# The Roles of Monoamine Oxidase-A and p38(MAPK) in Breast Cancer

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# ABSTRACT

Monoamine oxidase-A (MAO-A) is an enzyme that has historically been linked to major depressive disorder (MDD). The prevalence of MDD among breast cancer patients is almost 25%, but realistically it is underdiagnosed within this patient population. Most breast cancer is deemed estrogen receptor positive [ER(+)] and is commonly treated with the anti-estrogenic chemotherapeutic compound tamoxifen. Resistance to tamoxifen has been associated with a paradoxical activation of the stress-associated kinase, p38(MAPK) (normally associated with cell death). Our research group has recently demonstrated that p38(MAPK) can regulate the function of MAO-A in glial cells. Taken together, MAO-A, depression and p38(MAPK) are all associated with a poor prognosis in breast cancer patients, particularly those with an ER(+) status. Several mechanisms have been proposed in each respect and we hope to further elucidate this relationship by focussing on the interaction between MAO-A and p38(MAPK) in the context of breast cancer.

The **hypothesis** states that a functional interaction between the p38(MAPK) and MAO-A systems alters breast cancer cells in an ER-dependent manner.

The proposed objectives of this project are to determine what might be influencing MAO-A function in breast cancer cells, and how changes in MAO-A function affect cell phenotype. Using pharmacological approaches (i.e. antidepressant drugs), we investigated the role of MAO-A and p38(MAPK) on selected characteristics of ER(+) (e.g. MCF-7) and ER(-) (e.g. MDA-MB-231) breast cancer cells under four treatment conditions, which include clorgyline (CLG), an antidepressant MAO-A inhibitor, and SB203580, an inhibitor of p38(MAPK).

Our results indicate that the very high MAO-A activity in MDA-MB-231 (MB-231) cells was partly dependent on p38(MAPK) activity. The tumourigenic properties (e.g. anchorage-independent growth, migration) of MB-231 cells depended on both MAO-A and p38(MAPK) functions, although the effects were not additive suggesting that both inhibitors were exerting their respective effects via common signalling targets. The role of MAO-A and p38(MAPK) on MB-231 mitochondrial function and cell growth was negligible. In contrast, MAO-A and p38(MAPK) only influenced mitochondrial function in MCF-7 cells and did not affect any of the

other tumourigenic properties measured. Immunocytochemical methods, supported by Western blotting, revealed an increase in E-cadherin expression in both cell lines. This suggested that MAO-A and p38(MAPK) could be influencing transitions between epithelial and mesenchymal phenotypes.

Our *in vitro* findings suggest that MAO-A and p38(MAPK) might contribute to a common mechanism in breast cancer cell lines, but that their influence on cell phenotype is less dependent on the respective cell's ER status and perhaps more so dependent on the cell's metastatic potential. If this is the case, then the contribution of MAO-A and p38(MAPK) to [clinical] metastatic breast cancer should be duly considered. Our ongoing investigations are focussing on the influence of clinically relevant antidepressants on breast cancer cell phenotype *in vitro*.

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# LIST OF ABBREVIATIONS

**4-OH-TAM**, 4-hydroxy-tamoxifen

**5-HT**, 5-hydroxytryptamine (Serotonin)

**ACS**, Aqueous Counting Scintillant

**ADP**, adenosine diphosphate

**APS**, Ammonium Persulfate

**ATCC**, American Type Culture Collection

**BCA**, bicinchoninic acid

**BrdU**, 5-bromo-2-deoxyuridine

**$BS^3$**, bis[sulfosuccinimydyl] suberate

**CLG**, clorgyline

**CNS**, central nervous system

**CYP**, cytochrome P450

**DMEM**, dulbecco's modified eagle medium

**DMSO**, dimethyl sulfoxide

**DSM-IV**, Diagnostic and Statistical Manual of Mental Disorders-Fourth Edition

**DSS**, disuccinimidyl suberate

**DTT**, dithiothreitol

**ECL**, enhanced chemiluminscence

**EDTA**, ethylenediaminetetraacetic acid

**EMT**, epithelial-to-mesenchymal transition

**ER**, estrogen receptor

**ERK**, extracellular signal-regulated kinase

**FAD**, flavin adenine dinucleotide

**FBS,** fetal bovine serum

**GFP**, green fluorescent protein

**HEPES**, hydroxyethyl piperazineethanesulfonic acid

**HER2**, human epidermal growth factor receptor 2

**HRP**, horse radish peroxidase

**JNK,** c-Jun N-terminal kinases

**kDa**, kiloDalton

**MAO**, monoamine oxidase

**MAOI**, monoamine oxidase inhibitor

**MAPK**, mitogen-activated protein kinase

**MB-231**, MDA-MB-231

**MDD,** major depressive disorder

**MEM**, minimum essential medium

**MET**, mesenchymal-to-epithelial transition

**MTT**, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

**MW**, molecular weight

**NHS**, *N*-hydroxysuccinimide

**PBS**, phosphate buffered saline

**PIC**, protease inhibitor cocktail

**PR**, progesterone receptor

**RT**, room temperature

**SB**, SB203580

**SDS**, Sodium Dodecyl Sulfate

**SDS-PAGE**, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

**SERM**, selective estrogen receptor modulator

**SNRI**, serotonin-norepinephrine reuptake inhibitors

**SSRI**, selective serotonin reuptake inhibitors

**T/E**, trypsin/EDTA

# 1 INTRODUCTION

## 1.1 Major Depressive Disorder

Mood disorders are among the most commonly identified mental illnesses (1). According to the Public Health Agency of Canada approximately 8% of adults will experience major depression within their lifetime (1). On an international scale, major depressive disorder (MDD) is considered to be the leading cause of 'years lived with disability' (1). Depression is recognized as a heterogeneous disorder with many different subtypes developing from a variety of etiologies (2, 3); however, the term 'depression' will be used throughout this document, with the understanding that any given context could encompass any or all subtypes. Clinical depression or major depressive disorder is a mood disorder in which a person will experience intense feelings of distress over an extended period of time resulting in hopelessness, insomnia, and an aversion to perform daily activities and engage in social interactions (4). Initial detection of depression by a primary care physician typically involves assessing answers given by the patient to a specific series of questions (5) that will normally indicate the need for a more detailed assessment. Further analysis may require the use of symptom-based rating scales and diagnostic criteria that is commonly extracted from the Diagnostic and Statistical Manual of Mental Disorders-Fourth Edition, Text Revision (DSM-IV-TR) (6). A number of specific symptoms must be observed before categorizing a condition as MDD in order to ensure proper treatment and ultimate recovery. The DSM-IV provides four categories of symptoms for diagnosis: affective, behavioural, cognitive, and somatic symptoms (6). Frequently, studies will report patients as experiencing depressive symptoms which could include any number of signs, for example, feelings of helplessness (affective/mood), preferred isolation (behavioural), lack of focus (cognitive) and insomnia (somatic/physical) (7). At least one major depressive episode lasting two or more weeks, during which time a minimum of four depressive symptoms are displayed, qualifies for a diagnosis of MDD (8). Over half of those who have experienced only a single episode of major depression will suffer recurrence, thereby identifying a previous history of depression as a very strong predictor of future episodes (9).

Depression is not limited to a specific demographic; although it has been consistently documented that women experience depression at higher rates than men (10). Within a lifetime, the onset of depression can reflect turning points within a typical life cycle and is quite common during adolescence and early adulthood (11). However, those over the age of 80 also experience depression at increased rates as a result of increased physical dysfunction, loss of independence, and social isolation (11). Research has yet to uncover the precise aetiology of the condition, but it is currently believed to be brought on by a combination of genetic factors and environmental triggers (12) that contribute to a chemical imbalance in the central nervous system (CNS) (13-15).

### 1.1.1 Depression and the Serotonin System

MDD has long been associated with the serotonergic system, thus explaining the underlying action of several antidepressant medications (13, 16, 17). Serotonin (5-hydroxytryptamine; 5-HT) is a mood-regulating hormone, which at reduced levels has been implicated in the onset of different psychopathological conditions (18). 5-HT is a tryptophan derivative best known for its role as a neurotransmitter (19). Interestingly, however, only 1% of 5-HT is localized in the central nervous system (19); the remainder serves as an active hormone in the peripheral immune, circulatory, reproductive, and gastrointestinal systems (20). Nevertheless, various pharmaceutical agents have been designed to increase the availability of CNS 5-HT to provide relief of depressive symptoms. For instance, selective serotonin reuptake inhibitors (SSRIs) are frequently prescribed for the treatment of depression and inhibit 5-HT reuptake into the presynaptic neuron. This allows for an accumulation of 5-HT within the synaptic cleft and, thus, it is available to postsynaptic receptors (21). An alternative pharmacological approach led to the synthesis of agents which inhibit the metabolic action of enzymes that degrade 5-HT, such as monoamine oxidase (MAO) (22).

### 1.1.2 Monoamine oxidase

In humans there are two functionally distinct forms of the MAO enzyme: MAO-A and MAO-B, which are both integral outer mitochondrial membrane proteins. Their dependence on a flavin co-factor for full activation places them in the family of flavoproteins (23, 24). The existence of multiple forms of the protein became generally accepted in 1971 (25) and it would only later be discovered that MAO-A and MAO-B are encoded by two separate genes (26), despite having a 70% amino acid sequence homology (27). The primary structure of these two distinct proteins consists of 526 (MAO-A) and 520 (MAO-B) amino acid residues with respective molecular weights of 59.7 kDa and 58.8 kDa (28). There are four highly conserved regions between the MAO isozymes (Figure 1); these include (1) an adenosine diphosphate (ADP)-binding site; (2) a substrate-binding domain; (3) a C-terminus region, which possibly forms the transmembrane domain; and (4) a covalent- flavin adenine dinucleotide (FAD) binding site (29). MAOs share no sequence similarity with other known proteins, with the exception of the ADP-binding sequence, which is typically present in other FAD-dependent enzymes (30). This region is predicted to fold into a βαβ conformation, forming a site for interaction with the ADP moiety of FAD (31). FAD is covalently linked to Cys406 in MAO-A and is a functional component of the overall substrate oxidation reaction (32).

MAO-A and B also display differences in tissue distribution, structure and substrate preference. These isozymes are present in most mammalian tissue, but there are species-specific differences (33-35). In humans, both MAO-A and -B are expressed in most tissues with the exclusion of the blood platelets (which expresses MAO-B exclusively) (34). MAO-A and MAO-B are most abundant in the liver and placenta and have the lowest levels of expression in the spleen (35). The lungs, placenta and small intestines have higher MAO-A activity as opposed to the myocardium in which MAO-B is most active (35). The presence of MAO isozymes extend to many areas of the human brain. MAO-A is predominantly localized in catecholaminergic neurons whereas serotonergic neurons appear to contain MAO-B (36).

Both enzymes catalyze the oxidation of diverse amines, including 5-HT, dopamine and epinephrine (37). However, whereas MAO-B has a greater affinity for the trace amine, phenethylamine, MAO-A is more selective and likely to degrade 5-HT, epinephrine and norepinephrine (14, 33). Binda and colleagues (38) provide a concise representation of the

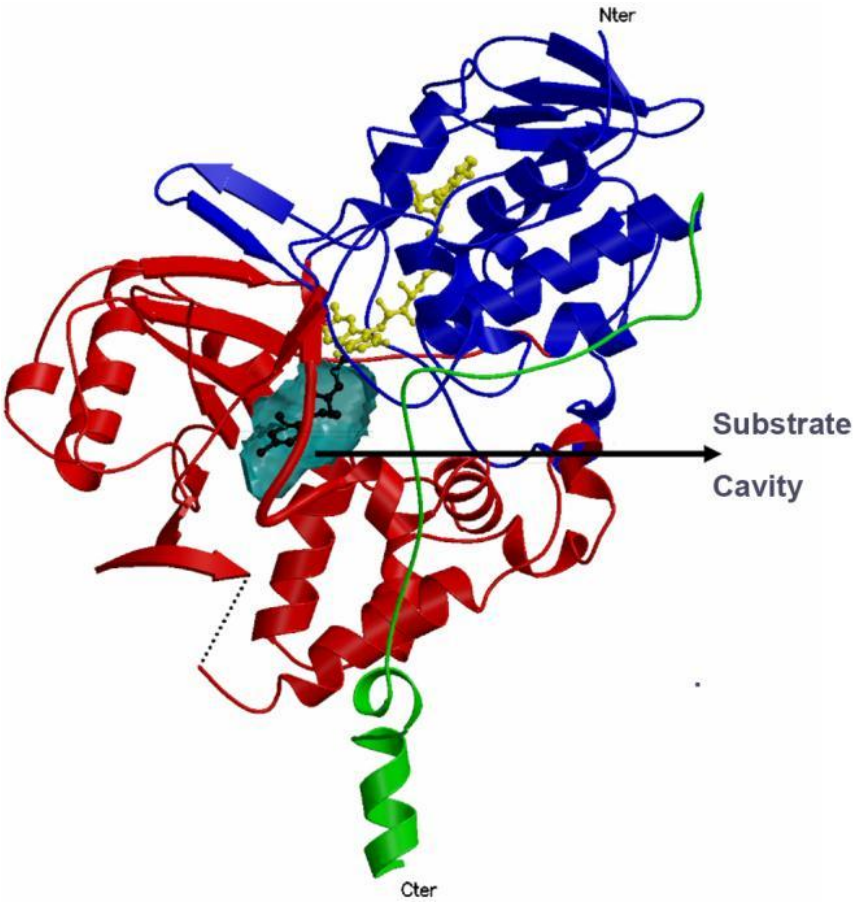



**Figure 1. Human MAO-A structure.** The ribbon diagram shows the flavin binding domain (blue), the substrate domain (red) and the C-terminus membrane binding domain (green). The covalent flavin moiety is shown in yellow as a ball and stick model (39). Reprinted from (39), with permission from Elsevier.

reaction catalyzed by MAOs. The researchers explain that the oxidative deamination reaction is initiated by the transfer of electrons from the amine nitrogen to oxygen coupled with the reduction of FAD to FADH. The resulting intermediate imine is spontaneously hydrolyzed to the corresponding aldehyde and ammonia. The cofactor is reoxidized by molecular oxygen, which itself is then reduced to hydrogen peroxide ($H_2O_2$) (33, 38, 40) (Figure 2). The $H_2O_2$ by-product of this reaction is a concern as it is a source of hydroxyl radicals, and thus contributes to oxidative stress (41, 42).

### 1.1.3 MAO-A

The primary role of MAO-A is to regulate the levels of aromatic monoamines in the central nervous system and peripheral organs (43). As mentioned, this involves the degradation of biogenic amines including the classical neurotransmitter 5-HT (44). The interaction between MAO-A and classical neurotransmitters has led to the extensive study of the enzyme in the context of several neurological conditions, including MDD (36). This has resulted in the development of MAO-Inhibitors (MAOIs), most relevant to this discussion are a class of antidepressants that target MAO-A to allow for an increase in 5-HT availability (45).

### 1.1.4 MAOIs: Clorgyline

MAOIs were the first class of drugs to demonstrate true antidepressant effects (46) and clorgyline was among the first acetylenic inhibitors of monoamine oxidase to be synthesized (47). Clorgyline [N-methyl-N-propargyl-3-(2,4-dichlorophenoxy) propylamine] is a propargylamine compound that selectively binds to MAO-A resulting in irreversible inactivation (48) and the subsequent rise in neurological monoamine concentrations, particularly the target substrate 5-HT (49). Clorgyline covalently binds to MAO-A in the substrate binding site. The mechanism-based inhibition by this ‘suicide’ inhibitor occurs following the binding of clorgyline to the N(5) atom of the FAD cofactor, explains Finberg and colleagues (46). This binding triggers MAO-A to process clorgyline as if it were a substrate. A reactive intermediate forms covalent bonds within the FAD-binding domain and this blocks subsequent access by substrates,

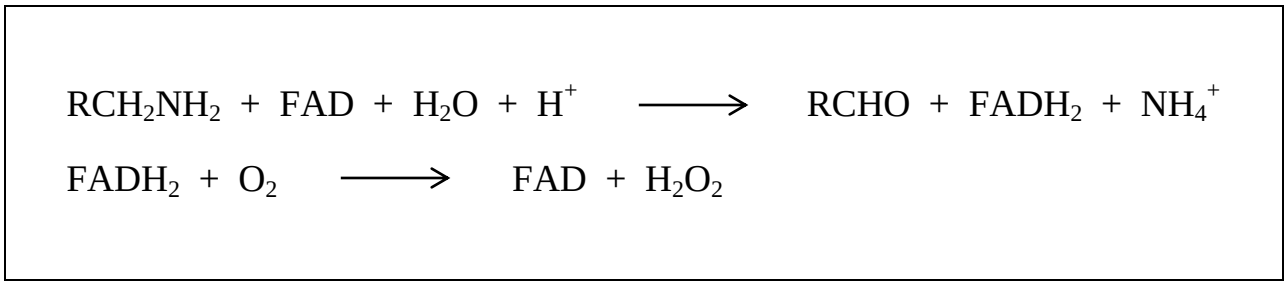



**Figure 2. The oxidation of amines by monoamine oxidases.** The amine substrate is oxidized while FAD is reduced to FADH. The intermediate imine is then hydrolyzed resulting in an aldehyde, $FADH_2$, and an amine. In the next phase of the reaction, $FADH_2$ is oxidized back to FAD and oxygen is reduced to $H_2O_2$ (38).

thereby irreversibly inhibiting MAO function (50). This irreversibility underlies the use of the label ‘suicide’.

## 1.2 Depression in Cancer Patients

The interdependent relation of comorbid cancer and depression is receiving greater attention along with the adverse consequences of this interaction. The coexistence of depression in cancer patients is almost 40% (51, 52); however, incidence rates of depression vary considerably between the different forms of cancer (53). Pancreatic and oropharyngeal cancers are associated with a strikingly high incidence of clinical depression (54), but the factors responsible for this stronger association are unknown and may be attributed to a pathophysiological effect, adverse side effects of treatment or an as yet unidentified variable (53). Moreover, the scarcity of peer-reviewed studies, high rates of patient drop-out in clinical studies, and the low number of total patients analyzed in the past 30 years precludes arriving at any definitive inferences (55). Fann and colleagues (56) performed a literature review on the prevalence of depression in women with breast cancer in the last 20 years. This study confirmed that, with the exception of pancreatic and oropharyngeal cancers, breast cancer patients have the leading incidence of depression compared to all other forms of cancer (51, 56). In an observational cohort study done out of London, England (57), over 200 female participants were assessed for depression in early-stage breast cancer. Almost 50% of the women experienced depression, anxiety or both in the first year following the initial diagnosis.

Regardless of primary tumour site, depression is three times more common in cancer patients compared to those in the general population (55). Although depressive symptoms frequently manifest within the cancer patient population, depression continues to be underdiagnosed throughout this demographic and is overlooked for a variety of reasons (58). For instance, experiencing psychological distress is expected in those who have been diagnosed with a terminal illness, to a certain degree. Furthermore, the threshold separating appropriate sadness and depression is difficult to detect among cancer sufferers (59). It becomes a challenge to attribute the onset of somatic symptoms, for example, either to cancer and the associated treatments or to depression (60). This is understandably a difficult task; however, treating a

depressive illness in a cancer patient is critical and can yield substantial benefits including maintenance of a stronger immune system (61) and extension of overall survival (62). If left untreated, however, depressed cancer patients demonstrate reduced medical compliance (63), extended inpatient care (64), and a poorer quality of life (65) coupled with increased mortality rates (53). Bultz and Carlson (66) recognize the impact of psychological distress within a cancer setting and emphasize the importance of monitoring emotional affliction as closely as the attention given to traditional vital signs. As was alluded to, the benefits of timely treatment of the comorbidity has been well-documented and reiterated in several studies throughout the literature (66). In cancer patients, antidepressants and psychotherapeutic interventions are recognized as effective treatments for clinical depression (67).

### 1.2.1 MAO-A in Cancer

MAO-A has been historically implicated in depression; however, recent findings have now also implicated this enzyme in cancer (19, 68-70). In a comparative analysis of gene expression in multiple cancers among various species, Rybaczyk and colleagues (19) demonstrated a consistent decrease in MAO-A mRNA expression in 95.4% of human cancer patients compared to normal controls. In human breast tumours, the researchers report a 5.6 fold decrease in MAO-A compared to normal controls. Based on this, these authors and others propose MAO-A as a biomarker of cancer progression (19, 71). The literature, however, remains inconclusive regarding the involvement of 5-HT metabolism in carcinoigenesis (19). The role of 5-HT in the context of cancer is ambiguous as a drug-induced rise in 5-HT may contribute to a protective mechanism in some forms of cancer (72, 73), whereas *in vitro* results propose that an increase in 5-HT appears to promote mitosis (74). Epidemiological studies have also supported an association between risk of cancer development and the use of antidepressants (75). As a result of the downregulation of MAO-A mRNA observed in cancerous tissues, it is legitimate to question how MAOIs may contribute to cancer risk. Indeed, increased cell proliferation has been observed in animal models treated with MAOIs (76, 77); however, this effect of MAOIs has not been substantiated in humans (19).

## 1.3 Breast Cancer

Breast cancer has become one of the most relevant cancers due to its incidence and mortality rates worldwide. Breast cancer has the highest incidence among females compared to all other cancers and is the second leading cause of death from cancer among women (78). Although breast cancer is one of the most common malignancies in women, 1% of all cases occur in men (79).

Similar to most cancerous tumour cells, breast cancer cells exhibit unique characteristics that arise from genetic mutations and contribute to a pathological phenotype (80) involving uncontrolled rapid cell division and immortalization, which refers to the ability of tumour cells to grow *indefinitely* (81). The transformation from a normal cell type to a cancerous or neoplastic cell is attributed to a number of factors including, gene amplification (82), changes in membrane components, and reorganization of the cytoskeleton (83). This multistep process is generally referred to as tumourigenesis (84). During tumourigenesis, cancer cells acquire the ability to overcome growth-inhibitory signals and divide indefinitely in the absence of growth-stimulating signals undetected by the host immune responses (84). As the cancer cell continually replicates, oxygen and nutrient supplies are maintained through tumour angiogenesis (85). These events mark the formation of malignant tumors that develop in the breast tissue and can subsequently invade adjacent normal tissue and disseminate to distant sites (86).

Malignant breast tumours are commonly differentiated based on estrogen receptor (ER) status, progesterone receptor (PR) status and human epidermal growth factor receptor 2 (HER2) status (87). This is a necessary assessment as it can provide critical information towards the selection of the most effective method of targeted adjuvant therapy, which can significantly impact overall prognosis.

### 1.3.1 Estrogen Receptor Status

Fifty to eighty percent of breast cancer cases are classified as ER-positive [ER(+)] (88), and of the treatments used against ER(+) breast cancer cells, tamoxifen is among the most widely prescribed drugs (89). Tamoxifen functions as a competitive inhibitor to hinder cell growth

stimulated by estrogen (90). There are two forms of the estrogen receptor that respond to 17β-estradiol (estrogen), ERα and ERβ, the latter generally being less relevant to breast cancer (91). In breast tissue the main effect of estrogen is to cause cells to grow and divide, which occurs in epithelial cells lining mammary glands in preparation for lactation (92). This is a normal and beneficial function of estrogen but by triggering cell division in these cells, the risk of breast cancer development also increases (93). The mutations in genes that regulate cell division are not caused by the presence of estrogen. However, because estrogen stimulates proliferation, the cells with existing mutations will also proliferate and may acquire additional mutations in DNA to eventually become cancerous (94). In any case, regardless of whether mutated cells are present, the exposure to estrogen in normal cells creates a greater opportunity for genetic errors to occur during DNA replication. Furthermore, once a tumour develops the cancerous cells will continue to respond to estrogen-stimulated growth (95).

The classic nuclear estrogen receptors, ERα and ERβ (96), bind to estrogen resulting in a conformational change to allow the complex to translocate to the nucleus and interact with DNA at specific sites known as estrogen response elements (97). This attachment initiates the binding of coactivator proteins resulting in the activation of genes, which will synthesize proteins that, in breast cells, will influence cell proliferation (98). This process is restricted by tamoxifen, which competitively binds to estrogen receptors forming a nuclear complex that reduces DNA synthesis (99).

### 1.3.2 Tamoxifen

Tamoxifen is designed as a pro-drug, meaning that the compound is administered in an inactive form that is chemically transformed *in vivo* through metabolic processes in the liver to an active form (100). The conversion of tamoxifen to its active form by the cytochrome P450 (CYP) system produces active metabolites such as 4-hydroxytamoxifen (101), which is an ER antagonist with a far greater binding affinity than tamoxifen. The ER/tamoxifen complex recruits co-repressor proteins to effectively inhibit the transcription of estrogen-responsive genes (102). The anti-estrogenic properties of this hormonal therapy force cells to arrest in the G0 and G1

phase of the cell cycle (103). Hence, tamoxifen does not destroy cells but prevents cell division and is therefore considered a cytostatic as opposed to a cytocidal substance (103).

Tamoxifen is classified as a selective estrogen receptor modulator (SERM), meaning that it can selectively block *or* stimulate estrogen receptors depending on the target tissue (104). The structure of the estrogen receptor is slightly modified depending on the tissue in which it is located (105, 106). In breast cells the gene activation for specific growth-promoting proteins is blocked by tamoxifen. However, tamoxifen can mimic estrogen in other areas of the body, which can have both positive and negative outcomes (107). For instance, in the uterus, estrogen promotes cell proliferation and tamoxifen yields the same effects (108, 109). Similar to estrogen's role in breast cancer development, tamoxifen can increase the risk of endometrial cancer (107). Thus, the complex properties of tamoxifen still require further investigation (108, 109).

Not all breast cancer cells express the estrogen receptor. These ER(-) breast cancers tend to be more aggressive and are not susceptible to the therapeutic effects of tamoxifen (110). Furthermore, in 10% of the general population the enzyme CYP2D6 (member of the cytochrome P450 super family) that converts tamoxifen to its active form is functionally impaired, thus preventing tamoxifen from providing maximum chemotherapeutic benefits (111). When considering tamoxifen as a potential treatment option, CYP2D6 enzyme function can be tested, but its metabolic abilities are also altered by certain medications. For instance, the activity of CYP2D6 is blocked in the presence of some commonly used antidepressants and antihistamines namely, SSRIs and diphenhydramine (Benadryl), respectively (112).

### 1.3.3 Antidepressants and Tamoxifen

As a pro-drug, tamoxifen is relatively inactive prior to hepatic metabolism by the CYP450 system (111). A number of metabolites are produced following primary and secondary metabolism; the most pharmacologically relevant of these products are 4-hydroxytamoxifen (4-OH-TAM) as well as endoxifen (113). In the past tamoxifen's anti-estrogenic action has been attributed to the increased potency of the 4-OH-TAM metabolite; however, more recent evidence has indicated endoxifen to be more clinically efficacious in chronic tamoxifen-treated patients

(114). Endoxifen is generated at the highest concentration following oxidation of tamoxifen's most abundant metabolite, N-desmethyltamoxifen, by the CYP2D6 enzyme (114).

Major antidepressants, such as the SSRIs fluoxetine and paroxetine, are potent CYP2D6 inhibitors. These drugs directly affect endoxifen concentrations in patients treated with tamoxifen by converting moderate metabolizers into poor metabolizers (115). By acting as competitive inhibitors these antidepressants interrupt the biotransformation of tamoxifen into its active metabolites (Figure 3); thereby, significantly reducing the antineoplastic effects of this chemotherapeutic agent (103). Yet this does not hold true for all antidepressants. For example, venlafaxine is only a weak inhibitor of the CYP2D6 enzyme, and thus it has no significant effect on the cancer-preventing benefits of tamoxifen (116).

### 1.3.4 Tamoxifen Resistance

The ability of cancer cells to escape apoptosis is considered an essential "hallmark of cancer" (117). Laboratory and clinical investigations have implicated certain stress signalling pathways in *de novo* and acquired tamoxifen resistance. For instance, elevated levels of phosphorylated c-Jun N-terminal kinases (JNK) and activated p38(MAPK) are associated with the acquired resistance to tamoxifen (118). A suggested mechanism of resistance associated with activated p38(MAPK) has been described in endometrial adenocarcinoma cells. Lee and Bai (119) reported an increase in p38(MAPK) activity by both tamoxifen and estrogen, resulting in the inhibition of ER nuclear export, enhancement of ER-coactivator interactions, phosphorylation of the ER-α protein, and increases in the uterine-specific agonist activity of tamoxifen. Each of these events contributes to an overall enhancement of the growth-stimulating activity of estrogens in endometrial cancer.

As mentioned, estrogen receptor expression is presently a defining feature of breast cancer and provides an efficient target for treatment. Estrogen has become associated with an increasing number of signalling pathways including both MAO-A (120) and p38(MAPK) (119, 121). The regulation of MAO-A is multifactorial and there is also evidence for the role of estrogen in this process (120). In a similar fashion to MAOIs, estrogen reduces MAO-A activity to raise levels of 5-HT and catecholamines in the brain (122). Estrogen-induced stimulation of cell proliferation

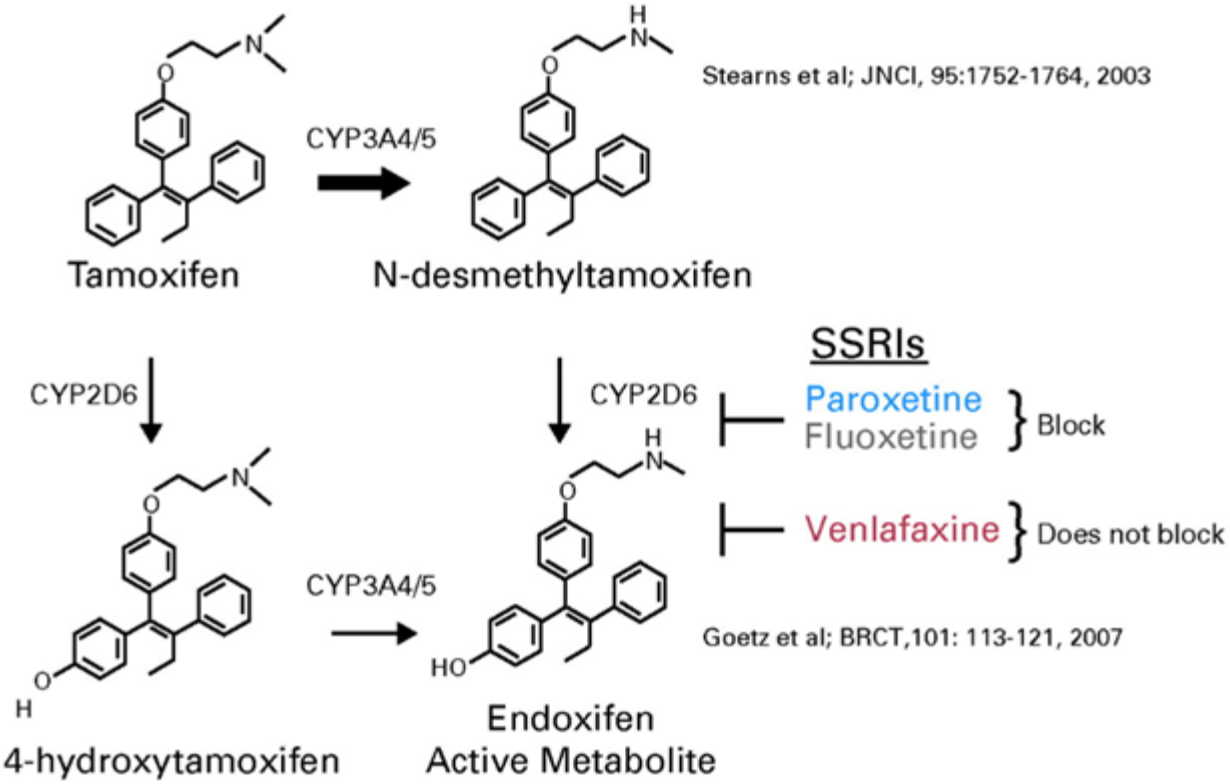



**Figure 3. The effects of certain SSRIs on tamoxifen metabolism.** The metabolism of tamoxifen to its active metabolite, endoxifen, is dependent on the SSRI being administered (123). Reprinted with permission. © (2008) American Society of Clinical Oncology. All rights reserved.

has long been a recognized factor in the development of breast cancer. Therefore, the influence of the regulation of MAO-A by estrogen could reveal a mechanism that can be applied to the understanding of tamoxifen resistance in breast cancer patients.

An explanation for the development of tamoxifen resistance would consequently allow physicians to identify the patients most likely to benefit from tamoxifen treatment. Gutierrez and colleagues (124) conducted a study to investigate the molecular changes associated with tamoxifen-resistant breast cancer and concluded that the biological pathways influencing tumor growth are altered as the tumor continues to progress. These findings highlight the complexities involved in understanding tumourigenicity. Interestingly, the group also observed increased p38(MAPK) activity in tamoxifen-resistant tumour samples from both animal and human tissues. Studies have also documented that an increase in extracellular signal-regulated kinases (ERKs) and p38(MAPK) signalling hinders the anti-proliferative effect of tamoxifen (118, 124). In breast cancer patients receiving tamoxifen, elevated p38(MAPK) activity was predictive of a poor prognosis (118). Tamoxifen-resistant cells showed increased basal levels of total and phosphorylated p38(MAPK) and it was then demonstrated that blocking the effects of p38(MAPK) with the specific inhibitor SB203580 reduced cellular proliferation in MCF-7 cells regardless of the cells' sensitivity status to tamoxifen (118). These studies consistently identify p38(MAPK) activity as an important factor to consider when attempting to explain tamoxifen resistance.

## 1.4 p38(MAPK)

### 1.4.1 MAPK Family

The mitogen-activated protein kinases (MAPKs) represent a family of highly conserved signal transducing enzymes that regulate cellular responses to various external stimuli (125). MAPKs consist of three major subgroups that selectively respond to upstream signalling events and independently perform an extensive range of functional roles (126) (4). Firstly, extracellular signal-regulated kinases (ERK) preferentially respond to growth factors to produce pro-survival signals and promote cell proliferation (127). The remaining two cascades interact significantly

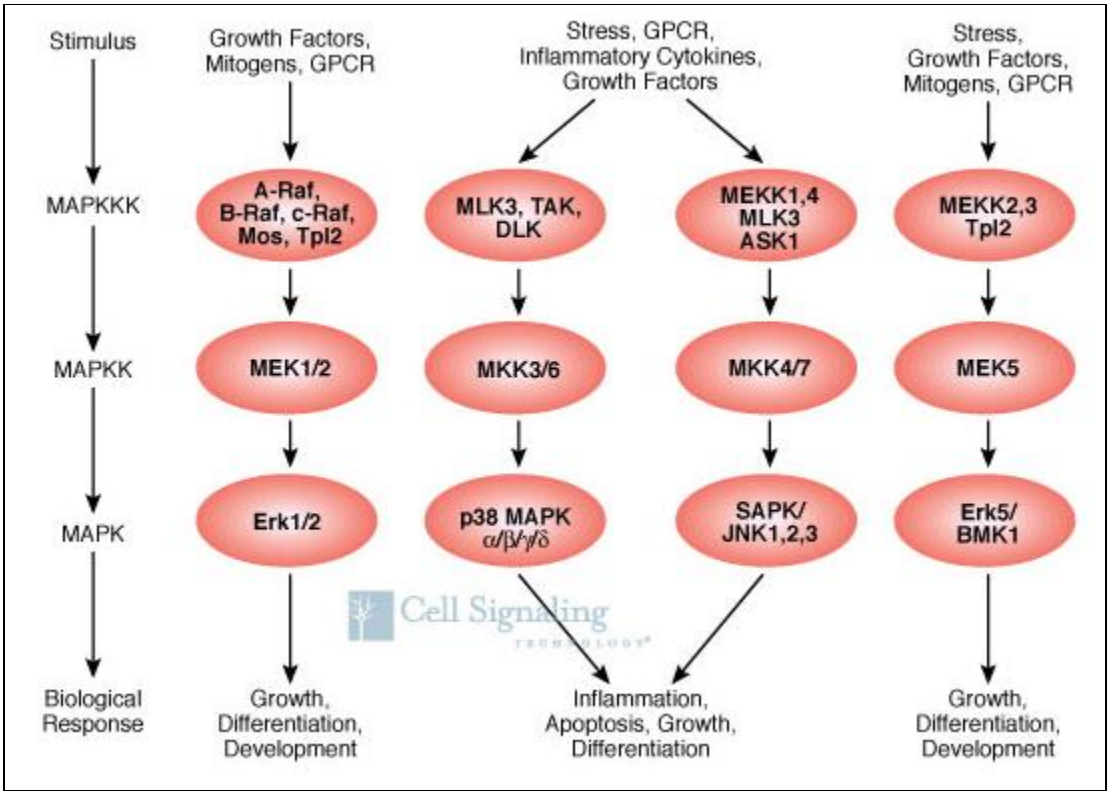



**Figure 4. Mitogen-Activated Protein Kinase (MAPK) cascades**. Pathway diagram reproduced courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com).

with each other and include the stress-associated kinases, JNK and p38(MAPK), which can promote inflammation and initiate apoptosis (126). Despite having different functions, these three classical signalling cascades undergo an equivalent activation mechanism whereby MAPK kinase (MAPKK) catalyzes the phosphorylation of both a Thr (Threonine) and a Tyr (Tyrosine) residue on its downstream targets (128).

### 1.4.2 p38(MAPK)

The p38(MAPK) cascade was first described in 1994 (129) and plays a critical role in cytokine production and the stress response (127). It is known to be activated by a variety of extracellular stressors including proinflammatory cytokines, osmotic shock, UV irradiation, bacterial lipopolysaccharides (LPS) and hydrogen peroxide induced oxidative stress (130). As a result, p38(MAPK) can directly influence a range of cellular processes including cytokine production, transcriptional regulation, cytoskeletal reorganization and apoptosis (131). The dual phosphorylation of p38(MAPK)'s $Thr^{180}$ and $Tyr^{182}$ of the Thr-Gly-Tyr (TGY) tripeptide motif by MAPK kinase-3 or -6 (MKK3 and MKK6) leads to the rapid activation of the enzyme (132); otherwise, p38(MAPK) is relatively inactive in its non-phosphorylated form (129). There exists four isoforms of p38(MAPK), α, β, γ and δ, each with differential tissue expression (133). The p38(MAPK)-α and -β isoforms are expressed in a wide variety of tissues in the body (134) whereas the γ and δ isoforms display a more restricted tissue distribution. There are high levels of p38(MAPK)-γ expression in the skeletal muscles, heart, lungs, thymus and testes (135). Similarly, p38(MAPK)-δ is expressed in the lungs and testes but also in the pancreas, small intestines, kidneys and epidermis (136). In addition, similar to ERK and JNK, each isoform of p38(MAPK) is recognized for its selective affinity for specific upstream activators and downstream effectors (137).

### 1.4.3 SB203580

SB203580 is a pyridinyl imidazole inhibitor commonly used in an experimental setting to elucidate the role of p38(MAPK) in cellular signalling (138). It is well-established that the

primary function of SB203580 is to block the catalytic activity of p38(MAPK) by acting as an ATP-competitive antagonist (139). The compound selectively inhibits the α and β isoforms of p38(MAPK) with high affinity (140). Most protein kinases are insensitive to SB203580, which accounts for its specific action on particular p38(MAPK) isoforms (141). Successful inhibition by the drug is dependent on the presence of a threonine residue at position 106 to accommodate the 4-flourophenyl moiety of SB203580 (142). Both p38(MAPK)-δ and -γ have large side-chains on the residues that occupy this site and although other protein kinases do possess a residue that is threonine or smaller at the 106 position, the inhibitory effect of the drug is less potent (143). SB203580 is capable of binding to both the activated and inactivated forms of p38(MAPK) with equal affinity (144). Several studies have demonstrated that the presence of SB203580 has no measurable effect on p38(MAPK) activation as indicated by the continued phosphorylation of $Thr^{180}$ and $Tyr^{182}$ by upstream signalling events (143, 145, 146).

### 1.4.4 p38(MAPK) and MAO-A

The phosphorylation of p38(MAPK) can be stimulated by reactive oxygen species such as hydrogen peroxide (147). Since the reaction catalyzed by MAO-A activity produces hydrogen peroxide (43), it is possible that this may, in turn, stimulate p38(MAPK) activity. Interestingly, our research group has shown in several CNS-derived clonal cell lines that activated p38(MAPK) inhibits MAO-A by phosphorylation of the Serine 209 residue in MAO-A (148). This feedback between MAO-A-generated reactive oxygen species, activation of p38(MAPK), and the subsequent de-activation of MAO-A by p38(MAPK) suggests an adaptive, beneficial response of p38(MAPK) activation. As a stress-activated protein, p38(MAPK) is well-recognized as an effector and regulator of many apoptotic pathways (149). Therefore, the suggested inverse relationship between these two proteins (and the pro-survival role it might play), is not consistent with the understood role of p38(MAPK) as a pro-apoptotic kinase. Perhaps this supports the p38(MAPK)-mediated feedback regulation of MAO-A in a manner reminiscent of classical antidepressants (148). If a reduction in MAO-A, and possibly the subsequent increase in 5-HT, does in fact indicate progression towards cancer as suggested by

Rybaczyk et al. (19) then serious consideration must be given to the use of antidepressants among cancer patients and those at high risk of developing certain forms of cancer.

## 1.5 Cancer Progression

### 1.5.1 Metastasis

The survival rate for breast cancer patients has significantly improved in the last 20 years (150). This is attributed to a rising public awareness and the development of enhanced diagnostic and treatment strategies, among other factors (150). Unfortunately, metastasis, which represents a spreading of the tumour from the primary site to other sites within the body, is often associated with increasing malignancy and a significantly poorer prognosis (151). In breast cancer, cells most commonly colonize at secondary locations in the lungs, brain, liver and bone (152). Metastasis is a series of events that involves the sloughing of cells from the primary tumour and their re-localization to a secondary site (153, 154). This multistep process begins with local invasion of tumour cells into the healthy surrounding tissue. Subsequently, these cells infiltrate neighbouring circulatory vessels in a process known as *intravasation* (155). Fortunately, less than 0.05% of circulating tumour cells will survive travel through the circulation (156). Before forming stable metastases, circulating tumour cells must overcome the mechanical stress of circulatory transit, adapt to a dynamic environment and escape immune-mediated destruction (155). These tumour cells must then exit the blood or lymphatic system (*extravasation*) (157). At this stage undetected tumour cells establish themselves in the new site and may remain dormant before proliferating to form a secondary tumour (Figure 5). Efforts to understand the signalling events that occur during metastasis have only led to a descriptive understanding of tumour progression (158). This task has proven to be difficult, because metastasis is detected at the end stages of pathological advancement. The end stage of the disease is marked by genetic instability; therefore, the tumour cell population is heterogeneous (159). Under these conditions metastatic carcinoma cells, which at this point have minimal presence, are difficult to detect (158). To further complicate the issue, during tumour expansion there are extensive interactions

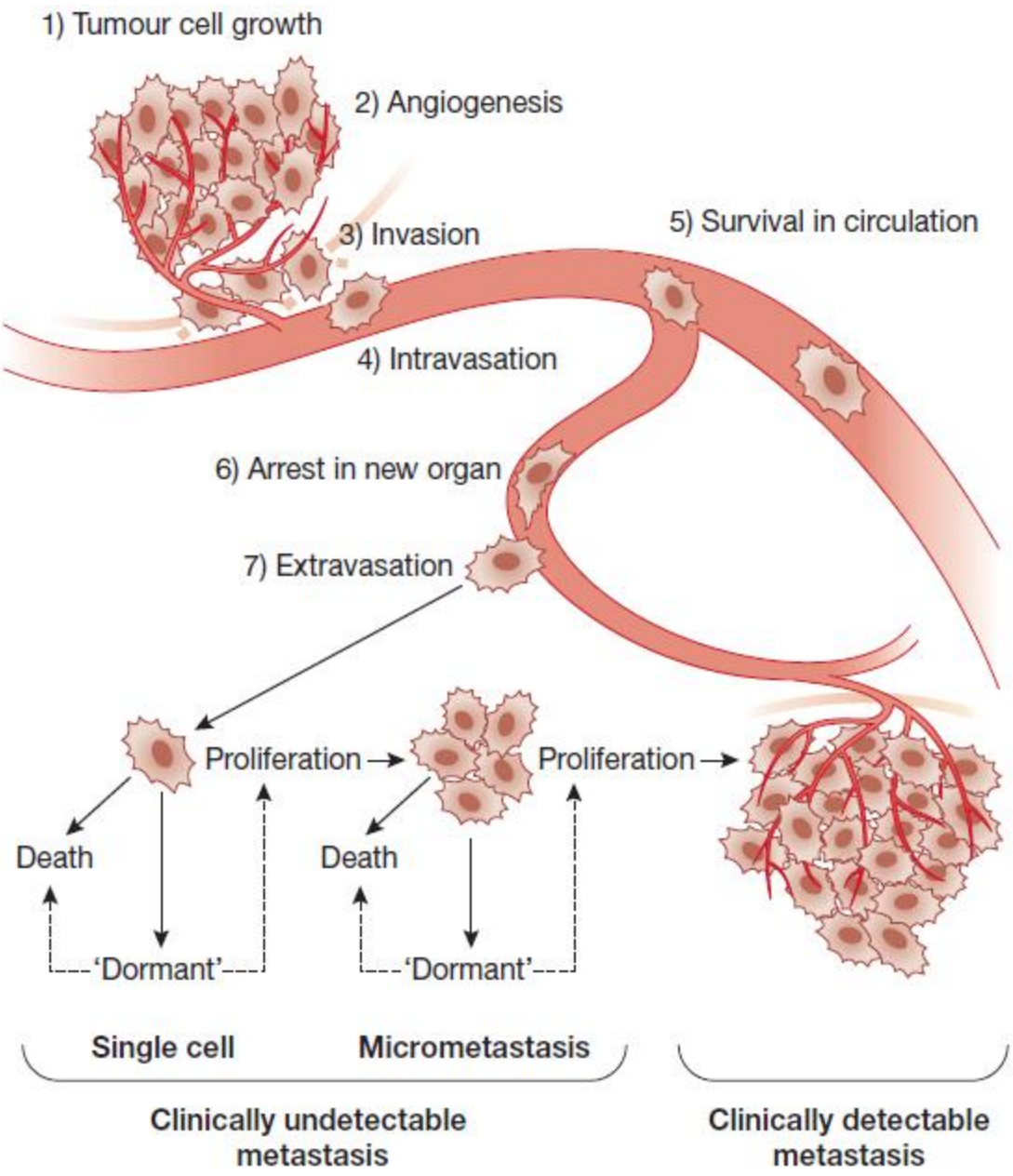



**Figure 5. The metastatic process.** Cancer cells spread to other parts of the body in a complex multistep process known as metastasis. There is first local invasion of cancer cells which intravasate into nearby circulatory vessels and are transported to a distant site. The cancer cell arrests at the new location and undergoes extravasation before migrating into the surrounding tissue and proliferating to form micrometastases (small tumours). Image reproduced with permission from reference (160).

between tumour cells and surrounding tissues (158). This makes it challenging to isolate and observe these cells, and to fully characterize the sequence of molecular events contributing to their metastatic behaviour.

Treatments such as chemotherapy do help to prolong life, and the selection process regarding which therapy would best benefit a given patient is increasingly more accurate due to the identification of biomarkers (161). The analysis of protein expression at tumour sites has proven to be a successful method of better understanding the disease and its advancement. As such, a major focus of breast cancer research is the identification of additional markers, particularly markers involved in breast cancer progression during the epithelial-mesenchymal transition (EMT).

### 1.5.2 Epithelial-Mesenchymal Transition

*In vitro* observations in human breast cancer cell lines clearly demonstrate that a loss of epithelial properties preceding the acquisition of mesenchymal features will lead to increasing disease aggressiveness (162). It is proposed that EMT, which occurs during embryonic development strongly resembles the actions of cancer cells as they undergo metastasis (163). In brief, during embryonic development cells that are initially part of the neural tube disengage from the parent epithelium and migrate throughout the embryo to specific sites prior to differentiation. The mechanism of cell migration and re-establishment in a distant location, however, is not entirely lost in adulthood as EMT is also implicated in tissue regeneration, wound-healing, organ fibrosis, and perhaps metastasis (164).

EMT refers to the series of morphological events that involve the cells abandoning the primary tumour site to migrate to another location in the body and form a secondary tumour (165). Epithelial and mesenchymal cells are the two cell phenotypes that are part of EMT (Figure 6). Epithelial cells form densely associated tissue layers with minimal intercellular space and strong connective junctions along multiple fronts, which is a characteristic of human breast cancer tumour cells (166). These cells are described as having apico-basal polarization, meaning that membrane proteins are asymmetrically distributed according to placement on either the apical (exposed to the extracellular space) or basal (attached to basal lamina) domains of the

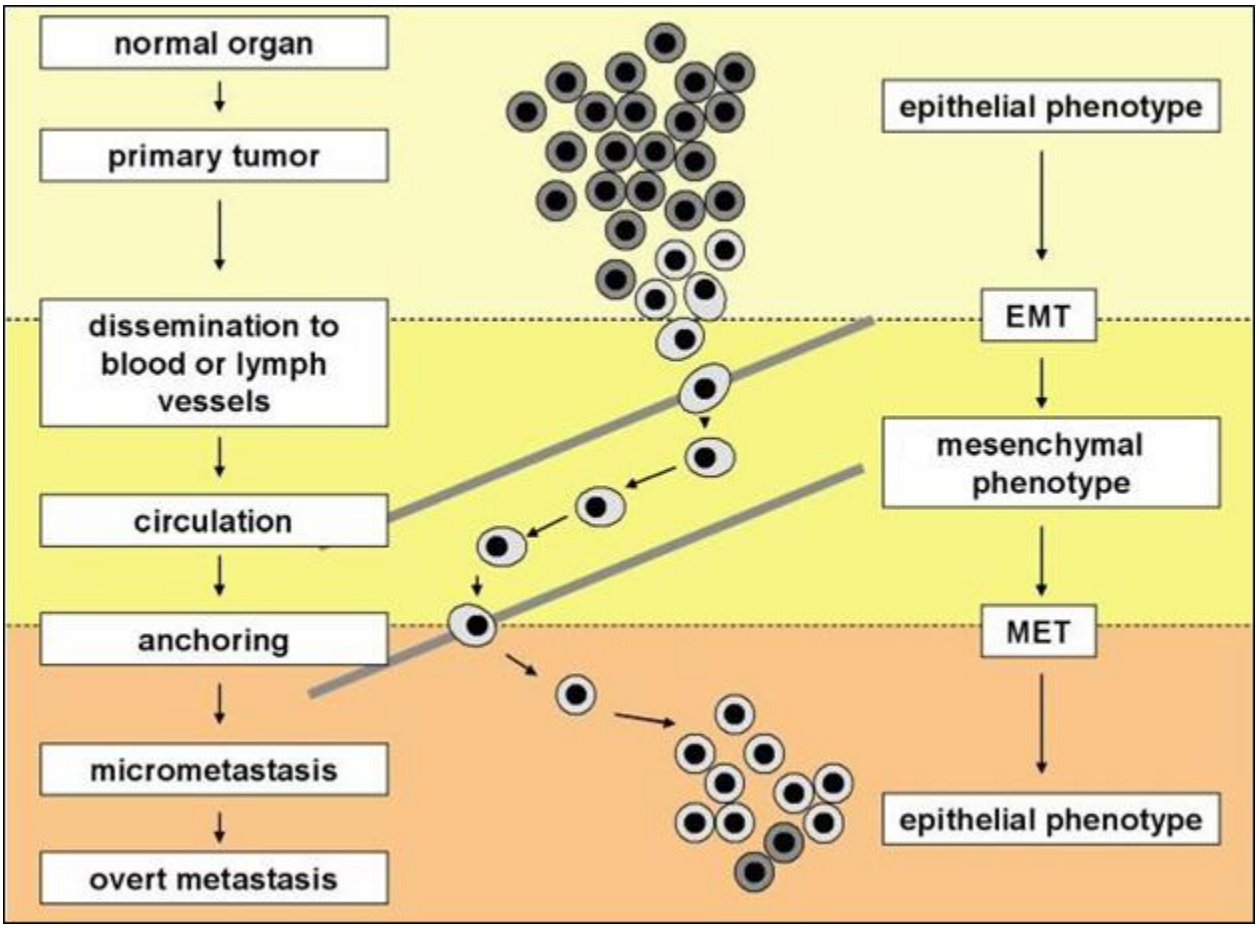



**Figure 6. The epithelial-mesenchymal transition.** Cancerous epithelial cells lose polarity and assume a mesenchymal phenotype to gain entry into the vasculature. The highly motile mesenchymal-like cancer cells eventually arrest at secondary sites. At this stage cells may remain dormant or revert back to the epithelial phenotype.

plasma membrane (167). The structure of epithelial sheets is maintained by a regulated adhesive junction arrangement, which is contingent on the layout of the cytoskeleton and the location of cadherin molecules on the plasma membrane. This rigid organization forms a diffusion barrier between individual cells (168). During EMT epithelial cells lose cell-to-cell contacts and undergo an extensive remodelling of the cytoskeleton. These events are concurrent with the expression of mesenchymal elements, which promote a migratory phenotype (169). Mesenchymal cells display front-back polarity and are completely surrounded by the extracellular matrix (170). The cells develop properties that facilitate motility, such as the appearance of long thin processes that extend from a small cell body (167).

The transition of cells into a mesenchymal form is a reversible process; conversely, the expression of adhesion molecules permit dispersed unattached cells to aggregate (171). Reverse EMT, commonly referred to as mesenchymal-epithelial transition (MET), is of equal importance in normal embryonic development as well as cancer progression (158). For example, following extravasation during metastasis, cells experience brief local migration before undergoing MET and initiating growth at a secondary tumour site. This system of events is coordinated by a series of gene regulatory components including: Slug, Snail, and Twist, of which E-cadherin is a downstream target (172). E-cadherin associated with β-catenin, as well as vimentin are generally acknowledged for their differential expression and specific roles in epithelial and mesenchymal phenotypes (173).

### 1.5.3 E-cadherin

E-cadherin is a transmembrane molecule expressed in all epithelial cells (174). It is a classical cadherin protein associated with adheren junctions and maintains strong mechanical attachments between cells (175). This transmembrane adhesion protein links the cytoskeleton to extracellular structures to accomplish cell-to-cell anchorage (175). This is an indirect connection, because the internal linkage is supported by intracellular anchor proteins, such as β-catenin (176). Conservation of the epithelial cell shape relies on the proper functioning of this complex otherwise disruption may accelerate tumour metastasis (177). The multifunctional cytoplasmic protein, β-catenin, regulates cell-cell adhesion by anchoring cadherins to the actin cytoskeleton

and may also contribute to the development of invasive carcinoma cells (176). E-cadherin, specifically, plays a crucial role in the maintaining epithelial cell interactions and thus has been identified as a key suppressor of metastasis (160).

### 1.5.4 Vimentin

The expression of vimentin tends to coincide with a reduction in E-cadherin presence along the plasma membrane (178). Vimentin is an intermediate filament, thus a major cytoskeletal component that forms highly dynamic structures in animal cells and imparts mechanical stability in cells of mesenchymal origin (179). Hence, it is a mesenchymal marker and its presence is correlated with the EMT (179). Evidently, this suggests a role for vimentin in facilitating cell migration, which is further supported by the expression of vimentin-like intermediate filament proteins at sites of elongation (180). In mammals vimentin is the most prevalent intermediate filament protein (180). Kokkinos and colleagues (181) compared three human breast cancer cell lines: (1) MCF-7 (poorly invasive, non-metastatic); (2) MDA-MB-231 (moderately invasive and metastatic); and (3) MDA-MB-435 (highly invasive and metastatic). Immunostaining revealed that the more aggressive the cell line, the greater the expression of vimentin.

Histological analyses have identified vimentin in over 90% of grade three (most aggressive) breast carcinomas (182). Vimentin expression in mammary glandular tissues has not been as consistent, and this is attributed to the variable expression of the protein in micrometastases versus macrometastases (181). There is a possibility that the mesenchymal properties featured in undetectable newly formed tumours are lost as the mass of cells becomes progressively larger and an epithelial phenotype becomes increasingly beneficial for tumour establishment (occurrence of the MET) (183). Although studies have proposed that vimentin is not only an indication of EMT, but also of invasive breast cancers, experts remain uncertain about its prognostic values (184, 185). Vimentin may be one marker for EMT; however, more specific markers are necessary to influence outcome predictions.

### 1.5.5 Tumour heterogeneity

To further complicate matters, breast cancer researchers have become well aware of the heterogeneous collection of cancer cells within a single breast tumour (159, 186-188). Immunohistochemical evaluations have undisputedly revealed that mammary tumours frequently consist of cells with varying levels of tumour marker expression, thus distinct morphology and behaviour (186). In a study conducted by Wild and colleagues (187), it was demonstrated that 97% of the epithelial breast carcinomas observed, displayed tumour heterogeneity. Moreover, tumour cells exhibited genetic evolution according to host pressures and treatment interventions (189). Intra-tumour heterogeneity provides a possible explanation for acquired drug resistance particularly in advanced stages of cancer. Over time, the genetic instability of tumour cells gives rise to new characteristics contributing to subsequent growth advantages (159). Shen and colleagues (188) reported that double-stranded breaks influenced chromosomal instability giving rise to new cellular characteristics in later stages of cancer. Consistent with these findings, Goldie and Coldman (190) established that the size of the tumour was positively correlated with the rate of development of tumour resistance, because of the greater potential for the occurrence of drug resistant cancer cells in a larger tumour population.

## 1.6 Rationale, Hypothesis & Objectives

Overall, there are three main studies which inspired us to examine the novel interaction of MAO-A and p38(MAPK) in breast cancer. The first was a recent report by Rybaczyk and colleagues that indicated significant losses of MAO-A status [e.g. mRNA] in 95% (!) of human cancers. The second was a group of studies implicating the activation of p38(MAPK) in breast cancer progression. The last study came from our research group in which we reported the regulation of MAO-A by p38(MAPK) in glial cells. Given these findings we questioned whether the activation of p38(MAPK) inhibits MAO-A function in breast cancer cells leading to the progression of the disease (Figure 7). The **hypothesis** states that a functional interaction between the p38(MAPK) and MAO-A systems alters breast cancer cells in an ER-dependent manner.

The objectives of this thesis are supported by the literature and include:

(i) To determine the extent that the chemical or molecular inhibition of MAO-A and/or p38(MAPK) affects breast cancer cell tumourigenic or metastatic potential *in vitro*.

(ii) If MAO-A and/or p38(MAPK) inhibition affects cell phenotype, then to determine whether p38(MAPK) regulates MAO-A-sensitive phenotypes in an ER-dependent manner.

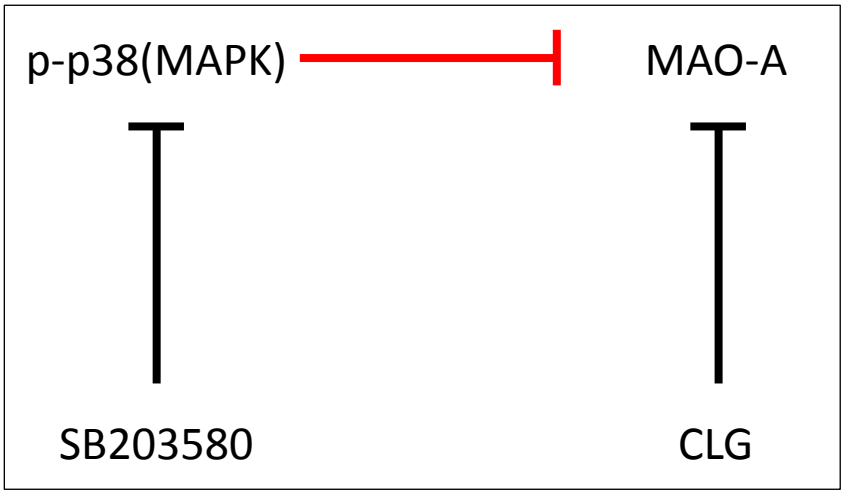



**Figure 7. Proposed interaction between p38(MAPK) and MAO-A.** Based on previous work in the literature we suggest that MAO-A is inhibited by both CLG and p38(MAPK) (148). The inhibition of activated p38(MAPK) by SB203580 should induce opposite effects of CLG. We expect to see this interaction particularly in ER(+) breast cancer cells.

# 2 MATERIALS AND METHODS

All reagents and materials were obtained from commercial sources (Table 1) unless otherwise indicated. The antibodies used are presented in Table 2.

**Table 1. Reagents and commercial suppliers.**

| Reagents & Materials | Company | Address |
|---|---|---|
| Acrylamide | Bio-Rad Laboratories | Hercules, CA, USA |
| Agarose | Invitrogen | Carlsbad, CA, USA |
| APS | Sigma-Aldrich | St. Louis, MO, USA |
| Aqueous Counting Scintillant | Amersham Biosciences | Piscataway, NJ, USA |
| BCA Protein Assay Kit | Pierce | Rockford, IL, USA |
| Bovine Insulin | Sigma-Aldrich | St. Louis, MO, USA |
| Bovine Serum Albumin | Sigma-Aldrich | St. Louis, MO, USA |
| Bromophenol Blue | Sigma-Aldrich | St. Louis, MO, USA |
| Cell Freezing Media | Invitrogen | Carlsbad, CA, USA |
| Clorgyline | Research Biochemical Int'l | Natick, MA, USA |
| [$^{14}$C]-radiolabeled serotonin | PerkinElmer | Waltham, MA, USA |
| DAPI Nucleic Acid Stain | Invitrogen | Carlsbad, CA, USA |
| DMSO | EM Science | Gibbstown, NJ, USA |
| DTT | Cell Signaling Technology | Danvers, MA, USA |
| Enhanced Chemiluminescence | GE Healthcare | Uppsala, Sweden |
| FBS | Invitrogen | Carlsbad, CA, USA |
| Film (X-OMAT LS) | KODAK | Rochester, NY, USA |
| Formalin | EMD Chemicals | Gibbstown, NJ, USA |
| Glycerol | J.T. Baker | Phillipsburg, NJ, USA |

| | | |
|---|---|---|
| Glycine | Sigma-Aldrich | St. Louis, MO, USA |
| Haemotoxylin | Invitrogen | Carlsbad, CA, USA |
| HEPES | Invitrogen | Carlsbad, CA, USA |
| L-glutamine | Invitrogen | Carlsbad, CA, USA |
| Matrigel Basement Membrane Matrix | BD Biosciences | Mississauga, ON, Canada |
| α-MEM | Sigma-Aldrich | St. Louis, MO, USA |
| MEM Vitamin Solution | Invitrogen | Carlsbad, CA, USA |
| 2-Mercaptoethanol | Sigma-Aldrich | St. Louis, MO, USA |
| Methanol | Fisher Scientific | Fair Lawn, NJ, USA |
| Dako Mounting Media | Dako | Carpinteria, CA,USA |
| MTT | Sigma-Aldrich | St. Louis, MO, USA |
| NaCl | Fisher Scientific | Toronto, ON, Canada |
| Nitrocellulose Membrane | Bio-Rad Laboratories | Hercules, CA, USA |
| PageRuler Protein Ladder | Thermoscientific | Logan, UT, USA |
| Pen-Strep | Invitrogen | Carlsbad, CA, USA |
| PIC | Sigma-Aldrich | St. Louis, MO, USA |
| RPMI-1640 | Sigma-Aldrich | St. Louis, MO, USA |
| SB203580 | Invitrogen | Carlsbad, CA, USA |
| SDS | J.T. Baker | Phillipsburg, NJ, USA |
| Tween® 20 | EM Science | Gibbstown, NJ, USA |
| TEMED | Bio-Rad Laboratories | Hercules, CA, USA |
| Tris-Base | Fisher Scientific | Toronto, ON, Canada |
| Tris-HCl | ICN Biomedicals | Aurora, OH, USA |
| Triton X-100 | Sigma-Aldrich | St. Louis, MO, USA |

| | | |
|---|---|---|
| Trypsin-EDTA | Sigma-Aldrich | St. Louis, MO, USA |

**Table 2. Antibodies and commercial suppliers.**

| Primary Antibody | Dilution | Company | Address |
|---|---|---|---|
| MAO-A (H-70) | 1:250 | Santa Cruz Biotechnology | Santa Cruz, CA, USA |
| E-cadherin (24E10) | 1:1000 | Cell Signaling Technology | Danvers, MA, USA |
| Monoclonal Anti-Vimentin | 1:1000 | Sigma-Aldrich | St. Louis, MO, USA |
| p-p38(MAPK) (T180/Y182) (28B10) | 1:200 | Cell Signaling Technology | Danvers, MA, USA |
| β-Catenin | 1:100 | Cell Signaling Technology | Danvers, MA, USA |
| Monoclonal Anti-β-actin | 1:3000 | Sigma-Aldrich | St. Louis, MO, USA |
| p-GSK3β (Ser9): Sc 11757R | 1:2000 | Santa Cruz Biotechnology | Santa Cruz, CA, USA |
| p38 MAP Kinase 9212 | 1:1000 | Cell Signaling Technology | Danvers, MA, USA |
| **Secondary Antibody** | **Dilution** | **Company** | **Address** |
| Goat-Anti-Mouse IgG | 1:2000 | Bio-Rad Laboratories | Hercules, CA, USA |
| Goat-Anti-Rabbit IgG | 1:2000 | Bio-Rad Laboratories | Hercules, CA, USA |

**Table 3. Reagents and components of preparation.**

| Reagents | Preparation |
|---|---|
| **4X Loading Buffer** | 8% SDS; 40% glycerol; 10% β-mercaptoethanol; 0.02% bromophenol blue; 0.25 M Tris-HCl (pH 6.8) |
| **Scott's Water** | 1g $NaHCO_3$; 5g $MgSO_4$; 500 mL $H_2O$ |
| **Lysis Buffer** | 1% Triton-X 100; 20 mM Tris, pH 7.5; 10% glycerol; 1 mM EDTA; containing 100X protease inhibitor cocktail |
| **PLC Lysis Buffer** | 50mM HEPES pH 7.5, 50 mM NaCl; 10% Glycerol; 1% Triton X-100; 1.5 mM $MgCl_2$; 1 mM EGTA; 10 mM Na-PPi; 100 mM NaF |
| **10X Running Buffer** | 30.3 g Tris; 144 g glycine; 10 g SDS; 1 L $H_2O$ |
| **K+ Buffer** | 181.6 mL 1M $K_2HPO_4$; 18.4 mL 1M $KH_2PO_4$; taken to 1 L with $H_2O$; pH 7.85 |
| **Buffer A** | 18.5 g Tris-HCl; 77 g Tris; 2 g SDS pH 8.8 (per 500 mL) |
| **Buffer C** | 30.0 g Tris-base; 2 g SDS pH 6.8 (per 500 mL) |
| **1X Transfer Buffer** | 3 g Tris-base; 14.4 g glycine; 37.5 mg SDS; 200 mL methanol; 800 mL $H_2O$ |
| **TBS** | 3 g Tris-base; 8 g NaCl; taken to 1000 mL with $H_2O$ |
| **TBST** | 500 µL Tween® 20; 1 L of 1X TBS |

**Table 4. Characterization of MCF-7 and MDA-MB-231 breast cancer cell lines.**

| Cell line | ER | PR | HER-2 | E-cadherin | Vimentin | Invasiveness |
|---|---|---|---|---|---|---|
| **MCF-7** | positive | positive | negative | positive | negative | non-invasive |
| **MDA-MB-231** | negative | negative | negative | negative | positive | highly-invasive |

## 2.1 Overview

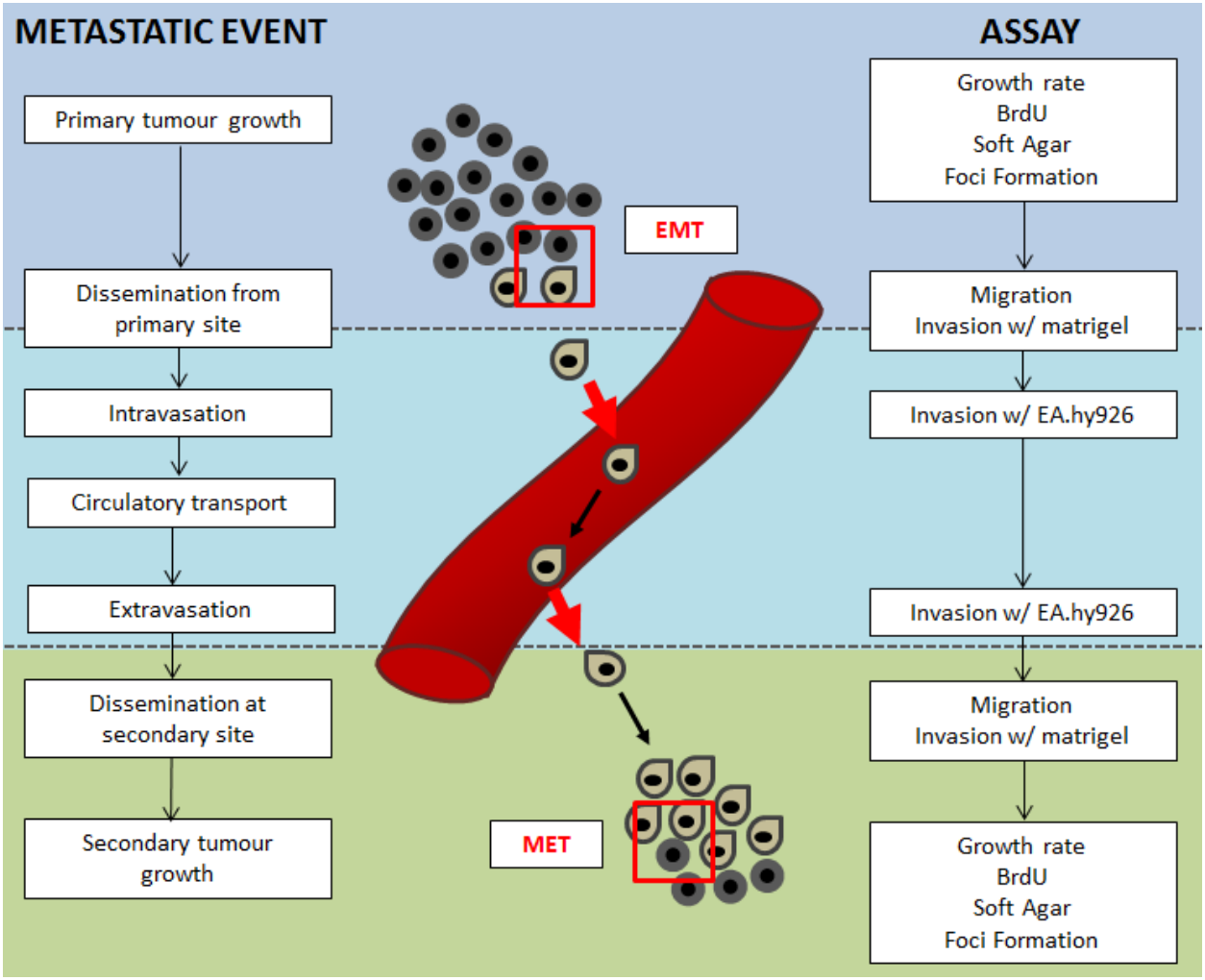



**Figure 8. *In vitro* assays used to analyze cancerous cell behaviour and phenotype**. The assays displayed on the right are those experiments that are used to study the adjacent metastatic event represented on the left. In this study, the EMT and MET have been examined using western blot and immunocytochemical analyses.

## 2.2 Subculture Protocol

The estrogen receptor-positive human breast carcinoma MCF-7 cell line and estrogen receptor-negative human breast carcinoma MDA-MB-231 cell line were obtained from the American Type Culture Collection (ATCC). The cells were maintained according to ATCC standards at 37°C and 5% $CO_2$ in a humidified atmosphere. MCF-7 cells were cultured in Alpha Minimum Essential Medium (α-MEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% L-glutamine, 1% Pen-strep and 10 µg/µL bovine insulin. The MB-231 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% Pen-Strep. We chose to use these two cell lines so as to align with other studies and reports that often compare and contrast the two.

The same method of cell passage was used for each cell line. The volumes described in this protocol are for cells kept in 100 mm cell culture dishes.

1. Warm media and 0.25% (w/v) Trypsin-0.53 mM EDTA (T/E) solution in a 37°C water bath.
2. Aspirate off the culture media from the culture dish.
3. Add 3 mL of the T/E solution to the flask and incubate at 37°C for 5 min or until cells detach.
4. Add 3 mL of complete growth medium and gently wash cells off plate.
5. Transfer the cell suspension to a 15 mL centrifuge tube. Centrifuge at 1000 *g* for 5 min at RT. Discard the supernatant.
6. Resuspend the cell pellet in 1 mL of fresh growth medium. Add appropriate aliquots of the cell suspension to sterile 100 mm culture dishes containing 10 mL of fresh growth media.
7. Tilt dish side-to-side to ensure even distribution of cells on growth surface and then place in the humidified incubator.
8. If necessary, cells can be stored in Cell Freezing Media and placed in liquid nitrogen for long-term storage.

## 2.3 MAO-A Activity Assay

The MAO-A activity within a given sample can be measured by introducing [$^{14}$C]-radiolabeled serotonin [5-hydroxytryptamine (5-HT)] and analyzing the rate of 5-hydroxytryptamine conversion to its acid metabolite [$^{14}$C]-5-hydroxyindole acetaldehyde.

1. Bubble oxygen through the potassium phosphate buffer for at least 20 min at RT.
2. Collect and harvest cells. Resuspend the pellet in 100-200 µL of $K^+$ buffer, depending on the size of the pellet, and use multiple up-and-down passages through a 22Gi syringe to disrupt the cell membrane.
3. Perform the BCA protein assay to determine the protein concentration of each sample and dilute to equal concentrations of 1-2 µg/µL in $K^+$ buffer.
4. In a scintillation vial prepare the working solution (WS: 1X total 5HT substrate) in the designated radioactive work area. Note that each sample tested should include one blank and three replicates.

   Sample calculation for WS:

   9 samples x (1 blank + 3 reps) = 36 samples
   36 samples x 50 µL WS/ sample = 1 800 µL WS required
   1 800 µL / 100 = 18 µL
   18 µL / 2 = 9 µL

   Therefore, you will require 9 µL of 5-HT standard and 9 µL of [$^{14}$C]-radioisotope (1:1 ratio). Bring to volume with 1 782 µL of $K^+$ buffer.

5. Add 25 µL of 3M HCl to each 'blank' (this will inactivate any enzyme function and will provide a 'background' for the radioenzymatic assay). Place all samples in a 37°C water bath at the same time, for 10 min.
6. Add 25 µL of 3M HCl to all sample tubes except the blanks.
7. Remove samples from water bath and add 1 mL of a 1:1 $H_2O$ saturated ethyl acetate: toluene solution.
8. Briefly vortex each sample and centrifuge (high speed) for 30 sec at RT.
9. Remove 700 µL of the upper layer and place into separate scintillation vials.
10. Add 4 mL of ACS cocktail to each scintillation vial and vortex.
11. Measure $C^{14}$ activity using scintillation spectrometer.

## 2.4 MTT Assay

This technique can be applied to measure cell viability by assessing the amount of metabolically active cells within a sample. It is a recognized method for monitoring the effect of drugs on cell viability or function. A yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT), is enzymatically reduced to insoluble purple formazan crystals. By using a detergent to solubilize the crystals, the resulting coloured solution can be analyzed by spectrophotometry. The resulting optical density values are relative to the conversion of MTT and ultimately to cell function.

1. Seed 1.0 *x* $10^4$ cells in 200 µL of media into each well of a 96-well plate. Incubate at 37°C and 5% $CO_2$ for 2 hours to allow cells to adhere to surface.
2. Replace media with treatment media (CLG: 1 µM; SB203580: 10 µM) and incubate overnight at 37°C and 5% $CO_2$.
3. Prepare MTT solution at a final concentration of 5 mg/mL dissolved in DMEM supplemented with 1% FBS.
4. Replace media in wells with 50 µL of the MTT solution. Blank (background) wells will hold 150 µL of dimethyl sulfoxide (DMSO).
5. Gently agitate plate to mix and then incubate at 37°C for 2 hours for metabolism to occur.
6. Add 150 µL of DMSO to all wells except blanks which will receive 50 µL of the MTT solution. Gently agitate plate and incubate at 37°C for 30-45 min.
7. Read optical density at 570 nm.

## 2.5 Growth Curves Protocol

The rate at which a tumour is able to grow is an indicator of the aggressiveness of the cancer cells. In order to analyze the cell's growth rate, a series of growth curves were produced as follows:

1. Seed equal numbers of cells to 100 mm culture dishes and treat accordingly (Vehicle; CLG, 1 µM; SB203580, 10 µM; CLG + SB203580).

2. Treat cells on Day 1 and replace with new growth media (including appropriate drug concentrations) every 48 hours.
3. Cells were harvested and counted using a hemocytometer. The concentration of cells in a 1 mL cell suspension was determined. The hemocytometer consists of nine 1 mm squares. The average of four 1 mm squares was determined and multiplied by $1.0 \times 10^4$ to calculate the number of cells per mL. This was done every 24 hours for a total of 4-7 days.

## 2.6 BrdU Assay

During the DNA synthesizing phase, or S-phase, of cell division, the synthetic thymidine analog, 5-bromo-2-deoxyuridine (BrdU), will be substituted into a cell's DNA in place of the endogenous thymidine nucleotide. The incorporation of this reagent allows for the detection of newly synthesized DNA based on immunodetection of the incorporated BrdU using a specific antibody.

1. Cells are plated on an 8-well chamber slide at $5.0 \times 10^3$ cells per well and incubated at 37°C at 5% $CO_2$ overnight.
2. Prepare the BrdU solution to a final concentration of 10 µM in culture media.
3. Aspirate media from chambers being careful not to disrupt the cell layer. Replace with 300 µL of the BrdU solution and incubate at 37°C at 5% $CO_2$ 2 hours.
4. Remove the BrdU-labelling reagent and wash very gently with PBS without rocking.
5. Fix cells with 10% formalin, rocking slowly at RT for 20 min.
6. Wash with PBS in 1% Triton X-100 at RT for 15 min.
7. Add 200 µL of 2N HCl to denature the DNA and incubate at 37°C for 1 hour.
8. Aspirate the 2N HCl from each well and neutralize the cells with borate buffer, pH 8.5, by washing 3X at RT for 10 min intervals.
9. Wash cells with PBS and incubate in blocking solution (PBS in 0.3% Triton X-100, 5% goat serum) rocking at RT for 30 min.
10. Prepare the fluorescent conjugated anti-BrdU antibody at a 1:500 dilution in blocking solution. Incubate the cells at 4°C overnight.
11. Wash cells 3X in PBS at RT for 10 min intervals.

12. Incubate cells at RT for 2 hours in secondary goat anti-mouse (visualized as a red colour upon fluorescing) at a 1:250 dilution in blocking solution.
13. Wash 3X in PBS at RT for 10 min intervals.
14. Counterstain with DAPI nucleic acid stain (visualized as 'blue') at RT for 5 min. Wash 3X in PBS at RT for 10 min intervals.
15. Mount dry samples with Dako mounting medium and analyze slide with a fluorescence microscope.

## 2.7 Foci Formation Protocol

Foci formation assays assess contact-inhibited growth of breast tumour cells. Once 100% confluence is reached and a monolayer of cells is formed, transformed cells will grow on top of one another to eventually form foci.

1. Seed $1.0 \times 10^5$ cells per 100 mm plate and maintain in recommended growth media for 10-14 days depending on the cell line. Ensure an even distribution of cells on the plate surface after seeding.
2. Change the media every 72 hours and more frequently with increasing confluence. Once 100% confluence is reached, the cells are grown for an additional 5 days to allow for foci formation upon the monolayer.
3. Place the plates on ice and wash carefully with ice-cold 1X PBS by adding it slowly down the side of the plate to avoid lifting of the cell monolayer.
4. Fix the cells in 4 mL of ice-cold methanol and store overnight at 4°C.
5. Remove the methanol and leave plates to air dry on ice.
6. Add 4 mL of a filtered 1:10 dilution of crystal violet to each plate and incubate at RT rocking gently overnight.
7. Rinse the plates using $ddH_2O$ until such time as all the stain is removed and then leave the plates to dry at RT.
8. Examine the plates under a BX71 Olympus microscope for the presence of foci (cells that have grown over each other to form a cluster or focus). Dark purple spots represent such a focus.

## 2.8 Soft Agar Assay

This assay was conducted to determine the potential for contact inhibition and anchorage-independent growth in the breast cancer cell lines. Normal cells require contact (i.e. need to be 'anchored') with a solid surface to allow for cell division. When cells are suspended between two gel surfaces the lack of contact with a solid surface will impede growth of a normal cell, but not the growth of a transformed cell. Normal cells will also grow to a monolayer and then once they come in contact with surrounding cells, will stop dividing. Thus, *in vitro* models of cellular transformation involve the acquisition of cancerous properties such as the ability of cells to grow over each other (due to a loss of contact inhibition) and to form colonies in semi-solid agar (anchorage-independence growth).

1. In a 60 mm petri dish, add the bottom agar layer (0.61%) (Table 5) which provides a nutrient base. To ensure that the agar is completely and evenly solidified, incubate plates at RT for 30 min. Particular attention should be used to avoid disturbing the high-percentage agar layers in these plates.
2. Collect cells and suspend 5.0 $x$ $10^4$cells per plate in the (0.36%) (Table 5) semisolid agar top layer mixture, including appropriate drug treatments (CLG: 1 µM; SB203580: 10 µM). Include at least three replicates per treatment group.
3. To provide additional nutritional supplements and prevent drying of the cells, 2 mL of top agar solution supplemented with indicated drug concentrations (excluding the agar) are added to each plate every 7 days.
4. Incubate plates for 30 days at 37°C in a 5% $CO_2$ incubator. At the end of 30 days, the cells are counted using a BX71 Olympus microscope at 10X magnification. The average colony count is based on three randomly chosen fields per experiment.

Determine the number of plates that are required and prepare the appropriate amount of bottom and top agar mixtures as instructed below, based on having 5 mL per plate. The volumes indicated are for 4 (60 mm) cell culture dishes.

**Table 5. Soft agar assay.** Top and bottom agar volumes and materials.

| | **Bottom Agar (0.61%)** | **Top Agar (0.36%)** |
|---|---|---|
| **Growth Media (with drug)** | 10.2 mL | 4 mL |
| **100 X MEM** | 1 mL | 0.3 mL |
| **$dH_2O$** | - | 1.3 mL |
| **FBS** | 2 mL | 0.8 mL |
| **1.8 % Agar** | 6.8 mL | 1.6 mL |

## 2.9 Migration and Invasion Assay

Migration and invasion assays follow essentially the same protocol, the only exception is that in a migration assay cells are migrated across a porous membrane (Figure 9A), whereas invasion requires the migration through a matrix barrier, typically matrigel or an endothelial monolayer. EA.hy926 cells are a human endothelial cell line used to observe transendothelial migration. The use of matrigel models the movement of cancer cells through the basement membrane, which a cancer cell must do once it detaches from the primary tumour and invades the surrounding tissue on its way to the circulatory system to begin the pathway to metastasis. Similarly, an endothelial cell layer between the breast cancer cells and the supporting filter membrane is used as a model of the barrier that these cells must breach when moving into (intravasation) and out of (extravasation) circulatory vessels (Figure 9B).

The human endothelial cell line, EA.hy926, was provided by Dr. Paul Mellor (Cancer Research Unit, Saskatoon). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS.

a) Invasion: seed the EA.hy 926 cells at $5.0 \times 10^5$ cells per Transwell filter 72 hours before the migration assay to allow the formation of a complete endothelial monolayer.

b) Matrigel: prior to migration, coat the filters with 50 µL of 1 mg/mL matrigel and incubate at 37°C for 4 hours.

1. The breast cancer cells were serum starved for 24 hours before performing the migration assay by replacing cell media with 0.5% FBS containing media.
2. Before seeding cells into the upper chamber carefully aspirate off EA.hy926 cell media/ remaining matrigel liquid being cautious not to puncture the monolayer/matrigel.
3. Collect serum-starved breast cancer cells (incubated in 0.5% FBS, 24 h) and resuspend in 0.5% FBS media at a concentration of $4.0 \times 10^5$cells/mL. Aliquots (500 µL) of this cell suspension are then layered onto each filter.

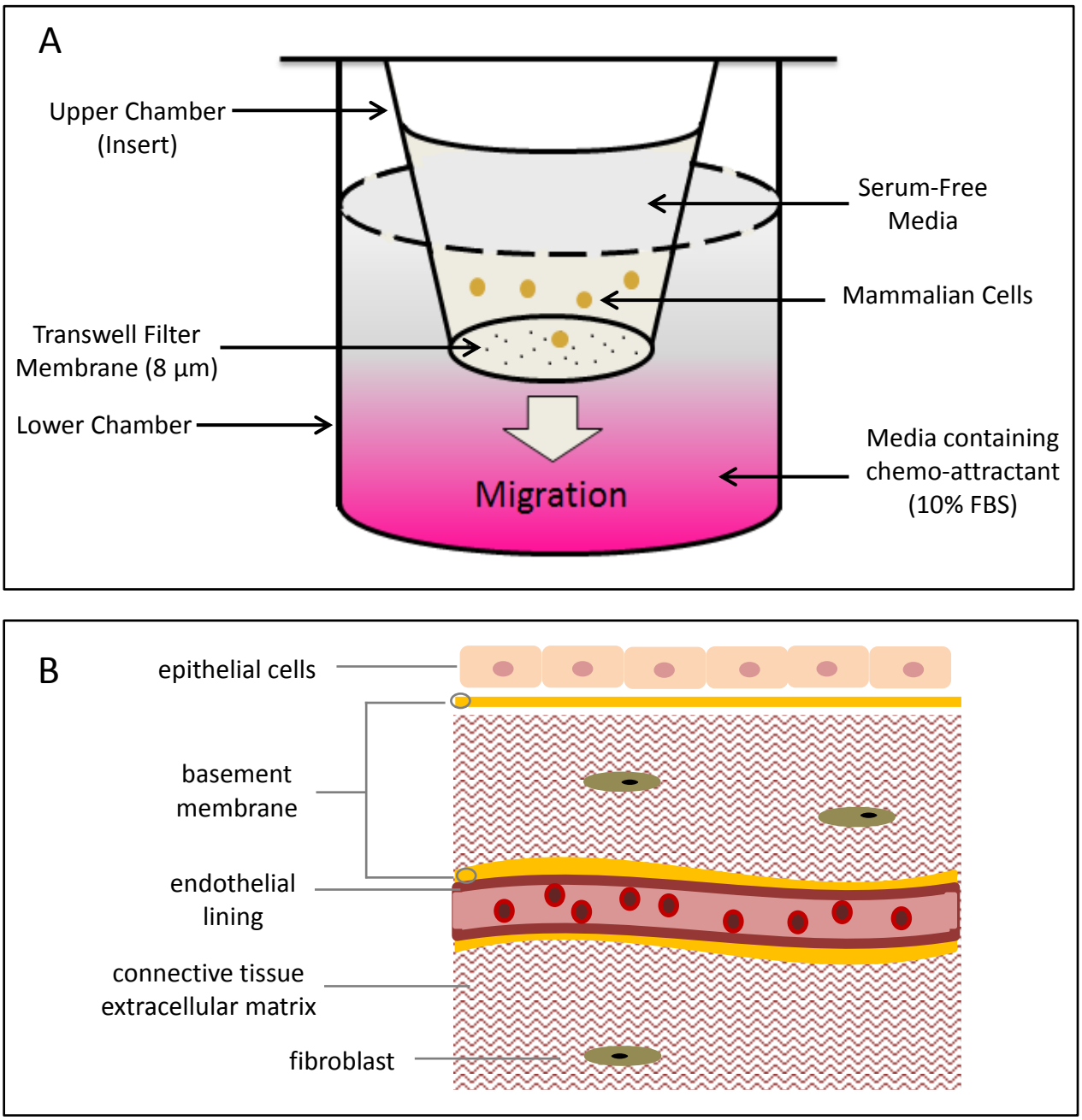



**Figure 9. *In vitro* and *in vivo* migration and invasion.** (A) In a Transwell system, the cancer cells migrate through the filter membrane along the concentration gradient toward the chemokine. (B) During carcinogenesis, epithelial cells alter their adhesion properties to facilitate expansion into adjacent tissues. These cells must degrade the basement membrane and undergo transendothelial migration prior to renewed proliferation at a secondary site.

4. Incubate cells for 2 hours allowing cells to adhere to the surface and then administer appropriate drug treatments. Incubate the treated cells for a 24 hour (EA.hy926) or 72 hour (matrigel) period.
5. To assess cell migration, the media is removed from the upper chamber. Cells that did not migrate are carefully removed from the upper side of the filter membrane using a cotton swab. The inserts are then immersed in ice cold methanol and stored at -20°C overnight.
6. Remove the methanol from the upper chamber and rinse in Scott's water. The cells are stained with haemotoxylin for 30 min at RT.
7. After emptying the insert, it is again washed in Scott's water for 5 min at RT.
8. The inserts are then dehydrated through increasing ethanol concentrations of 50%, 75%, 90% and 95% for 1 minute intervals. The filters are left to air dry completely before removing the filter membranes and mounting them onto a microscope slide with Dako Flourescent Mounting Media.
9. Using a BX71 Olympus microscope count migrated cells across 5 fields at 20X magnification.

## 2.10 Immunoblotting Protocol

Immunoblotting (also known as Western blotting) is based on the separation of proteins by gel electrophoresis as a function of their molecular weight (MW) and then transferring the separated (resolved) proteins to a membrane. Proteins are identified using a primary antibody specific for that protein which is then detected by a secondary antibody that not only recognizes the first antibody, but that is also conjugated to an enzyme. The enzyme, most often horse radish peroxidase (HRP), converts a substrate to a light-emitting product that can then be detected using film. The identification and/or detection of the protein(s) is then compared to a protein ladder which has been run on the same gel and that contains proteins of known molecular weight.

A. Sample Preparation

1. Harvest and lyse cells using appropriate amounts of lysis buffer.
2. Incubate on ice for 30 min.

3. Centrifuge samples for 10 min at 12 000 *g* at 4°C. Use this pre-cleared sample (the supernatant) to perform the bicinchoninic acid (BCA) assay to determine the total protein concentration for each cell lysate.
4. Prepare aliquots of 1-2 µg/µL by diluting the sample with PLC lysis buffer and 4X loading buffer.
5. Boil the samples for 5 min at 95°C (to denature the proteins so that they migrate freely during the gel electrophoresis) and store at -20°C until needed.

B. Gel electrophoresis

1. Standard immunoblotting protocols are based on protein separation according to molecular weight using SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis). The polyacrylamide gel is prepared as follows in Table 6.
2. Bathe the gel apparatus in 1X running buffer diluted from a 10X stock solution added into the gel holder.
3. To determine protein size and to monitor protein migration along the gel, a molecular weight marker (PageRuler Protein) was loaded onto the gel. Equal amounts of protein are loaded into each well (usually between 20-40 µg).
4. Apply a constant voltage of 110 V to the resolving gel apparatus and allow proteins to resolve for approximately 90 minutes.

C. Transfer

The proteins are transferred from the gel onto a nitrocellulose membrane.

1. Place the nitrocellulose membrane next to the gel and together they are sandwiched between absorbent paper and sponges (see Figure 10).
2. Remove air bubbles and submerge the sandwich in 1X transfer buffer. The transfer buffer is constantly stirred and kept ice cold so as to keep the temperature of the solution from heating (as would be expected from running the system at 0.23 A for 90 min).

D. Immunodetection

Once the proteins have been transferred to the nitrocellulose membrane, remove the membrane and incubate it in a blocking solution (5% milk casein in 1X TBS; 1 h, RT) to block all non-specific binding sites on the membrane.

**Table 6. Preparation of SDS polyacrylamide gel.** Volumes listed are for a 1 mm gel thickness.

| | **10% Resolving Gel** | **4% Stacking Gel** |
|---|---|---|
| **dd$H_2O$** | 4.01 mL | 3.00 mL |
| **Buffer A** | 2.50 mL | - |
| **Buffer C** | - | 1.25 mL |
| **Acrylamide*** | 3.33 mL | 0.67 mL |
| **10% APS** | 50 µL | 25 µL |
| **10% SDS** | 100 µL | 50 µL |
| **TEMED** | 10 µL | 5 µL |

*30% Acrylamide/Bis solution (37.5:1 acryl/bis) (commercial source)

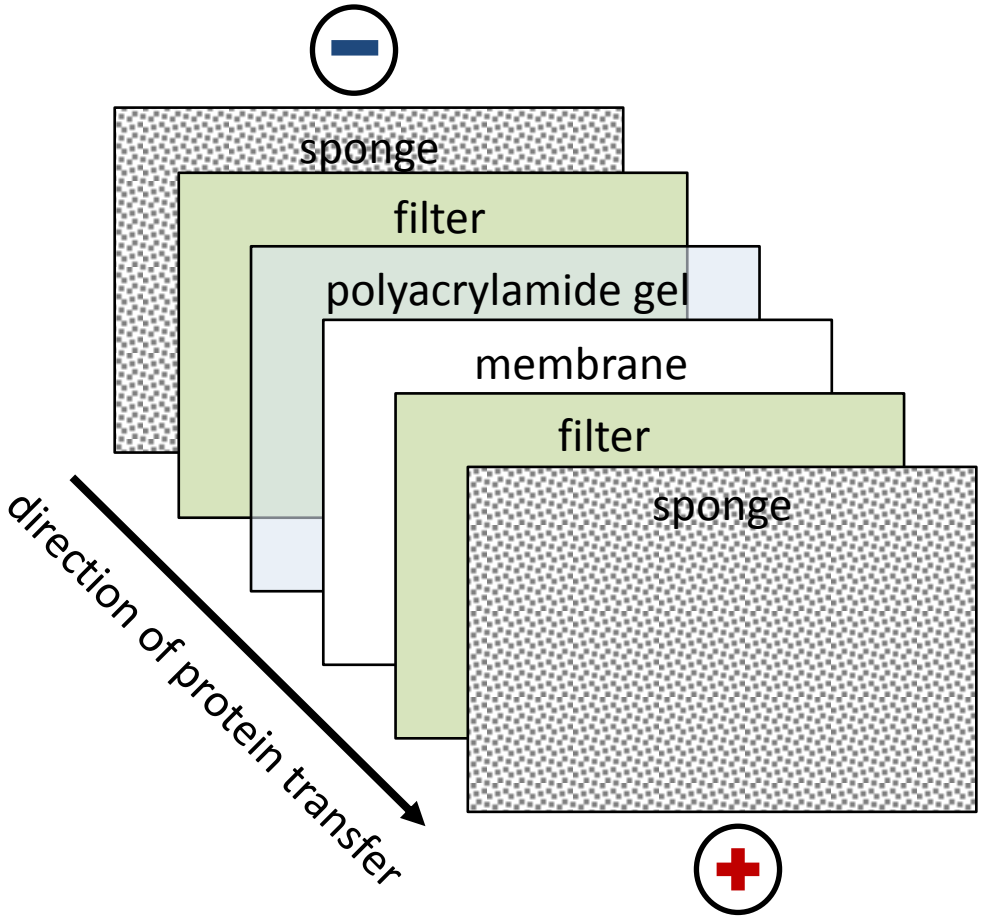



**Figure 10. Assembly of the blotting sandwich during protein transfer.** Proteins are transferred in an electrical field from the gel onto the membrane. The negatively charged proteins travel towards the cathode and are bound to the nitrocellulose membrane.

1. Dilute the primary antibody in 1X TBST at the concentration suggested by the individual commercial sources. The membrane/antibody solution is sealed in a fitted plastic bag and placed between two glass plates (the pressure from the plates keeps the bag flat and the solution evenly distributed over the membrane). This is incubated overnight at 4°C.
2. Wash the membrane 3X in 1X TBST at RT rocking for 10-15 min.
3. Add the HRP-conjugated secondary antibody to 20 mL of 5% milk in TBST at the suggested dilution and rock the membrane in this solution at RT for 1-2 hours.
4. Wash the membrane 3X in TBST at RT for 15-20 min.

E. Detection

1. Prepare ECL (enhanced chemiluminscence) detection reagent and allow it to warm to RT.
2. Incubate the membrane in the ECL reagent for 1 min, then remove it and allow excess ECL solution to drain off. Place the membrane in a film cassette and cover with a thin piece of transparent plastic (keeps the film from getting wet) and then expose to x-ray film.
3. Expose the film and if needed re-expose the membrane to more film for varying amounts of time to optimize the detection of the protein band.

Each immunoblotting experiment also includes the detection of a housekeeping protein, e.g. β-actin, as an 'internal control' to demonstrate that equal amounts of cellular protein was loaded into each lane on the gel.

## 2.11 Immunocytochemistry

Immunofluorescence techniques were used to examine the expression and distribution of markers of the epithelial-mesenchymal transition (EMT) in treated cell cultures: These markers include E-cadherin, vimentin, and β-catenin.

1. Seed $5.0 \times 10^3$ cells per well of an 8-well chamber slide and incubate at 37°C in 5% $CO_2$ for 2-4 hours to allow cells to adhere to the slide surface.
2. Without disrupting the cells, gently wash cultures in 1X PBS.
3. Fix cells with 10% formalin, rocking slowly at RT for 20 min.

4. Gently wash cells twice in 1X PBS before adding blocking solution (1.5 mL donkey serum, 900 µL 10% Triton X-100, 27.6 mL 1X PBS). Block cells rocking at RT for 30 min.
5. Prepare the primary antibody in blocking solution. Incubate cells in primary antibody rocking at 4°C overnight.
6. Wash the cells 3X in 1X PBS at 10 min each.
7. Prepare the fluorescent secondary antibody at a 1:200 dilution. Incubate cultures for 2 hours rocking at RT.
8. Wash the cells 3X in 1X PBS at 10 min each.
9. Counterstain with DAPI at RT for 5 min. Wash 3X in PBS at RT for 10 min each.
10. Mount dry samples with Dako mounting medium and analyze slide for the distribution and intensity of the target proteins.
11. The distribution of E-cadherin and vimentin within cells was analyzed by collecting the fluorescence intensity along a bisecting line centered on the nucleus and the relative quantity of proteins (based on intensity) in the nucleus/cytoplasm was quantitated using ImageJ 1.32j (http://rsb.info.nih.gov/ij/).

### 2.11.1 Statistical Analysis

Data are expressed as mean ± SEM. Significance (set at $P<0.05$) was assessed either by Student's t-test (for comparing two means) or by analysis of variance (ANOVA: for comparison of 3 or more means) with post-hoc analysis relying on Bonferroni's Multiple Comparison Test (GraphPad Software, Inc., San Diego, CA).

## 2.12 Nuclear Fraction Protocol

This subcellular fractionation procedure allows for the separation of membrane, cytoplasmic and nuclear cell fractions (Figure 11). This protocol should be carried out at 4°C at all times.

1. Cells were grown on 100 mm plates.
2. Cells are transferred to an eppendorf tube using T/E and original growth media to neutralize and transfer. This portion of the protocol is non-sterile and can be performed at RT.

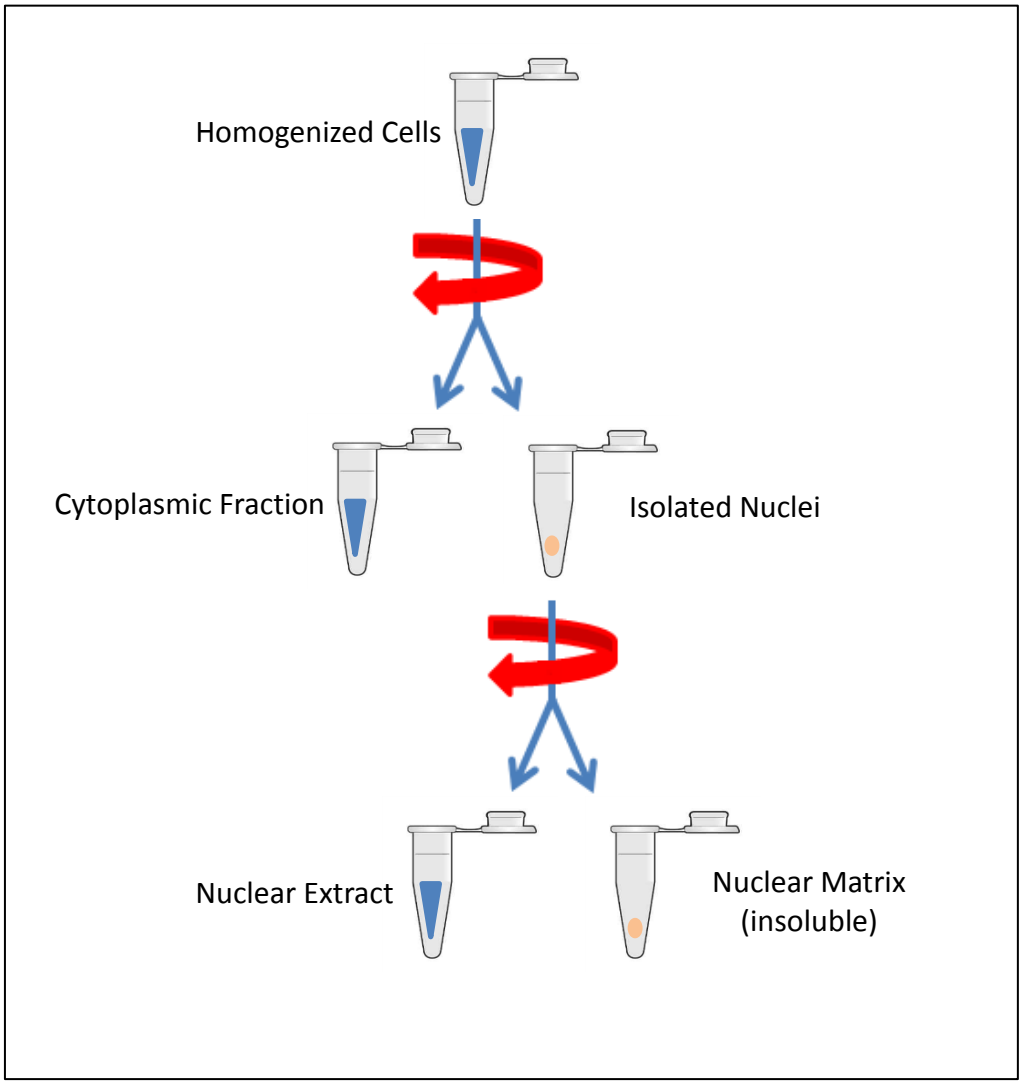



**Figure 11. Nuclear protein fractionation.** The application of centrifugation methods (indicated by the red arrows) to separate cytoplasmic and nuclear fractions.

3. Make up the hypotonic and extraction buffers according to the total volume required, depending on the pellet size (Table 7).
4. Depending on the size of the pellet add the appropriate volume of hypotonic buffer to each tube and disrupt cells using a pestle with the same number of strokes in each tube (10-20).
5. Centrifuge sample at 3000 rpm for 5 min at 4°C. Then collect the supernatant in a new eppendorf tube, this is the cytoplasmic fraction (cytosolic proteins).
6. Resuspend pellet in the extraction buffer mixing before adding to each tube. Rock for 20 min at 4°C before centrifuging samples at 15 000 rpm for 10 min at 4°C.
7. The supernatant contains the nuclear extract (nucleic acid binding proteins) and the pellet contains the nuclear matrix (insoluble nuclear proteins).

## 2.13 Transfection protocol

The introduction of foreign DNA into mammalian cells allows for the expression of the protein encoded by that DNA. We introduced DNA that encodes for the green fluorescent protein (GFP) and MAO-A variants to visualize cells using fluorescence microscopy. This was accomplished using the following transfection protocol:

1. Prior to transfection cells were seeded in a 100 mm culture dish in 10 mL of growth medium (plate should be over 75-80% confluent at the time of transfection).
2. At the time of transfection:
    a. Dilute 24 µg of plasmid DNA in 1.5 mL of Opti-MEM I Reduced Serum Medium without serum and gently mix.
    b. Gently mix Lipofectamine™ 2000 before diluting 60 µL in 1.5 mL of Opti-MEM I Medium. Gently mix and incubate at RT for 5 minutes.

3. Combine the diluted plasmid DNA with the diluted Lipofectamine™ 2000 solution, mix gently and incubate at RT for 20 min to allow DNA- Lipofectamine™ 2000 complexes to form.

**Table 7. Nuclear protein fractionation.** Concentrations of reagents for the hypotonic and extraction buffers.

| Reagents | Final Concentration |
|---|---|
| 1 M HEPES pH7.0 | 20 mM |
| 1 M KCl | 10 mM |
| 1 M DTT | 0.5 µM |
| 10% Triton X-100 | 0.1% |
| 100% Glycerol | 20% |
| 500 mM PMSF | 2 mM |
| 1 mg/mL Aprotinin | 5 µg/mL |
| 1 mg/mL Leupeptin | 5 µg/mL |
| 5 N NaCl | 420 mM* |
| $H_2O$ | -to final volume- |

*Both buffers are exactly the same with the exception that 5N NaCl is only added to the extraction buffer.

4. 3 mL of the solution is added to each culture plate containing the adhered cells and medium. To ensure that the solution is evenly distributed gently rock the plates back and forth.
5. Following 4-6 hours of incubation at 37°C replace media with new growth media.
6. Incubate cells for 24 hours at 37°C in a 5% $CO_2$ incubator.
7. To use the transfected cells for separate assays, harvest the cells from each culture dish. The pellet can then be used immediately in experimental procedures.

## 2.14 DSS and $BS^3$ Cross-linker Protocol

A crosslinking experiment was conducted to investigate the subcellular localization of MAO-A in MB-231 versus MCF-7 cells. Proteins, such as MAO-A, have several primary amines as part of the side chain of lysine (K) residues and in the N-terminus of each polypeptide. These primary amino groups (-$NH_2$) are targeted by such *N*-hydroxysuccinimide (NHS) esters as disuccinimidyl suberate (DSS) and its analog bis[sulfosuccinimydyl] suberate ($BS^3$) (191). NHS esters form stable amide bonds with primary amino groups in pH 7-9 buffers, resulting in the release of *N*-hydroxysuccinimide. DSS is hydrophobic and membrane-permeable allowing for intracellular conjugations, whereas $BS^3$ is hydrophilic and useful for cell-surface protein crosslinking (191). Both water-soluble and insoluble forms have essentially identical reactivity with primary amines.

1. Cells were treated for 24 hours with CLG and/or SB203580 at 1 µM and 10 µM, respectively prior to harvest. Crosslinking is performed on cells in suspension, following the collection of a cell pellet (2.5 $x$ $10^7$ cells/mL) in an eppendorf tube (Subculture Protocol).
2. The pellet is washed 3X in ice-cold PBS to remove amine-containing culture media.
3. Crosslinkers are prepared by:
    a) dissolving $BS^3$ in $H_2O$ in the required volume at a 25 mM concentration.
    b) dissolving DSS in DMSO in the required volume at a 25 mM concentration.
4. The pellet is resuspended in the DSS or $BS^3$ solution made in PBS to a final concentration of 1 mM.

5. The reaction mixture is incubated at RT for 30 min.
6. Add glycine to a final concentration of 10 mM to quench the reaction and incubate at RT for 15 min.

Pellet undergoes a final wash in PBS preceding protein determination and western blot analysis.

# 3 RESULTS

Please note that the 'Results' section includes a discussion of the results at each step for ease of interpretation and to provide rationale for the choice of subsequent experiments. A general discussion is presented following this section.

The MCF-7 and MB-231 breast cancer cell lines are both derived from malignant human adenocarcinomas isolated from pleural effusions (192, 193) and are the most widely used breast cancer cell lines in research (194). These cell lines are largely studied to examine hormone dependence in breast cancer. The MCF-7 cells are classified as ER(+) [ER(+), PR(+), HER-2(-)] (195), whereas the MB-231 cells are classified as a triple negative breast cancer [ER(-), PR(-), HER-2(-)] (196). We chose these cell lines so as to align with other reports that often compare and contrast the two.

An initial investigation corroborated that the MB-231 cells represent a more aggressive cancer cell line as observed in other studies (197). For instance, an indication of aggressiveness is the spindle-shaped morphology of MB-231 cells, which can be associated with a mesenchymal-like phenotype supportive of invasive behaviour (171).

MAO-A and p38(MAPK) protein expression was compared between the MCF-7 and MB-231 cell lines using immunoblotting. The MB-231 cells clearly express higher MAO-A expression than the MCF-7 cells (Figure 12A). The ratio between total and phosphorylated p38(MAPK) is greater in the MCF-7 cell line compared to the MB-231 cell line (Figure 12B).

The **hypothesis** states that a functional interaction between the p38(MAPK) and MAO-A systems alters breast cancer cells in an ER-dependent manner. In glial cells, MAO-A activity is inhibited by p38(MAPK) (198) and we expected to find a similar effect in breast cancer cells. If this pattern holds, then CLG and SB203580 should induce opposite effects within each assay since CLG is an irreversible inhibitor of MAO-A and SB203580 inhibits p38(MAPK). Additionally, inhibition of p38(MAPK) should prevent any inhibition of MAO-A by p38(MAPK) (Figure 5 in 'Introduction' section).We examined how p38(MAPK) inhibition (using SB203580) affected MAO-A activity in MCF-7 and MB-231 cells.

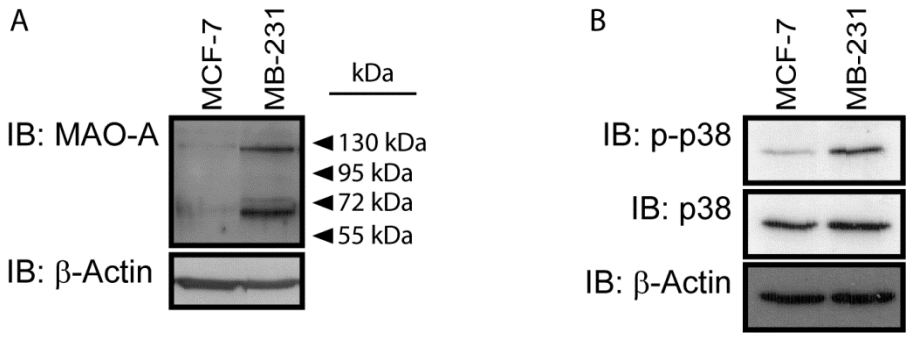



**Figure 12. Endogenous MAO-A and p38(MAPK) expression in MCF-7 versus MB-231 cells.** Using immunoblotting techniques, endogenous MAO-A protein (A) expression was higher in the MB-231 cells compared to MCF-7 cells. The ratio of total to phosphorylated p38(MAPK) protein expression (B) was greater in the MCF-7 cell line compared to the MB-231 cells.

## 3.1 Impact of p38(MAPK) inhibition on MAO-A enzymatic activity

We determined the effects of CLG and SB203580 on the enzymatic activity of MAO-A in both MCF-7 and MB-231 cells. CLG (1 µM, 24 h) treatment decreased MAO-A activity in MB-231 cells [t = 22.27, df = 46, $P < 0.0001$] (Figure 13) as well as in MCF-7 cells [t = 37.65, df = 4, $P < 0.0001$] (Figure 13). In MB-231 cells treated with SB203580 (10 µM, 24 h), there was a decrease in MAO-A activity in comparison to the activity observed in the vehicle control group [t = 3.39, df = 6, $P = 0.0148$] (Figure 14). In MCF-7 cells, SB203580 (10 µM, 24 h), as expected, increased MAO-A activity [t = 4.82, df = 6, $P = 0.0029$] (Figure 14).

The effect of p38(MAPK) inhibition was not consistent in both cell lines. This indicates *a priori* that while p38(MAPK) could influence MAO-A in these cells lines, the mechanisms involved were not the same. In addition, this assay demonstrated that the innate activity of MAO-A in the MB-231 cells was very high compared to that in the MCF-7 cells. These data supported the use of these two cell lines for comparison in this study.

## 3.2 The effects of overexpression and inhibition of MAO-A and inhibition of p38(MAPK) on mitochondrial function

To determine whether the two inhibitors, i.e. CLG and SB203580, exerted any effect on the general 'health' of the cells, we treated both cell lines with combinations of the inhibitors and measured the reduction of MTT to formazan crystals. Note that the MTT assay provides quantitative data regarding cell viability, proliferation, and/or mitochondrial function, but does not necessarily reveal specific cellular mechanisms.

MTT conversion was used to evaluate mitochondrial function/cell viability in MB-231 and MCF-7 cells after treatment with CLG (1 µM) and/or SB203580 (10 µM) for 24 hours. A reduction in MTT conversion was observed in MCF-7 cells treated with CLG and SB203580 compared to the vehicle control group [$F(3,23) = 7.441$, $P = 0.0016$]. There was no effect of either inhibitor on MTT conversion in MB-231 cells [$F(3,23) = 0.880$, $P = 0.4682$] (Figure 15).

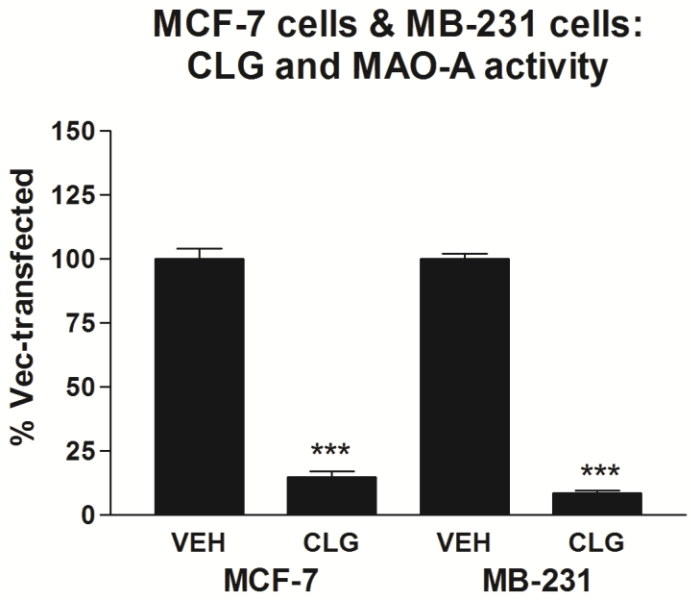



**Figure 13. MAO-A enzymatic activity in MB-231 and MCF-7 cells treated with clorgyline.** MAO-A activity was significantly inhibited by CLG (1 µM, 24 h). in both cell lines (n=3). ***: $P < 0.0001$ versus the respective vehicle (Veh)-treated control group.

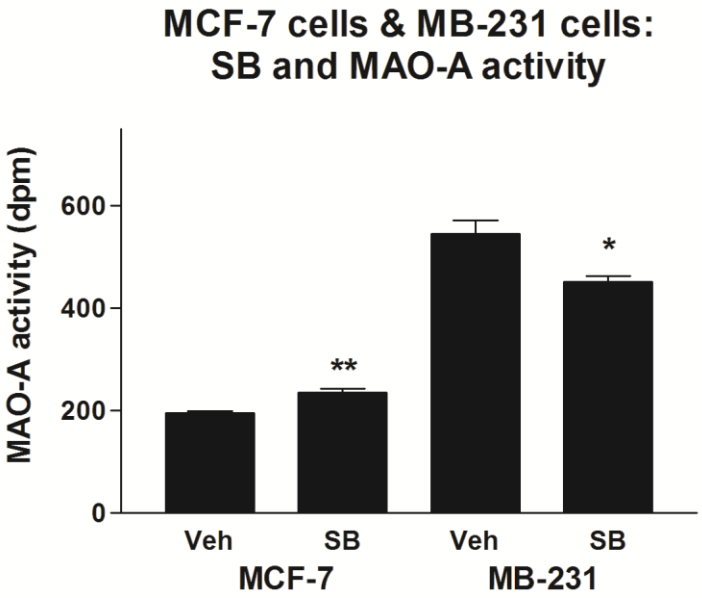
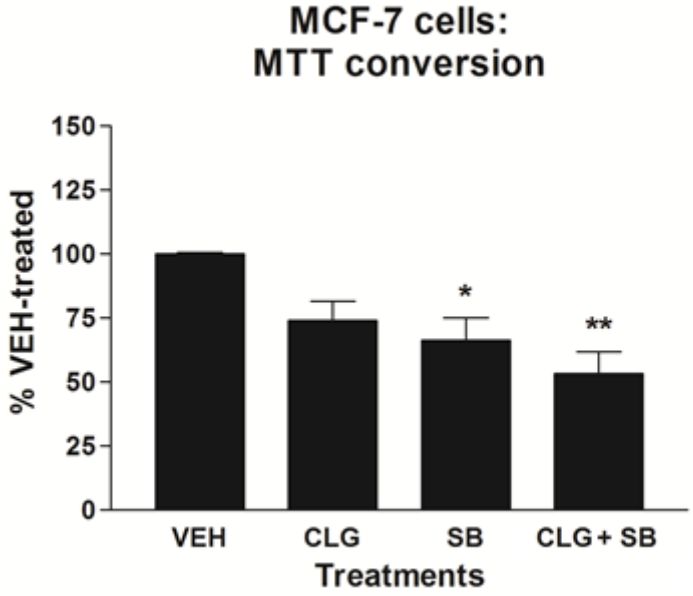



**Figure 14. MAO-A enzymatic activity in MB-231 and MCF-7 cells treated with SB203580.** Treatment of MB-231 cells with SB203580 (10 µM, 24 h) resulted in a reduction in MAO-A activity, whereas treatment of MCF-7 cells with SB203580 (10 µM, 24 h) resulted in an increase in MAO-A activity (n≥4). *: $P$ <0.05 & **: $P$ <0.01, versus the respective vehicle (Veh)-treated control group.

**A**




**B**

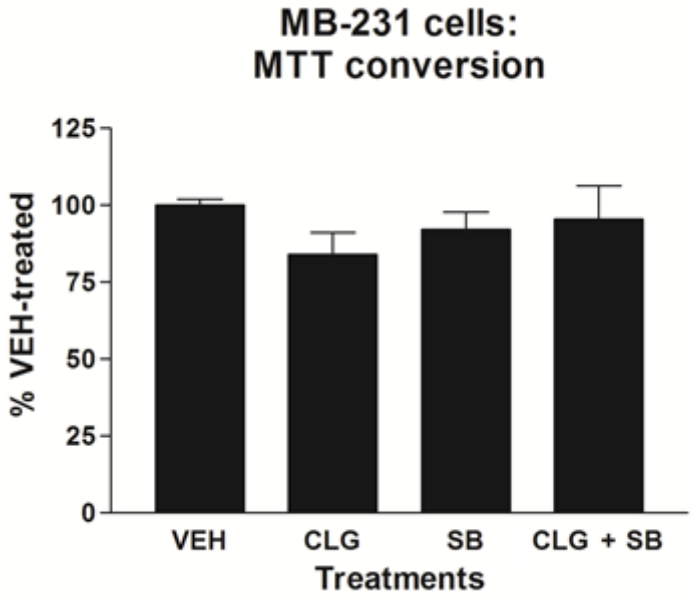



**Figure 15. MTT conversion in MCF-7 and MB-231 cells treated with CLG and SB203580.** Cells were treated with SB203580 (10 μM, 24 h) and/or CLG (1 μM, 24 h). (A) The effect of CLG on MTT conversion was not statistically significant in MCF-7, but SB203580 had a pronounced effect in these cells. (B) Neither inhibitor had an effect in MB-231 cells. *: $P < 0.05$ & **: $P < 0.01$ versus vehicle (VEH)-treated control cells (n=6-9).

A previous M.Sc. student in our research group, Xia Cao, originally tested the effects of MAO-A overexpression on MTT conversion. The overexpressed MAO-A results could also help to explain the inhibitor effects on MTT conversion. There was no effect of MAO-A overexpression on MTT conversion in the MB-231 cultures [t = 1.945, df = 13, $P$ = 0.0737], perhaps because these cells already have high innate MAO-A activity. In contrast, the overexpression of MAO-A in MCF-7 cells increased MTT conversion [t = 5.891, df = 13, $P$ < 0.0001] (Figure 16). This was an unanticipated observation as we observed that treatment of MCF-7 with SB203580 induced MAO-A activity (Figure 14) and inhibited MTT conversion (Figure 15), yet overexpression of MAO-A induced MTT conversion (Figure 16). As already indicated, MTT results are not specific to any single cellular event and could represent multiple events, with a predominant effect overshadowing minimal effects. It is also possible that an MTT result could represent the cumulative output of several unrelated mechanisms. Although the results of the MTT conversion assay were perplexing, they do confirm that the p38(MAPK) and MAO-A systems do influence cell function, that the two cell lines are indeed different, and, as such, that they represent valid models for comparing and contrasting the effects of p38(MAPK) and MAO-A inhibition in the context of breast cancer. We chose to next examine the role of p38(MAPK) and MAO-A on tumourigenic properties of these two cell lines.

## 3.3 Impact of MAO-A and p38(MAPK) inhibition on proliferation rate

The ability of a cell line to form tumours is critical in breast cancer progression; hence we chose to first determine if CLG and/or SB203580 were affecting cell proliferation. Two-way analysis of variance revealed that the proliferation (based on cytometer cell counts) of vehicle-treated MCF-7 cells was significantly increased over the 6-day test period [F(3,56) = 412.5, $P$ < 0.0001]. There was a modest effect of treatment [F(4,56) = 3.688, $P$ = 0.0171] and neither was the interaction between days X treatments [F(12,56) = 1.167]. A similar trend was seen for MB-231 cells over the 6-day test period [F(3,56) = 942.2, $P$ < 0.0001]. In contrast with MCF-7 cells, however, there was a significant effect of SB203580 or CLG on their own (both showed significantly reduced proliferation on day 6), but this was cancelled out in the combined treatment group [F(4,56) = 45.00, $P$ < 0.0001] and the interaction between days X treatments

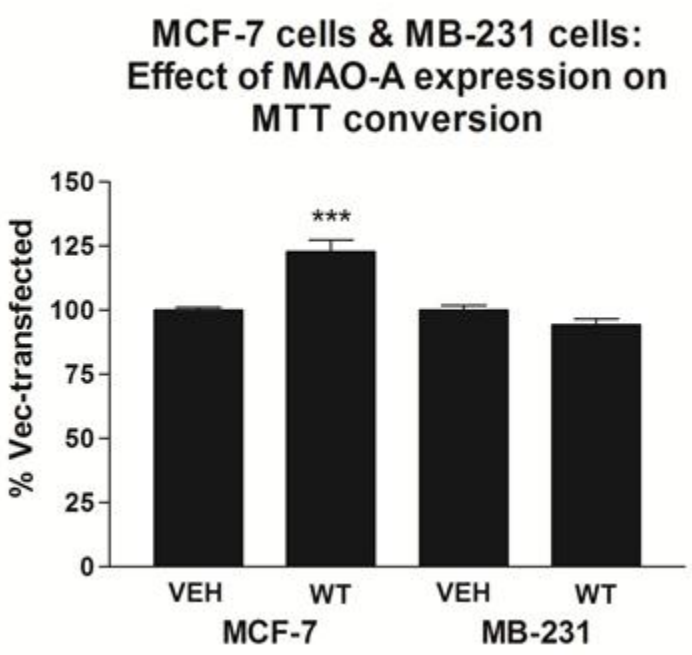



**Figure 16. MTT conversion in MCF-7 and MB-231 cells overexpressing MAO-A**. Compared to the empty vector (Vec), overexpression of wildtype (WT) MAO-A in MCF-7 cells significantly increased MTT conversion. There was no effect of MAO-A overexpression in MB-231 cells. ***: $P < 0.001$ versus Vec-transfected control cells (n=6-9).

was also significant [F(12,56) = 47.69] (Figure 17). The growth curves clearly show differences in the cell lines' response to manipulation of the p38(MAPK) and MAO-A systems. It is also clear that the effects of the individual inhibitors (and, by extension, the individual systems) exert one effect when used independently, but the concurrent manipulation of both systems exerts an altogether novel effect. Clearly these systems do interact, but perhaps not in the way that was originally anticipated. The growth rates also do not corroborate the results of the MTT assay, which confirms the limitations of the interpretation of data generated by the MTT assay (i.e. in this case a one-day MTT assay is not necessarily a good indicator of what is transpiring within the cell). To confirm the validity of changes we observed in the growth rate assays, we chose to examine BrdU incorporation as another means of quantitating cell proliferation in these cell lines.

Bromodeoxyuridine (BrdU) is a synthetic thymidine analog that is incorporated into a cell's DNA during cell division and is therefore a valid means of identifying proliferating cells (199). We did not observe any significant changes between the four treatment groups in either the MCF-7 [F(3,15) = 1.979, $P$ = 0.1710] or MB-231 [F(3,11) = 0.4314, $P$ = 0.7363] cell lines (Figure 18). Although this would initially suggest that neither the MAO-A or p38(MAPK) systems are significantly impacting proliferation rate, it should be borne in mind that these results were obtained over a 24 hour period (BrdU is toxic and is often only tested over short test periods). We are currently examining whether BrdU staining can be monitored over a longer test period (i.e. 96 and 144 h). At this point, we chose to examine how these two systems affected foci formation in the two cell lines.

## 3.5 Absence of foci formation with MCF-7 and MB-231 cells

We assayed for foci formation in order to assess to what extent contact-inhibited growth of MB-231 and MCF-7 breast tumour cells would be affected by treatment with CLG and/or SB203580. Under normal circumstances once cells have grown to full confluence, i.e. there is a monolayer of cells (referred to as 100% confluence), those cells that are 'transformed' (i.e. that are more tumour-like) will grow over each other to eventually form clumps of cells, or foci (200). Our initial attempt was not successful simply due to technical difficulties, i.e. the

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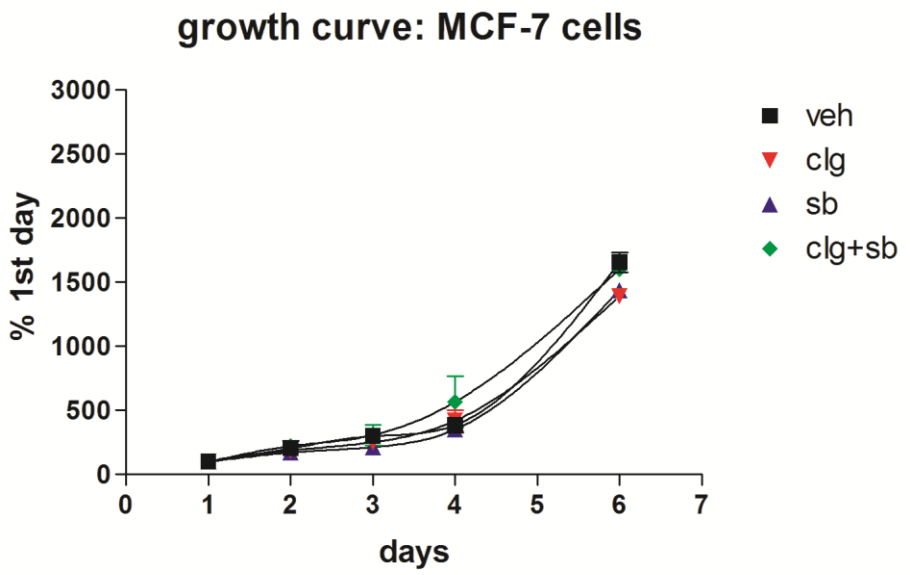


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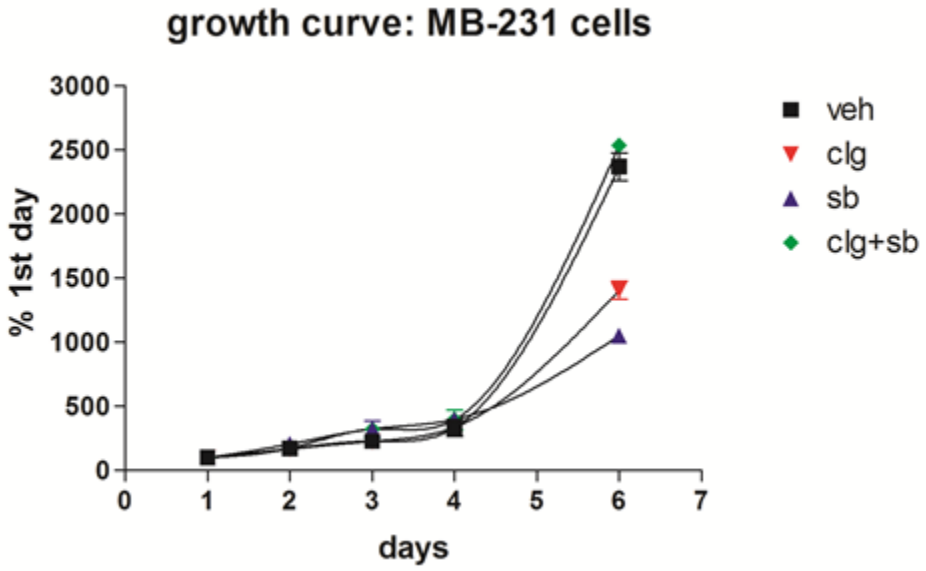


**Figure 17. The growth rate of MCF-7 and MB-231 cells with p38(MAPK) and MAO-A inhibition.** (A) Treatment with CLG and SB203580 did not exert a very profound effect in MCF-7 cells, (B) but the inhibitors on their own significantly inhibited the MB-231 growth rate. The growth rate of MB-231 cells treated with the combination of CLG+ SB203580 was not significantly different from the growth rate of vehicle (Veh)-treated MB-231 cells (n=4).

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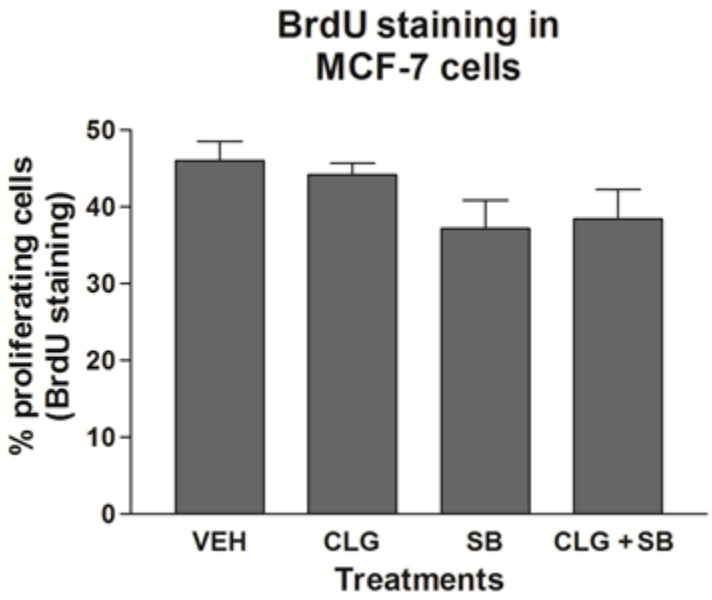


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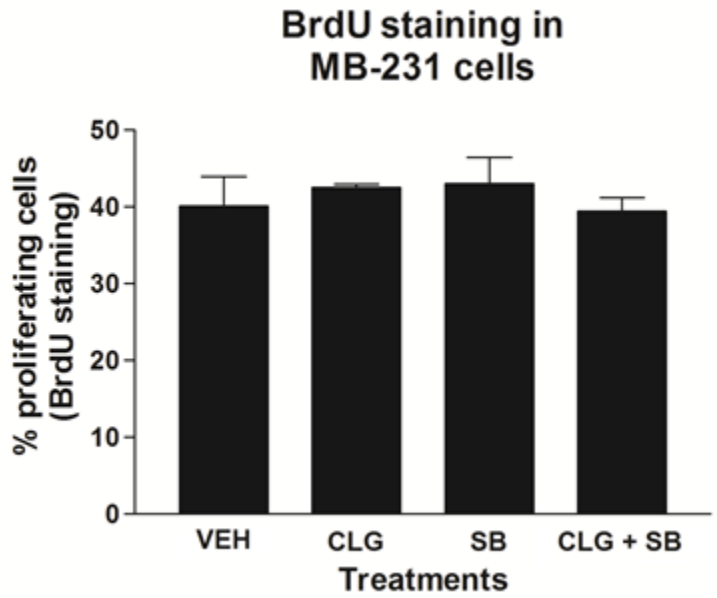


**Figure 18. BrdU incorporation in MCF-7 (A) and MB-231 (B) cells with CLG and SB203580**. The cell lines were treated with CLG (1 µM) and SB203580 (10 µM) for 24 hours and then stained for BrdU incorporation. No effects were observed under any test conditions (n=3-4).

monolayer lifted off of the plate during the wash step and the cells were subsequently lost, an improved technique was adopted to reduce lifting of the monolayer and also to improve the de-staining step so that visualization of the foci would be easier. In our hands, MB-231and MCF-7 cells did not form foci under any conditions even after a two week growth period. The results of this assay were not reproducible and inconclusive; therefore, the data has not been presented. Future attempts to perform this assay should follow the protocol outlined ('Materials and Methods' section), which was eventually developed, with the help of Jennifer Nyarko (Cancer Research Unit, Saskatoon), over several trial assays. We chose to use another approach to assess anchorage-independent growth, namely the soft agar assay.

## 3.6 Effects on anchorage-independent growth following MAO-A and p38(MAPK) inhibition are restricted to the MB-231 cell line

The formation of colonies in soft agar was used as a means of determining anchorage-independent growth, another common sign of tumourigenicity.

While we expected, based on the MTT results (Figure 15, above), that the inhibitors should have a more significant effect in the MCF-7 cell line, this was not what was observed [$F(3,35) = 1.384$, $P = 0.2656$]. In contrast, although we expected no effect of the inhibitors in MB-231 cells (based on the MTT assay), we actually observed a significant reduction in the growth of MB-231 colonies in soft agar with both inhibitors, either alone or in combination [$F(3,27) = 5.000$, $P = 0.0078$] (Figure 19). The reduced colony formation in MB-231 cells treated with either CLG or SB203580 suggested that the cells were less inclined to form tumours under these treatment conditions. This could be indicating that these cells are being directed to a phenotype that is less tumourigenic. In support of this notion, the literature reports that one of the reasons that the MB-231 cell line is often studied is because it is a metastatic cell line (201, 202). At this point, the inhibitors appear to have a more significant effect on the MB-231 cells; however, tumour formation (soft agar assay) and rapid cell growth (growth curves) are decreased during MAO-A and p38(MAPK) inhibition. Tumour cells typically exhibit these characteristics

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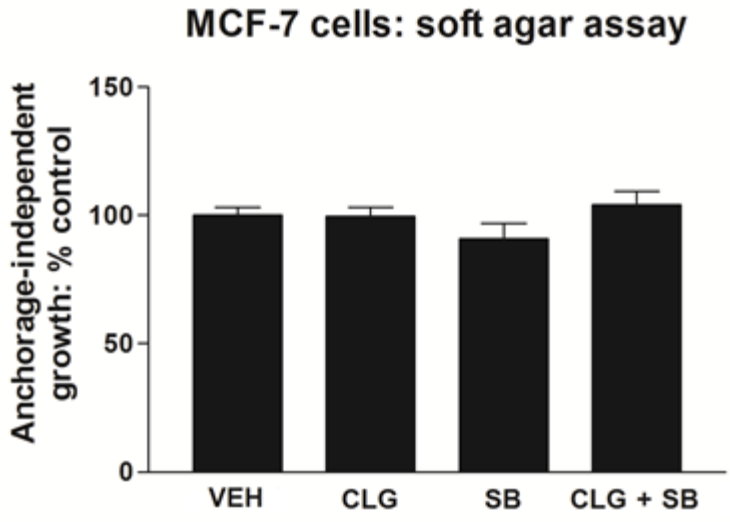


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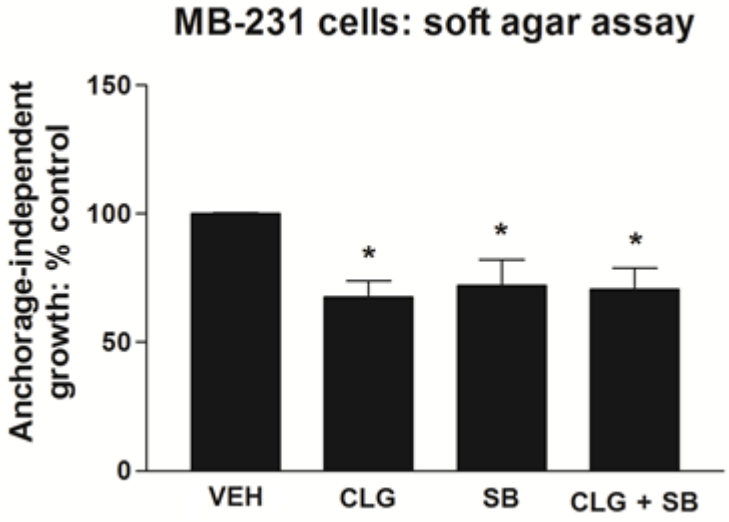


**Figure 19. Anchorage-independent growth in MCF-7 and MB-231 cells with CLG and SB203580.** (A) Whereas, colony formation in the MCF-7 cell line did not differ significantly between treatment groups or in comparison to the vehicle control group (VEH), (B) SB203580 and CLG significantly reduced anchorage-independent growth of MB-231 cells. *: $P < 0.05$ versus VEH-treated cultures. Mean colony count for MCF-7 cells (12±3 colonies, n=8) versus the MB-231 cells (81.2±10.4 colonies, n=9).

Tumour cells typically exhibit these characteristics (soft agar colony formation and rapid growth), and tumour progression would be indicated by an *increase* in these parameters. This is clearly not the case and suggests that perhaps we should examine these two cells lines based on their metastatic potential rather than simply on their ER status, this concept will be further discussed ('Discussion' section). Metastatic cells are more likely to migrate and invade surrounding tissue. We conducted a series of assays to assess the influence of the p38(MAPK) and MAO-A systems on the migratory and invasive potential of both cell lines.

## 3.7 Migration & Invasion Assays

### 3.7.1 Migration Assay

Cell migration is based on chemotaxis, e.g. the movement along a chemical concentration gradient (203); chemotaxis of tumour cells is very important for tumour cells to disperse during metastasis (203). This assay was based on the number of cells that pass through a porous membrane; in this case we used the *in vitro* Transwell insert system (Figure 9A). The lower chamber contained 1 mL of DMEM supplemented with 10% FBS (chemoattractant); this volume allowed for contact with the underside of the Transwell insert (porous membrane) and established a concentration gradient between the upper and lower chamber. Each experiment included, as a control, a parallel set of Transwell inserts that contained 0.1% FBS in DMEM in the lower chamber. Of note, passive diffusion of cells through the filter membrane does not occur readily because pores of the filter are smaller than the size of the breast cancer cells. The MB-231 and MCF-7 cells normally display entirely different morphologies; however this is not apparent once they have migrated (Figure 20A) as all cells typically have a slightly altered appearance and exhibit an irregular shape following migration.

The MB-231 cells are a more aggressive lineage and therefore showed a higher rate of migration in comparison to the MCF-7 cells. Migration rates were not significantly different between treatment groups of the MCF-7 cells [$F(3,11) = 0.1399$, $P = 0.9333$]. Of note, however, the MB-231 cells that received the MAO-A-inhibitor (independently or in combination) did display statistically significant increased migration (Figure 20B). The inhibition of p38(MAPK) had only a partial effect on migration in the MB-231 cells. Furthermore, when SB was used in combination with CLG the effect on migration was not additive. Taken together with the reduced

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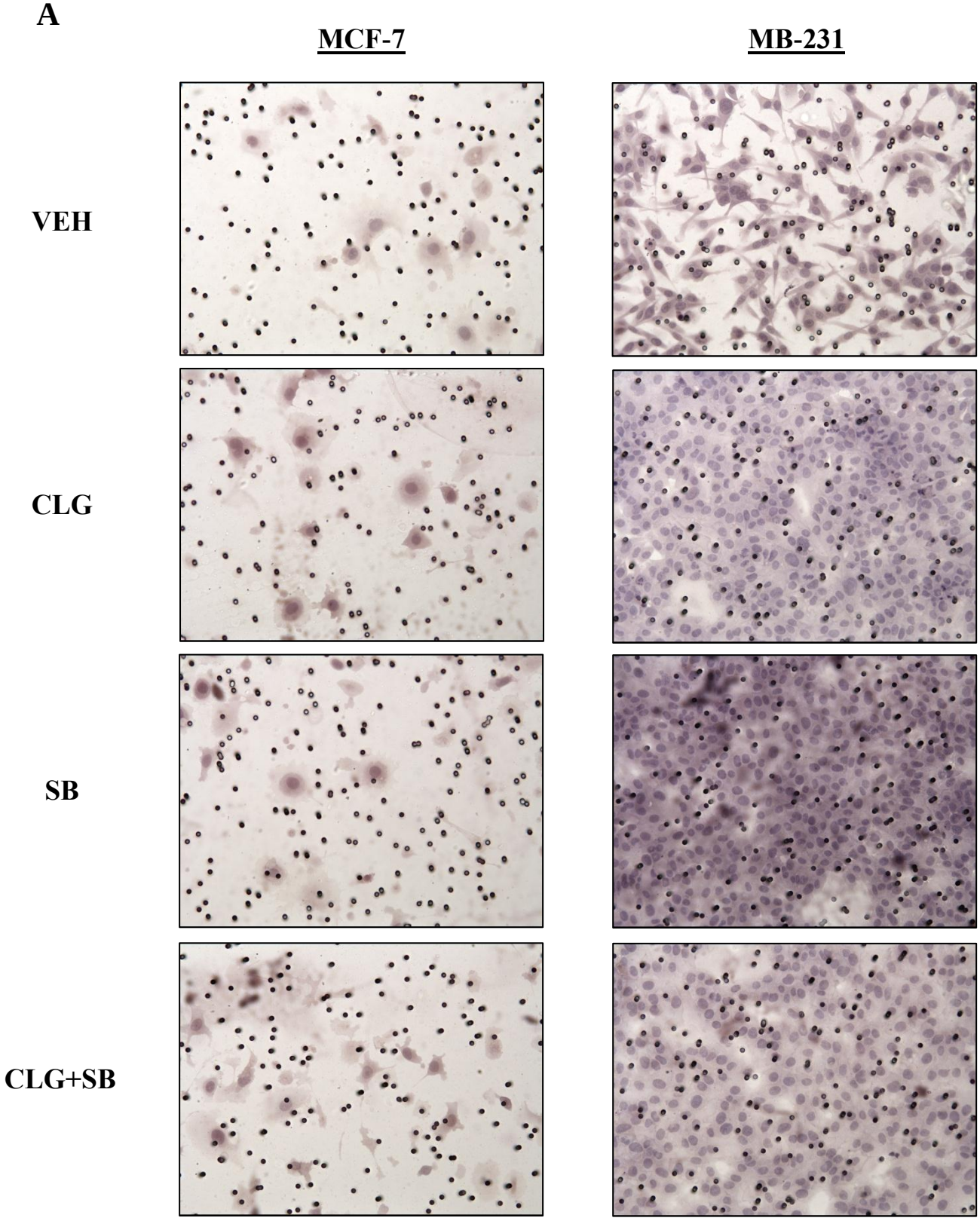

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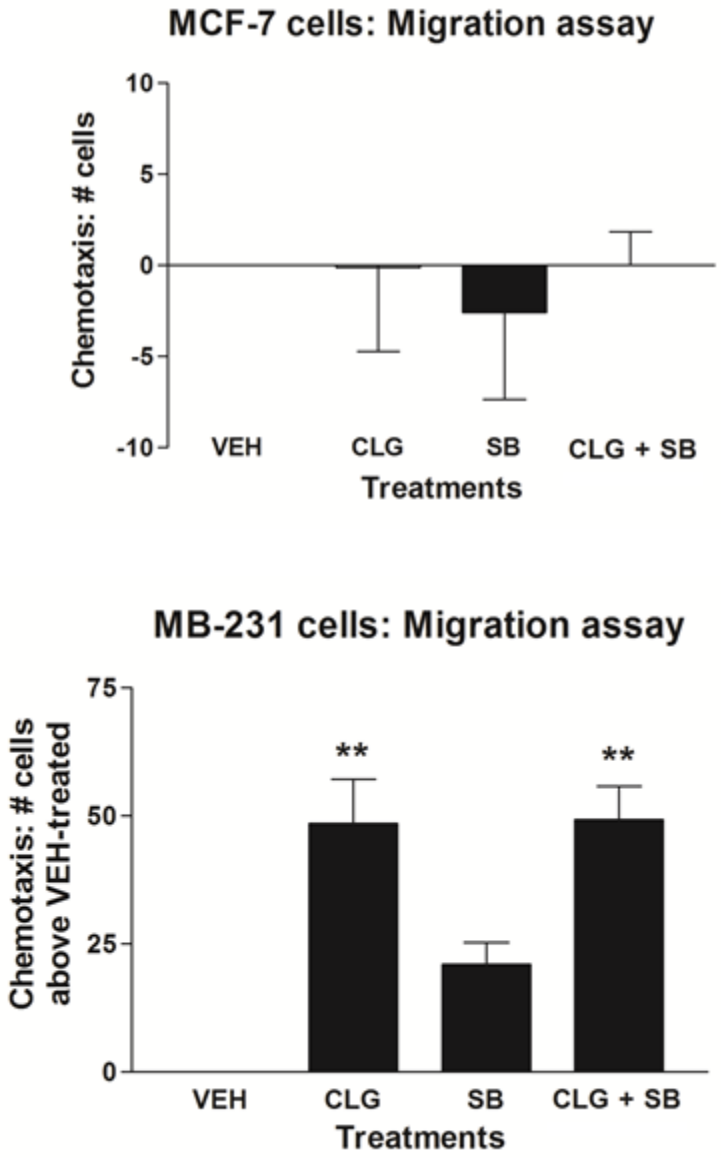


**Figure 20. Migration of MCF-7 and MB-231 cells treated with CLG and/or SB203580.** (A) Representative images of migrating MB-231 and MCF-7 cells (preceding page). The cells are a purple shade; the small black spots are pores in the filter. (B) Cell migration was quantified and expressed as # of cells migrating with treatment over # cells in (VEH)-treated control group. Top panel: the inhibitor treatments had no effect on the migration of MCF-7 cells. Bottom panel: the inhibition of MAO-A (1 µM; 20h) and p38(MAPK) (10 µM; 20h) resulted in increased migration in MB-231 cells, but the effect was greatest with CLG treatment. $P<0.01$ versus vehicle VEH-treated group (n=3, in triplicate).

colony formation in treated MB-231 cells observed from the soft agar assay, the increased migration seen in treated groups reflects a tendency towards an increased metastatic potential in this cell line in response to the drug treatments. To further investigate the metastatic potential of these cell lines following treatment, we examined models of cell invasion through various physiological barriers, i.e models of the endothelial cells of the vasculature and the basement membrane.

### 3.7.2 Invasion through a monolayer of EA.hy926 cells (model of the vasculature)

In order to assess the ability of MCF-7 and MB-231 cells to undergo intra/extravasation, we wanted to determine their ability to migrate through, or invade, a layer of endothelial cells (EA.hy926) before migrating through a Transwell chamber filter (the filter acts as a platform for counting cells). Our initial attempt did not provide any conclusive result as we suspected that some of the EA.hy926 cells were migrating through the filter along with the breast cancer cells. In order to test this hypothesis, we assayed the migration of EA.hy926 cells in the absence of breast cancer cells and, unfortunately, determined that there was migration of these cells in response to the chemoattractant (Figure 21). In retrospect this is not surprising as the literature identifies EA.hy926 cells as a hybrid cell line between human endothelial-like cells derived by fusing human umbilical vein endothelial cells and human lung adenocarcinoma cells (A549 cells) (204). These cancer-endothelial hybrids (EA.hy926) have been used to examine mechanisms of cancer metastasis and invasion (205) and a recent report by Lu and colleagues (205) indicated that EA.hy926 cells had a greater migratory potential than the parental A549 cells, which are highly metastatic epithelial lung cancer cells.

Several alternative approaches were examined. We thought that EA.hy926 cells could be differentiated from MB-231 and MCF-7 cells based on their morphology, but this proved invalid as all cells were irregular and elongated following migration. We also attempted to identify cell types based on their differential expression of specific proteins, i.e. E-cadherin and vimentin (based on concurrent studies being done with these proteins; see section ‘Immunocytochemistry’ on page 74). Unfortunately, MB-231 and EA.hy926 cells both express high levels of vimentin and low levels of E-cadherin. The MCF-7 cells did not display sufficient migratory capacity in this assay; as such they were not included in this investigation. We are currently using MB-231 cells that stably express the green fluorescent protein (GFP) (a gift from Dr. Andrew Freywald,

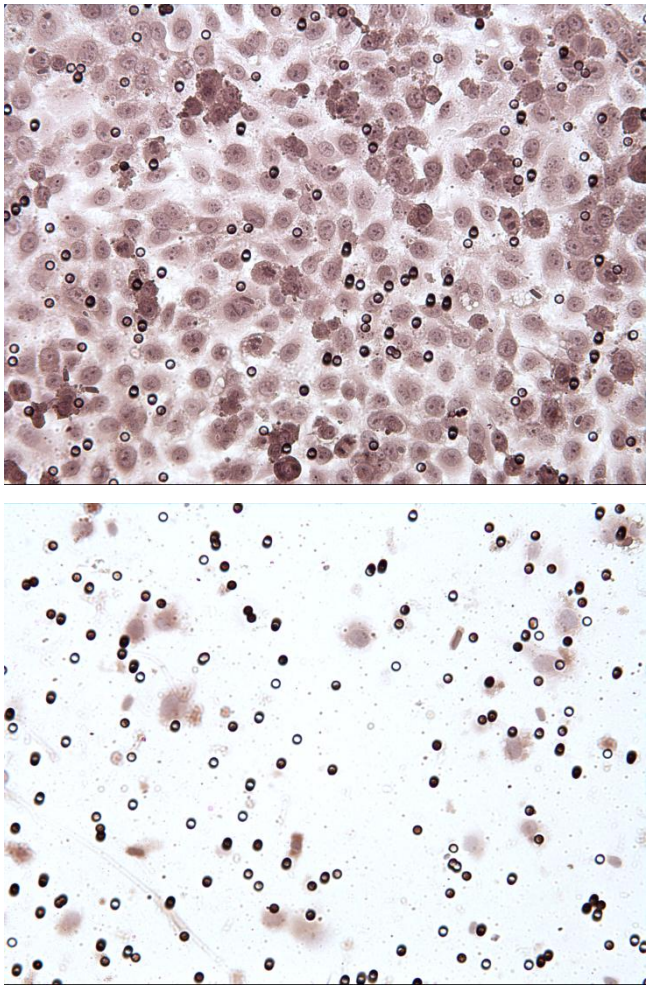

**Figure 21. EA.hy926 cell migration using Transwell chambers.** Each of the invasion chambers were seeded with $5x10^5$ EA.hy926 cells and cells migrating onto the lower surface of the filter were stained using hematoxylin. These are representative images of migrated EA.hy926 cells following a 96-hour incubation period (top panel) with a chemotactic concentration (10%) of FBS and (bottom panel) without; i.e. 0.5% FBS.

Biochemistry, University of Saskatchewan) and we will use this fluorescent marker to differentiate the MB-231 cells from any migrated EA.hy926 cells.

### 3.7.3 Invasion through matrigel (model of the basement membrane)

The protocol for the invasion assay using matrigel (an artificial matrix that is used to model the extracellular matrix) is dependent on the cell lines being investigated. Because of the variability in the rates of invasion, it was necessary to determine the optimal conditions for each cell line. Several trials and control experiments were conducted using a range of matrigel concentrations (2 mg/mL vs. 1 mg/mL), matrigel volumes (25 µL, 50 µL, 75 µL or 100 µL) and the test period for the migration itself (24, 48 or 72 hours). Once the optimal conditions were established (e.g. 50 µl of 1 mg/mL matrigel, with a 72 hour invasion period), the effects of CLG and SB203580 on the invasive capacity of MB-231 cells was assessed.

Treatment of MB-231 cells with SB203580 and CLG, either alone or in combination, induced an increase of 50% in the invasive capacity for these cells, although the variability in the data rendered the increase not statistically significant [$F(3,15) = 2.821$, $P = 0.0839$] (Figure 22). The tendency for an increase in invasiveness in the cells treated with the inhibitors is expected given that active migration of tumour cells (as seen in Figure 20) is a prerequisite for tumour-cell invasion.

Given the metastatic nature of MB-231 cells, the observed increases in migration and invasion strongly suggest an effect beyond the behaviour expected if it were acting as a primary site tumour cell line. It is important to understand that ER(-) cells are still very capable of forming tumours, but the MB-231 cells were not giving any indication of exhibiting tumour-like characteristics during p38(MAPK) or MAO-A inhibition. At this point, we started to consider that the metastatic potential of this cell line is playing a role in the cell's reaction to SB203580 and CLG, rather than its actual ER status. If these drugs are exerting an influence that is more dependent on the cell's metastatic status, then we would expect changes in proteins including E-cadherin and vimentin which are associated more with phenotypes beyond the primary tumour stage.

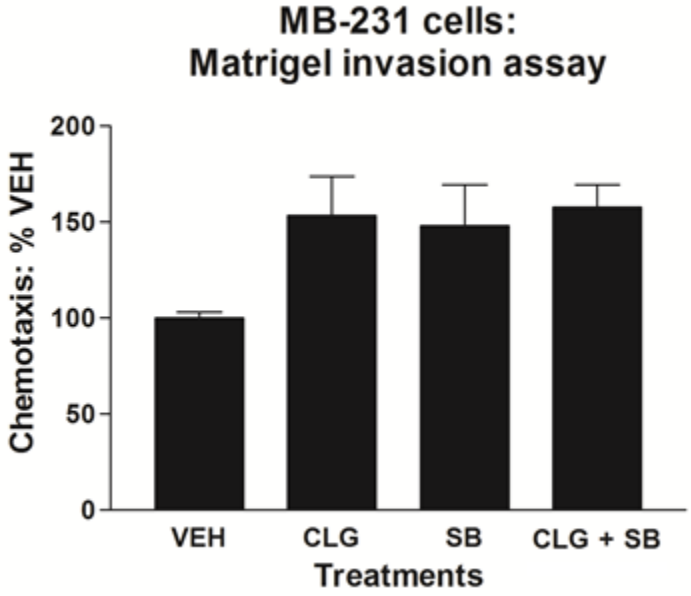


**Figure 22. Invasiveness of MB-231 cells treated with CLG and SB203580.** Although the treatments resulted in an increased invasiveness of the MB-231 cells, this was not statistically significant (n = 4).

## 3.8 Immunocytochemistry

The expression of intercellular junction and membrane cytoskeletal proteins has been extensively investigated in the context of cancer progression (206). Mechanisms contributing to invasive and metastatic potential may involve alterations in such proteins as E-cadherin, vimentin and β-catenin. The expression of E-cadherin and vimentin in MB-231 and MCF-7 cells, in the absence of MAO-A and/or p38(MAPK) activity, was evaluated using immunocytochemistry.

The distribution patterns of E-cadherin and vimentin were different between the two cell lines. The expression of vimentin was modest and appeared to be restricted to specific patterns within MCF-7 cells and its expression did not appear to be altered following any of the treatments with inhibitors (Figure 23). In these same cells, E-cadherin was segregated in areas of cell-cell contact and was significantly up regulated by treatment with SB203580 or CLG, either alone or in combination (Figure 23). We confirmed the expression of vimentin in MB-231 cells (207) and demonstrated that it was clearly localized to the plasma membrane (and allowed for imaging of the cell body and extensions). As with MCF-7 cells none of the treatments had any overt effect on vimentin expression in MB-231 cells. In contrast, MB-231 cells, which are known to be E-cadherin null (208), responded quite uncharacteristically to the inhibitor treatments by a modest induction of E-cadherin expression (Figure 24).

The distribution of protein expression in both MCF-7 and MB-231 cell lines was confirmed using quantitative analysis of the images using densitometry techniques. The image processing software, ImageJ, produces densitometric data which can then be graphically represented. The line profile plot method creates a graphical representation of pixel intensities based on a bisecting line centre of the nucleus (‘50’) and extending beyond the edge of the cell (‘0’ on the x-axis) (see Figure 25 for an example). In the MCF-7 cells, vimentin is concentrated around the nucleus (i.e. the line on the graph is higher nearer the ‘50’). E-cadherin expression in these same cells is more in proximity with the plasma membrane (between ‘30-40’) (Figure 26A).

The changes in protein expression observed using immunocytochemistry results were confirmed by western blot analysis (Figure 27). These results indicate a reduction of vimentin

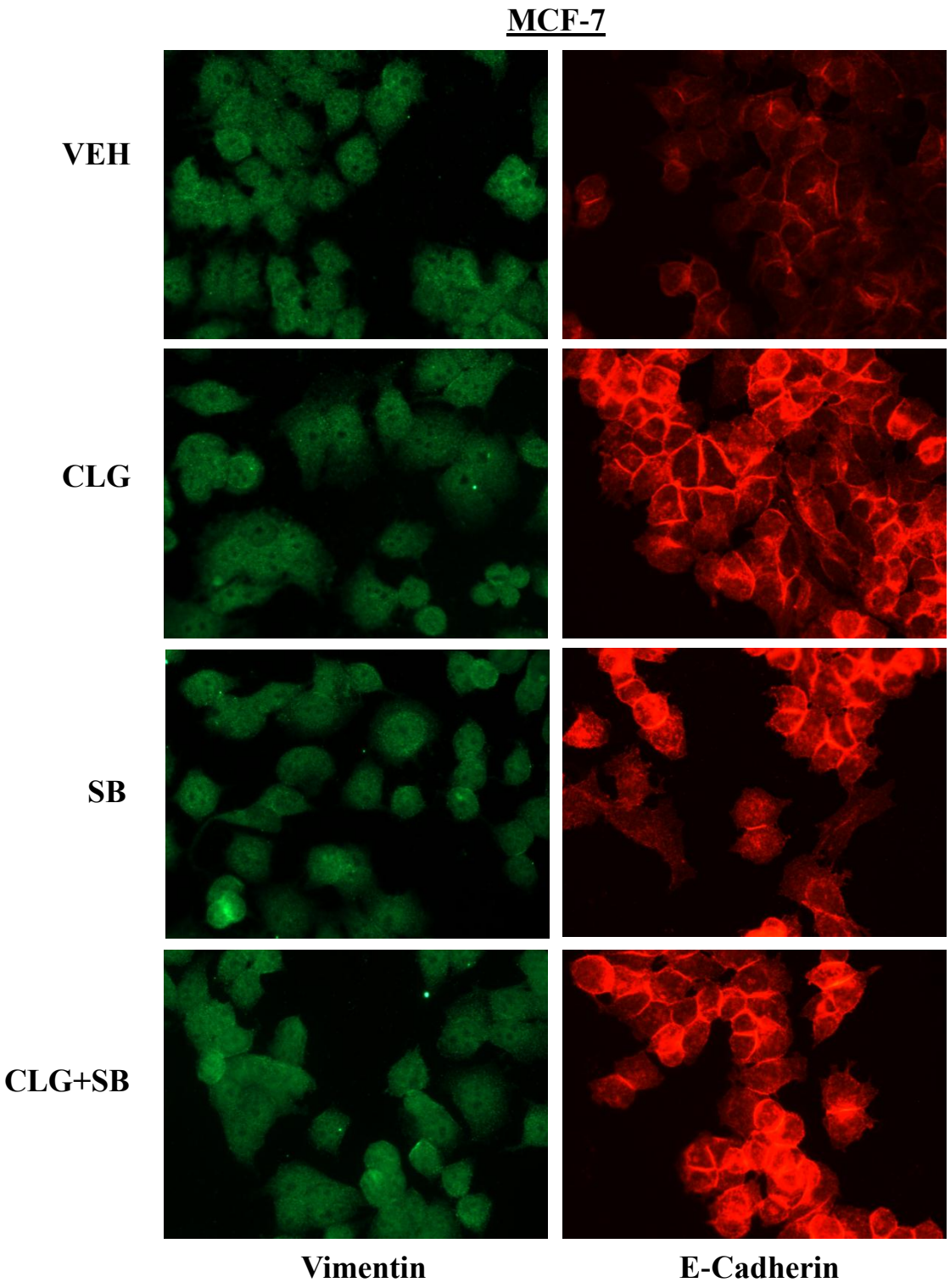


**Figure 23. Vimentin and E-cadherin expression in MCF-7 cells treated with CLG and SB203580.** Immunofluoresecence was used to detect vimentin and E-cadherin expression in MCF-7 treated with CLG (1 µM) and/or SB203580 (10 µM) for 24 h.

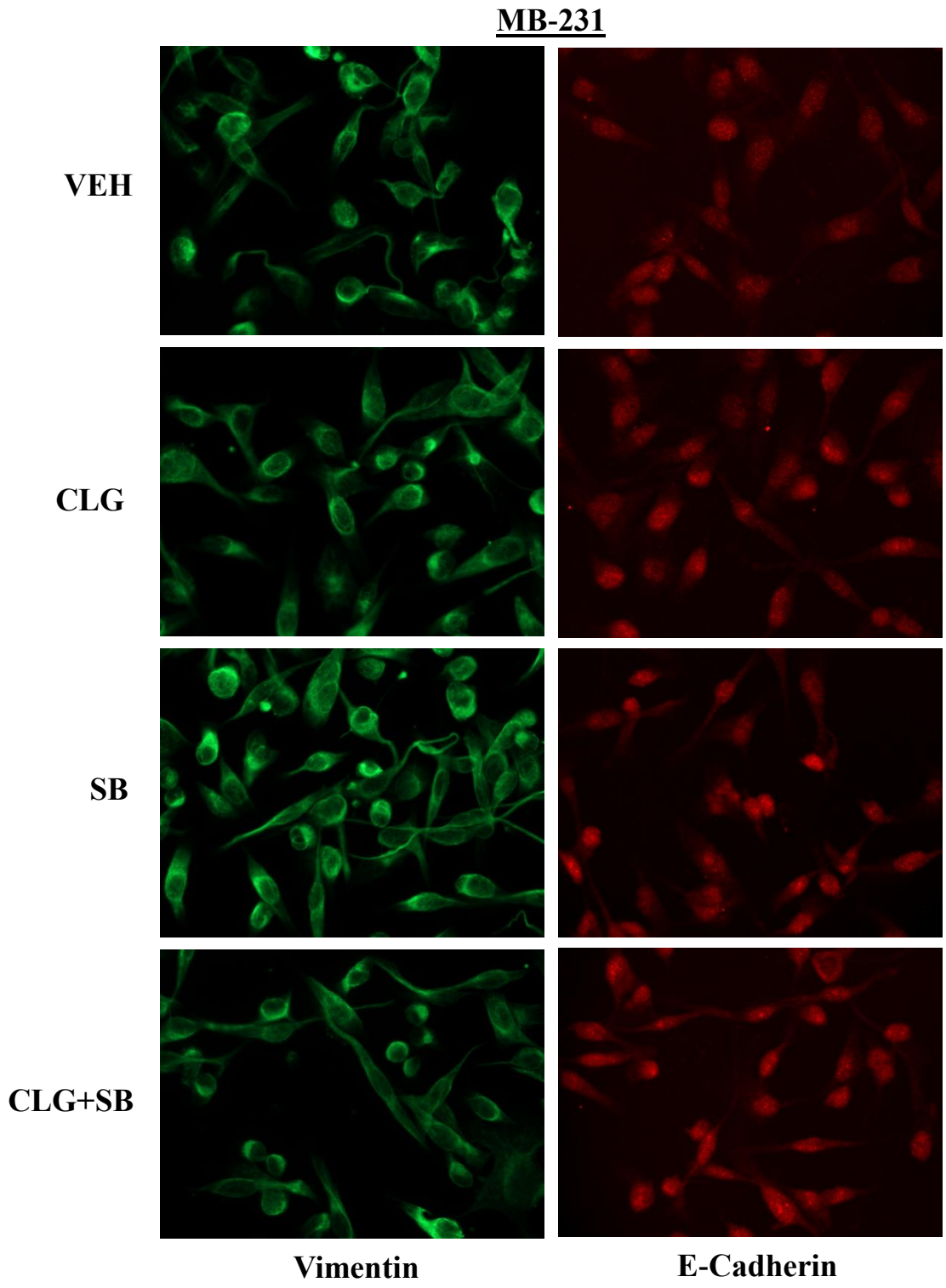


**Figure 24. Vimentin and E-cadherin expression in MB-231 cells treated with CLG and SB203580.** Immunofluoresecence was used to detect vimentin and E-cadherin expression in MB-231 treated with CLG (1 µM) and/or SB203580 (10 µM) for 24 h.

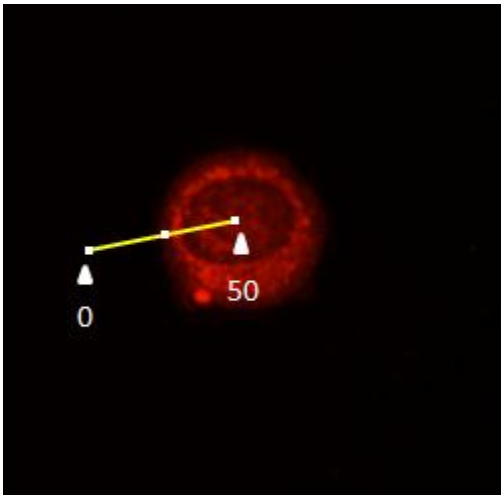


**Figure 25. An example of a bisecting line centered on the nucleus.** Using ImageJ one can produce a two-dimensional graph of the intensities of pixels along a line drawn within an image. This is illustrated using an image of an MCF-7 cell and using immunocytochemical techniques to quantitate the distribution of E-cadherin expression. The line starts outside of the cell ('0') and terminates in the centre of the nucleus ('50').

protein expression in the CLG+SB203580 group of MCF-7 and a rise in vimentin in the group treated only with SB203580. In these same cells, there was an increase in E-cadherin cleavage following drug treatment as seen by the appearance of the stronger lower bands in the 55-65 kDa range (Figure 27). In MB-231 cells, vimentin was reduced in the SB203580-treated group (Figure 27), thus confirming the results of our densitometric analyses (Figure 28). As expected, E-cadherin expression was not detectable in the vehicle-treated control MB-231 cells, but was induced by SB-203580 treatment (with or without CLG); the latter observation confirmed our densitometric analyses.

The epithelial-to-mesenchymal transition (EMT) is now acknowledged for its central role in tumour progression (169). E-cadherin is also a critical switch during EMT in early embryonic development during which time downregulation of E-cadherin results in the acquisition of a fibroblastic phenotype causing dissociation from the parent epithelium and subsequent migration (163). This re-organization of the epithelial structure (209, 210) and the loss of adhesion between epithelial tumour cells can reflect a loss of E-cadherin expression in the plasma membrane (211). A closer look at our immunocytochemistry results (Figure 24) and the corresponding densitometry (Figure 28) suggests that this newly expressed E-cadherin in MB-231 cells is closely associated with the nucleus. To confirm this notion, we undertook nuclear fractionation studies in combination with western blot analysis.

## 3.9 E-cadherin expression in cytosolic versus nuclear subcellular fractions

The fractionation studies confirm the increase in E-cadherin expression in the cytosol of SB203580-treated MCF-7 cells and also reveal a doublet at 130 kDa in the nuclear extract (soluble fraction of the nucleus). The top band of the doublet disappears with SB203580 treatment and the bottom band appears stronger, although we did not quantify this (Figure 29A). In these same fractions we looked for the expression of GSK3β , a protein which, when phospho-inhibited, stimulates the transcription of Slug and Snail (repressors of E-cadherin expression) and ultimately induces EMT (212). A similar pattern, i.e. increased E-cadherin concurrent with the detection of a phospho-GSK3β immunodetectable band was also evident in MB-231 cell cytosol

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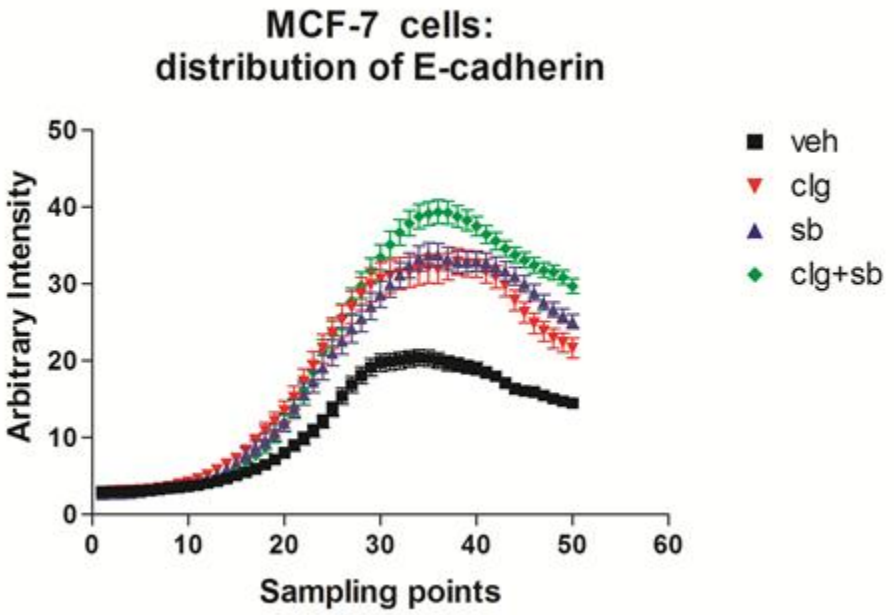


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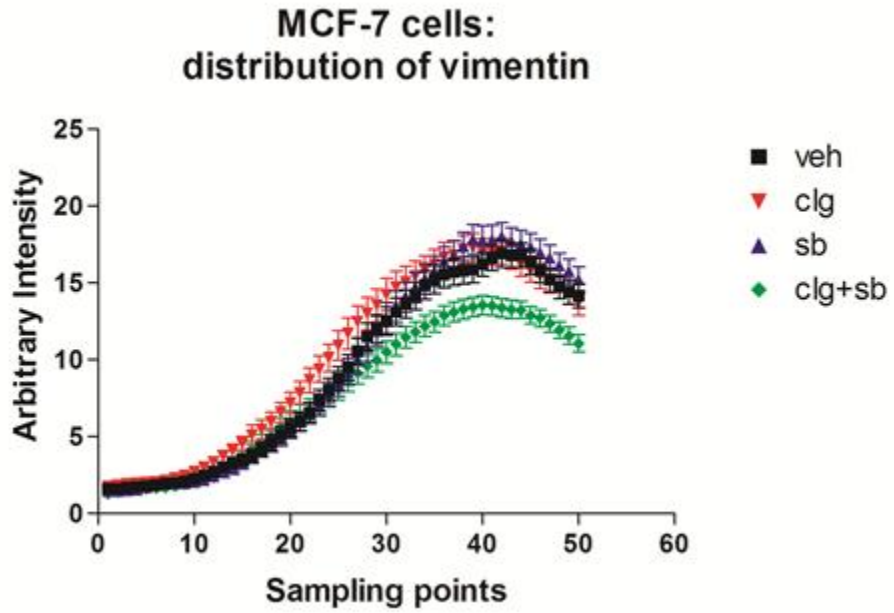



**Figure 26. Quantification of E-cadherin and Vimentin localization in treated MCF-7 cells.** The distribution E-cadherin (A) and vimentin (B) in MCF-7 cells treated with CLG (1 µM) and/or SB203580 (10 µM) for 96 hours determined through densitometric analysis of images (using ImageJ) obtained using immunofluorescence. The data points are based on a line starting outside of the cell ('0' on the x-axis) and terminating at the centre of the nucleus ('50').

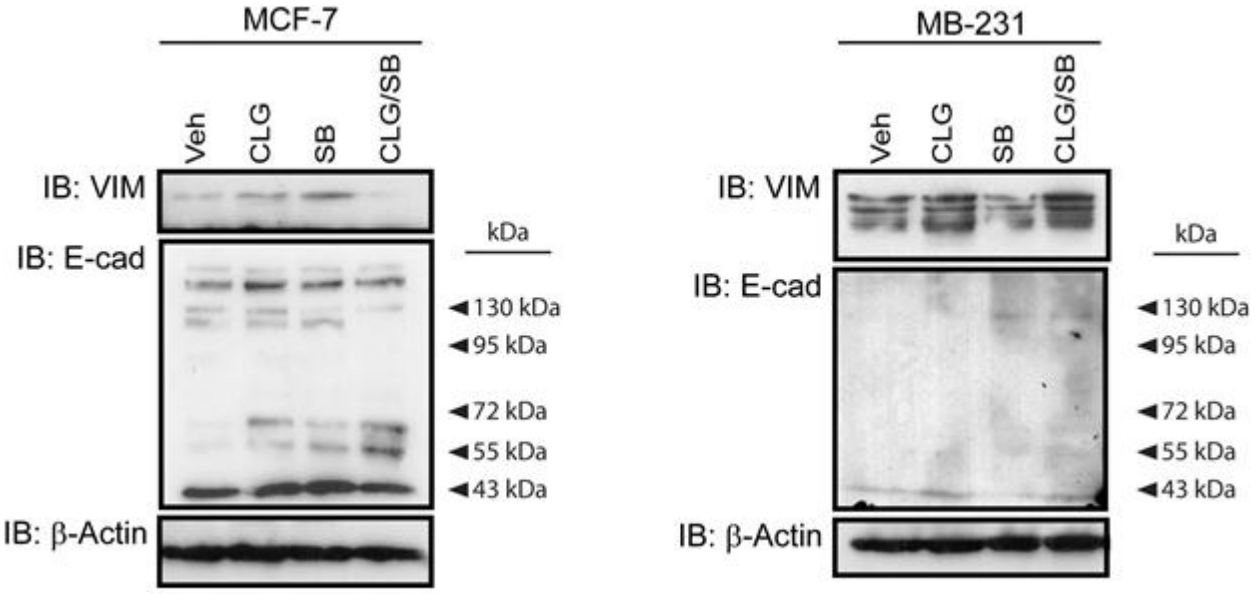
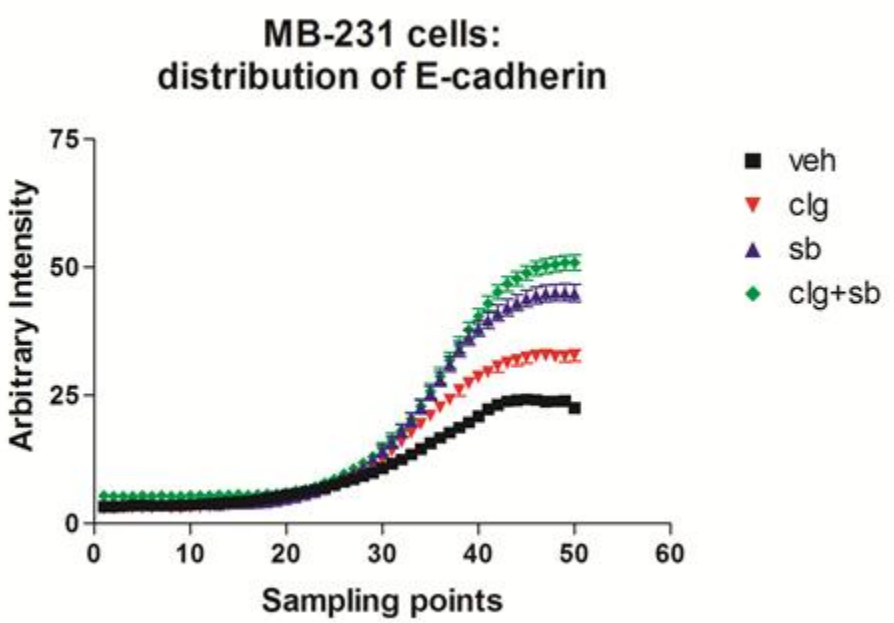



**Figure 27. Western blots of E-cadherin and vimentin expression in MCF-7 and MB-231 cells with CLG and SB203580.** The expression of vimentin (58 kDa) and E-cadherin (estimated 135 kDa) in cell lysate of MCF-7 cells was increased (with the exception of vimentin in CLG+SB203580 cultures). In contrast, the expression of vimentin was unchanged in MB-231 lysates regardless of treatment, but the expression of E-cadherin was detectable in lysates from cells treated with SB203580 (either alone or in combination with CLG).

**A**




**B**

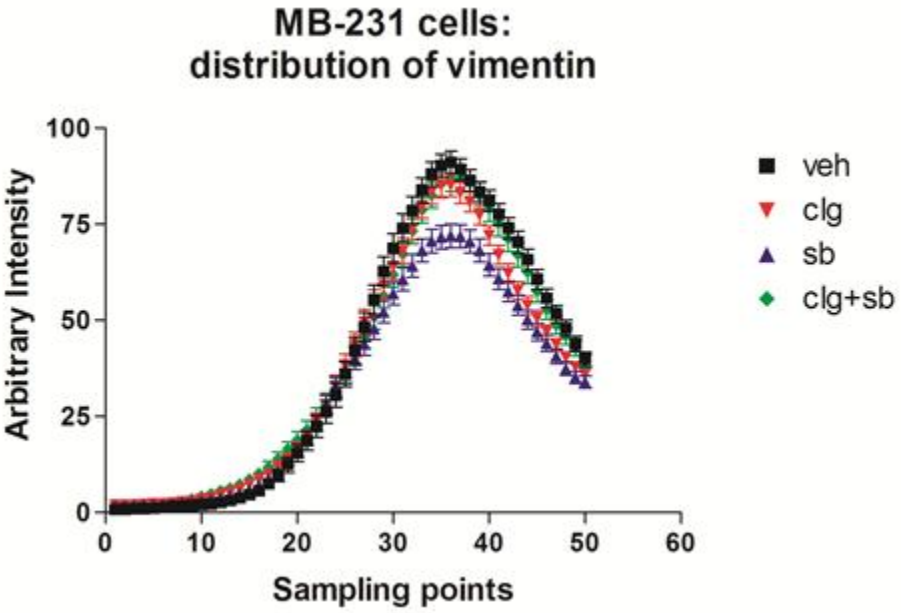
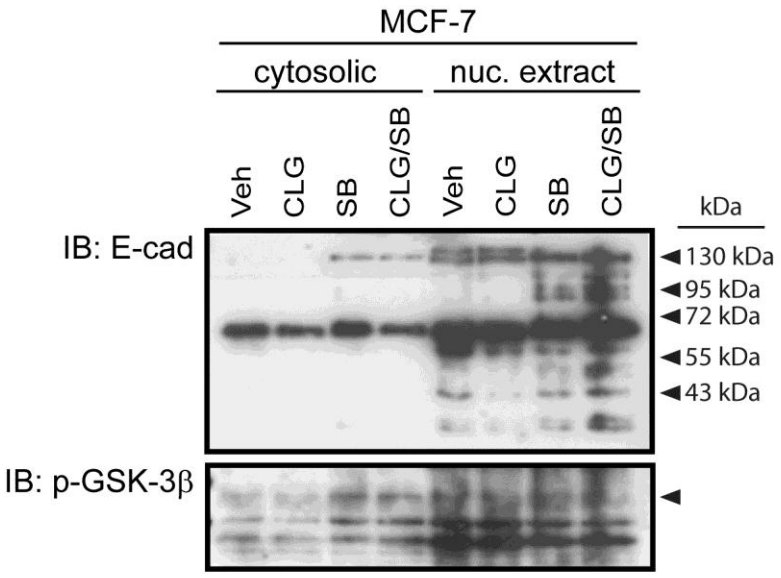
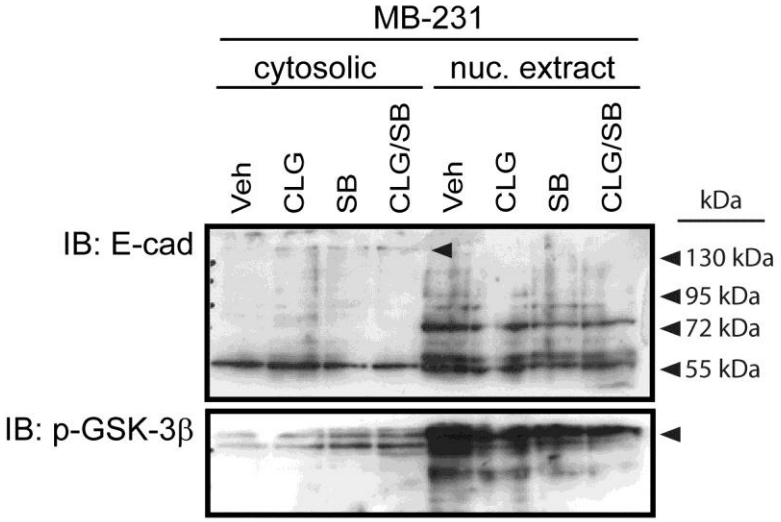



**Figure 28. Quantification of E-cadherin and Vimentin localization in treated MB-231 cells.** The distribution E-cadherin and vimentin in MB-231 cells treated with CLG (1 µM) and/or SB203580 (10 µM) for 96 hours determined through densitometric analysis of images obtained using immunofluorescence. Details as in Figure 22.

**A**


MCF-7
cytosolic
nuc. extract
Veh
CLG
SB
CLG/SB
Veh
CLG
SB
CLG/SB
kDa
IB: E-cad
130 kDa
95 kDa
72 kDa
55 kDa
43 kDa
IB: p-GSK-3β

**B**


MB-231
cytosolic
nuc. extract
Veh
CLG
SB
CLG/SB
Veh
CLG
SB
CLG/SB
kDa
IB: E-cad
130 kDa
95 kDa
72 kDa
55 kDa
IB: p-GSK-3β

**C**

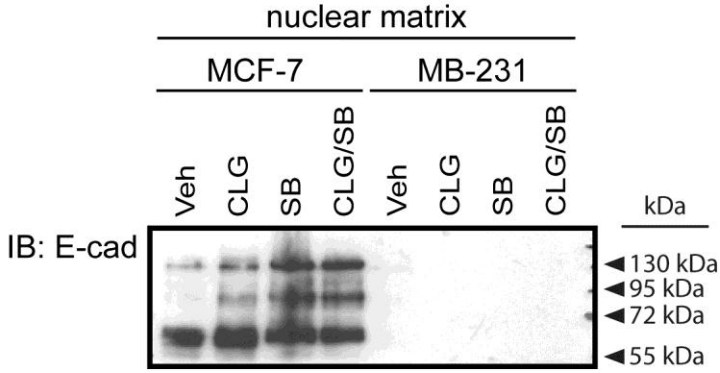

nuclear matrix
MCF-7
MB-231
Veh
CLG
SB
CLG/SB
Veh
CLG
SB
CLG/SB
kDa
IB: E-cad
130 kDa
95 kDa
72 kDa
55 kDa

**Figure 29. E-cadherin expression in nuclear and cytosolic fractions of MCF-7 and MB-231 cells** (previous page)**.** (A) E-cadherin (estimated 135 kDa) and phospho-GSK3β (p-GSK3β) (47 kDa) expression was monitored in the cytosolic and nuclear extracts of treated MCF-7 cells. Note the appearance of E-cadherin in the cytosolic fractions and the loss of the upper band in the doublet in the nuclear matrix of SB203580 (SB)-treated cells. Levels of p-GSK3β are increased in corresponding cytosolic fractions. (B) E-cadherin and p-GSK3β expressions were monitored in the cytosolic and nuclear extracts of treated MB-231 cells. Note the appearance of E-cadherin in the cytosolic fractions of CLG- and/or SB203580 (SB)-treated cells. Levels of p-GSK3β are increased in corresponding cytosolic fractions. (C) An immunodetectable E-cadherin band at 130 kDa appears to associate with the nuclear matrix in MCF-7 cells (insoluble fraction). There was no detectable E-cadherin in corresponding fractions from treated MB-231 cells. (kDa/kiloDalton: protein ladder).

(there was no detectable E-cadherin signal in corresponding nuclear extracts) (Figure 29B). We did observe differences in the cleavage pattern of E-cadherin (exists as a 135 kDa inactive precursor protein (213) and in its mature form as a 120-124 kDa protein (214)). Subcellular fractionation revealed that the band lost from the E-cadherin doublet in MCF-7 cells appears to be associated with the nuclear matrix (insoluble fraction) (Figure 29C). We are currently unclear as to the relevance of this observation. β-Catenin levels were not significantly affected by treatment in either of the two cell lines (Figure 30).

## 3.10 Cross-linking Studies

MCF-7 and MB-231 cells were examined for MAO-A expression and it was revealed that MAO-A expression in MCF-7 is predominantly perinuclear, whereas the expression of MAO-A is more diffuse in MB-231 cells. In addition, a portion of this MAO-A is associated with the cell membrane (Figure 31A). Parallel cultures were harvested and aliquoted to different 1.7 mL Eppendorf tubes. These cell suspensions were then treated with either DSS (disuccinimidyl suberate: a non-cleavable and membrane permeable cross-linker) or $BS^3$ (bis(sulfosuccinimidyl)suberate: a non-cleavable and membrane impermeable cross-linker). Proteins were then extracted and resolved for standard western blot analysis and probed for MAO-A (Figure 31B). The results of this experiment confirmed that MAO-A exists in different pools. In MCF-7 cells, the reduced MAO-A levels that are expressed are primarily intracellular. In contrast, in MB-231 cells, there is a significant pool of MAO-A that appears in a higher molecular weight complex following treatment with $BS^3$, which confirms (given that $BS^3$ is cell impermeable) that the MAO-A must be associated with the plasma membrane. This was confirmed by a reduction in MAO-A activity in similarly treated cell homogenates (Figure 31C) and suggests that the role of MAO-A in cancer cell progression might be more complex than originally anticipated.

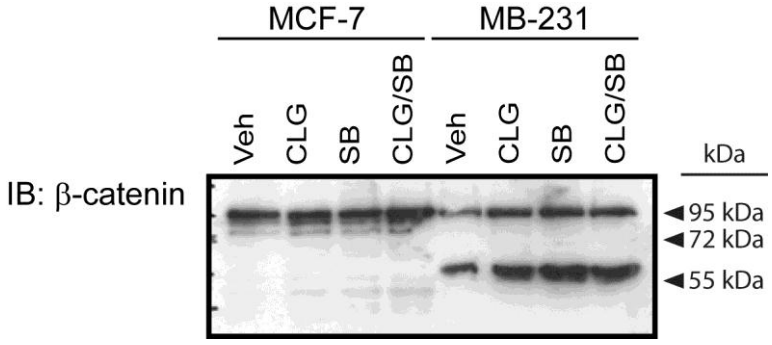
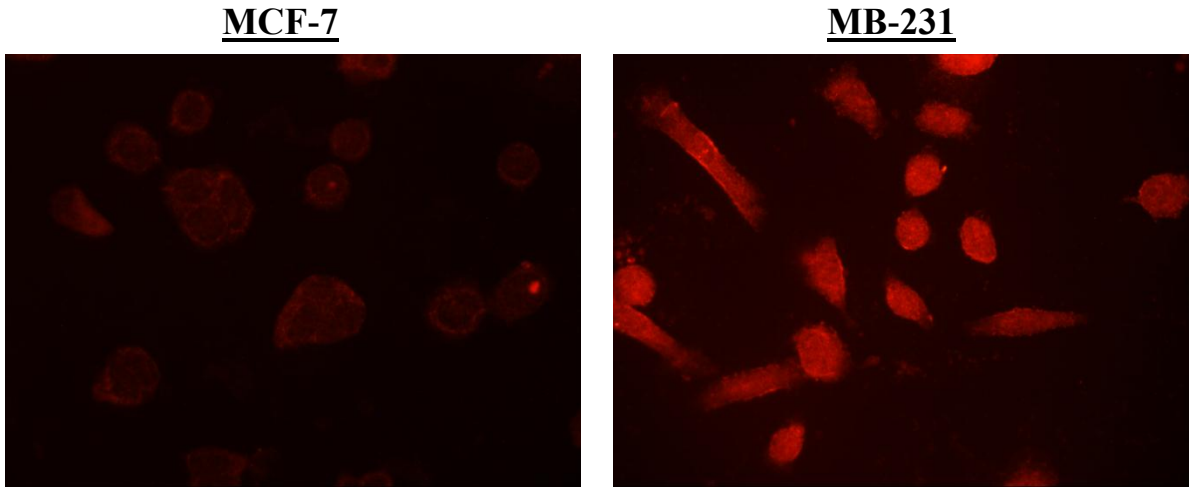
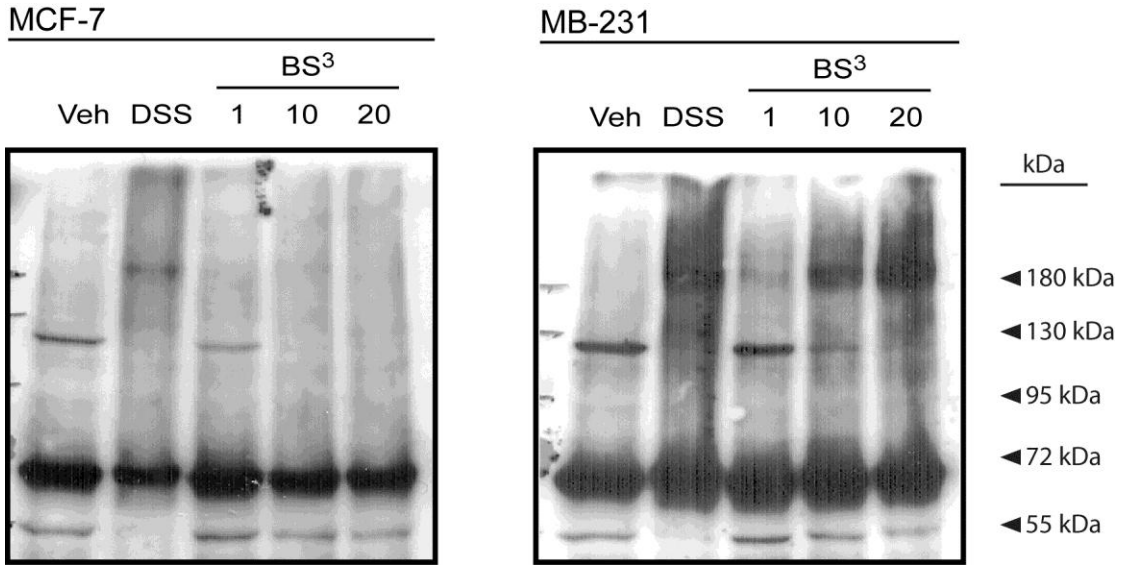



**Figure 30. Western blot of β-catenin protein expression in treated MB-231 and MCF-7 cells.** Cell lysate of CLG- and/or SB203580 (SB)-treated MCF-7 and MB-231 cells were probed for the expression of β-catenin (92 kDa). There were no significant effects of any treatment in either cell line.

**A**


MCF-7
MB-231

**B**


MCF-7
BS3
Veh
DSS
1
10
20
MB-231
BS3
Veh
DSS
1
10
20
kDa
180 kDa
130 kDa
95 kDa
72 kDa
55 kDa

**C**

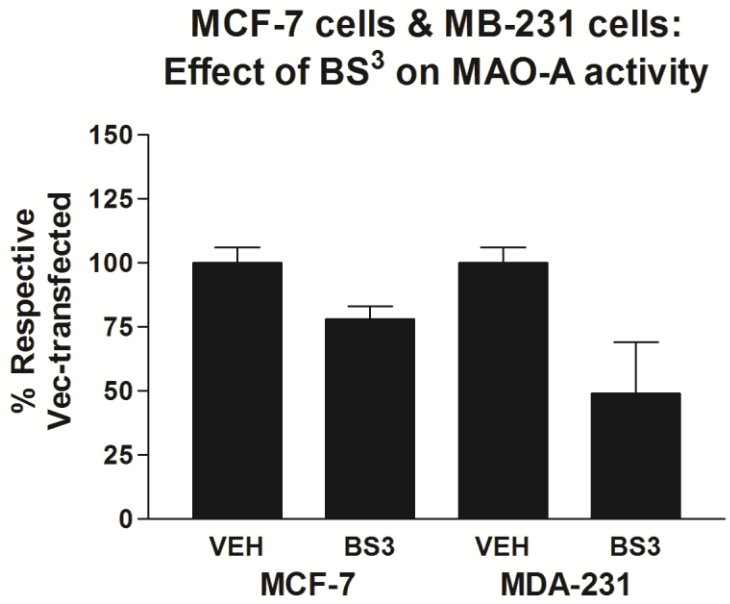



**Figure 31. MAO-A in MCF-7 and MB-231 cells with immunofluorescence and immunoblotting.** Multiple pools of MAO-A exist in MCF-7 and MB-231 cells. (A) Immunofluorescence confirms that the levels of expression of MAO-A protein in MCF-7 and MB-231 cells corresponds to the differences in innate activities determined above (see Figure 9). While the MAO-A in MCF-7 cells appears to concentrate in the perinuclear region (mitochondria and endoplasmic reticulum), in MB-231 cells MAO-A expression is more diffuse and is also associated with the plasma membrane. (B) Cross-linking studies with DSS (a cell-permeable cross-linking reagent) and $BS^3$ (a cell impermeable reagent) reveals a very strong band in $BS^3$-treated homogenates, suggesting that at least a portion of MAO-A is expressed on the cell membrane. (C) A reduction in MAO-A activity confirmed MAO-A crosslinking in $BS^3$-treated homogenates of both MCF-7 and MB-231 cells.

# 4 DISCUSSION

The MCF-7 cell line is a well-characterized ER(+) cell line that is weakly invasive, while the MB-231 cell line is ER(-) and highly invasive (215) and incurs a post-epithelial-mesenchymal transition (post-EMT) classification (216). These two breast cancer cell lines exhibit very different phenotypes making them ideal candidates for comparison, thus providing a means of understanding what might be occurring in diverse cell populations. We chose to study the MCF-7 and MB-231 breast cancer cell lines so as to align with other studies and reports that often compare and contrast the two.

The current study sought to investigate if a functional interaction between the p38(MAPK) and MAO-A systems exists in breast cancer cells. This working hypothesis was based on the conclusion drawn from previous work from our laboratory that p38(MAPK) directly inhibits MAO-A (148). This was an unanticipated conclusion as the activation of both MAO-A, as an $H_2O_2$-generating enzyme, and p38(MAPK), as a 'stress-associated' kinase, is most often associated with an apoptotic phenotype. Others, however, have reported on the constitutive activation of p38(MAPK) in brain, which suggests a role for this kinase in normal physiology (217, 218). Ultimately, we determined that the Serine209 residue in MAO-A was a target for p38(MAPK) and that the inhibition of MAO-A by transient p38(MAPK) activation was a pro-survival event that permitted the cell to survive a minor or transient toxic event. In contrast, prolonged activation of p38(MAPK) would initiate cell death cascades, as expected of this pro-apoptotic kinase (219). During a review of the literature we found a report of significant losses of MAO-A status [e.g. mRNA] in 95% (!) of human cancers (19), particularly in pre-cancerous states. These authors proposed that the loss of MAO-A status (and the possible increase in serotonin that would follow the loss of this catabolic enzyme) could indicate progression towards cancer. Thus, the loss of MAO-A status could be a useful biomarker and could be a powerful means for testing for the potential onset of cancer.

Breast cancer is one of the most prevalent malignancies in woman. The expression of receptors for female hormones, particularly estrogen, is currently used as a defining feature in breast cancers. Indeed, it is now generally acknowledged that approximately 60% of premenopausal and 75% of postmenopausal women with breast cancer have an estrogen receptor

positive [ER(+)] subtype (220). In addition, the approximate 30% prevalence of mood disorders, particularly depression, in breast cancer patients is two to three times greater than that in the general population (221, 222). This suggests a role for MAO-A (given its clear link to depression) in breast cancer, as does the report that the use of antidepressants, including MAO inhibitors, can increase the risk of breast cancer (223, 224). Furthermore, the loss of MAO-A status in breast cancer cells could explain why MAOIs diminish the effects of cisplatin and irradiation therapy in tumour cells compared to normal non-tumourigenic cells (225). In contrast, others have found that increased MAO-A activity is linked to malignancy (at least experimentally) (226), while inhibition of MAO has been linked to a *better* prognosis in prostate (227) and colorectal (228) cancers. The evidence for a link between the use of newer-generation antidepressants, such as the selective serotonin reuptake inhibitors (SSRIs) (which do not target MAO), prior to (223, 229) or after (230) diagnosis and breast cancer recurrence is also ambivalent. Needless to say, the literature is inconclusive regarding the role of MAO-A in cancer, but this provides an opportunity for new research, such as our own, to elucidate the topic.

Our interest was piqued further by the observation that tamoxifen, a selective estrogen receptor modulator used in both pre- and postmenopausal women with ER(+) breast cancer (231, 232), has also been linked to MAO-A (220) as well as with a significant risk for developing depression (233, 234). As stated above, MAO-A function is regulated, in part, by the p38(MAPK) pathway. Our work as well as that of others based on observations in CNS-derived cell lines strongly suggests that the effect of p38(MAPK) on MAO-A is cell line-dependent, with potential contribution by both transcription-dependent and -independent mechanisms (148, 198, 235-237). Interestingly, resveratrol, a polyphenolic compound isolated from red grapes, is a potent MAO-A inhibitor (238) that induces p38(MAPK)-dependent (239) and p38(MAPK)-independent (240) cell death in the ER(+) MCF-7 cell line and in the ER(-) MDA-MB-231 cell line, respectively. The role of p38(MAPK) in cell growth, invasive potential and a tamoxifen-resistant phenotype is clear in MCF-7 cells (118, 241-243). Interestingly, to further relate tamoxifen, p38(MAPK) and MAO-A, the expression of MAO-A has long been known to be tightly regulated by the estrogen [receptor] system (244, 245). The regulation of MAO-A by estrogen is tissue-specific; for instance in the brain estrogen decreases MAO-A (246). This is consistent with the effects of estrogen-replacement therapy, which decreases MAO-A to increase available 5HT, associated with improvements in mood (247).

As there has not been any single characterization of the roles of both p38(MAPK) and MAO-A in these cell lines, we chose to compare and contrast the influence of these two systems on breast cancer cell tumourigenicity. To do this we used two pharmacological inhibitors that are known to be specific for p38(MAPK) and MAO-A, namely SB203580 and clorgyline (CLG), respectively. We found that the innate levels of MAO-A activity in the two cell lines differed, with MB-231 cells having a high level of MAO-A activity and MCF-7 cells having a significantly lower level of MAO-A activity. We also confirmed the inhibitory potential of the MAO-A inhibitor CLG in these cell lines. Given the previous work from our laboratory (148), we expected that the inhibition of p38(MAPK) would increase MAO-A activity. While the results using the MCF-7 cells supported this notion, the effect of SB203580 in MB-231 cells did not. Furthermore, both SB203580 and CLG had a negative effect on MTT conversion in MCF-7 cells. While the effect of CLG on MCF-7 cells was corroborated by an increase in MTT conversion following the ectopic expression of MAO-A protein, the effects of SB203580 and CLG in either cell line were not corroborative of our proposed model. In other words, if SB203580 was inhibiting p38(MAPK) and activating MAO-A in MCF-7 cells (as hypothesized), then why would it exert a similar effect as an MAO-A inhibitor on MTT conversion? Clearly, the interaction between p38(MAPK) and MAO-A function was cell line-dependent. Yet, the fact that tamoxifen is known to exert an effect on MAO-A (220) and is effective against ER(+) cells, such as the MCF-7 cell line (248), suggests that the effects of MAO-A would be important in ER(+) cells, our data do not support this hypothesis. In fact our data suggest that the cell's ER status might not be the primary factor that dictates whether it is influenced by MAO-A-dependent mechanisms. Thus, it was clear that the cell’s phenotype, if influenced by these two proteins/enzymes, was not going to simply rely on an interaction between the two proteins and the regulation of MAO-A catalytic activity.

One possibility for these inconsistent findings could be a limitation of the MTT conversion assay. This assay is thought to reflect mitochondrial metabolic rate and is a widely accepted technique employed to indirectly reflect viable cell counts (249, 250). Yet, while it represents a simple, inexpensive and efficient method, it may not be entirely accurate given that metabolic activity can be altered by different conditions and chemical treatments and, thus, introduces considerable variation in the results reported from such assays (251). Since the key processes differentiating cancer cells from normal cells is their ability to divide indefinitely, to

form a malignant tumour and to invade other tissues (84, 86), we undertook several other assays designed to study specific aspects of breast cancer cell function and tumourigenicity.

We determined that MB-231 cells grew in soft agar much more readily than MCF-7 cells, therefore cell-line dependent effects of the inhibitors were once again observed. Indeed, the inhibitors decreased MB-231 cell growth in soft agar, but had no effect on MCF-7 cells in this assay. Given the ability of SB203580 and CLG to reduce the growth rate of MB-231 cells and the ability of these inhibitors to also reduce growth on soft agar, this could be indicating that these cells are adopting a migratory phenotype. In support of this change in phenotype, we demonstrated that treated MB-231 cells were more likely to migrate (e.g. through a Transwell filter) and showed a more invasive phenotype, i.e. by migrating through matrigel (used as a model of the basement membrane). It is known that anchorage-*dependent* cell growth is a mechanism that guards *against* metastasis. Of all the cells that are shed by tumours and capable of migrating and invading the circulation, only those few that are not restricted by anchorage-dependent growth can re-establish at secondary sites and metastasize (252). The MB-231 cell line is frequently studied as a representative metastatic cell line (201, 202) and is designated as a post-EMT cell line (207, 216) which suggests a state characterized by a loss of adhesion and increased cell mobility (165).

As we had already questioned whether the ER status of the MCF-7 cell line was a factor in the roles of p38(MAPK) and MAO-A, it is quite possible that what we were observing were phenotypic changes that were more so dependent on the cell's metastatic potential. With this in mind, we chose to examine these cells for the levels of expression of E-cadherin and vimentin, two markers which have been extensively implicated in the induction of EMT. A reduction in E-cadherin levels and an increase in vimentin expression are distinct features of the EMT and the reverse is true during the MET (152). Using both immunofluorescence and western blot analysis, we observed a significant increase in E-cadherin expression in both cell lines following treatment with the p38(MAPK) and/or the MAO-A inhibitors. While this might prevent the metastatic conversion of epithelial cells during cancer progression, in cells which have already undergone the transition, such as MB-231 cells (post-EMT), the atypical expression of E-cadherin could be indicative of the MET. During the end stages of metastasis the MET would lead to the establishment of micrometastases at the secondary site (158, 253).

Mechanistically, the presence of E-cadherin in the E-cadherin-null MB-231 cell line (254) could be due to changes in GSK3β signalling. E-cadherin is integral to the maintenance of the epithelial state and its expression is suppressed by the Snail protein (255), a transcriptional regulator in cancer development (256). The non-phosphorylated form of GSK3β prevents the action of Snail (255), thereby indirectly regulating E-cadherin expression. E-cadherin is synthesized as a 135 kDa inactive precursor protein (213) and the mature form of E-cadherin is a 120-124 kDa protein (214). Proteolytic cleavage of full-length E-cadherin at a site adjacent to the transmembrane domain releases an extracellular N-terminal fragment (80 kDa) (257) that has been implicated in the induction of cancer cell invasion (258, 259). In addition, when E-cadherin is cleaved by the γ-secretase complex the resulting cytosolic C-terminal fragment 2 (E-cad/CTF2) can translocate to the nucleus (260), which supports another potential pathway for increased motility in metastatic cells. The translocation of E-cadherin to the nucleus has also been shown to be of paramount importance for pituitary adenoma tumour invasion (261). While the phospho-inhibition of GSK3β would be expected to repress E-cadherin, this was not the case under our experimental conditions. However, it would appear that the post-translational regulation of E-cadherin could depend on a GSK3β-dependent event. In addition, the E-cadherin processing (or, perhaps more importantly, its subcellular localization rather than its expression levels) might have more impact in our treated cell lines. The multiple band pattern that was apparent on the films may correlate with the multiple species of E-cadherin mentioned throughout the literature. The mechanism which mediates E-cadherin cleavage in these breast cancer cell lines requires further investigation. The nuclear fractionation also revealed that the band lost from the E-cadherin doublet in MCF-7 cells appears to be associated with the nuclear matrix (insoluble fraction) (Figure 24, bottom panel), however, we are unclear of the importance, or lack thereof, of this observation.

While it is still unclear exactly *how* the p38(MAPK) and MAO-A systems are exerting their effects in these two cell lines, it is becoming clear that these two systems are potentially playing a very specific and apparently very subtle role during a specific window in the metastatic process. According to the molecular and phenotypic changes in these cells under the current laboratory conditions, they appear to be in an invasive/migratory state in preparation for establishing tumour growth. According to Kalluri and Weinberg (165), the MB-231 cells in our study are exhibiting an intermediate phenotype which manifests during the EMT/MET transition.

If we extrapolate from the molecular and cellular analyses of EMT/MET features outlined by Blick and colleagues (254), these events would be best placed in the extravasation step of metastasis.

While p38(MAPK) inhibitors do not form part of the normal battery of chemotherapeutics associated with breast cancer in the clinic, antidepressants do. Clearly they are not used to treat the breast cancer *per se*, but secondary or reactive depression (to a diagnosis of breast cancer) is a serious issue in this patient population. Since most antidepressants used currently fall primarily into the class of drugs known as selective serotonin reuptake inhibitors (SSRIs), whose mechanism of action, as the name implies, involves the blockade of the reuptake (clearance) of serotonin from the synaptic cleft, MAO-A might not be a significant consideration. However, the one SSRI which is contraindicated in breast cancer is paroxetine (262). Studies suggest that the increase in death associated with this drug in the breast cancer population is due to its ability to inhibit CYP2D6-mediated activation of tamoxifen, thereby mitigating its therapeutic effect (100). Yet preliminary *in vitro* investigations using purified human MAO-A indicates that paroxetine is also a relatively potent MAO-A inhibitor (A. Holt, G.B. Baker & D.D. Mousseau, unpublished data). Therefore, if MAO-A inhibition is having significant effects at the secondary site, as our results suggests, then this could have serious clinical implications. During early stage breast cancer the antidepressants may not have a detectable effect, i.e. at stages when tumour cells resemble the MCF-7 cell line. However, if antidepressants are administered in the later stages when the cancer has metastasized (and more so resemble MB-231 cells), then the drugs might trigger MET in circulating tumour cells and indirectly promote the establishment of the tumour cell at a secondary location in the body.

On a final note, as part of our ongoing characterization of MAO-A and in the hope of understanding why MAO-A could be exerting such distinct effects in these two cell types, we determined where MAO-A was being expressed within the cell as there is quite a substantial literature that indicates that it is not expressed exclusively on the outer mitochondrial membrane. Using immunocytochemistry, we confirmed that MCF-7 cells express low levels of MAO-A and that the MAO-A appears to be localized to the perinuclear region, where most of the mitochondria would be found. In contrast, MB-231 cells express much higher levels of MAO-A, which is in keeping with the higher levels of innate MAO-A activity in this cell line. However,

we also observed that the distribution of MAO-A is much more diffuse and that a portion of the MAO-A signal seems to be associated with the plasma membrane. This is not unexpected as some of the older literature shows clear evidence of a plasma membrane-associated pool of MAO in various tissues (263). We confirmed the expression of MAO-A on the plasma membrane by cross-linking studies using a cell impermeable amine-amine cross-linking reagent that would clearly only cross-link those proteins exposed to the outside of the cell. $BS^3$ is the hydrophilic analog of DSS at the same concentration (1 mM) the crosslinking reagents yielded different results which supports our findings that $BS^3$ is a membrane impermeable reagent that is interacting with MAO-A. While we did detect a modest high molecular weight band (on western blot) in the MCF-7 cell line, suggesting that a minor pool of MAO-A is expressed on the plasma membrane, parallel studies using the MB-231 cell line revealed a very strong high molecular weight band. In parallel cultures, MAO-A activity was decreased following similar cross-linking studies, which would be expected of a protein that is exposed on the outside of the cell and inactivated by irreversible cross-linking of amine groups.

Perhaps these distinct pools of MAO-A contribute to different cellular events and it is only the cumulative effects of all MAO-A-mediated events that is detectable as a *phenotype*. For example, a generalized decrease in *mao-A* mRNA is observed in most cancers (19), yet MAO-A activity is increased in experimental breast cancer in rats (226) and paradoxical increases in 5-HT (which would not be the anticipated consequence of increased MAO-A availability) in human breast cancer are thought to support tumour growth (264). Similarly, MAO-A protein is clearly induced in prostate cancer (227), yet, again paradoxically, serotonin is concurrently increased in this cancer (265) to the point that it is also proposed as a valid marker for tumour progression (266). This clearly suggests that the MAO-A protein, while present in the cell, does not necessarily promote a phenotype that is based strictly on the catalytic activity of the MAO-A protein. Again, subtle and, as yet, unclear mechanisms are clearly implicated.

While it is clear that the p38(MAPK) and MAO-A systems do interact in these cell lines, it is clear that they do not interact in the manner that was hypothesized (based on our previous work using neuronal and glial cell lines). In addition, while some of the effects observed in this study may have been modest, it is quite possible that their effect(s) *in vivo* would be very subtle and would accumulate and become more pronounced after long-term MAO-A inhibition. This is

important because medications being used to alleviate depressive symptoms are meant to alter conditions within the brain; however these substances are delivered systemically and their effects may be induced on other cell types as well. The effects of the substance could be the same as in neuronal cells, opposite or entirely novel and unknown. As a result, it is important to recognize that treatment with antidepressants not only alter brain function but also influence the entire system.

# 5 PROSPECTIVE INVESTIGATIONS

Another approach may be to perform similar tumour and invasion assays using a catalytic dead species of MAO-A so as to provide another level of potential differentiation. It is possible that MAO-A function may extend to effect cellular processes independent of its catalytic activity as our recent studies have suggested. We hope to better understand MAO-A function by building on current crosslinking studies to confirm our present findings. It would also be very interesting to examine more fully the mechanisms and pathways involved in the context of MAO-A and p38(MAPK) in breast cancer and determine the upstream activators and downstream targets involved. This study may further benefit from the use of more clinically relevant antidepressants, namely moclobemide (reversible MAO-A inhibitor) and newer-generation SSRIs, such as fluoxetine (Prozac) to treat cells and determine effects of these drugs on cell phenotype, viability, etc. and what role MAO-A has in these phenotypes.

It is also our intention to conduct *in vivo* studies to understand some ambiguities that have surfaced from *in vitro* results. We have recently obtained an MB-231 cell line that stably expresses the green fluorescent protein (GFP). This will allow for visual identification of MB-231 cells following their injection into nude mice. Nude mice are often used for the study of tumourigenicity as they are immunocompromised and, thus, do not 'reject' foreign transplanted tissues such as human breast cancer cells. Properties such as tumour growth (measurement of tumour size) and metastatic potential (appearance of secondary tumours in, for example, lungs) will be monitored using the MB-231-GFP cells in both vehicle-treated control mice and antidepressant-treated mice. In addition, the strategy to target MAO-A with both acute and chronic drug treatments will provide an advantage in clarifying how MAO-A modulating drugs may be influencing breast cancer prognosis. Moreover, it is reasonable to anticipate that these results could facilitate future investigations in understanding the role of p38(MAPK) and MAO-A in the tamoxifen-resistance profile in breast cancers.

Our results indicate clear differences between MCF 7 (ER+) and MB-231 (ER-) breast cancer cell lines in response to shifting levels of MAO-A and p38(MAPK), which could have significant bearing on breast cancer cell prognosis. Our *in vitro* findings strongly suggest that the influence of MAO-A on cell phenotype is less dependent on the respective cell's ER status and

perhaps more so dependent on the cell's metastatic potential. If this is the case, then the contribution of MAO-A to [clinical] metastatic breast cancer should be duly considered when considering an antidepressant drug treatment for depression in this (and other) cancer patient population.

# 6 REFERENCES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Michael Miracle
Senior Web Designer
Cell Signaling Technology
mmiracle@cellsignal.com
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