dimmock, Jane Alcorn

Pharmacokinetic Studies of Selected Curcumin Analogues in Male Wistar Rats

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Abstract

A subsequent step to in vitro pharmacokinetic (PK) assays in drug discovery is the determination of in vivo PK in rodents. Dimmock and co-workers developed a new series of 1,5-diaryl-3-oxo-1,4-pentadienyl containing curcumin analogues, which exhibited potent cytotoxicity against various colon cancer cell lines. The curcumin analogues, NC 2314, NC 2315, NC 2453 and NC 2454 were selected for in vivo PK evaluation in rodents based on their high apparent permeability in the Caco-2 model of intestinal absorption. Intravenous and oral administration of these compounds at 1 mg/kg and 10 mg/kg, respectively, led to acute toxicity and death within several hours of administration. HPLC-UV analysis of blood samples collected before death identified very low to undetectable levels of NC compounds in rat serum at all time points evaluated and for both routes of administration. These outcomes indicate that these compounds are highly toxic in vivo with death occurring at very low systemic levels of the compounds. Any further advancement of this series of molecules requires identification of the toxicophore and the exact mechanism of toxicity.

Keywords: Curcumin analogues; Pharmacokinetics; rodents

Introduction

Historically, poor pharmacokinetic characteristics, particularly low oral bioavailability and poor half-lifes, were a significant cause of loss of compounds from the drug discovery and development pipeline. Today, given the early integration of PK evaluations in the drug discovery process, the major reasons included lack of efficacy and toxicity(1, 2). Rodents are a widely used model in drug discovery for estimation of pharmacokinetic characteristics of promising compounds. Typically, molecules exhibiting favourable in vitro PK characteristics (i.e. good cellular permeability and metabolic stability) are followed by the in vivo PK evaluations in rodents (3, 4)(5, 6). The pre-clinical pharmacokinetics identifies the clinical pharmacokinetic issues likely to be faced and helps to determine the starting dose for clinical trials (3, 4).

Dimmock and co-workers are interested in the development of 1,5-diaryl-3-oxo-1,4-pentadienyl containing curcumin analogues as potential anticancer drugs (7, 8). Although curcumin, one of the active constituents of turmeric, exhibits anticancer activity in colon cancer curcumin demonstrates poor aqueous solubility, low bioavailability and low efficacy(9, 10). To improve upon these drawbacks the β-diketone moiety of curcumin was replaced with a ketone to generate 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore. The curcumin analogues with this new phamacophore exhibit high in vitro cytotoxicity against various colon cancer cell lines.

In vitro permeability assessment of this series of curcumin analogues identified four analogues (NC 2314, NC 2315, NC 2453 and NC 2454) that exhibited high apparent permeability in the Caco-2 model system. Earlier experiments showed that these highly cytotoxic compounds did not adversely affect Caco-2 monolayer integrity (see chapter 4) and thus, the apparent permeability values in Caco-2 were considered in the selection of curcumin analogues for PK assessment in rats. The selected curcumin analogues (Figure 5.1) exhibit high apparent apical-to-basal permeability in the Caco-2 model. As a subsequent step in drug discovery, we conducted intravenous and oral pharmacokinetic evaluations in male Wistar rats, which is reported herein.

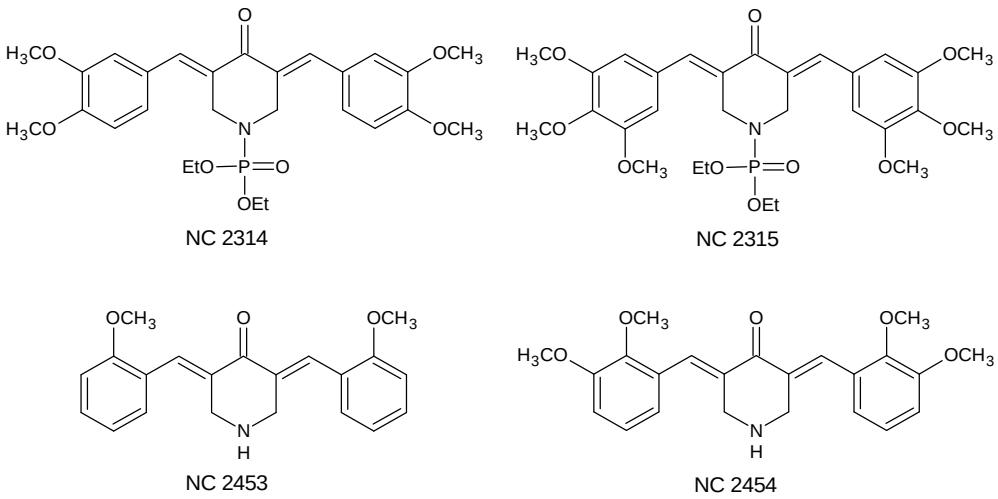

Figure 5.1: Structure of 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore containing curcumin analogues selected for pharmacokinetic evaluations in male Wistar rats.

Materials and Methods

Chemicals and Reagents:

Animals were procured from the Animal Resources Center, University of Saskatchewan. The silastic cannula was obtained from VWR International (Mississauga, ON, Canada). The curcumin analogues (Figure 5.1) were synthesized at the College of Pharmacy and Nutrition, University of Saskatchewan by reported procedures (7, 11). MilliQ water at 18.2 MΩ resistance was obtained from a MilliQ water purification system (Millipore, MA, USA). The vacutainers were obtained from BD biosciences (Mississauga, ON, Canada). All the chemicals unless stated otherwise were procured from Sigma-Aldrich (Oakville, ON, Canada)

Single Oral and Intravenous Bolus Dose Pharmacokinetic Studies:

Male Wistar rats (N=3) with a mean weight of 225±25 g were housed in a temperature and humidity controlled facility (22°C ± 2°C) on a 12-hour light:dark cycle (0700 h – 1900 h) and had free access to standard rodent diet and tap water. One day before dose administration, silastic cannulas (internal diameter and outer diameter, 0.63 mm and 1.19 mm, respectively) (VWR) were surgically implanted under isoflurane anaesthesia into the right jugular and left femoral veins for intravenous bolus dosing study, whereas only a right jugular vein was cannulated for the oral bolus dosing study. Curcumin analogues namely NC 2314, NC 2315, NC 2453 and NC 2454 were administered orally (10 mg/kg) as a suspension in a vehicle containing DMSO, PEG

300, ethanol, Tween and saline mixed in the ratio of 1, 20, 10, 20, and 49 percent, respectively (dose volume <0.5 mL). Blood samples were collected into vacutainer glass tubes at 0 (pre-dose), 15, 30, 45 min, and at 1, 2, 4, 8, 12 and 24 h post dosing. Samples were left to coagulate for 40 min in the dark and centrifuged at 10,000 rpm for 5 min to separate serum. Serum was transferred to microcentrifuge tubes and stored at -80°C until analysis using a previously developed and validated HPLC-UV (high pressure liquid chromatography-ultraviolet) method (12). NC 2083 was dosed intravenously at 2 mg/kg dissolved in DMSO, PEG 300, Tween 80 and ethanol mixed in equal proportions via the femoral cannula. The blood sampling schedule (blood collection from the jugular cannula) was 0 (pre-dose), 5, 10, 20, 30, 45 min, and at 1, 2, 4, 8, 12 and 24 h post dosing. This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

Results and Discussion

In order to evaluate the in vivo PK characteristics of 1,5-diaryl-3-oxo-1,4-pentadienyl containing curcumin analogues, four compounds (NC 2314, NC 2315, NC 2453 and NC 2454) with high Caco-2 permeability were selected. These curcumin analogues at 1 mg/kg (iv) and 10 mg/kg (oral) were fatal to the rats while administration of vehicle did not cause toxicity. Intravenous administration of these compounds at 1 mg/kg killed rats within 5 minutes to 2 hours postadministration. Early blood samples collected before death showed very low plasma concentrations (<100 ng/mL) and exhibited a rapid decline (Half-life <30 min) in the concentration. The number of data points was not sufficient for PK modeling. Rats survived for a longer duration (8-24 h) following oral administration. The concentration of compounds were below the LLOQ (50 ng/mL) in all serum samples collected from orally administered rats. In general, the rats were sluggish after administration of the compounds and the death was immediately preceded by the clawing, pawing, burying and jumping.

The apparent reasons for the toxicity by these compounds are not clear. Given the very rapid decline in serum concentrations, these compounds may be expected to rapidly partition into tissue and accumulate into vital organs such as brain to exhibit acute cytotoxicity. Alternatively, the compounds may undergo rapid transformation into toxic metabolites that cause fatal toxicity. However, an understanding of the mechanism of toxicity needs further investigation. Upon close

examination of the in vitro cytotoxicity evaluations of these compounds in HCT-116 and SW620 cells the inhibition curve exhibited a very steep slope. About 100% loss in cell viability was achieved within a 2 to 4 fold concentration range indicating an apparent narrow therapeutic index for these compounds. A more precise dose selection (i.e. lower dose) coupled with a more highly sensitive analytical method (i.e.tandem MS) may facilitate an understanding of the pharmacokinetics/toxicokinetics of these compounds.

Conclusion

The tested curcumin analogues, NC 2314, NC 2315, NC 2453 and NC 2454 are fatal to animals at the intravenous and oral doses of 1 mg/kg and 10 mg/kg, respectively. These outcomes indicate that these compounds are highly toxic in vivo with death occurring at very low systemic levels of the compounds. Any further advancement of this series of molecules requires identification of the toxicophore and the exact mechanism of toxicity

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CHAPTER 6

OPTIMUM PHYSICOCHEMICAL SPACE FOR CURCUMIN ANALOGUES CONTAINING 1,5-DIARYL-3-OXO-1,4-PENTADIENYL PHARMACOPHORE

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Optimum Physicochemical Space for Curcumin Analogues Containing 1,5-Diaryl-3-Oxo-1,4-Pentadienyl Pharmacophore

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Abstract:

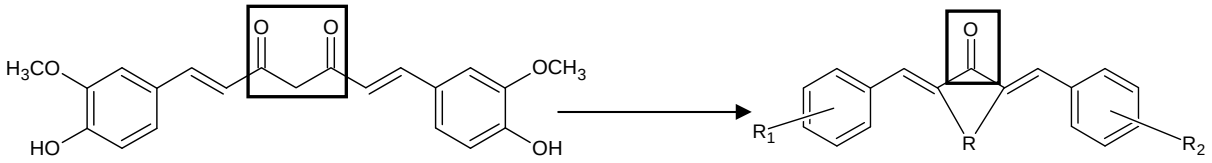
Identification of the optimal physicochemical space for a series of compounds is necessary to aid the drug design process. The Lipinski's rule of five (RO5) and Veber's rule were proposed to provide general guidance; however, these rules do not apply universally to all series of compounds. Dimmock and coworkers are interested in the development of curcumin analogues containing 1,5-diaryl-3-oxo-1,4-pentadienyl group as anticancer agents. Recent pharmacokinetic studies in our laboratory on these curcumin analogues identified poor pharmacokinetic properties. To explain these outcomes, we closely examined the optimal physicochemical space for curcumin analogues containing 1,5-diaryl-3-oxo-1,4-pentadienyl group by comparing compounds synthesized in Dimmock's laboratory with sets of compounds synthesized at three unrelated laboratories who reported promising lead compounds in this analogue series. The analysis identified that the physicochemical characteristics of the promising leads conformed strictly to the RO5. Compounds with Log P values, molecular weights and molecular volumes inconsistent with the RO5 exhibited poor pharmacokinetic characteristics. We conclude that curcumin analogues containing 1,5-diaryl-3-oxo-1,4-pentadienyl group should conform to the RO5 to increase the likelihood of successful identification of promising lead compounds.

Keywords: Physicochemical properties; curcumin analogues; Lipinski's rule of five,

Introduction:

The increase in our understanding of physicochemical properties and physiological processes significantly improved the design of drugs in the last decade. Based on the study on large number of molecules, Lipinski proposed 'rule of five' (RO5) (1), which states that the molecule to be orally viable should conform to at least three of the four rules, which are (i) molecular weight ≤ 500 (ii) LogP ≤ 5 (iii) number of hydrogen bond donors ≤10 (iv) number of hydrogen bond acceptors ≤ 5. Later Veber identified that the compounds should have total polar surface area ≤ 140 Å2 and number of rotatable bonds ≤ 10 to have good oral bioavailability (2). The implementations of these general rules along with pharmacokinetic screening in drug design and drug discovery processes improved the attrition rate of molecules (3, 4). However, these rules seem not to be universally applicable and the optimum physicochemical range varies with different series of compounds (5).

Dimmock and coworkers are interested in the development of curcumin analogues containing 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore. These analogues demonstrate high cytotoxicity against various colon cancer cells (6). Curcumin, one of the constituent of turmeric, exhibits benefits in a variety of disease conditions including colon cancer; however, the low oral bioavailability of curcumin is a major concern (7-10). One reason for curcumin's low oral bioavailability is its instability at neutral and basic pH due to the presence of β-diketone moiety(11). The replacement of β-diketone with a keto group resulted in a novel series of curcumin analogues containing 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore (Figure 6.1) (12-14).



Curcumin

1,5-Diaryl-3-oxo-1,4-pentadienyl containing curcumin analogues

Figure 6.1: Structure of curcumin and scaffold of 1,4-diaryl-3-oxo-1,4-pentadienyl containing curcumin analogues. Box represents the part of the structure (β-diketone moiety) replaced with a group to generate the new curcumin analogues

Some compounds synthesized in our laboratory exhibited very high in vitro cytotoxicity but failed to exhibit any effect in vivo (6, 15-19). Retrospective pharmacokinetic investigations on one of the most cytotoxic compounds of the series, NC 2083, which failed to demonstrate efficacy in a tumor xenograft mouse model, suggested low membrane permeability, high plasma protein binding and high red blood cell partitioning as principal causes for lack of in vivo efficacy. Given the association of pharmacokinetic properties with physicochemical characteristics, we wish to incorporate optimum physicochemical properties in the design of our compounds to improve pharmacokinetic characteristics; however, the optimum physicochemical spaces for this class of compounds are yet not known. In our current work, we have identified the optimum ranges of physicochemical characteristics for curcumin analogues containing 1,5-diaryl-3-oxo-1,4-pentadienyl group, which may lead to improved success rates in drug discovery.

Method

Selection of the Compounds

A substructure search for compounds containing 1,5-diaryl-3-oxo-1,4-pentadienyl group was conducted using SciFinder web edition (Chemical Abstract Service, Columbus, Ohio, USA) and references were extracted. Subsequently, the references were refined by the name of the author who reported activity of curcumin analogues containing 1,5-diaryl-3-oxo-1,4-pentadienyl group. Our search identified three additional laboratories that reported in vivo activity for these curcumin analogues. All the references reporting synthesis of curcumin analogues containing 1,5-diaryl-3-oxo-1,4-pentadienyl group from these laboratories were selected for the determination physicochemical characteristics. For our laboratory, we selected compounds from the database of compounds synthesized after 2006.

Calculation of Molecular Properties

The structures of the compounds from our and other laboratories were drawn in Chemsketch 12.01 (Advanced Chemistry Development Inc., Toronto, ON, Canada) and simplified molecular input line entry specifications (SMILEs) were generated. The SMILEs were provided as the input for molinspiration free online cheminformatics services (http://www.molinspiration.com ,Molinspiration Cheminformatics Slovensky Grob, Slovak Republic) to calculate physicochemical parameters including logP, total polar surface area

(TPSA), number of rotatable bonds (nrotb), number of hydrogen bond donors (nOHNH), number hydrogen bond acceptors (nON), molecular weight (MW).

Data Analysis

The measures of central tendency (mean, median) and variability (standard deviation) of the data were calculated using Microsoft Excel 2007 (Microsoft corporation, Redmond, WA, USA). The plots were generated using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego California, USA).

Results and Discussion

The implementation of appropriate predicted physicochemical characteristics in the design of molecules has decreased attrition rates attributed to poor pharmacokinetics in drug discovery (3, 20, 21). Lipinski's RO5 and Veber's rules(2) were elaborated to identify the optimal physicochemical space necessary to have good pharmacokinetic characteristics, but these rules are not equally applicable to every series of molecules. Since physicochemical properties in conjunction with preclinical pharmacokinetics are used to predict human pharmacokinetics (22) and toxicity (23), knowledge of the optimal physicochemical space for a series of compounds is necessary to inform the drug design efforts to increase the likelihood of designing successful candidates. In order to identify the optimal physicochemical space for curcumin analogues containing 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore, we undertook the current study whereby we compared the physicochemical space of our molecules with those from laboratories that generated successful candidates.

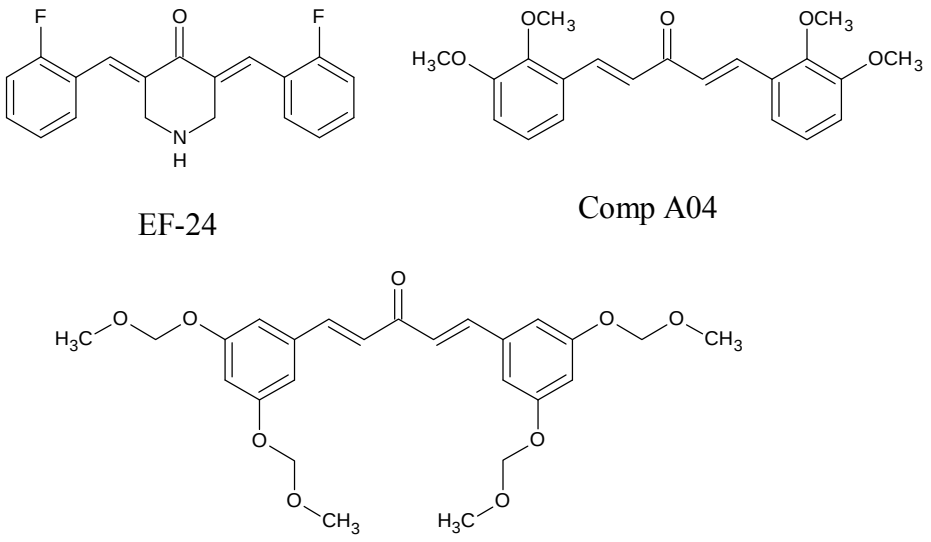


Figure 6.2: Curcumin analogues containing the 1,5-diaryl-3-oxo-1,4-pentadienyl group that exhibit anticancer efficacy in pre-clinical models.

Three laboratories, Snyder's lab (Lab-1) (14), Shibata's lab (Lab-2) (24)and Yang's lab (Lab-3) (25), reported inhibition of tumour growth or regression in mice following administration of EF-24, GO-Y030 and A-04 (Figure 6.2), respectively. The predicted physicochemical characteristics (Table 6.1) of these compounds do not violate Lipinski's RO5, but GO-Y030 has more number of rotatable bonds than Veber's rules recommend. The molecular weight of these compounds ranged between 311.3 and 474.5 D and the range of observed miLogP values was 3.2-4.2. Our analysis of the physicochemical characteristics of successful candidates suggests that compounds with lower molecular weights and logP values have a greater potential to succeed in subsequent evaluations. However, the minimum molecular weight of curcumin analogues containing the 1,5-diaryl-3-oxo-1,4-pentadienyl group is 234.3, and attempts to limit the molecular weight of compounds within RO5 may be quite challenging. As well, given the interdependence between molecular weight and logP, the logP values of this series also tends to be higher, which create further challenges to keep analogues within RO5 recommendations.

Table 6.1: Mean±SD and Median of physicochemical parameters of molecules that shows in vivo efficacy in mice

Physicochemical Parameters*	EF-24(14)	GO-Y030	A-4	Mean±SD
miLogP	3.2	4.2	3.5	3.6±0.5
TPSA ($Å^2$)	29.1	91.0	54.0	58.0±31.1
natoms	23	34	26	27.7±5.7
MW (Da)	311.3	474.5	354.4	380.1±84.6
nON	2	9	5	5.3±3.5
nOHNH	1	0	0	0.3±0.6
nviol	0	0	0	0.0±0.0
nrotb	2	16	8	8.7±7.0
Volume ($Å^3$)	274.3	434.6	314.0	341.0±83.5

*miLogP = logP predicted by molinspiration cheminformatics tool; TPSA = Total polar surface area; natoms = number of atoms in the molecule; MW = Molecular weight; nON = number of hydrogen bond acceptors; nOHNH = number of hydrogen bond donors; nviol = number of violations; nrotb = number of rotatable bonds

All published molecules from our laboratory and the three other laboraties were used to predict the physicochemical space of the curcumin analogues. Assumptions made in meta-analysis included (i) all these labs have published all compounds synthesized in this series, (ii) all labs intend to discover and develop an anticancer curcumin analogue, and (iii) all labs have similar academic constraints and expertise. While Lab-1, Lab-2 and Lab-3 reported the synthesis of a total of 11, 31 and 57 molecules, respectively, we (Lab-4) reported 92 compounds synthesized since 2006 (Table 6.2). Lab-1 showed the highest (9.1%) pre-clinical success rate followed by Lab-2 and Lab-3 with 3.2 and 1.8% success rate, respectively. Lab-4 reported no pre-clinical success. An important drawback of the meta-analysis is this small sample size of successful molecules. We excluded compounds from labs who failed to report in vivo efficacy, as defined, efficacy in a tumor xenograft mouse model. This series of curcumin analogues have largely garnered attention from academia and, therefore, is not unsurprising that few reports of successful candidates were found.

On close examination of the data, compounds from Lab-4 on average possess higher molecular weights, logP values and molecular volumes as compared with the other laboratories (Table 6.2). Although the mean logP values from Lab-3 were very similar to that of Lab-4, the molecular weights and total molecular volumes were significantly lower than Lab-4 (Table 6.2

and 6.3). Since molecular weight and logP are inversely related with the oral bioavailability, compounds from Lab-4 are likely to demonstrate poor oral bioavailability. Furthermore, given the effect of logP and molecular volume on the passive permeability, these compounds are also expected to show low passive permeability (26). Permeability assessments in the Caco-2 model indicated low permeability coefficients despite the possible involvement of active uptake process (Chapter 5). The molecules from lab-4 possess high logP, molecular weight (Figure 6.3) and shows lowest percent of compounds that do not violate the Lipinski's rule. Expectedly, the success rate of lab-4 is least, which emphasizes the necessity to conform to the Lipinski's RO5 for this series of curcumin analogues.

Table 6.2: Mean±SD of physicochemical parameters of compounds from different laboratories predicted by molinspiration chemiinformatics tool (www.molinspiration.com).

Physicochemical Parameters*	LAB-1 Mean±SD	LAB-2 Mean±SD	LAB-3 Mean±SD	LAB-4 Mean±SD
miLogP	3.6±0.5	3.7±0.9	5.1±1.2	5.1±1.8
TPSA ($Å^2$)	48.7±18.1	61.7±27.3	34.6±19.5	70.9±33.3
natoms	22.2±2.0	27.1±5.2	25.8±4.0	38.2±12.5
MW (Da)	297.6±27.6	379.8±75.9	362.8±54.3	522.5±173.2
nON	3.0±1.1	5.3±2.6	2.8±1.8	5.8±3.2
nOHNH	1.2±1.0	0.5±1.0	0.2±0.6	0.8±1.1
nrotb	3.6±2.0	8.4±4.3	5.1±3.0	5.9±3.5
Volume ($Å^3$)	270.7±25.0	344.5±68.5	330.5±58.7	473.8±158.3

*miLogP = logP predicted by molinspiration cheminformatics tool; TPSA = Total polar surface area; natoms = number of atoms in the molecule; MW = Molecular weight; nON = number of hydrogen bond acceptors; nOHNH = number of hydrogen bond donors; nviol = number of violations; nrotb = number of rotatable bonds

Table 6.3: Percent of curcumin analogues synthesized at different laboratories that do not conform to the RO5 and Veber's rule of drug likeliness.

Physicochemical parameters[b]	% of compounds			
	Lab 1	Lab 2	Lab 3	Lab 4
miLog P > 5	0	9.7	52.6	48.9
TPSA>140	0	0	0	5.4
molecular weight>500	0	3.2	0	44.6
nON>10	0	3.2	0	6.5
nOHNH>5	0	0	0	0
Nviol = 0	100	87.1	47.4	37
Nviol = 1	0	9.7	52.6	28.3
Nviol = 2	0	3.2	0	32.6
Nviol = 3	0	0	0	2.2
nrotb>10	0	25.8	7	6.5
Total number of compounds	11	31	57	92
Percent success rate[a] (pre-clinical efficacy)	9.1	3.2	1.8	0

[a]Percent success rate was calculated by dividing the number of active compounds with total number of compounds synthesized

[b]miLogP = logP predicted by molinspiration cheminformatics tool; TPSA = Total polar surface area ($Å^2$); MW = Molecular weight (Da); nON = number of hydrogen bond acceptors; nOHNH = number of hydrogen bond donors; Nviol = number of violations; nrotb = number of rotatable bonds

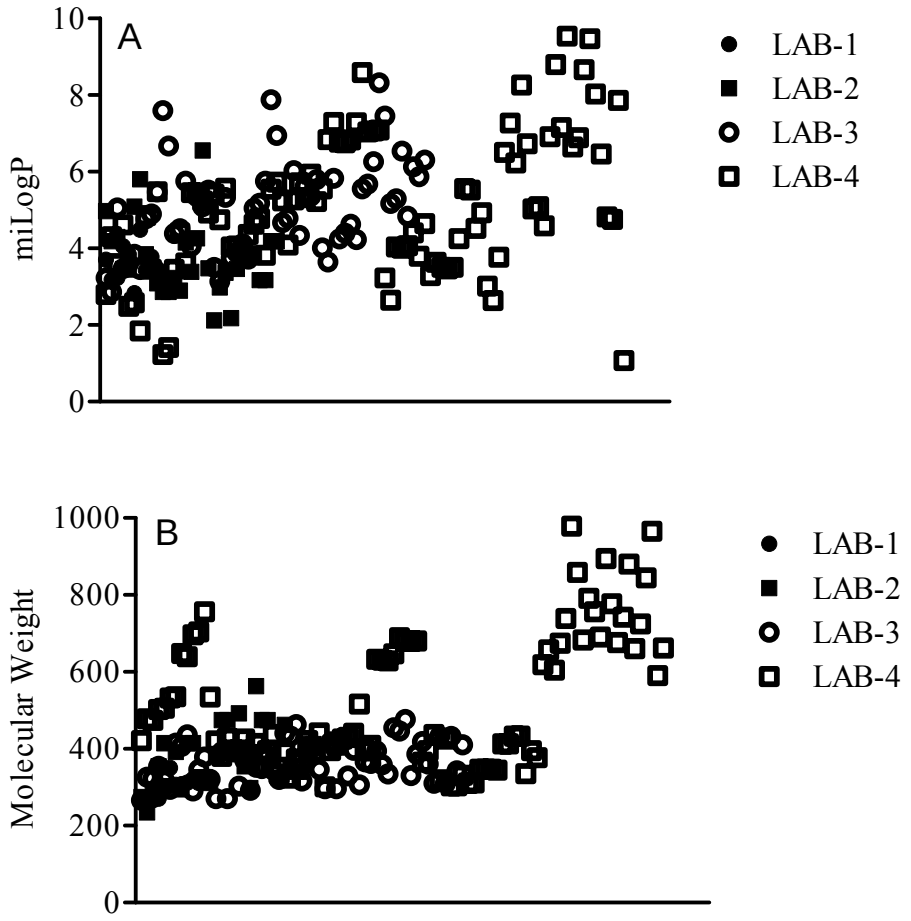


Figure 6.3: Distribution of miLogP (A) and Molecular Weight (B) of curcumin analogues containing 1,5-diaryl-3-oxo-1,4-pentadienyl group synthesized at different laboratories (LAB 1-4).

Conclusion

A meta-analysis of the physicochemical space of curcumin analogues containing the 1,5-diaryl-3-oxo-1,4-pentadienyl group synthesized at different laboratories identifies the dependence of success rates with strict conformity to Lipinski's RO5. Given the high molecular weight of the basic pharmacophore, the 1,5-diaryl-3-oxo-1,4-pentadienyl group, designing compounds without violating RO5 will be challenging. Despite the limited sample size as a potential drawback of the meta-analysis, this analysis has provided important guidance for our future design efforts involving curcumin analogues with this pharmacophore. Similar analysis on

other series of new molecules will contribute in the design and improve the success rate.

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CHAPTER 7

ANTI-ANGIOGENIC EFFECT OF CURCUMIN ANALOGUES IN HCT-116 AND SW620 COLON CANCER CELL LINES

Ravi Shankar Prasad Singh, Umashankar Das, Jonathan R. Dimmock, Jane Alcorn

Anti-Angiogenic Effect of Curcumin Analogues in HCT-116 and SW620 Colon Cancer Cell Lines

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Abstract

Angiogenesis is an essential process for the survival and growth of tumours. Biomarkers including vascular endothelial growth factor (VEGF), interleukin 8 (IL8) and cycooxygenase (COX-2) play important roles in angiogenic processes. Similarly, COX-2 and glyoxalase systems mediate inflammation and carcinogenesis begins at the site of chronic inflammation. Curcumin inhibits the expression of these essential proteins that mediate angiogenesis and inflammation. The antiangiogenic effect of 1,5-diaryl-3-oxo-1,4-pentadienyl containing curcumin analogues is not known. In order to identify the targets of 1,5-diaryl-3-oxo-1,4-pentadienyl containing curcumin analogues, our current study aims to study the effect of these curcumin analogues on some common angiogenic and inflammatory biomarkers in metastatic SW620 and non-metastatic HCT-116 cell lines. We selected four 1,5-diaryl-3-oxo-1,4-pentadienyl containing curcumin analogues and treated HCT-116 and SW620 cells for 24 h at 0.3 μM and subsequently measured gene and protein expression. These curcumin analogues did not affect glyoxalase I and II in both of the cell lines used. In general, these curcumin analogues inhibited VEGF and COX-2 but unexpectedly induced proangiogenic chemokine IL8. Given the inhibitory effect on VEGF and COX-2, these compounds may act as anti-angiogenic agent.

Key words: Curcumin; angiogenesis; inflammation; VEGF; COX-2; IL8

Introduction

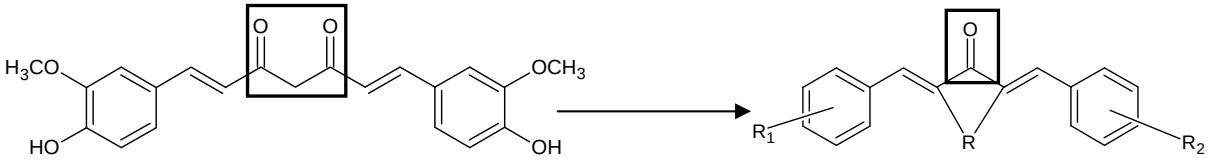
Tumour angiogenesis, which is the formation of capillaries in tumours, is essential for tumour growth beyond a few millimetres in size (1). Tumour cells need accessibility to blood to access nutrients for growth and to provide a means to metastasize (2). Inhibition of angiogenesis results in the cessation of tumour growth and this has been exploited for anticancer chemotherapy. Bevacizumab is the first angiogenesis inhibitor to be approved for the use in anticancer chemotherapy (3). Retrospective analysis further identified that some currently marketed drugs including taxol, herceptin, celecoxib, rosiglitazone also demonstrate anti-angiogenic activities. (4).

Several essential angiogenesis biomarkers play important roles in angiogenesis. Inhibition of those essential components that drive angiogenic pathways results in the inhibition of tumour growth. The major biomarkers involved in angiogenesis in tumours are vascular endothelial growth factor-A (VEGFA), tumour necrosis factor-α (TNF-α), Interleukin-8 and cyclooxygenase-2 (1, 5-7). VEGF is vital for vasculogenesis and angiogenesis as evident by the fact that animals with even a single missing allele failed to survive (8). The antiangiogenesis drug, Bevacizumab, inhibits VEGF to exhibit its effect (9, 10). The second biomarker, interleukin 8 (IL8), is a chemokine that plays an important role in angiogenesis by modulation of cell adhesive molecules including integrins and cadherins (11, 12). High concentration of IL8 in solid tumours is associated with the tumour survival and growth (13). The third biomarker, cyclooxygenase (COX-2), is an enzyme responsible for conversion of arachidonic acid to prostaglandins. Inhibition of COX-2 inhibits gene and protein expression of other angiogenic biomarkers (14) including VEGF, osteopontin (OPN) (15), matrix metalloprotease (MMP)-2 (16) and angiopoetin-1 (17). Additionally, COX-2 plays an important role in carcinogen activation, tumourigenesis, prevention of apoptosis and metastasis (18).

Susceptibility to carcinogenesis increases with duration of inflammation (19). Chronic inflammation resulting from human papilloma virus and hepatitis B virus infection initiates cervical and hepatocellular carcinogenesis, respectively (20, 21). Prolonged exposure to chemical irritants such as cigarette smoke, asbestos and silica results in chronic inflammation with subsequent initiation of carcinogenesis (22, 23). The use of NSAIDs as a chemopreventive agent arises from the association of inflammation and carcinogenesis (24, 25). Glyoxalase enzyme system (Glyoxalase I and II), which is responsible for detoxification of methylglyoxal, is

believed to be a possible link between inflammation and cancer (24).

Given the anticancer and chemopreventive activity of curcumin in different cancers including colorectal cancer (26), Dimmock and coworkers modified curcumin analogues to improve efficacy, solubility and bioavailability, the major concerns in the clinical use of curcumin (27-29). Replacement of the β-diketone moiety of curcumin with a ketone group is likely to increase the stability of the molecule at neutral and basic pH (30, 31) and therefore, improve bioavailability. This replacement resulted in a new pharmacophore, 1,5-diaryl-3-oxo-1,4-pentadienyl, that contains conjugated α,β-unsaturated ketone (Figure 7.1)(32, 33). Many analogues containing this pharmacophore demonstrate high efficacy against various cancer cell lines in the National Cancer Institute (NCI) screening and increased solubility as compared with curcumin (32, 34). The drug discovery program at the University of Saskatchewan is interested in the development of these curcumin analogues as anticancer agents. These compounds have also garnered attention from several different labs across the globe (35-37).



Curcumin

1,5-Diaryl-3-oxo-1,4-pentadienyl containing curcumin analogues

Figure 7.1: Structure of curcumin and scaffold of 1,4-diaryl-3-oxo-1,4-pentadienyl containing curcumin analogues. Box represents the part of the structure (β-diketone moiety) replaced with a group to generate the new curcumin analogues

Curcumin demonstrates activity against neoplasms via a variety of mechanisms including inhibition of angiogenesis (38). While curcumin inhibits tumour angiogenesis via inhibition of essential proteins such as VEGF (39), IL8 (40)and COX-2 (41), it exhibits chemopreventive activity via inhibition of inflammatory enzymes such as glyoxalase I and II and COX-2 (24, 42). A curcumin analogue under development, EF 24, inhibited angiogenesis via inhibition of VEGF, IL8 and COX-2 (43). The objectives of our current research were (a) whether the selected curcumin analogues (NC 2314, NC 2315, NC 2453 and NC 2454) (Figure 7.2) possessing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore demonstrate inhibition of common angiogenesis and inflammation mediators and (b) whether these curcumin analogues act via the same mechanisms in metastatic as well as non-metastatic cell lines.

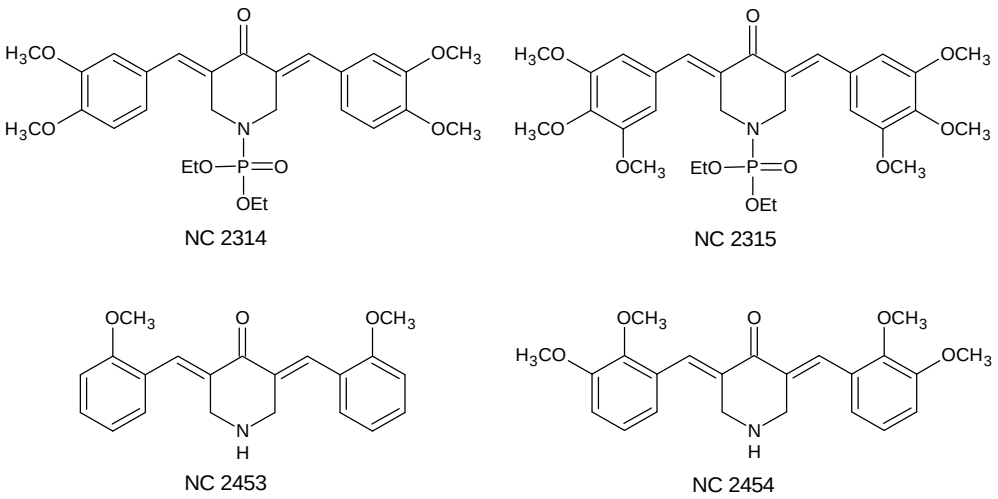


Figure 7.2: Structure of selected 1,5-ediaryl-3-oxo-1,4-pentadienyl pharmacophore containing curcumin analogues.

Materials and Methods:

Chemicals and Reagents:

The synthesis of NC 2314 and NC 2315 has been reported previously (34) while the preparation of NC 2453 and NC 2454 will be described in due course. 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 and Leibovitz medium were purchased from ATCC (American Type Culture Collection, Rockville, USA). Penicillin-streptomycin antibiotic solution and all other chemicals unless otherwise indicated were purchased from Sigma (Oakville, ON, Canada). MilliQ water at 18.2 MΩ resistance was obtained from a MilliQ water purification system (Millipore, MA, USA).

Cell Culture

Dr. Keith Bonham, Saskatoon Cancer Center, Saskatoon gifted HCT-116 and SW 620 cell lines. The HCT-116 and SW620 cell lines were subcultured in McCoy's 5A (ATCC cat No: 30-2007) and Leibovitz L-15 media (ATCC cat No: 30-2008), respectively, supplemented with 10% fetal bovine serum and 1% penicllin-streptomycin antibiotic solution. The HCT-116 cells were grown in an atmosphere of 95% O_2 and 5% CO_2 with 95% humidity whereas SW620 cells were grown at 37°C in 100 % atmospheric air.

Cytotoxicity Assay

Cytotoxicity of these curcumin analogues were determined using sulforhodamine B assay as reported by Skehan et. al. (44, 45). Cells growing in their exponential phase were harvested using 0.25 % trypsin in versene and the cell count was determined using trypan blue exclusion method. In a 96-well plate, about 5×103 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 different concentrations of test compounds and 1% DMSO (control) were added and incubated for 72 hours. Subsequently, 50 µL of 50% w/v trichloroacetic acid in water was added to each well and incubated at 4°C for one hour for the fixation of the cells. A plate was fixed at the time of treatment of the cells to obtain the number of cells at time zero (Tz). The plates were washed with tap water four times and air dried. The fixed cells were stained for 10 minutes with 0.4% w/v sulforhodamine in 1% v/v acetic acid and subsequently 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 absorbance was read at 515 nm. The percent growth was calculated using equation 9.1. The IC50 was derived by fitting four-parameter curve into percent cell growth versus log concentration data using GraphPad Prism 5.0 for windows (GraphPad Software, San Diego, California, USA).

$$\%\ \text{cell growth} = \frac{\mathrm{OD}_{\mathrm{sample}-}\mathrm{OD}_{\mathrm{Tz}}}{\mathrm{OD}_{\mathrm{control}-}\mathrm{OD}_{\mathrm{Tz}}} \times 100 \qquad \text{(Equation 7.1)}$$

Real Time PCR

Total mRNA was extracted from the cells by using a RNeasy Mini kit (Qiagen Inc., Mississauga, ON) according to manufacturer instructions. The quantity and purity of RNA was ascertained spectrophotometrically by the measurement of OD at 260 nm and OD ratio 260/280 nm, respectively, using a nanoview spectrophotometer (GE Healthcare Life Sciences,. Baie d'Urfe Quebec, Canada) cDNA was synthesized from total RNA using QuantiTect Reverse Transcription kit (Qiagen Inc., Mississuaga, ON) according to manufacturer's instruction and stored at -80°C until analysis. Specific primers to the gene sequences obtained from National Center for Biotechnology Information Gene Bank were designed using Primer3 software (Whitehead Institute for Medical Research. Primer 3). The designed specific primers were subsequently validated for the specificity of amplification, amplification efficiency over a

concentration range and consistency with amplification efficiency of housekeeping gene. Quantitative Real Time-PCR analysis on the cDNA samples was carried out using Power SYBR Green PCR master mix (Applied Biosystems, Streetsville, ON) and Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems, Streetsville, ON). Three stage PCR reactions were set up. In the initial activation stage, the samples were given 1 cycle of 15 min at 94°C. In the second stage of amplification 40 cycles of three step thermal cycling (denaturing at 94°C for 15 seconds, annealing at 60°C for 30 seconds and primer extension at 60°C for 30 seconds) was given and in the final stage, the melt curve analysis was performed by heating at 65°C-95°C at 0.5°C/second. The fold change in expression was determined using ΔΔCT method.

Validation of Primers

Single melting peak and a single band with appropriate size range on 2% agarose gel electrophoresis ascertained the specificity of amplification. The PCR conditions were optimized to result in an efficiency of 1.9-2.1 (slope of -2.9 to -3.5) over a minimum of three-log concentration range. A similar determination of specificity and amplification efficiency was conducted on housekeeping gene (beta microglobulin). The similar amplification efficiency of gene of interests with housekeeping genes was confirmed by assuring a slope of less than 0.1 when ΔCT (the difference between target gene CT and housekeeping gene CT) was plotted against log concentration of RNA. Primers that resulted in almost 100% amplification efficiency and produced similar efficiency of amplification with housekeeping gene were selected for the analysis. The selection of housekeeping gene was also based on a minimal variation among the treated and untreated samples.

ELISA

Cells, as grown above, were treated with 2 μM of NC 2314, NC 2315, NC 2453, NC 2454 and 1% DMSO (control) for 24 hours. Supernatants were collected and analyzed for IL8 and VEGF using human IL8 and VEGF colorimetric ELISA Kits (Pierce, Fisher Canada, Nepean, ON), respectively, according to manufacturer's instructions.

COX-2 Activity Assay

Cells, as grown above, were treated with 2 μM of NC 2314, NC 2315, NC 2453, NC 2454

and 1% DMSO (control) for 24 hours. Cells were washed with phosphate buffered saline (pH 7.4) and harvested using rubber policeman. Cell pellets containing about 1×108 cells were obtained after centrifugation at 1000×g for 15 minutes at 4°C. Cell pellets were homogenized in homogenization buffer (0.1 M Tris Hydrochloride containing 1mM EDTA, pH 7.8) containing 10 µL of protease inhibitor cocktail (Sigma, Oakville, ON) and centrifuged at 10,000×g for 15 minutes at 4°C. Supernatants were analyzed for COX-2 activity assay using Cayman COX-activity assay kit (Cayman Chemical Company, Ann Arbor, MI, USA). The protein content was determined using Bradford assay.

Statistical Analysis

One way ANOVA with Dunett's post hoc test was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA) to test the significance of change in the inhibition or induction of protein expression of IL8 andVEGF and the enzyme activity of COX-2 by the treatment of curcumin analogues in HCT-116 and SW620.

Results:

Cytotoxicity Assay

In order to determine the suitable concentration cell line exposure to the NC compounds we conducted a cytotoxicity assay. The concentration demonstrating 50% inhibition of cell growth (IC50) determined in HCT-116 and SW620 cell lines are given in Table 7.1. The IC50 values of these compounds against both of these cell lines were very similar. Based on these values 0.30 µM concentration was chosen for the treatment of each cell line.

Table 7.1: IC_{50} values of curcumin analogues against colon cancer cells (HCT-116 and SW620) using the sulforhodamine B assay.

Curcumin analogues	IC_{50} (µM)	
	HCT-116	SW-620
NC 2314	0.33±0.02	0.32±0.03
NC 2315	0.27±0.02	0.29±0.03
NC 2453	0.17±0.02	0.33±0.01
NC 2454	0.12±0.01	0.29±0.01

Quantitative Real Time RT-PCR

We conducted a gene expression analysis of VEGFA, IL8, Glyoxalase 1 (GLO-1), Glyoxalase II (GLO-2) and COX-2 using quantitative Real Time-PCR. The primers (Table 7.2) designed against the mRNA sequence of these targets using primer 3 software exhibited amplification efficiency between 95-105%. The melting curve analysis of the product showed a single peak. Additionally, 2% agarose gel electrophoresis of the PCR product exhibited the presence of single product band and the absence of primer dimer band. The change in mRNA expression was analyzed using ΔΔCT method with beta-2 microglobulin as housekeeping gene. Except for an induction of VEGFA by NC 2314 in the SW620 cell line, no significant change was observed in mRNA expression of any of the selected targets (Table 7.3). However, these compounds significantly inhibited COX-2 and VEGFA in HCT-116 cell line. Glyoxalase I and II was not inhibited significantly in HCT-116 as well as SW620 colon cancer cell lines and NC 2314 had no effect on VEGFA mRNA expression. Surprisingly, these compounds significantly increased (82%-1214%) the mRNA expression of interleukin 8 in HCT-116 cell line.

Table 7.2: The sequence of validated primers that exhibited specific amplification with 95-105% amplification efficiency of the targets (the angiogenesis proteins) in the two-step real-time PCR (details in the method section).

Target*	Forward Primer	Reverse Primer
VEGFA	AGCGCAAGAAATCCCGGTA	TGCTTTCTCCGCTCTGAGC
B2MG	GAGTGCTGTCTCCATGTTTGATG	CTCTAAGTTGCCAGCCCTCCT
COX-2	AGGGTTGCTGGTGGTAGG	TTCATCTGCCTGCTCTGG
GLO1	CCGCCATGATTCACATTTGA	GTTGGCATGGCCTTTCCA
GLO2	ACGTCAAGTGCCTGGCGACC	AGAACTTCCCGCAGCCAGCC
IL8	TTGGCAGCCTTCCTGATTTC	AACTTCTCCACAACCCTCTG

*VEGFA = Vascular endothelial growth factor – A; B2MG = ß-2 microglobulin; COX-2 = Cyclooxygenase 2; GLO1 = Glyoxalase 1; GLO2 = Glyoxalase 2; IL8 = Interleukin 8

Table 7.3: Percent change in mRNA expression levels relative to control of angiogenesis and inflammatory biomarkers in HCT-116 and SW620 colon cancer cell lines after treatment with 0.3 μM of NC 2314, NC 2315, NC 2453, NC 2454 and 1% DMSO (control) in respective media for 24 hours.

Cell Lines	Compound code	% Change in mRNA expression*				
		COX-2	GLO-1	GLO-2	IL-8	VEGFA
SW620	NC 2314	↑12	↓6	↓18	↓15	↑76
	NC 2315	↑13	↑9	↓8	↓17	↑17
	NC 2453	↑18	↑13	↓16	↑6	↑5
	NC 2454	↓2	↓5	↑18	↑12	↓19
HCT-116	NC 2314	↓54	↔	↓5	↑82	↓16
	NC 2315	↓41	↓5	↓12	↑152	↓60
	NC 2453	↓40	↑19	↓12	↑941	↓74
	NC 2454	↓30	↓6	↓6	↑1214	↓72

*VEGFA = Vascular endothelial growth factor – A; B2MG = ß-2 microglobulin; COX-2 = Cyclooxygenase 2; GLO1 = Glyoxalase 1; GLO2 = Glyoxalase 2; IL8 = Interleukin 8

ELISA and Activity Assays

To understand the translation of the change in gene expression into the protein we conducted enzyme linked immunosorbant assay (ELISA) for IL8 and VEGF and COX-2 activity assay. The inhibition of COX-2 enzyme in HCT-116 cells by NC 2314, NC 2315, NC 2453 and NC 2454 was 93, 82, 80 and 38 percent, respectively (Figure 7.3A). The decrease in cycloxygenase activity in HCT-116 was significant ($p<0.001$) after 24 h of treatment with 0.3 μM of curcumin analogues (NC 2314, NC 2315, NC 2453 and NC 2454); however, none of these curcumin analogues modulated COX-2 activity significantly ($p=0.3887$) in SW 620 (Figure 7.3B).

While NC 2314 did not exhibit any effect on the VEGF expression in HCT-116 cell line, other curcumin analogues NC 2315, NC 2453 and NC 2454, demonstrated about 21% decrease in the VEGF expression (Figure 7.4A). In contrast, NC 2314 induced the expression of VEGF in SW620 cell line while other tested curcumin analogues did not demonstrate any inhibitory effect (Figure 7.4B).

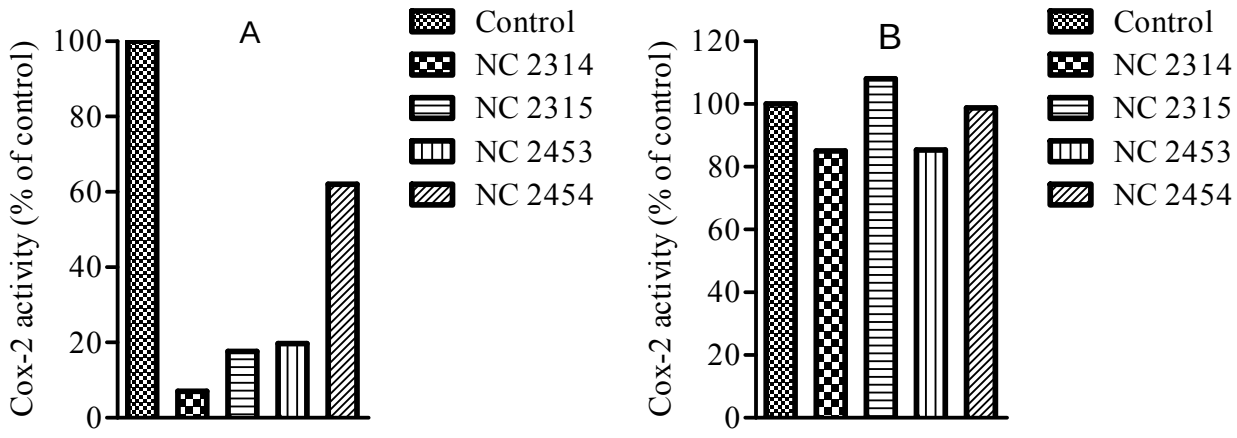


Figure 7.3: Cycloxygenase activity in HCT-116 (A) and SW620 (B) after treatment with 0.3 µM of NC 2314, NC 2315, NC 2453, NC 2454 and 1% DMSO (control) in respective media for 24 hours. The decrease in cycloxygenase activity in HCT-116 was significant ($p<0.001$) but insignificant ($p=0.3887$) in SW 620.

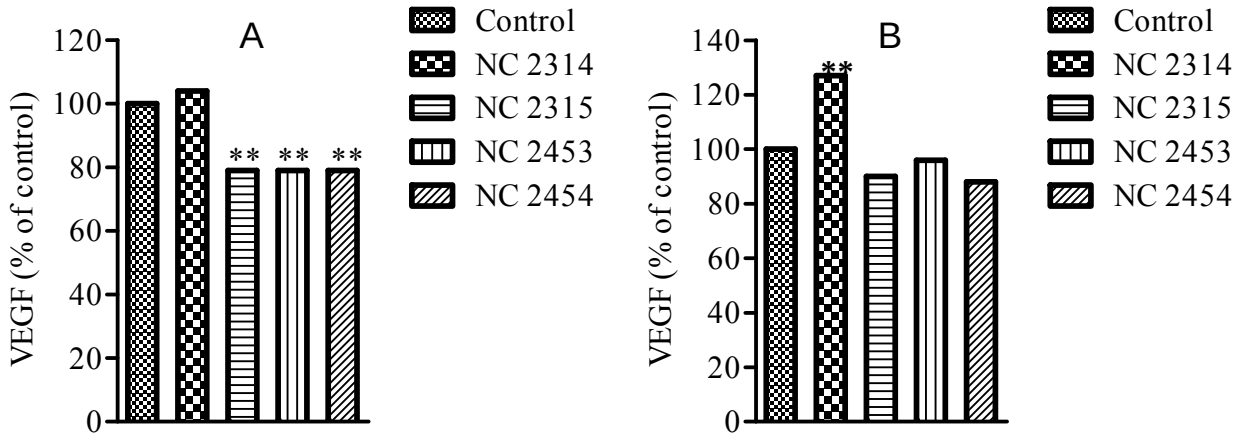


Figure 7.4: Expression of VEGF in HCT-116 (A) and SW620 (B) after treatment with 0.3 µM of NC 2314, NC 2315, NC 2453, NC 2454 and 1% DMSO (control) in respective media for 24 hours. (**) denotes the significant change in protein expression of VEGF with respect to control.

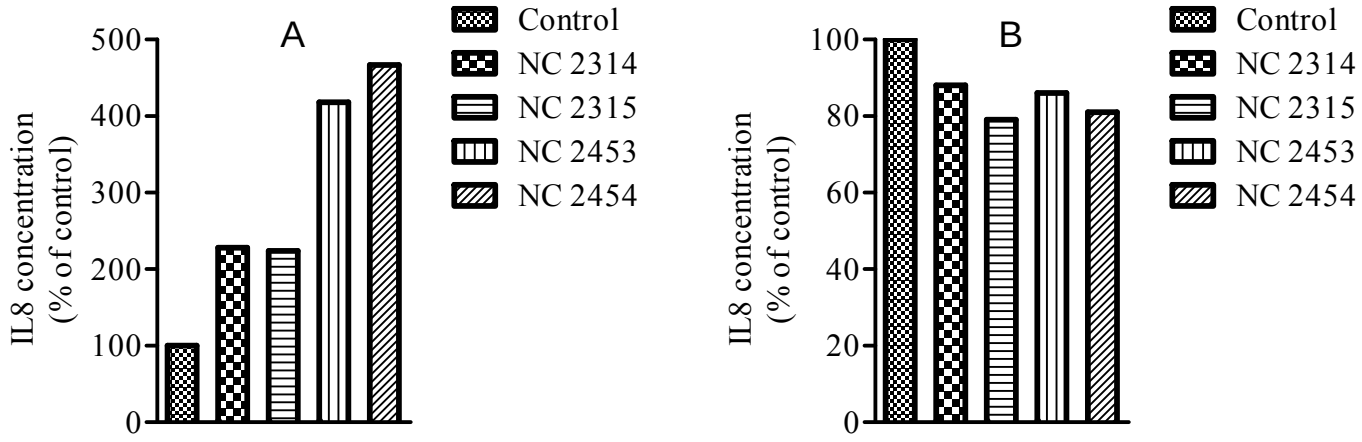


Figure 7.5: Expression of IL8 in HCT-116 (A) and SW620 (B) after treatment with 0.3 μM of NC 2314, NC 2315, NC 2453, NC 2454 and 1% DMSO (control) in respective media for 24 hours. The IL8 expression in HCT-116 significantly ($p<0.001$) increased whereas it decreased significantly in SW620 ($p<0.001$) in SW 620.

Unexpectedly, the curcumin analogues significantly increased the expression of IL8 in HCT-116 cells while no change was observed in SW620 cell lines. The change in the protein expression of IL8 in HCT-116 after 24 h of treatment with 0.3 μM Curcumin analogues NC 2314, NC 2315, NC 2453 and NC 2454 by 228, 224, 418 and 467 percent, respectively (Figure 7.5A). However, none of the curcumin analogues modulated the expression of IL8 in SW620 cell line (Figure 7.5B).

Discussion:

Given its promising efficacy in a variety of diseases including cancer, curcumin has garnered much attention in research. however, the bioavailability remain the major concerns in the development of curcumin as a drug. Modification of curcumin into 1,5-diaryl-3-oxo-1,4-pentadienyl containing curcumin analogues increased the efficacy in various cancer cell lines. Some studies on a few lead compounds identified in other labs have indicated that these curcumin analogues act via a variety of mechanisms including inhibition of angiogenesis (43, 46-48). Curcumin and one of its analogue, EF24, inhibit VEGF, IL8 and COX-2 mediated tumour angiogenesis, which is vital for tumour survival and growth (43). However, it was not clear whether other 1,5-diaryl-3-oxo-1,4-pentadienyl containing compounds demonstrate similar

activity and whether this activity is consistent across metastatic and non-metastatic colon cancer cell lines. Therefore, we selected four different compounds with promising cytotoxic activity to determine their effects on gene and protein expression of VEGF, IL8, COX-2. Additionally, we tested the effect of these curcumin analogues on gene expression of glyoxalase I and II, a common link between cancer and inflammation (24), to determine whether these curcumin analogues can also act as a chemopreventive agent. Compounds were selected based on their in vitro efficacy in conjunction with improved aqueous solubility and permeability, which are likely to make them pharmacokinetically more viable.

Two colon cancer cell lines with different metastasizing potential were chosen. HCT-116 isolated from colorectal tumour has low potential for metastasis in comparison to SW620, which was isolated from metastasized colon cancer. The selected curcumin analogues (NC 2314, NC 2315, NC 2453 and NC 2454) exhibited similar IC50 values (~0.30 μM) in HCT-116 and SW620 cell lines. Thus, the concentration for the treatment was selected as 0.30 μM. The maximum effect was observed after 24 h of incubation in pilot experiments.

Except for the genetic expression of glyoxalase I and II, modulations of other angiogenic and inflammatory markers were cell line specific, which may be attributed to the differential cellular microenvironment and gene expression pattern (49, 50). In contrast to curcumin, these curcumin analogues did not affect mRNA expression of glyoxalase I and II in both of the colon cancer cell lines and, therefore, we did not test protein expression of glyoxalase enzymes. Such differences in the activity may be attributed to the structural changes; however, exact pharmacophore responsible for the modulation of Glyoxalase I and II remains to be established. In general, these curcumin analogues did not modulate gene or protein expression of VEGF, IL8 and COX-2 in SW620 cell line except that NC 2314 increased expression of VEGF. While these compounds did not affect the selected essential angiogenic and inflammatory proteins in SW620, the modulation effect was significant in HCT-116. COX-2 and VEGF was inhibited significantly by these curcumin analogues except NC 2314 which did not exhibit any effect on VEGF expression. Unexpectedly, these curcumin analogues induced mRNA and protein levels of the proangiogenic chemokine, IL8. Since curcumin induces ROS generation (51), which subsequently induces IL8 production, IL8 induction is likely to be mediated via increased ROS generation in HCT-116 cell line. In earlier reports, ROS mediated the increase in IL8 expression in A549 cells, which is an adenocarcinomic human alveolar basal epithelial cell line (52),

gingival fibroblast and submadibular gland carcinomic cells (53). Our colleague also found increased ROS production in HCT-116 cell lines after the treatment with 1,5-diaryl-3-oxo-1,4-pentadienyl containing compounds (unpublished). However, the exact mechanism of IL8 induction by these curcumin analogues is not yet clear and needs elucidation. Given that the angiogenic effect of IL8 is mediated via the induction of VEGF (54), the inhibition of VEGF, a downstream target, by curcumin analogues is likely to demonstrate anti-angiogenic effect.

Conclusion:

Despite exhibiting similar IC50, the curcumin analogues, NC 2314, NC 2315, NC 2453 and NC 2454 show differential mechanism of action in HCT-116 and SW620 cell lines. Since HCT-116 was more responsive to the curcumin analogues, we conclude HCT-116 as a better model to test the angiogenic effect of curcumin analogues. The curcumin analogues inhibit COX-2 and VEGF but induce the proangiogenic cytokine IL8 in HCT-116 cell line presumably via increase in ROS generation. Given the inhibitory effect on VEGF, a vital angiogenesis protein and downstream target to the IL8, the curcumin analogues are likely to exhibit anti-angiogenic effect. The study identifies the modulatory effect on some of the common essential proteins involved in the angiogenesis; however, the translation of this effect in animal models remains to be seen.

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CHAPTER 8
GENERAL DISCUSSION

8.1 Discussion

The emphasis on translational research by major funding organizations resulted in academia's incursion into drug discovery, which was once pharmaceutical industry's prerogative. Only 13 of 78 academic drug discovery units in the USA were set up prior to 2000 while other units mushroomed within the last 10 years (1). A decade ago, pharmaceutical industries delved into high-stake drug discovery business while academia focused on hypothesis driven basic research providing logical support to the drug discovery industries. Academia avoided drug discovery due to a variety of reasons including financial constraints, resource constraints, conflicting objectives and lack of hypothesis driven approaches. Brown and Goldstein's identification of the role of HMG-CoA reductase (2) in the cholesterol biosynthetic pathway provided logical support for Merck to develop lovastatin and simvastatin (3). Recently, the emphasis placed on translational research by major funding agencies such as NIH and CIHR compelled academic institutions to start drug discovery programs (4). The University of Saskatchewan is one of the Canadian universities to begin a drug discovery program, which is still in its nascent stage.

Dimmock and coworkers are interested in the development of curcumin analogues as anti-colon cancer agents (5-7). Curcumin, a constituent of turmeric powder, exhibits anticancer properties and epidemiological association exists between turmeric intake and the lower prevalence of colon cancer (8, 9). However, the major barriers in the development of curcumin as an anticancer drug remain its poor solubility, low bioavailability and inadequate efficacy (10-12). In order to improve upon these major concerns, Dimmock and co-workers replaced the β-diketone moiety with a ketone group to generate a new series of compounds with the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore (5). This new series of curcumin analogues demonstrated very high *in vitro* cytotoxicity against various cancer cell lines, in particular colon cancer cell lines, thus generating interest in the development of these curcumin analogues as a potential anticancer agent (13). This series has received attention from different labs for a variety of indications including infections, malaria and cancer (14-16).

One of the curcumin analogues, NC 2083, exhibited high efficacy against various colon cancer cell lines, (17) but failed to effect tumour regression in SW620 tumour implanted nude

mice. Given the high *in vitro* efficacy of this compound, the reasons for the failure of NC 2083 to cause tumour regression in mouse tumor models were not clear. Therefore, our first purpose was to determine the reason for NC 2083's failure in order to provide feedback to the drug design group at the University of Saskatchewan. Based on unfavourable *in silico* predicted physicochemical characteristics we predicted poor PK properties to be responsible for its lack of efficacy (18). We conducted a battery of pharmacokinetic studies to understand whether the lack of efficacy of NC 2083 is due to unfavourable PKs.

We required a validated analytical method suitable for the analysis of samples generated during PK assays. We developed a general reverse phase isocratic HPLC method using ultraviolet detection (chapter 3) for the quantification of NC 2083 and other curcumin analogues in different rat biomatrices and validated using pre-defined USFDA criteria (19). At the inception of this project mass spectrometry was not available, necessitating the use of a less sensitive analytical approach. NC 2083 was unstable in light and on bench-top at room temperature for 6h but showed stability when stored on ice in the dark. Also, given the affinity of NC 2083 to thiol containing proteins, we investigated its potential covalent binding with serum albumin but we observed minimal covalent binding. This method was tested for its suitability to 13 other curcumin analogues with different logP values currently under study at the University of Saskatchewan. With slight modification in chromatographic conditions, this method was also suitable for the quantification of several curcumin analogues with variable lipophilicity. Given its ability to quantify different new molecules in widely used biomatrices, the method was suitable for the sample analysis of a variety of ADME studies with different analogues. The versatility of method is important to carry out a variety of pharmacokinetic studies without spending time on method development and validations.

A battery of pharmacokinetic assays including permeability assay, plasma protein binding, metabolic stability in rat liver microsomes and in vivo pharmacokinetic studies were conducted to identify the reason for lack of efficacy. NC 2083 exhibited low permeability, extensive plasma protein binding and intermediate hepatic clearance in Caco-2, rat plasma, and rat liver microsomes, respectively. The concentration of NC 2083 in serum samples collected after intravenous and per oral administration was below the limit of quantification (<50 ng/mL). While the low permeability and intermediate hepatic clearance explained the low serum concentration in oral pharmacokinetic samples, the low serum concentration in intravenous

serum samples remained elusive. A blood cell partitioning experiment suggested that NC 2083 rapidly and extensively (~80% within 5 minutes) partitions into blood cells and remains in equilibrium with the plasma. Other anticancer drugs including oxaliplatin (20), ifosphamide (21), topotecan (22) and irinotecan (23) exhibit high partitioning into red blood cells yet remain viable therapeutic options in the treatment of cancer.

On the whole these pharmacokinetic studies suggested that the lack of NC 2083 efficacy *in vivo* was due to its low permeability, high plasma protein binding and high RBC partitioning characteristics. Given the affinity of 1,5-diaryl-3-oxo-1,4-pentadienyl containing curcumin analogues to alkylate thiol containing proteins, we tested the intra-erythrocytic depletion of glutathione. Our results suggested that the NC 2083 at 30 μM did not decrease the glutathione content significantly. Given the rapid partitioning of NC 2083 into blood cells, we tested for anti-malarial activities of NC 2083 and a series of compounds structurally related with NC 2083. The partitioning of antimalarial compounds into red blood cells is desirable because malarial sporozoites spend a part of their life cycle in erythrocytes. Many of these compounds were found active against *Plasmodium falciparum*. Recently, other laboratories also identified curcumin analogues containing 1,5-diaryl-3-oxo-1,4-pentadienyl group as having antimalarial activity (14). Interestingly, our compounds were also effective against drug resistant malarial parasites and thus can be sought as a potential solution to the new emerging drug resistance in malarial therapy (24, 25). Although the literature indicates that anticancer agents such as methotrexate may have potential as antimalarial agents (26) our pharmacokinetic directed discovery of the anti-malarial property of this series of compounds was unique, i.e. the identification of certain pharmacokinetic characteristics led us to explore the potential of our analogues as antimalarial agents.

Dimmock and co-workers have synthesized a plethora of 1,5-diaryl-3-oxo-1,4 pentadienyl containing curcumin analogues exhibiting high efficacy against various colon cancer cell lines, and with recent investigations, a number of these analogues also demonstrate putative antimalarial activity particularly against resistant *P. Falciparum* strains . Hence, a strategy to identify analogues with more promising pharmacokinetic and pharmacodynamic properties was required. Since our investigation of NC 2083 suggested that lack of *in vivo* efficacy was due to unfavourable pharmacokinetic characteristics, a pharmacokinetic screening of curcumin analogues was our next priority. Since poor absorption characteristics was a major stumbling

block for NC 2083, we set up a permeability-screening assay to provide rapid *in vitro* assessments of intestinal permeation.

The two widely used cell monolayer models, Caco-2 and MDCK, were selected due to their morphological and physiological similarity with the small intestine (27-29); however, their susceptibility to highly cytotoxic agents had not been studied. Highly cytotoxic agents may adversely affect cell monolayer integrity. Compounds such as *Clostridium difficile* toxin A (30) and piperine, (31, 32) are known to influence the tight junction and modulate permeability across the intestinal polarized epithelium. In order to select a model that is least affected by curcumin analogues, we tested curcumin analogues and related chalcones (another series of promising anticancer compounds) in both models. While exposure of Caco-2 with curcumin analogues did not alter the permeability of Lucifer Yellow (LY) (a paracellular marker), the analogues significantly increased the paracellular permeability of LY in MDCK. This increase in LY permeability correlated with the loss of MDCK cell viability indicating that these curcumin analogues affect MDCK cells adversely. Interestingly, chalcone and curcumin analogues without piperidine ring did not adversely effect either cell line. To the best of our knowledge the literature has not reported a differential effect on cell viability between the two model systems depending upon the analogue series. Despite these effects, the permeability rankings of compounds were very similar between MDCK and Caco-2 models. Interestingly, the passive permeability of analogues in MDCK failed to correlate with clogP. Altogether, we concluded that MDCK is not suitable for the permeability assessment of curcumin analogues containing piperidine ring; however, it may be used for the permeability assessment of non-piperidine based curcumin analogues and chalcones. Our studies suggest that researchers should take care in the selection of the appropriate *in vitro* absorption model and assure that appropriate optimization experiments are conducted in advance to ensure that their compounds do not adversely affect monolayer integrity.

Based on the permeability screening assay we identified NC 2314, NC 2315, NC 2453 and NC 2454 as highly permeable curcumin analogues with acceptable solubility characteristics. Subsequently, we evaluated these compounds in rats to determine their pharmacokinetic characteristics. Rats died within hours of administration of a 10 mg/kg oral dose and 1 mg/kg intravenous dose. The concentration of these curcumin analogues in early plasma samples collected before death after IV administration were very low whereas the concentration in oral

samples were below the limit of quantification. This suggests rapid distribution of these compounds in the body (and red blood cells) and most likely into vital organs such as brain or heart to cause acute toxicity. However, further investigation is necessary to identify the mechanism of toxicity. Apparently, these curcumin analogues have a low therapeutic index, which is corroborated by the *in vitro* inhibition profile of these compounds in HCT-116 and SW620 cell lines. The inhibition profile showed cell viability changed from 100 to 0 percent within a 2- to 4-fold change in concentration. In my work, poor analytical sensitivity (50 ng/mL) of HPLC-UV precluded PK evaluation at lower doses; however, an analytical method with improved analytical sensitivity using LC-MS/MS would allow PK evaluations at lower, less toxic, doses

Additionally, in order to identify potential pharmacological targets of the curcumin analogues containing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore, we tested the inhibitory potential of these compounds on the genetic expression and protein expression of various key angiogenesis and inflammatory biomarkers using real time RT-PCR and ELISA or activity assays, respectively. Curcumin is known to inhibit tumour angiogenesis, which is the formation of blood vessels that is essential for the growth of the tumour (33, 34). In order to test whether these 1,5-diaryl-3-oxo-1,4-pentadienyl curcumin analogues inhibit angiogenesis and inflammatory biomarkers similar to curcumin, we conducted a gene and protein expression analysis of angiogenesis biomarkers (VEGF, IL8 and COX-2) and inflammatory biomarkers (Glyoxalase I, Glyoxalase II) in the metastatic SW 620 and non metastatic HCT-116 cell lines. Similar to curcumin, these curcumin analogues inhibited VEGF and COX-2 in HCT-116 cell line without any alteration in the SW 620 cell line. The lack of alteration in VEGF and COX-2 expression in SW 620 cells is interesting as the curcumin analogues demonstrated similar IC_{50} values in both cell lines. This clearly indicated the mechanism of cell growth inhibition is cell line specific. HCT-116 was a better model than SW620 to study the effect of these curcumin analogues on angiogenesis biomarkers. However, the curcumin analogues should be tested for angiogenesis inhibition in pre-clinical animals to understand whether the modulation of angiogenesis biomarkers results in *in vivo* efficacy. Additionally, these curcumin analogues did not modulate glyoxalase I or II in any of the cell lines and therefore, these are not likely to show anti-inflammatory activity.

The design and synthesis of curcumin analogues by Dimmock and coworkers focused to

increase *in vitro* efficacy without due attention to the physicochemical properties (that would assure favourable PK properties) of the molecules and, in the process, increased molecular obesity (35) (molecular obesity refers to molecules with high logP values and molecular weights). Given the association of physicochemical properties with pharmacokinetic parameters, we investigated the optimal physicochemical space for this series of compounds as Lipinski's RO5 and Veber's rule is not universally applicable for all series of molecules (36). We conducted a comparison between our laboratory and other laboratories involved in the synthesis of curcumin analogues containing 1,5-diaryl-3-oxo-1,4-pentadienyl group that have successfully identified one molecule exhibiting *in vivo* efficacy in pre-clinical model. The comparison suggests that the success rate of the lab correlated with the number of conformities to Linpinski's RO5. In Dimmock's lab, violation of RO5 was more abundant as ~50% of the compound synthesized violated RO5. Since, none of the successful candidate violated any of the RO5 criteria, the future design of molecules with this pharmacophore should strictly conform to RO5.

Similar to other academic drug discovery units (ADDU), our Drug Design and Discovery Research Group (DDDRG) at the University of Saskatchewan also face numerous challenges and need to develop strategies to overcome them. First, the most common challenge for any ADDU is to arrange funding. In a survey by Frye et. al. (1) of academic drug discovery units in the USA, 68% of the labs identified both amount and stability of funding as a major concern. Financial constraint does not allow ADDUs to develop a molecule and bring it into the market; only three labs in USA have been able to take their molecule to Phase II clinical trials (3) and none of the molecules have moved to Phase III. Similar to other ADDUs, the DDDRG also encounters funding as the biggest deterrent in the drug design and discovery program. Therefore, a more practical target for the DDDRG would be to out license the molecule after pre-clinical studies. Limited funding restricts the DDDRG's ability to employ qualified personnel or to procure high throughput screening (HTS) instruments to expedite the research program. Although some of the other ADDUs have procured HTS (37) the results of high throughput screening are being questioned as such screening has given more false positive molecules, termed as pan assay interference compounds (PAINS) (38). This issue is attributed to the lack of experience of academia in making choices, which is another major challenge that ADDUs face today (39).

Second, to gain academic benefits in terms of tenure, promotion and merit increases, rapid publication of results are required. Expectedly, publication received a score of 6.9/8 as a priority

for ADDUs in USA (1). This publication pressure not only jeopardizes patent processes but also results in more PAINS in the drug discovery pipeline and publications (39). Thirdly, graduate students that have little industrial experience largely carry out the current drug discovery program of the DDDRG at the University of Saskatchewan as DDDRG cannot hire dedicated staff due to financial constraints. These graduate students require a lengthy training period and then leave the ADDUs upon completion of the graduate program with a limited contribution to the drug discovery program. This reliance on graduate students slows down the productivity of the unit. In contrast, pharmaceutical industries have dedicated staffs that receive rapid training in a specialized area and continue to work for prolonged periods within the industry to add value to the organizations that trained them. Lastly, the non-hypothesis driven nature of drug discovery usually receives unjustified comments from grant application reviewers and other academic units.

The challenges that we face today should be dealt with at different levels. First, the issues of conflicting interests for ADDUs personnel with drug discovery program should be dealt at the University management level by modifying the criteria for promotion and merit pay for ADDU staff. This may ensure that the conflicting interests do not jeopardize the process of drug discovery. Second, collaboration with pharmaceutical industries to gain expertise and funding is advisable. University of Alabama formed a collaborative partnership with Southern Research Institute to share expertise and support their drug discovery program (40). More recently, pharmaceutical industries are seeking out more collaboration with smaller industries and academia to deconstruct (41) their drug discovery programs and decrease the cost of drug development. The DDDRG should actively seek collaboration with industry and form a symbiotic relationship. Finally, the DDDRG should arrange seminars and conferences to educate other people about the drug discovery processes. The education of other researchers may stop unjustified comments on grant applications and increase the potential to receive funding. Despite the challenges it faces today in drug discovery, academia will make its niche in the pharmaceutical industry's domain in future.

8.2 Challenges and Limitations

My current work faced several challenges and had a few limitations. One of the major challenges was the lack of identified lead molecule. Except NC 2083, all other compounds were

tested only for *in vitro* efficacy and no information on *in vivo* efficacy was available. Due to the lack of in house established anticancer efficacy screening models, the promising compound could not be screened readily. In the absence of clearly identified lead molecule, the utility of complete pharmacokinetic characterizations could not be established and unamibiguous decisions could not be taken. The second major challenge was the poor aqueous solubility of selected series of curcumin analogues as this posed a challenge in the selection of candidate compounds for comparison between Caco-2 and MDCK. Several compounds were screened for their solubility in the permeability assay medium containing 1% DMSO. Other approaches to improve solubility were not employed to avoid any intereferences on the quality of Caco-2 and MDCK monolayers and permeability characteristics of the candidate compounds. The third major challenge was the low sensitivity of the analytical method. Given the tendency of these piperidone analogues toward blood cells, the sensitivity of HPLC-UV method was not sufficient to quantitate the concentrations in serum and generate a concentration versus time profile. Determination of concentrations in whole blood might facilitate the pharmacokinetic characterization. As well, the unavailability of more sensitive analytical technology such as LC-MS/MS was a major limitation. This technology may have allowed a complete pharmacokinetic characterization. Another challenge was the lack of manpower and resources, which was required to gather information on different aspects of molecules and help in decision making.

8.3 Conclusions

My current work is a first step towards the establishment of a pharmacokinetic and pharmacodynamic screening process in the drug discovery endeavours at the University of Saskatchewan. My current work identified the inadequacy in the current model adopted in the drug design and drug discovery of anti-cancer curcumin analogues at the College of Pharmacy and Nutrition. The pharmacokinetic and pharmacodynamic evaluations with the curcumin analogues laid down some fundamental building blocks for anti-cancer curcumin analogues drug discovery efforts in the College of Pharmacy and Nutrition. My work suggests the need to give the due consideration to physicochemical characteristics in the design of curcumin analogues to incorporate desirable pharmacokinetic and pharmacodynamic characteristics. A model of drug discovery has been proposed, which is likely to be more efficient; however, the efficiency of the model remains to be seen.

8.4 Future Directions

My current work was a first step in the pursuit of establishing the drug design and discovery strategy at the University of Saskatchewan. Given the multifaceted nature of the drug discovery research, the future research may take different directions. However, future research that is most relevant to our lab are summarized here.

8.4.1 Rational Drug Design and Screening Strategies

Currently, Dimmock and coworkers design and synthesize molecules to enhance *in vitro* efficacy and in the process, the molecules are gaining molecular obesity (high molecular weight and logP) that results in the poor pharmacokinetic properties. Our analysis with the different laboratories (chapter 7) suggests the molecule's conformity to Lipinski's RO5 is likely to improve the success rate and, therefore, the design of future molecules should conform to RO5. Currently in Dimmock's lab, a large number of compounds are designed simultaneously prior to efficacy and pharmacokinetic studies. The current model of drug discovery and development generates many unpromising molecules, which can be reduced by the feedback based drug design and discovery model (Figure 8.1).

In a feedback based drug design and discovery model, a small number of molecules are designed with the conformity to RO5 and subjected to *in vitro* efficacy, physicochemical characterization and pharmacokinetic studies. Based on the results of these molecules, the next set of molecules should be designed to optimize pharmacokinetic properties before *in vivo* efficacy and toxicity studies are undertaken.

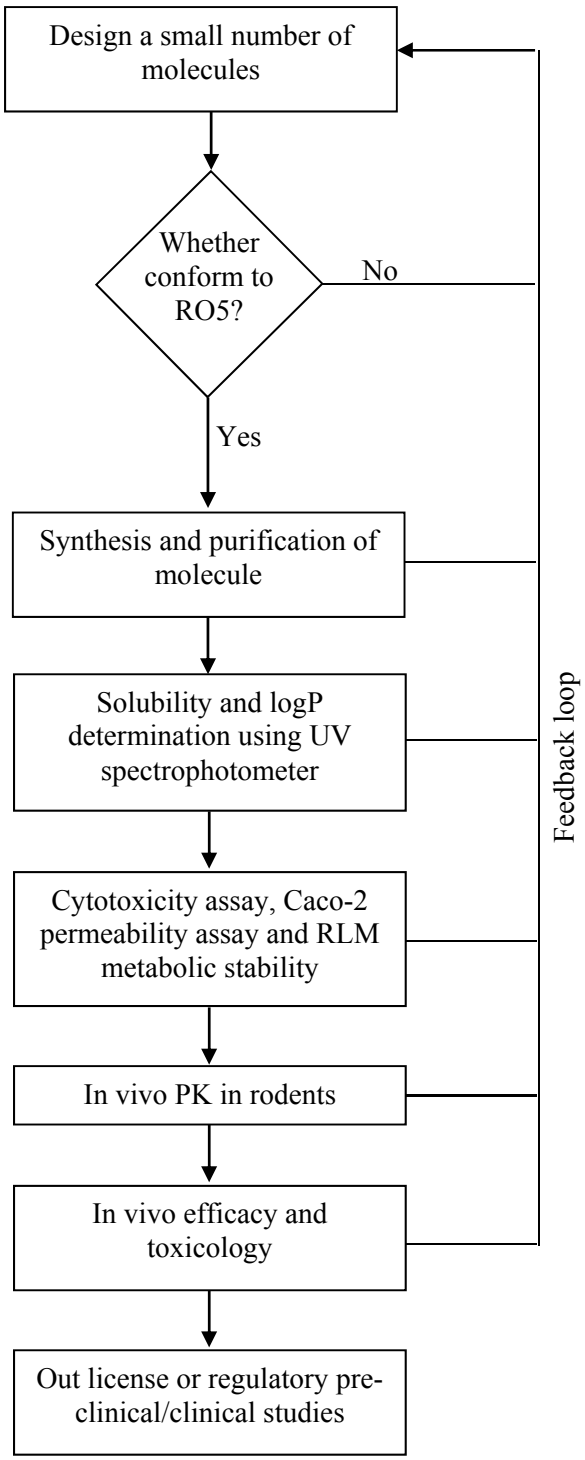

Figure 8.1: Schematic diagram of proposed feedback based drug design and discovery model for anticancer curcumin analogues containing 1,5-diaryl-3-oxo-1,4-pentadienyl group at the University of Saskatchewan

8.4.2 Efficacy and Toxicological Assessment

As the curcumin analogues with high permeability, NC 2314, NC 2315, NC 2453 and NC 2454, were fatal to rats upon oral administration at 10 mg/kg, we need to investigate their mechanism of toxicity and establish the toxicophore, a part of the molecule responsible to cause toxicity. NC 2083 did not exhibit toxicity, which may be attributed to the low oral bioavailability of the molecule. As we selected highly permeable molecules for pharmacokinetic studies that are likely to reach systemic circulation, the molecules exhibited more severe toxicity presumably due to the increased absorption. The toxicity of the pharmacophore, 1,5-diaryl-3-oxo-1,4-pentadienyl group, is not known at the moment. We will need to establish the acute and chronic toxicity of the basic pharmacophore in pre-clinical animals to understand the minimal toxicity caused due to the presence of pharmacophore and subsequently develop strategies to avoid them. Such information will allow us to identify the toxicophore and assure that it is avoided in the further design of the curcumin analogues.

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