Modulation of human melatonin MT_1 receptor by valproic acid and its effects in combination with melatonin on human breast cancer

"Modulation of human melatonin MT_1 receptor by valproic acid and its effects in combination with melatonin on human breast cancer"

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ABSTRACT

The MT_1 receptor is involved in the oncostatic action of melatonin and valproic acid (VPA) in human MCF-7 breast cancer cells and VPA can upregulate this receptor in C6 glioma cells. Therefore, the effect of VPA on the expression of the MT_1 was examined in MCF-7 cells. Treatment of MCF-7 cells in low serum conditions with VPA (0.5 or 1mM) for 24 or 72 h caused a significant increase in MT_1 receptor expression, as shown by reverse transcription-polymerase chain reaction analysis (RT-PCR). MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays performed in high serum conditions revealed a significant concentration-dependent inhibition of MCF-7 cell proliferation by VPA (0.5 - 5 mM), whereas melatonin (1 or 10 nM) showed modest effects alone. However, a combination of VPA and melatonin produced a marked synergistic inhibition of cell proliferation. In subsequent experiments, under high serum conditions, VPA treatment for 24h on these cells resulted in a significant decline of MT_1 mRNA while the protein levels were still increasing, as seen by RT-PCR and western blotting respectively. The involvement of multiple biochemical events, such as: induction of the p53 tumor suppressor gene and repression of the estrogen receptor (ER)-alpha might be responsible for the synergistic inhibition of these cells after the simultaneous exposure to VPA and melatonin. These results indicate that clinically relevant concentrations of VPA upregulate melatonin MT_1 receptor expression in human breast cancer cells. Moreover, the enhanced antiproliferative effect observed with a combination of VPA and melatonin suggests that a similar therapeutic approach may be beneficial in human breast cancer.

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LIST OF ABBREVIATIONS

4P-PDOT	4-phenyl-2-propionamidotetraline
6-OHDA	6-hydroxydopamine
AC	adenyl cyclase
AIF	apoptosis inducing factor
ANOVA	analysis of variance
AP-1	activating protein-1 transcription factor
Bcl-2	b-cell lymphoma/leukemia 2-gene
BDNF	brain derived neurotrophic factor
bFGF	basic fibroblast growth factor
BZ	Benzodiazepine
cAMP	cyclic adenosine 3'-5'- monophosphate
CBHA	m-carboxycinnamic acid bis-hydroxamide
CNS	central nervous system
CSF	cerebrospinal fluid
DA	dopamine
DAG	diacylglycerol
DMEM	Dulbecco's Modified Eagle's Medium
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EGFR	EGF receptor
ER	estrogen receptor
ER	endoplasmic reticulum
ERE	estrogen response element
ERK	extracellular-regulated kinase
F ratio	The variation due to an experimental treatment or effect divided by the variation due to experimental error.
FGFR	FGF receptor
GABA	γ-aminobutyric acid
GABA-T	GABA transaminase
GAD	glutamate decarboxylase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDNF	glial cell line derived neurotrophic factor
G protein	GTP binding protein
GRP78	glucose-regulated proteins
GRP94	glucose-regulated proteins
GSH	glutathione
GSK-3ß	glycogen synthase kinase-3ß
HATs	histone acetyltransferases
HDAC	histone deacetylase
H_2O_2	hydrogen peroxide
HIOMT	hydroxy indole-O-methyl transferase
IP_3	inositol triphosphate

JNK/SAPK	c-Jun NH2-terminal kinase/stress-activated protein kinase
Luzindole	N-acetyl-2benzyltryptamine
MAPK	Mitogen-Activated Protein Kinase
MAPKKs	MAPK kinases
MAPKKK	MAPKK kinases
MEK	MAPK/ ERK kinase
Melatonin	N-acetyl-5 methoxytryptamine
MT_1	melatonin receptor subtype 1
MT_2	melatonin receptor subtype 2
mTOR	mammalian target of rapamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAT	N-acetyl transferase
NK	natural killer
OD	optical density
·OH	hydroxyl radical
$ONOO^-$	peroxynitrite anion
PIP2	Phosphatidylinositol bisphosphate
P450	aromatase
PBS	phosphate buffer saline
PCR	Standard Polymerase Chain Reaction
PD	Parkinson's disease
PKC	protein kinase C
PLC	phospholipase C
PT	pars tuberalis
RA	retinoic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT-PCR	Reverse transcription-polymerase chain reaction analysis
RZR/ROR	nuclear melatonin receptors
SAHA	suberoylanilide hydroxamic acid
SCF	stem cell factor
SCN	suprachiasmatic nuclei
sPNET	supratentorial primitive neuroectodermal tumor
SSA	succinate semialdehyde
TAM67	dominant negative form of c-jun
TBE	Tris/Borate/EDTA buffer solution
TH	tyrosine hydroxylase
TNF- α	tumour necrosis factor-α
TGF-ß	transforming growth factor-ß
TRE	TPA responsive element
TSA	Trichostatin A
VPA	Valproic acid
VPD	Valpromide

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SECTION 1: BACKGROUND

1.1: Melatonin Production

Melatonin, an indoleamine also known as N-acetyl-5 methoxytryptamine, is synthesized in pinealocytes (cell type derived from non-rod and non-cone photoreceptors) mostly during the dark phase and is secreted by the endocrine pineal gland. The rhythm of production is endogenous, generated in the suprachiasmatic nuclei (SCN), the major central rhythm generating system in mammals. The maximum production of melatonin occurs at night by a signal from the eye indicating the absence of light and hence it has been correctly known as the "hormone of darkness".

The precursor for this indole hormone is tryptophan. Both the enzymes, e.g., rate limiting enzyme, N-acetyl transferase (NAT) and the methylating enzyme, hydroxy indole-O-methyl transferase (HIOMT), regulate the cyclic production and metabolism of melatonin (Ravindra et al., 2006). Melatonin is metabolized in the liver by microsomal enzymes (Ravindra et al., 2006).

There is universal agreement that melatonin is quickly released into the bloodstream and then diffuses into other body fluids such as saliva, ovarian follicular fluid and semen (Reiter et al., 1991). Exceptionally high concentrations are documented in the cerebro-spinal fluid and bile (Tan, 1999). Melatonin crosses all morphophysiological barriers, e.g., the blood-brain barrier and placenta and spreads throughout the cell (Oaknin-Bendahan et al., 1995). A considerable inter-individual variability in the secretary activity of melatonin has been reported; it is between 18-40 pg/mL at night. Its half-life is less than 30 minutes (Brown et al., 1997).

The levels of melatonin at night are 10-20 times higher than concentrations of melatonin measured during the day (Reiter, 2003). Acute light exposure at night inhibits melatonin production while alternating periods of lights and darkness serve to synchronize the melatonin rhythm to 24 hours. Consequently, disrupting the 24 hours of the biological clock results in the alteration of the melatonin synthesis cycle (Reiter, 2003). A shift in the pattern and/or levels of melatonin secretion have been reported to coincide with sleep disorders, jetlag, depression, stress, reproductive activities, some forms of cancer and immunological disorders (Ravindra et al., 2006).

Melatonin synthesis is influenced by age, gender, and seasons. Its levels decrease with increase in age; however, in elderly women it is higher than in elderly men. The amplitude of the nocturnal melatonin increase can be severely attenuated over the years but there seems to be variations among individuals in the rate at which melatonin is lost (Reiter, 2003). Moreover, it is suggested that the reduction of the number of ß-adregenic receptors on the pineaolocytes affects synthetic ability of aging pineal gland (Reiter, 2003). A seasonal variation is also observed in humans; the levels are higher in winter than in summer.

In several pathophysiological conditions, like coronary heart disease (Brugger et al., 1995), orthostatic hypotension (Tetsuo et al., 1981), schizophrenia (Fanget et al., 1989), and Alzheimer's disease (Mishima et al., 1999), reduced concentrations of melatonin have been observed. Melatonin levels have been found to vary in different types of cancer including breast cancer (Schernhammer et al., 2005), brain tumors

(Mandera, 2003), colorectal cancer (Kos-Kudla et al., 2002), hepatocarcinoma (Qin et al., 2004), endometrial cancer (Grin et al., 1998), and prostate cancer (Sainz et al., 2005).

1.2: Physiological and Pharmacological actions of melatonin

Physiological effects of melatonin are associated with processes that involve normal circulating levels in the range of picomolar to nanomolar concentrations. Pharmacological actions of melatonin involve concentrations in the micromolar range, (10-20 mg/kg intraperitoneally), known to interact with $GABA_A$ and benzodiazepine (BZ) receptors (Armstrong et al., 2002). Studies suggest that at these concentrations, melatonin acts as a direct free radical scavenger (Mayo et al., 2003).

1.2.1: Free radical scavenger and antioxidant

Melatonin is a multifaceted free radical scavenger. It detoxifies a variety of free radicals and reactive oxygen intermediates including the hydroxyl radical/hydrogen peroxide, peroxy radicals, peroxynitrite anion, singlet oxygen, nitric oxide and lipid peroxidation (Reiter et al., 1996). The highly toxic hydroxyl radical is efficiently neutralized by melatonin resulting in N -Acetyl- N -formyl-5-metoxy kynuramine (Reiter et al., 1996).

Melatonin is thought to function via several ways which includes direct free radical scavenging; stimulation of antioxidative enzymes; increasing the efficiency of mitochondrial oxidative phosphorylation and enhancing the efficiency of other antioxidants (Mayo et al., 2003). This hormone is known to act as a direct scavenger by neutralizing reactive oxygen species (ROS), reactive nitrogen species (RNS), including

the hydroxyl radical (·OH), hydrogen peroxide (H_2O_2), and peroxynitrite anion ($ONOO^-$) (Mayo et al., 2003). When melatonin interacts with the hydroxyl radical, it becomes the indolyl radical. There is a net gain where a highly toxic reactant is replaced by a low toxicity radical. Furthermore, melatonin has indirect antioxidative actions such as stimulation of antioxidative enzymes (i.e. superoxide dismutase, glutathione peroxidase and reductase (Reiter, 2003; Rodriguez et al., 2004). According to recent findings, these antioxidant enzymes are the first line of defense against free oxidants by neutralizing them to harmless byproducts (Rodriguez et al., 2004). Studies suggest that physiological doses of melatonin stimulate synthesis of glutathione (GSH), which is another antioxidant and free-radical scavenger (Urata et al., 1999).

1.2.2: Immunomodulator

Among melatonin's versatile functions, immunomodulation has emerged as a major effect of the hormone. Many studies show that melatonin plays a fundamental role in enhancing the immune response. An important target of melatonin is the thymus, the central organ of the immune system (Maestroni et al., 1993). There is evidence suggesting the existence of membrane specific binding sites for melatonin in immune cells (Guerrero et al., 2000). Melatonin seems to bind to melatonin receptors in T helper cells or monocytes, stimulating the production of interferon gamma and interleukins 1, 2, 6 and 12, which in turn upregulate the immune response, restoring immunodeficiency states (Rosales-Corral et al., 2003; Garcia-Maurino et al., 1998). In *vitro* studies show that melatonin treatment increases natural killer (NK) cell activity in human and also upregulates several immunomodulatory cytokines including tumour necrosis factor-α

(TNF-α), transforming growth factor-ß (TGF-ß), and stem cell factor (SCF) by macrophages (Maestroni, 1993).

Furthermore, melatonin is thought to be a potent inhibitor of apoptosis in immune cells (Sainz et al., 1999). Studies suggest that T and B subsets of lymphocytes from rat thymus and spleen express melatonin receptor mRNA. Other studies suggest that human lymphocytes have machinery to synthesize and produce large amount of melatonin. Therefore, lymphoid cells maybe an important physiological source of melatonin which could be involved in the regulation of the human immune system (Carrillo-Vico et al., 2004; Maestroni, 1993).

1.2.3: Neuroprotective Agent

Melatonin has been shown to be a neuroprotective agent in many models of neurodegeneration. Studies suggest that in the 6-hydroxydopamine (6-OHDA) model of Parkinson's disease (PD), the destruction of catecholaminergic neurons likely happens through free radicals and therefore induces degeneration of the dopaminergic nigro-striatal pathway (Jin et al., 1998). It is thought that in PD patients, dopaminergic neurons are affected as a consequence of their high exposure to ROS leading to decrease in tyrosine hydroxylase (TH) enzyme activity and a dopamine (DA) deficiency in the striatum (Jin et al., 1998). Melatonin treatment after stimulation of PD symptoms results in increasing TH activity and dopamine levels (Jin et al., 1998)

Recent studies suggest that physiological concentrations of melatonin upregulates glial cell line derived neurotrophic factor (GDNF) mRNA and protein (Armstrong et al., 2002). GDNF is a neuroprotective agent which promotes survival and regeneration of

dopaminergic neurons in the brain. Intrastriatal injections of pharmacological doses of melatonin increase levels of GDNF mRNA in the rat striatum (Tang et al., 1998). More studies suggest that melatonin treatment upregulates GDNF mRNA and as a result diminishes iron-induced oxidative injury to the locus coerules of the rat brain (Chen et al., 2003).

In addition, pretreatment of SHSY5Y neuroblastoma cells with pharmacological doses of melatonin results in restoring brain derived neurotrophic factor (BDNF) secretion, trk receptor and mRNA expression to normal levels after stress induced ß-amyloid, a cytotoxic agent (Olivieri et al., 2003).

1.2.4: Oncostatic Agent

The mechanisms through which melatonin exerts its oncostatic properties are explained in a variety of ways based on its different known actions: (a) indirect neuroendocrine mechanisms, such as the melatonin down regulation of the neuroendocrine reproductive axis and the subsequent reduction of estrogen hormones responsible for abnormal growth of the mammary gland (Reiter et al., 1980); (b) as a result of melatonin's anti-estrogenic actions on the mammary epithelial cells (Cos et al., 1991; Molis et al., 1994); (c) as a biological modifier by increasing the expression of p53 and p21 gene in MCF-7 breast cancer cells (Mediavilla et al., 1999). Studies suggest that 48h of melatonin treatment causes a significant increase in the expression of p53 as well as p21WAF1 proteins (Mediavilla et al, 1999). P53 is an important tumour suppressor gene involved in cell cycle regulation. The p53 protein activates the expression of the p21 gene (also known as WAFZ/Cipl) by binding to its promoter. p21 inhibits cyclin

dependent kinases thus leading to a failure of the phosphorylation of the retinoblastoma protein (Rb). Upregulation of p21 results in G_O/G_1 arrest; (d) as a consequence of its anti-oxidant properties; (e) as an immunomodulator (Fraschini et al., 1998); (f) derived from its inhibitory effects on telomerase activity in tumor cells (Leon-Blanco et al., 2003). Telomerase inhibitors are a potentially a valuable tool because they lead to cancer cell death.

At physiological doses, this hormone is cytostatic and inhibits cancer cell proliferation in *vitro* via various cell cycle specific effects. At pharmacological doses, melatonin exerts cytotoxic activity in cancer cells. At both pharmacological and physiological doses, melatonin acts as a differentiating agent in some cancer cells and lowers their invasiveness and metastasis by alternating cell surface adhesion molecules (E-cadherin and beta1 integrin) and by maintenance of gap junctions' intercellular communication (Cos et al., 1998). In MCF-7 breast cancer cells, melatonin is known to exert its effects alone or in combination with other agents to cause cell death (Jawed et al., 2007).

Studies indicate that melatonin inhibits the growth of several types of cancer cells (Sainz et al., 2005). There is general agreement that melatonin, in *vitro*, inhibits breast cancer cell proliferation and invasiveness, whereas in *vivo*, prevents initiation and development of spontaneous or chemically induced mammary tumors in rodents (Sanchez-Barcelo et al., 2003; Cos et al., 2000).

1.3: Melatonin Receptors

Melatonin exerts its cellular effects on target tissues through specific binding proteins or receptors (Ram et al., 2002). Melatonin binding sites have been identified in various tissues from human and other mammals including the central nervous system, human granulosa cells, certain cells of immune system and breast tissue (Niles et al., 1999; Ram et al, 2002; Brzezinski et al., 1997).

Studies have shown that the physiological effects of melatonin are mediated by two Gi protein coupled MT_1 and MT_2 receptors in mammals (Dubocovich et al., 1998). The human MT_1 receptor is localized to chromosome 4q35.1 and the cDNA encodes a protein of 350 amino acids with a predicted molecular mass of 37-39 kDa (Reppert et al., 1994; Ram et al., 2002). The MT_1 receptor is found in the pars tuberalis of the pituitary (PT), paraventricular thalamus, striatum, cerebellum, and suprachiasmatic nucleus (SCN) of the hypothalamus that functions to mediate the circadian effects of melatonin (Dillon et al., 2002; Reppert et al., 1995; Roca et al., 1996; Reppert et al., 1994; Barrett et al., 1996). In addition, the MT_1 receptor has been detected in the peripheral tissues such as lung, liver, kidney, and ovary (Naji et al., 2004; Niles et al., 1999}, tumour cells such as breast cancer cells, C6 glioma cells, and melanoma cells (Castro et al., 2005; Ram et al., 2002; Pickering et al., 1992). Structural analysis of this receptor suggests that it belongs to the GPCR superfamily of receptors (Reppert et al., 1994). The structure of the MT_1 receptor consists of an extracellular ligand-binding domain located at the N-terminus, seven hydrophobic segments representing the transmembrane domain, and a C-terminal G-protein coupled intracellular domain (Reppert et al., 1994).

The human MT_2 receptor, on the other hand, is localized to chromosome 11q21-22 (Dillon et al., 2002; Reppert et al., 1995). This receptor is approximately 40 kDa (Reppert et al., 1994). The MT_2 receptor is found in the human retina, striatum, cerebellum (astrocytes and Bergmann glia), SCN of the hypothalamus, hippocampus, ovary, and lung (Reppert et al., 1995; Dubocovich et al., 1997; Hunt et al., 2001; Al-Ghoul et al., 1998). Studies suggest that C6 glioma cells express both MT_1 and MT_2 (Armstrong et al., 2002). The MT_1 receptor, but not the MT_2 receptor, is found in MCF-7 human breast cancer cells (Ram et al., 2002). Melatonin plays an important role in regulation of reproductive activity in seasonal breeders (Reiter, 1980). In seasonal breeders, animals that have one or more estrous cycles during certain periods of the year, high affinity receptors (MT_1) in the pars tuberalis (PT) of the pituitary are thought to mediate the effects of melatonin on reproductive function (Morgan et al., 1994). Studies suggest melatonin binding sites are present in human granulosa cells (Niles et al., 1999; Yie et al., 1995). In humans, melatonin stimulates progesterone production in granulosa cells through melatonin receptors (Niles et al., 1997; Woo et al., 2001).

Studies where MT_1, in mammalian SCN, is knocked out in mice, it is suggested that the MT_1 receptor is involved in inhibition of neuronal firing. In contrast, the MT_2 receptor is responsible for the phase shifting circadian activity rhythms in C3H/HeN mouse (Dubocovich et al., 1998).

1.4: Other melatonin binding sites

Nuclear (RZR/ROR) melatonin receptors have been found in peripheral tissues such as heart, lung, liver, and kidney (Naji et al., 2004). Studies also suggest that

melatonin binds directly to calcium-binding proteins like calmodulin and protein kinase C in the cytosol (Anton-Tay et al., 1998; Pozo et al., 1997; Romero et al., 1998).

1.5: Regulation of Melatonin receptors

Density of the melatonin receptors not only varies with species but in several tissues it is influenced by the light/dark cycle, location, developmental, endocrine status, and pharmacological conditions through desensitization and upregulation of its receptors (Vanecek et al., 1998).

The mechanisms underlying melatonin receptor desensitization, the process by which receptors become refractory to their agonist, may involve homologous regulation (agonist-specific i.e. melatonin) or heterologous regulation (agonist-nonspecific i.e. estrogen) (Witt-Enderby et al., 2003). As for homologous regulation of melatonin receptors, phosphorylation and receptor down-regulation appear to play a role. The studies performed to date determining the mechanisms underlying heterologous regulation of melatonin receptors, however, have primarily focused on changes in melatonin receptor density (Witt-Enderby et al., 2003).

Phosphorylation, uncoupling of the receptor with the G-protein, which triggers arrestin binding to the receptor, lead to desensitization, or agonist-induced sequestration of receptors which can occur within minutes. In contrast, down regulation occurs after exposure to an agonist for one or more hours (Niles et al., 1997). Melatonin receptor desensitization may be the result of internalization events. Removal of receptors from the membrane via clathrin-coated vesicles can serve to prevent agonists from activating their receptors. Internalization events may prove to be important components underlying

melatonin receptor regulation (von Gall et al., 2002). Studies suggest that when cultured or recombinant cells expressing melatonin receptors are exposed to melatonin chronically, the affinity of melatonin for its receptors decreases perhaps due to uncoupling of the receptor, receptor internalization, and/or due to the receptor down regulation (Witt-Enderby et al., 2003; Tenn et al., 1993).

Melatonin itself is known to be involved in the regulation of it high affinity receptors. In the rodent SCN and pars tuberalis (PT), MT_1 mRNA expression and ^{125}I-Mel binding exhibit elevated levels during the daytime when the circulating melatonin levels were low. In addition light exposure that is known to suppress melatonin synthesis also increased ^{125}I-Mel binding, synchronized with the suppression of melatonin synthesis (Guerrero et al., 1999; von Gall et al., 2002), consistent with the concept that ligand availability regulates receptors levels. There is also complex developmental regulation of melatonin receptors in Syrian hamsters, with a dramatic decline in SCN melatonin receptor binding levels after birth (Duncan et al., 1993). This binding can be attributed to the MT_1 receptor, as hamster species lack a functional MT_2 receptor gene, and the expression of the MT_1 receptor mRNA also undergoes a dramatic postnatal decline (von Gall et al., 2002; Gauer et al., 1998).

Upregulation of melatonin MT_1 can be induced by an increase in cyclic adenosine 3'-5'- monophosphate (cAMP). Studies suggest that cholera toxin which raises cAMP levels through the activation of stimulatory G protein increases MT_1 level significantly (Barrett et al., 1996). Application of cAMP activators such as forskolin to PT cells increased MT_1 mRNA in a dose-dependent manner (Barrett et al., 1996). In contrast,

studies suggest that melatonin inhibits cAMP synthesis and reverses the forskolin-induced increase of the MT_1 mRNA levels. Therefore, melatonin can act via cAMP-signal transduction pathway to regulate expression of its own receptor (Barrett et al., 1996).

1.6: Melatonin agonists and antagonists

Luzindole (N-acetyl-2benzyltryptamine) is a non selective antagonist for both G-protein coupled receptors, MT_1 and MT_2. It is known to exhibit a 25-fold higher affinity for the human MT_2 melatonin receptor than its affinity for the MT_1 subtype, as determined by the competition for 2-[^{125}I]iodomelatonin binding to human transfected receptors (Conway et al., 1997; Dubocovich et al., 1998; Dubocovich et al., 1997). There is a selective MT_2 antagonist, 4-phenyl-2-propionamidotetraline (4P-PDOT) (Dubocovich et al., 1998).

1.7: Melatonin Signaling

Activation of the MT_1 melatonin receptor has been reported to inhibit adenylyl cyclase (Reppert et al., 1994). The existence of receptor subtypes is a typical feature of G protein-coupled receptors and may present the following advantages to the cell: (1) selectivity for the natural ligand(s). These are a wide variety of ligands that use GPCRs to stimulate cytoplasmic and nuclear targets through heterotrimeric G-protein-dependent and -independent pathways (Luttrell et al., 1999; Della Rocca et al., 1999). Such signaling pathways regulate key biological functions such as cell proliferation, cell survival and angiogenesis; (2) signaling of melatonin receptors via the cAMP pathway

appears to be a general feature of melatonin signaling which may be, according to the subtype expressed, complemented by the modulation of the cGMP pathway; and (3) selective intracellular signaling due to coupling to different effectors and selective regulation of signaling by desensitization or sensitization (Petit et al., 1999; Petit, 1998).

1.7.1: Melatonin and estrogen signaling

An increased concentration of cAMP in estrogen receptor (ER)-responsive breast cancer cells is seen when estrogens, through their binding to high-affinity membrane binders, activate adenylate cyclase (AC); cAMP synergizes with E2/ER complex and enhances ER-mediated transcription with subsequent transcription of its receptor, ER-alpha. However, melatonin, after its binding to membrane receptors, inhibits adenyl cyclase (AC) and decreases cAMP, thus counteracting the effects of estrogen (Sanchez-Barcelo et al., 2003).

Moreover, melatonin is known to decrease the expression of ER-α as well as it inhibits the binding of the estradiol–ER complex to the estrogen response element (ERE) on DNA (Molis et al., 1994; Lawson et al., 1992). Melatonin inhibits both aromatase (P450) activity and expression in *vitro* (MCF-7 cells) as well as in *vivo*, thus behaving as a selective estrogen enzyme modulator (Gonzalez et al., 2007). In addition, in MT_1 transfected MCF-7 cells, melatonin treatment inhibited aromatase mRNA expression where 1nM melatonin induced a higher and significant down-regulation of aromatase mRNA expression ($p<0.05$) than in vector-transfected cells (Gonzalez et al., 2007). Melatonin mediates its effects by its binding to a high affinity membrane-bound receptor coupled to Gi proteins (Yuan et al., 2002; Ram et al., 2002; Treeck et al., 2004).

1.8: MCF-7 Cells: an estrogen receptor- positive breast cancer cell line

The MCF-7 cell line is derived from a pleural effusion of a human breast adenocarcinoma. The epithelial breast cancer derived MCF-7 cell line is one of the most frequently used model systems to study different aspects of breast tumour cell growth. The estrogen receptor (ER)-positive MCF-7 human breast cancer cell line has been used extensively for the study of estrogen-responsive human breast cancer. However, various levels of estrogen responsiveness have been described in different stocks of MCF-7 cells thus exhibiting a differential responsiveness to the anti-proliferative effects of melatonin and the possible mechanisms involved (Ram et al., 2000). In addition, recent studies suggest that different stocks of MCF-7 exhibit different levels of the MT_1 receptor. Differential response of melatonin on MCF-7 cells proliferation may also be a result of variation in the MT_1 levels (Jawed et al., 2007).

1.9: Valproic Acid

Valproic acid (VPA), an anticonvulsant, has been used for 30 years to treat epilepsy and also has been proposed as a mood stabilizer (Gottlicher et al., 2001). Epilepsy is associated with high-frequency firing of neurons and VPA is known to decrease seizures through inhibition of neuron firing (Johannessen, 2000). VPA decreases high-frequency repetitive firing of action potentials by enhancing Na channel inactivation (Macdonald et al., 1994). VPA is also known to reduce a low threshold (T-type) Ca-channel current (Macdonald et al., 1994). In neurons, high concentrations of VPA hyperpolarize the cell by increasing the potassium (K) conductance (Slater et al., 1978).

In *vitro* studies suggest that anti-epileptic properties of VPA may result from the increase of γ-aminobutyric acid (GABA) in the brain (Johannessen, 2000). There is one recent study which demonstrated that VPA at 300/uM significantly increases the synaptic GABA release from cultured neurons (Gram et al., 1988). Furthermore, indirect evidence for enhanced GABA release in response to VPA comes from studies showing GABA increases in cerebrospinal fluid (CSF) of dogs and humans (Loscher, 1979; Loscher et al., 1981). However, there are several other studies which relate the VPA induced increase in presynaptic GABA levels to reduced GABA turnover and/or release in response to a postsynaptic effect of VPA (Chapman et al., 1982; Kapetanovic et al., 1988).

In general, inhibition of GABAergic activity results in increase seizures and potentiation of GABAergic activity results in reduced seizures (Johannessen, 2000; Gurvich et al., 2002). It is suggested that VPA increase GABAergic singaling in the brain by inhibiting GABA transaminase (GABA-T), an enzyme that degrades GABA to succinate semialdehyde (SSA) (Gurvich et al., 2002). It is also shown that VPA can increase levels of glutamate decarboxylase (GAD), GABA biosynthetic enzyme that converts glutamate to GABA (Mesdjian et al., 1982). Studies suggest that VPA increases $GABA_B$ receptor and enhances $GABA_A$ agonist actions (Olpe et al., 1988)

The clinical dose range for VPA is about 0.4-1mM (Gurvich et al., 2002; Yuan et al., 2001). The side effects for VPA include teratogenicity (Robert et al., 1983). Administration of VPA during the pregnancy is associated with increase risk of spina bifidia, limb defects, and cardiac malformations (Robert et al., 1982; Robert et al., 1983).

1.10: Neuroprotection by VPA

Recently, a significant increase of the melatonin MT_1 receptor mRNA was observed in C6 glioma cells derived from rat brain following treatment with VPA at concentrations varying from 0.1mM-5.0mM, for 24 or 48 hours (Castro et al., 2005). Western analysis confirmed that the VPA-induced increase in MT_1 mRNA results in the upregulation of MT_1 protein expression (Castro et al., 2005). These findings suggest that the neuroprotective properties of VPA may play a role in the modulation of MT_1 melatonin receptor (Armstrong et al., 2002).

Chronic administration with VPA protects neuronal cells from damage caused by oxidative stress (Wang et al., 2003). Studies suggest that treatment of C6 glioma cells with VPA significantly increases endoplasmic reticulum (ER) stress proteins, glucose-regulated proteins, GRP78, GRP94, and calreticulin (Niles et al., 1999). Studies also suggest that treatment of C6 cells with VPA results in increasing molecular chaperone molecules capable of binding to Ca^{2+} and protect cells from oxidative stress (Niles et al., 1999). In addition, glutathione-S-transferase activity, protective factor of oxidative stress, increases after chronic VPA treatment (Qin et al., 2004).

1.11: VPA and Cancer

VPA suppresses tumour growth, metastasis, and induces tumour differentiation in *vitro* and in *vivo* (Blaheta et al., 2002). Several modes of action might be relevant for the biological activity of VPA: (1) It increases the DNA binding of activating protein-1 (AP-1) transcription factor, and the expression of genes regulated by the extracellular-regulated kinase (ERK)-AP-1 pathway; (2) It downregulates protein kinase C (PKC)

activity; (3) It inhibits glycogen synthase kinase-3ß (GSK-3ß), a negative regulator of the Wnt signaling pathway; (4) It blocks HDAC (histone deacetylase) causing hyperacetylation of histones and activation of transcription. Hyperacetylation of histones is also known to activate p53, a tumour suppressor (Blaheta et al., 2002). The identification of VPA as an inducer of cell differentiation and inhibitor of cell growth elucidate an important role of VPA for cancer therapy.

1.11.1: Modulation of HDAC activity

The modification of histone N-terminal tails by acetylation or deacetylation can alter the interaction between histones and DNA, and thus regulate gene expression. The enzymes responsible for reversible acetylation/-deacetylation processes are histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively (Grozinger et al., 1999). HATs act as transcriptional coactivators, and HDACs are part of transcriptional corepressor complexes (Kruhlak et al., 2001). VPA can directly inhibit histone deacetylase (HDAC) activity and cause the hyperacetylation of histones (Thiagalingam et al., 2003)

Mammalian HDACs can be divided into three classes according to sequence homology (Khochbin et al., 2001). Class I consists of the yeast Rpd3-like proteins (HDAC1, HDAC2, HDAC3, and HDAC8). Class II consists of the yeast Hda1-like proteins (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10) (Furumai et al., 2001). Class II HDACs have been implicated in the regulation of muscle differentiation (Fischle et al., 1999). The deacetylase activity of class II HDACs is regulated by

subcellular localization. Class III comprises the yeast Sir2-like proteins (Dressel et al., 2001).

Therapeutic concentrations of VPA are known to inhibit several HDAC isoforms (Phiel et al., 2001; Gottlicher et al., 2001) resulting in increased acetylation of histones H2A, H2B, H3, H4 in *vitro* and in *vivo* (Gottlicher et al., 2001; Phiel et al., 2001). Studies suggest that VPA inhibits HDAC activity by binding to the catalytic center and therefore blocking substrate access (Gottlicher et al., 2001).

Multiple molecular pathways including cell cycle arrest, apoptosis, angiogenesis, and senescence explain the anti-proliferative effect of valproic acid as an HDACI on prostate (PCa) cancer cells in *vitro* and in *vivo* (Shabbeer et al., 2007). Studies with HDACIs [valproic acid (VPA), trichostatin A (TSA), and sodium butyrate] against six endometrial cancer cell lines decreased the proportion of cells in S phase and increased the proportion of cells in the G(0)-G(1) and/or G(2)-M phases of the cell cycle (Takai et al., 2004). Terminal deoxynucleotidyl transferase-mediated nick end labeling assays showed that these HDACIs induced apoptosis (Takai et al., 2004). This was associated with altered expression of genes related to malignant phenotype, including an increase in p21(Waf1), p27(Kip7), and E-cadherin and a decrease in B-cell lymphoma/leukemia 2-gene (Bcl-2) and cyclin-D1 and cyclin-D2 (Takai et al., 2004). Studies on medulloblastoma and supratentorial primitive neuroectodermal tumor (sPNET) suggest that the antitumor activities of valproic acid are correlated with induction of histone (H3 and H4) hyperacetylation and activation of p21 (Blaheta et al., 2005).

1.11.2: Modulation of MAPK pathways

The Mitogen-Activated Protein Kinase (MAPK) pathways transduce a large variety of external signals, leading to a wide range of cellular responses, including growth, differentiation, inflammation and apoptosis. The MAPK-associated pathways are composed of a growth factor–responsive pathway, including ERK1/2, and two stress-responsive pathways, including c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38MAPK. The former comprises Ras, Raf-1, MAPK/ ERK kinase (MEK), and ERK 1/2. In cells stimulated by growth factors [e.g., epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF)], the Ras proto-oncogene is activated via receptor protein tyrosine kinases such as EGF receptor (EGFR) and FGF receptor (FGFR). Ras can associate with the plasma membrane by virtue of lipid modifications at its COOH terminus (Traverse et al., 1993). This recruitment to the plasma membrane transduces Ras from an inactive form (GDP-Ras) to an active form (GTP-Ras), and is essential for activation by growth factors (Traverse et al., 1993). GTP-Ras interacts with Raf-1, localizing the latter to the plasma membrane where it becomes activated by several kinases and phosphatases (Chang et al., 2003). Activated Raf-1 then induces the sequential activation of MEK in a phosphorylation-dependent manner. Activated ERK1/2 then translocate from the cytoplasm to the nucleus and activate a number of transcription factors associated with cell cycle progression, survival, development, and differentiation (Chang et al., 2003). These MAPK pathways are known to regulate AP-1 transcription factors (Kyriakis et al., 2001). Hence, the MAPK/ERK pathway plays a central role during cell growth and survival.

Studies suggest that therapeutic concentrations of VPA activate MAPK/ERK pathway in human SHSY5Y neuroblastoma cells. The ERK pathway is required for the VPA induced AP-1 transcription as seen by the blockade of ERK pathways with PD98059 (Yuan et al., 2001). In addition, studies suggest that ERK activation is required to promote neurite growth in human neuroblastoma cells (Yuan et al., 2001). These data are associated with human SH-SY5Y neuroblastoma cells only. Further studies are necessary to clarify whether VPA selectively activates the ERK-AP-1 pathway in other tumor cell types and if this activation also correlates with cell differentiation.

Recent studies suggest that apoptosis in MCF-7 cells occur through the modulation of pro-apoptotic Bcl-2 family proteins and the MEK/ERK signaling pathway (Jo et al., 2007). MAPKs such as extracellular signal-regulated kinase (ERK)1 and ERK2 are also rapidly stimulated by estrogens in MCF-7 cells (Zivadinovic et al., 2005). However, the specific relationship of these responses to the levels of ER-α in the membrane has rarely been investigated (Razandi et al., 2003). The idea that VPA may cause apoptosis in the MCF-7 cell line via MEK/ERK signaling pathways awaits further study.

1.11.3: Modulation of Activator Protein-1 Medicated Transcriptional Activation

Activator protein-1 (AP-1) is a collection of homodimeric and heterodimeric complexes composed of products from two transcription factor families, Fos and Jun. Fos and jun are the commonest constituent proteins in mammalian cells. AP-1 activity is induced by growth factors, cytokines and oncoproteins. AP1 binds to transcription response elements (TPA responsive element, TREs) in the promotor region of several

genes that regulate cell proliferation, survival, differentiation and transformation. (Gurvich et al., 2002). The genes known to be regulated by the AP-1 family of transcription factors in the brain include genes for various neuropeptides, neurotrophins, receptors, transcription factors, enzymes involved in neurotransmitter biosynthesis, and proteins that bind to cytoskeletal elements (Gurvich et al., 2002).

c-Jun/AP-1 has been linked to invasive properties of aggressive breast cancer (Zhang et al., 2007). Recently, it has been reported that overexpression of c-Jun in the breast cancer cell line MCF-7 results in increased AP-1 activity, motility and invasiveness of the cells in *vitro*, resulting in tumor formation in nude mice, and causes in *vivo* liver metastasis of MCF-7/c-Jun cells (Zhang et al., 2007). Studies also suggest that AP-1 blockade by overexpressing a dominant-negative form of cJun (cJun-DN, TAM67) inhibits breast cancer cell growth both in *vivo* and in *vitro* (Liu et al., 2004). TAM67 inhibits breast cancer growth predominantly by inducing inhibitors of cyclin-dependent kinases (such as p27) and by reducing the expression of the cyclins involved in transitioning from G1 into S phase of the cell cycle (Liu et al., 2004). More findings suggest that AP-1 blockade suppresses mitogenic signals from multiple different peptide growth factors as well as estrogen, and inhibits the growth of MCF-7 breast cancer cells both in *vitro* and in *vivo*. Therefore, preventing AP-1 gene activation may prove useful in restoring the endocrine responsiveness of such high-risk ER-positive breast cancers.

However, it has been demonstrated that VPA, at therapeutically relevant concentrations, increases AP-1 DNA binding activity in cultured cells in *vitro* (Chen et al., 1997). Incubation of rat C6 glioma cells with VPA resulted in 1–2 fold increases in

the DNA binding activity of AP-1 transcription factors in both a time- and concentration-dependent manner, and occurred within the drug's therapeutic range. Similarly, VPA-increases AP-1 DNA binding activity in human SH-SY5Y neuroblastoma cells and in rat brain (Asghari et al., 1998; Chen et al., 1997). These findings raise the possibility that VPA may produce its effects by regulating the expression of subsets of genes via its effects on the AP-1 family of transcription factors (Chen et al., 1999; Chen et al., 1997). Currently, there are no studies that focused on VPA effects on AP-1 DNA binding activity in cultured MCF-7 breast cancer cells.

1.11.4: VPA effects in Bcl-2 expression and other oncostatic genes

Apoptosis is an essential and highly conserved mode of cell death that is important for normal development, host defense, and the suppression of oncogenesis. Among the numerous proteins and genes involved, members of the caspase family and the Bcl-2 family play important roles in modulating apoptosis (Cohen, 1996). Apoptosis is mediated by the activation of caspases, which amplify the apoptotic signal and proteolytically process numerous cellular target molecules with various functions (Akgul et al., 2001). Bcl-2 family proteins are vital for the regulation of apoptosis by controlling the mitochondrial membrane potential to release cytochrome c and for activating caspase-9 and -3 (Akgul et al., 2001).

Previous studies suggest that chronic treatment with VPA increases anti-apoptotic Bcl-2 gene in rat cerebral cortex (Chen et al., 1999). Studies also suggest that five-day treatment of human neuroblastoma cells with VPA increases the expression of Bcl-2 genes as a result of MAPK/ERK signaling pathway (Yuan et al., 2001).

Other findings suggest that the TSA inhibits growth of small cell lung cancer cells by elevating p21, p27 along with decreasing Bcl-2 protein levels (Platta et al., 2007). Recent findings propose that 5mM concentration of VPA causes apoptosis in endometrial cancer cells and decreases Bcl-2 protein expression (Takai et al., 2004). The findings raise the possibility that VPA and other HDACIs may prove particularly effective in treatment of endometrial cancers.

1.11.5: Downregulation of Protein Kinase C

PKC isoforms are often overexpressed in cancer and are involved in regulation of angiogenesis, tumor proliferation, dedifferentiation, and/or invasion. Protein kinase C (PKC) delta, a member of the novel family of PKC serine-threonine kinases, has been implicated in negative regulation of proliferation and apoptosis in a large number of cell types, including breast cancer cell lines, and postulated as a tumor suppressor gene (Grossoni et al., 2007). PKC delta plays a crucial role in transducing the invasion-promoting effects of platelets in breast cancer cells, and the specific inhibition of PKC delta may be a strategy to decrease platelet-mediated cancer cell invasion (Alonso-Escolano et al., 2006). More findings implicate that PKC beta enhances growth and expression of cyclin D1 in human breast cancer cells (Li et al., 2006). Therefore, inhibitors of these isoforms may be useful in breast cancer chemoprevention or therapy. Recent findings suggest that PKC promotes apoptosis in LNCaP prostate cancer cells through activation of p38 MAPK and inhibition of the Akt survival pathway (Tanaka et al., 2003).

Chronic exposure of rat C6 glioma cells to ''therapeutic''concentrations of VPA have resulted in decreased PKC activity in both membrane and cytosolic fractions. Downregulation of PKC activity in the presence of VPA has also been observed in human U937 monocytic leukemia cells. PKC might be a novel target for enhancing the efficacy of histone deacetylase inhibitor in cancer therapy. However, to this date, no studies have been carried to study the effects of VPA on PKC activity on human breast cancer cells.

1.11.6: VPA and estrogen signaling pathway

A recent novel potential therapeutic strategy using histone deacetylase (HDAC) inhibitors to enhance the action of hormonal therapy agents in ER-α positive breast cancer has been reported. HDAC inhibitors [TSA, suberoylanilide hydroxamic acid (SAHA) and VPA], inhibited proliferation of MCF-7 breast cancer cells and, in combination with tamoxifen inhibited proliferation better than with either agent alone (Hodges-Gallagher et al., 2006). VPA enhanced tamoxifen action at doses within the concentration range (0.4mM-1mM) used for anti-convulsive therapy (Hodges-Gallagher et al., 2006). VPA promoted cell death via apoptosis without affecting cell cycling by inducing the pro-apoptotic gene Bik that is induced by antiestrogens, therefore, enhancing antiestrogen action (Hodges-Gallagher et al., 2006). These in *vitro* results suggest that VPA and other HDAC inhibitors have the potential as a therapeutic agent to treat ER- positive breast cancer.

1.12 Valpromide: A VPA analog

Valpromide (VPD), the primary amide of VPA, is approved as an antipsychotic agent in several European countries (Winkler et al., 2005; Badir et al., 1991). VPD crosses the blood–brain barrier (BBB) more readily leading to higher central nervous system (CNS) concentrations than VPA (Winkler et al., 2005). VPD is 3–5 times more potent than VPA and is not teratogenic in animal models (Winkler et al., 2005; Badir et al., 1991). Previous studies indicate that VPD can protect against chemically induced seizures in mice (Lampen et al., 1999). VPD does not inhibit HDAC activity, which makes it very useful for comparison studies with VPA, a potent HDAC inhibitor (Phiel et al, 2001).

1.13: Objectives and Hypothesis

VPA is known to modulate melatonin MT_1 receptor expression in MCF-7A cells under low (1%) serum conditions (Jawed et al., 2007). MC-7A breast cancer cells express lower basal levels of the MT_1 receptor, whereas, MCF-7B cells express higher basal levels of MT_1 receptor. In keeping with the foregoing, I hypothesized the following:

1. Treatment of estrogen dependent human breast cancer cells (MCF-7) with valproic acid (VPA) will regulate expression of the melatonin MT_1 receptor.
2. Given the anti-cancer property for the agents, VPA and melatonin, the effects of combinatorial drug-hormone treatment on cellular proliferation will be more potent than with either agent alone.

3. This enhanced antiproliferative effect will involve changes in the expression of certain oncostatic genes such as p53 and ER-alpha.

The three objectives that we used to conduct this study were as follows:

1. **To examine the effects of melatonin and VPA on MCF-7 cell proliferation.** MCF-7 cell proliferation was studied using the MTT assay and morphological changes of these cells were studied using Hoescht test.
2. **To study the effects of VPA on MT_1 receptor mRNA and protein expression in human MCF-7 cells.** The mRNA levels of MT_1 were studied at various time intervals (6h, 24h, or 72h) using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). MT_1 receptors were examined using western blotting.
3. **To observe the effects of VPA and melatonin alone and in combination on the expression of oncostatic genes such as P53 and ER-alpha.** The mRNA levels of p53, and ER-alpha were examined at various time intervals (6h, 24h, or 72h) using RT-PCR.

SECTION 2: MATERIALS AND METHODS

2.1: Cell Culture

All experiments were performed with MCF7-B subline (passages 13-22). MCF7-B cells have shown to have higher basal levels of MT_1 (Jawed et al., 2007). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% or 1% fetal bovine serum (FBS), 100 µl/ml penicillin, and 1.25 mg/ml streptomycin at 37°C in a 5% CO_2 humidified atmosphere. After about three days of growth, the medium was aspirated and changed to either of the two media conditions: (1) DMEM/1% FBS or (2) DMEM/10% FBS for examination of gene expression and/ or proliferation studies. When the cells reached 65-75% confluence, they were treated with valproic acid or melatonin as described in section 2.6-2.8. Valproic acid and melatonin were purchased from Sigma-Aldrich (Oakville, ON).

2.2: Culturing MCF-7

A cryovial stored in liquid nitrogen was thawed by placing it at 37 °C. The cells were transferred to a 15ml conical tube containing 2ml of 100%FBS media. The cells and the media were centrifuged for 3 minutes at 3000 rpm (~2000 x g). Following this, the supernatant was discarded and the pellet was resuspended using 10ml of either of the media conditions as described in section 2.1. The cell suspension was dispensed onto a 10cm Petri dish from SarStedt (Montreal, Quebec). The cells were then incubated at 37°C in a 5%CO_2/air humidified atmosphere.

2.3: Passaging MCF-7 cells

Cells were subcultured into 10cm Petri dishes when they reach 90-100% confluence. Previous media was aspirated and TrypLE^{TM}Express from Invitrogen (Burlington, ON), a cell dissociation enzyme, was used to detach the cells from the Petri dish. Then an aliquot of culture medium (1%FBS/DMEM or 10%FBS/DMEM) was added to inactivate the enzyme's activity. Cells were collected in a conical tube and with a Pasteur pipette resuspended in 1ml of media. Then 1ml of this cell suspension was supplemented to 9ml of culture medium into each 10cm Petri dishes.

2.4: Cell Counting

100% confluent cells were removed from the 10cm SarStedt Petri dish using a cell scraper. Cell counting was performed using the trypan blue exclusion assay (Sigma-Aldrich, Oakville, ON). Cells were resuspended in 1ml of culture medium (1%FBS/DMEM or 10%FBS/DMEM) and 20ul of cell suspension was added to 80ul of trypan blue solution. Using a hemocytometer, cells at the centre square and the four corner squares for both chambers were counted for a total of 10 squares. The number of cells per ml is equal to the average number of cells from the 10 squares multiplied by 10^4 X 5 (dilution factor).

2.5: Cryopreservation of MCF-7 cells

Cells were grown to confluence (90-100%) and harvested using a cell scraper in 6ml of freezing medium (10%DMSO, 10%FBS in DMEM). Cells were left overnight at -80°C and transferred to liquid nitrogen for cryopreservation.

2.6 Valproic Acid (VPA) treatment

VPA was prepared as a 50mM stock in DMEM. From this stock, 20µl, 100µl, 200µl, 600µl, or 1ml was directly diluted to the medium (1%FBS/DMEM or 10%FBS/DMEM) to give a final concentration of 0.1, 0.5, 1, 3, 5mM VPA. MCF-7 cells (60-80% confluence) were treated with VPA in either culture medium, as mentioned in section 2.1. After the 6h or 24h incubation, total RNA or protein was extracted as described in sections 2.9 and 2.16.

2.7: Melatonin treatment

Melatonin from Sigma-Aldrich (Oakville, ON) was prepared as a 10mM stock in 100% DMSO. From this stock, serial dilutions were done to get 1000nM, 100nM, 10nM melatonin concentrations. From these stocks, 1ml was directly diluted in the 10%FBS/DMEM medium to give final concentrations of 100nM, 10nM or 1nM melatonin. After 6 or 24h of incubation, total RNA was extracted as described in section 2.9.

2.8: VPA and melatonin treatment

VPA doses (section 2.6) were added first followed by 1ml of either concentration of melatonin (section 2.7). After 6 or 24h of incubation, total RNA was extracted as described in section 2.9.

2.9: RNA extraction

Total RNA was isolated from MCF-7 cells using TRIzol (Invitrogen, Oakville, ON) according to the manufacturer's instruction. The medium was removed from the 10cm SarStedt Petri dishes and 1ml of TRIzol was added per dish. Homogenization of samples was completed by using a 20-gauge needle and syringe followed by an incubation period of 5 min at room temperature. Then, 0.2ml of chloroform (ACP, Montreal) was added and samples were left at room temperature for another 15min. Samples were then centrifuged for 15min at 13, 000 rpm at 4°C to separate the solution into an aqueous phase and an organic phase. RNA remains in the top aqueous phase; the bottom organic phase contains DNA and proteins. Then the RNA top layer is transferred to a new tube with 0.5ml of isopropyl alcohol for 10 min at room temperature, allowing RNA to precipitate. After the incubation, samples were kept at -20°C overnight. The following day, samples were centrifuged for 10min at 13,000 rpm at 4°C. RNA remains at the bottom of the Eppendorf tube as a white pellet. The supernatant was discarded and the white pellet was resuspended in 1ml of 75% ethanol and centrifuged once again for 5min at 9, 000rpm at 4°C. The supernatant was removed and the RNA pellets were allowed to dry for 5min. Next, the RNA pellets were dissolved in 35µl of RNase-free water and subsequently incubated at 55°-60°C for 10 min. The RNA was kept at -80°C.

2.10: Yield and purity of RNA

The concentration of RNA was determined by measuring the absorbance at 260nm (A_{260}) in a Beckman spectrophotometer. An absorbance of 1 unit at 260nm corresponds to 40µg of RNA per ml (A_{260} of 1=40µg/ml). RNase free water was used to

calibrate the spectrophotometer. A sample of 2ul of RNA is added to 98µl of RNase-free water per reading. A total of three readings of each RNA sample were averaged to determine its yield (260nm). The RNA concentration was calculated using the following equation:

$$[RNA] = (A_{260}) * (40\mu g/ml/A_{260}) * 50(\text{dilution factor})$$

$$= (\mu g/ml) / 1000$$

$$= (\mu g/\mu l)$$

The ratio of the reading at 260nm and 280nm (A_{260}/A_{280}) provides an estimate of protein or excessive contamination. Pure RNA has an A_{260}/A_{280} ratio of 1.8-2.1.

2.11: DNase treatment

RNA samples were treated with DNase I (Invitrogen, Oakville, ON) to eliminate any DNA contamination. A total of 25µg of RNA per sample was treated with 2.5µl of DNAse I enzyme, 2.5µl of 10X DNAse I buffer and RNAse-free water to have a total volume of 25µl. The samples were then incubated at 37°C for 40min and 2µl of 25mM EDTA (pH 8.0) was added to inactivate the DNAse I enzyme. The reaction was heated for 10min at 65°C. Finally, the samples were stored at -80°C.

2.12: Reverse transcription (RT)

RT was performed with an Omniscript ^{TM} Reverse Transcriptase kit (Qiagen, Mississauga, ON) according to the manufacturer's instruction. Two µg of total DNAse treated-RNA was used to synthesize cDNA. The master mix was prepared by using 2µl of 10X buffer, 2µl of 5mM dNTP Mix (0.5mM final), 2µl of Oligo dT (0.5ug/ul) from MBI

Fermantes (Burlington, ON), 1μl of RNAse inhibitor (10units/ul) (Invitrogen, Oakville, ON), 1.0μl of Omniscript RT (4 units final) and a volume of RNAse-free water (~9.8μl) required to bring the final volume to 20μl after addition of RNA (~2.2μl). Subsequently, the samples were incubated for 60 minutes at 37°C, followed by heating for 5minutes at 93°C to inactivate the enzyme and finally chilled on ice. The RT product (cDNA) was stored at -20°C for perseveration.

2.13: Standard Polymerase Chain Reaction (PCR)

PCR was carried out using the HotStarTaq master mix kit (Qiagen Inc., Mississauga, ON). The HotStarTaq master mix consists of HotStarTaq DNA polymerase, PCR buffer with 2X $MgCl_2$, and 400μM of DNTPs (A, T, C, and G). First, the procedure used for semi-quantitative standard PCR was established by cycle dependent experiments of gene targets such as: MT_1, P53, ER-α (Table 1, Figure 1) for either of the media conditions as mentioned in section 2.1. Cycles were selected from a linear range within the mid-exponential phase. Next, the thermal cycler was programmed for initial temperature at 95°C for 15 minutes. This step activates HotStarTaq DNA polymerase. Then, the cDNA samples were first round amplified at the following settings: 94°C for 30 seconds, 55 °C (was optimized using the cycle dependency curve of each gene target and the T_m) for 30 seconds, and 72°C for 1 minute and the final incubation was at 72°C for 10 minutes as performed previously (Armstrong et al., 2002). Conditions used for each target gene can be found on table 2.

2.14: cDNA gel electrophoresis

Products from RT-PCR (section 2.13) were separated on 2% agarose gel in 1X TBE buffer. 1X TBE was prepared using 10X TBE (Tris Base: 108 g, Boric Acid: 55g, 0.5M EDTA 20 mL, and water to 1.0 L). Then, the gel was stained with 3µl of ethidium bromide (10mg/ml). A total of 2µl of loading buffer (6X), and 10µl of PCR products (cDNA) were loaded on each lane. Gels were run at 75V for 2 hours and scanned using Alpha imager ^{TM} 200 (Kodak). Optical density (OD) ratios of each gene target such as: MT_1, p53, ER-alpha, and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained for semi-quantitative statistical analysis as described in section 2.17.

2.15: MTT Assay

MCF-7 cells were spun down, resuspended and counted using a hemocytometer and a 0.4% Trypan Blue solution. Cells were seeded in 10%FBS/DMEM in triplicate on a 96-well plate at about 15000 cells per well for treatment, or in a range of 750 to 45,000 cells per well for the standard curve (Figure 2), and allowed to attach overnight. Cells were treated with VPA (0.1- 5 mM), or melatonin (1, 10 and 100 nM), or a combination of both agents simultaneously. In the last 2h of treatment 10µL of a 5mg/mL stock solution of MTT (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in phosphate buffer saline (PBS) was added to each well. Medium and MTT were then removed and cells were lysed using 100µL of 20% SDS/50% formamide solution, as reported previously (Crocker et al., 1996). Plates were left to incubate overnight and read the following day using a 595nm filter in a Titertek Multiskan Plus scanning spectrophotometer (ELISA reader).

2.16: Western blotting

Proteins were extracted using Ripa Buffer (Tris-HCl: 50 mM, pH 7.4, NP-40: 1%, Na-deoxycholate: 0.25%, NaCl: 150 mM, EDTA: 1 mM, PMSF: 1 mM, Aprotinin, leupeptin: 1 μg/ml each, Na_3VO_4: 1 mM). DC-protein assay protocol (Bio-Rad Laboratories (Canada) Ltd., Mississauga, ON) was obtained from instruction manual.

Next, 80μg of protein with 6x loading dye (1ml of 0.5M Tris-HCl, pH6.8, 800 μl glycerol, 1.6ml of 10% SDS, 400μl of 2-mercaptoethanol, 200μl of 0.05% bromphenol blue was loaded onto the SDS-PAGE (4% stacking, 12% separating gel). Stacking gel (4%) was prepared using: 1.25 ml 0.5M Tris-HCl, pH 6.8, 0.025 ml of 20% SDS, 0.67 ml of 30%/0.8% Acrylamide/Bis-acrylamide, 0.025 ml of 10% ammonium persulfate, 0.005ml of TEMED, and 3.1ml of water. Separating gel (12%) constituted of the following reagents: 2.5ml of 1.5 M Tris-HCl, pH 8.8, 0.05 ml of 10% SDS, 4.0 ml of 30%/0.8% Acrylamide/Bis-acrylamide, 0.05 ml of 10% ammonium persulfate, 0.005 ml of TEMED, and 3.4 ml of water.

MCF-7 membranes were subjected to SDS-PAGE for 3h at 75V and transblotted overnight at 4°C at 30V. The melatonin MT_1 receptor was detected using a 1:100 dilution of anti-human MT_1 antibody (CIDTech Research Inc., Cambridge, ON) incubated overnight at 4°C. After subsequent incubation with a 1:1000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for two hours, proteins were detected by ECL – enhanced chemiluminescent autoradiography, as reported previously (Castro, 2005). For internal controls, blots were stripped and reprobed with a 1:10,000 dilution of

monoclonal anti-mouse β-actin antibody (Sigma-Aldrich, Oakville, ON) and then incubated with a 1:20,000 dilution of HRP-conjugated anti-mouse secondary antibody (Sigma-Aldrich, Oakville, ON).

2.17: Hoescht Test

In order to detect possible drug-induced apoptotic changes, MCF- 7 cells were grown on a 24-well plate in DMEM/10% FBS with or without VPA (1 mM) for 24h. Cells were fixed in a 1% paraformaldehyde solution and washed with phosphate-buffered saline. The cells were stained with 0.2 mM of the Hoechst 33258 DNA dye (Sigma-Aldrich Oakville, ON) for 15 min and nuclear morphological changes were observed under a fluorescence microscope

2.18: Statistical Analysis

The optical density (OD) values for the target genes (MT_1, p53, ER-alpha) were first normalized to β-actin protein levels or GAPDH mRNA levels. The ratios were further normalized to account for other sources of variations by obtaining percentage values. A one-way ANOVA (analysis of variance) was performed followed by a Neuman-Keuls test or Dunnett's test to compare different groups. Statistical significance was considered when $P<0.05$.

Table 1: Nucleotide sequences and sizes for primers used to carry out RT-PCR reactions.

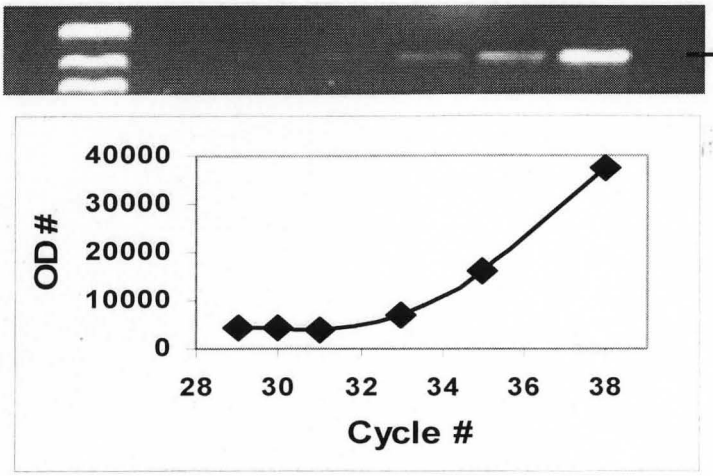
Gene	Primers (5'→3')	Nucleotides	Size (bp)
MT_1	agtcagtgggttcctgatgg	335	572
	cattgaggcagctgttgaaa	906	
P53	cctcaccatcatcacactgg	967	426
	tctgagtcaggcccttctgt	1392	
ER-alpha	agg cac ctg ctc atg gga caa	6079-6059	629
	tgt ggc gct caa ttg acc acc	1129-1149	
GAPDH	ttc acc acc atg gag aag gc	1147-1166	237
	ggc atg gac tgt ggt cat ga	1383-1364	

Table 2: RT-PCR conditions for selected genes

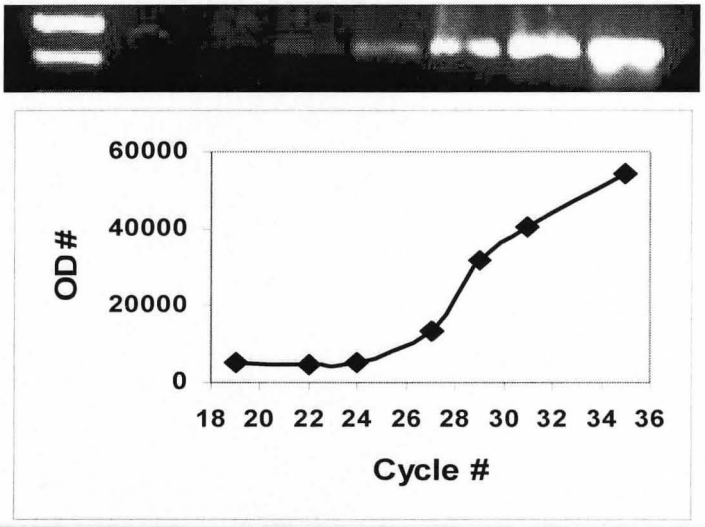
Gene	cDNA volume (µl)	Anealing Temperature (°C)	# Cycles
MT_1	3	55	34
P53	2	57	27
ER-alpha	2	61	27
GAPDH	1	55	25

Figure 1: RT-PCR cycle dependency optimization for MT_1, P53, and ER-alpha genes. (**A**) Top: Lane1= Marker, Lanes 2-7=29, 30, 31, 33, 35, 38. MT_1 size is 572bp. Bottom: Optical OD ratios as a function of cycle number. A cycle number of 34 was found within the exponential phase of MT_1 cycle dependency curve. (**B**) Top: Lane1= Marker, Lanes 2-8=19, 22, 24, 27, 29, 31, 36. P53 size is 426bp. Bottom: Optical OD ratios as a function of cycle number. A cycle number of 27 was found within the exponential phase of p53 cycle dependency curve. (**C**) Top: Lane1= Marker, Lanes 2-7=20, 23, 25, 27, 30, 36. ER-alpha size is 629bp. Bottom: Optical OD ratios as a function of cycle number. A cycle number of 27 was found within the exponential phase of ER-alpha cycle dependency curve.

A


MT1: 572 bp
40000
30000
20000
10000
0
OD#
28
30
32
34
36
38
Cycle #

B


P53: 426 bp
60000
40000
20000
0
OD#
18
20
22
24
26
28
30
32
34
36
Cycle #

C

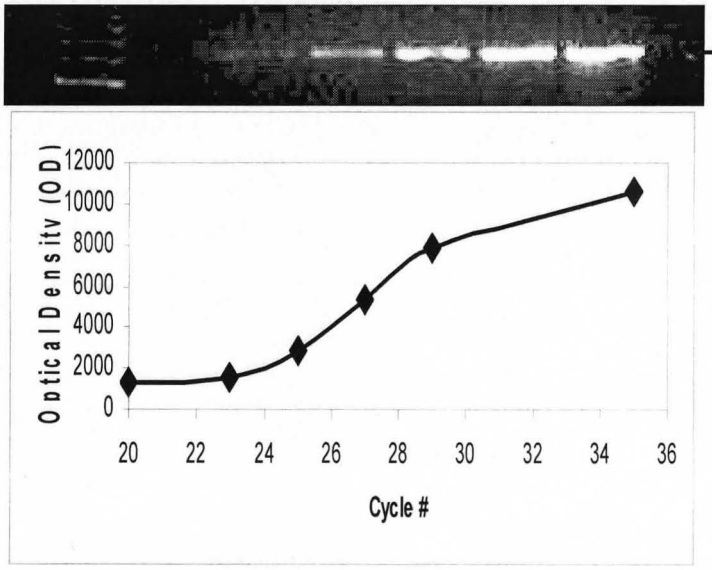
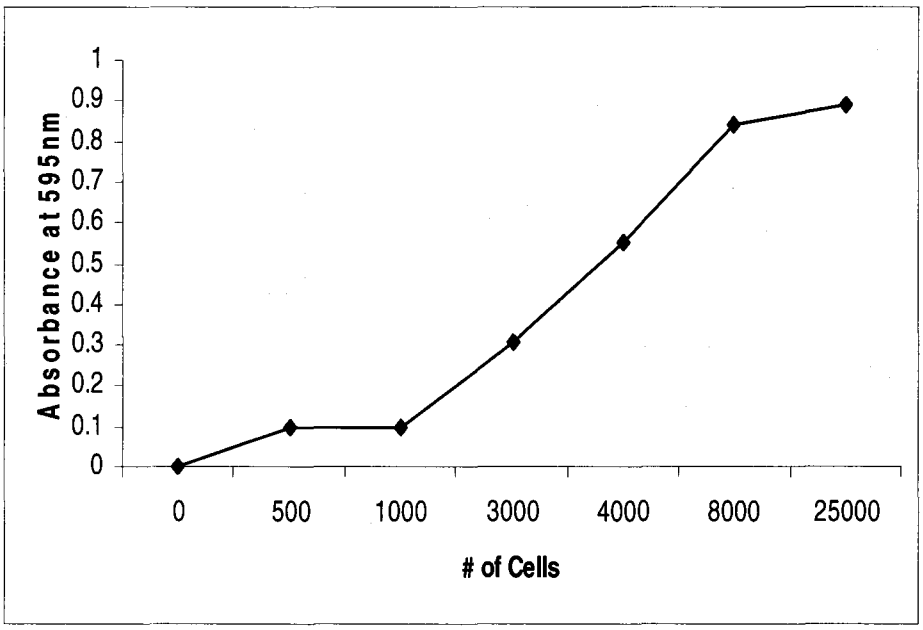

ER-alpha: 629 bp
Optical Density (OD)
12000
10000
8000
6000
4000
2000
0
20
22
24
26
28
30
32
34
36
Cycle #

Figure 2: MTT Standard Curve for MCF-7 breast cancer cells. (**A**) 10%FBS medium was used to plate 500-2000 cells overnight.

A


Absorbance at 595nm
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
500
1000
3000
4000
8000
25000
# of Cells

SECTION 3: RESULTS

3.1: Effects of VPA and melatonin on MCF-7 cell growth

Treatment of MCF-7 cells with VPA for 24h suppressed MCF-7 growth (Figure 3A). After 24h, melatonin (1 and 10 nM) inhibited growth by about 50% in one experiment, whereas about 5-20% inhibition was seen in two other experiments. However, when both VPA and melatonin were added to cultures simultaneously at the start of treatment, there was a significant inhibition of MCF-7 cell growth, which exceeded that seen with either agent alone, as shown in figure 3B. In order to determine whether the inhibitory effect of VPA on MCF-7 cell growth involved apoptosis, cells were stained with Hoescht 33258 and visualized under a fluorescent microscope. As shown in figure 4, VPA treatment for 24h, resulted in morphological changes which indicate apoptosis.

3.2: Effects of VPA Treatment on MT_1 mRNA expression in MCF-7B cells

3.2.1: 1%FBS medium

Treatment of MCF-7b cells with VPA (0.1, 0.5, 1.0, 3.0 and 5.0 mM) for 24h appeared to cause a dose-dependent increase in melatonin MT_1 receptor mRNA expression (Figure 5A). Longer treatment with VPA for 72h produced a significant induction of melatonin MT_1 receptor mRNA ($F= 5.826$, $p<0.0089$, $n=3$). Post hoc analysis by a Neuman-Keuls test indicated that melatonin MT_1 receptor mRNA expression was increased significantly ($p<0.05$) in cells exposed to 1.0, 3.0, or 1.0 mM VPA for 72h (Figure 5B).

3.2.2: 10%FBS medium condition

Treatment of MCF-7B cells with VPA (0.1, 0.5, 1.0, 3.0 and 5.0 mM) indicated that melatonin MT_1 receptor mRNA expression was decreased significantly ($p<0.01$) in cells exposed to 0.5 or 1.0 mM VPA for 24h (Figure 6C). However, one-way ANOVA did not indicate any significant effects when all the doses of VPA were included due to variance (Figure 6B).

To investigate if the significant decrease of MT_1 mRNA at clinical doses (0.5-1.0 mM) is upregulated in earlier time points, 6h of VPA treatment on MCF-7B cells was tested. Results suggest a U-shaped dose response with induction at lower doses and inhibition at higher doses. As shown in figure 6A, after exposure of MCF-7B cells to 1.0 mM VPA, there is a significant induction of MT_1 mRNA ($F=2.985$, $p<0.0432$).

Figure 3: Dose-dependent inhibition of MCF-7B cell growth by VP and melatonin. (**A,B**) Cells were treated with the indicated concentrations of VPA and/or melatonin for 24 h. Data shown are means ± S.E.M. (n = 3) of % values obtained from MTT analysis of cell proliferation, as compared with appropriate controls. Controls were DMEM/10% FBS for VPA, and the same medium containing 0.0001% or 0.001% DMSO for 1 and 10 nM melatonin respectively *$p<0.05$; **$p<0.01$ vs. controls.

Fig. 3

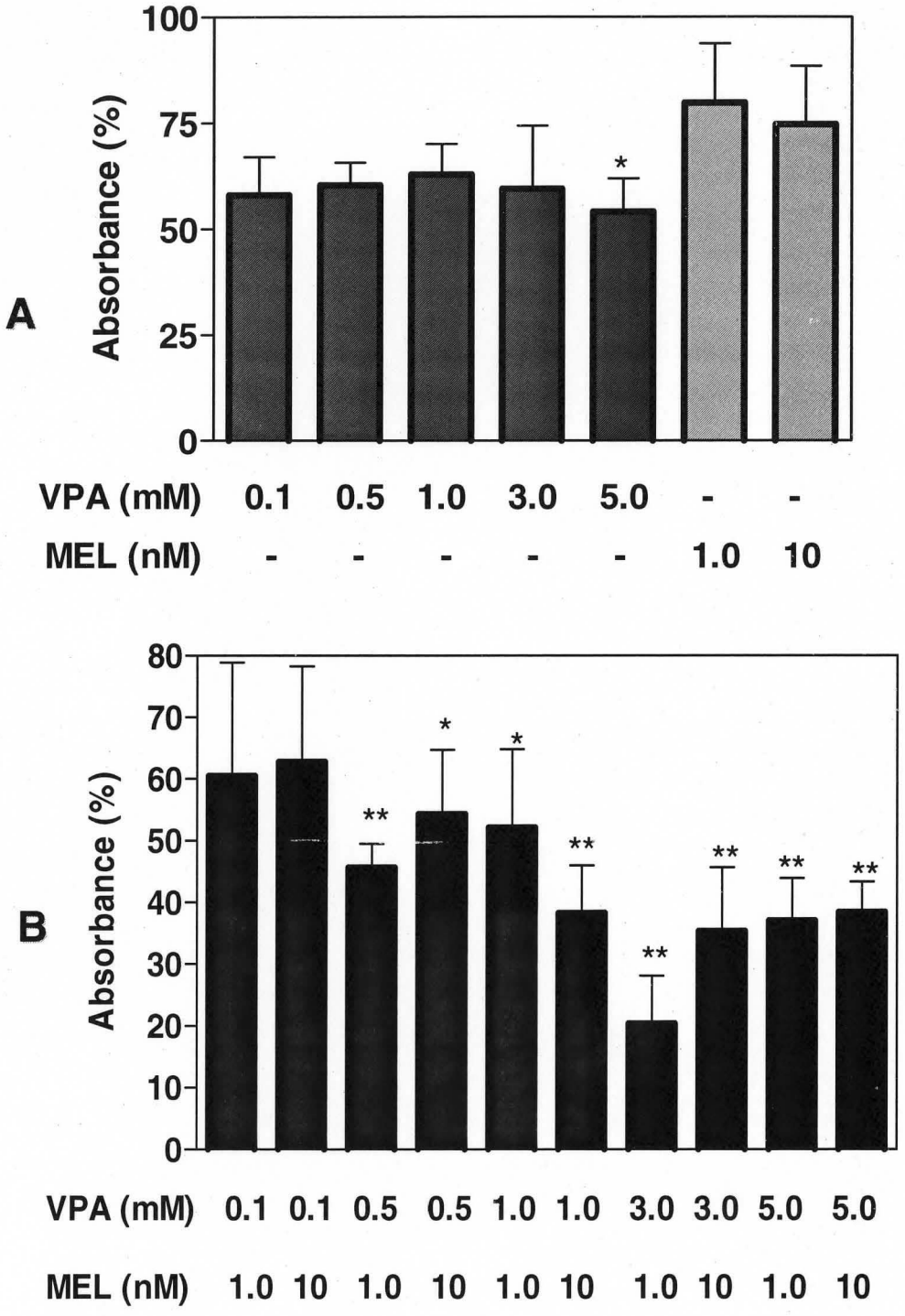
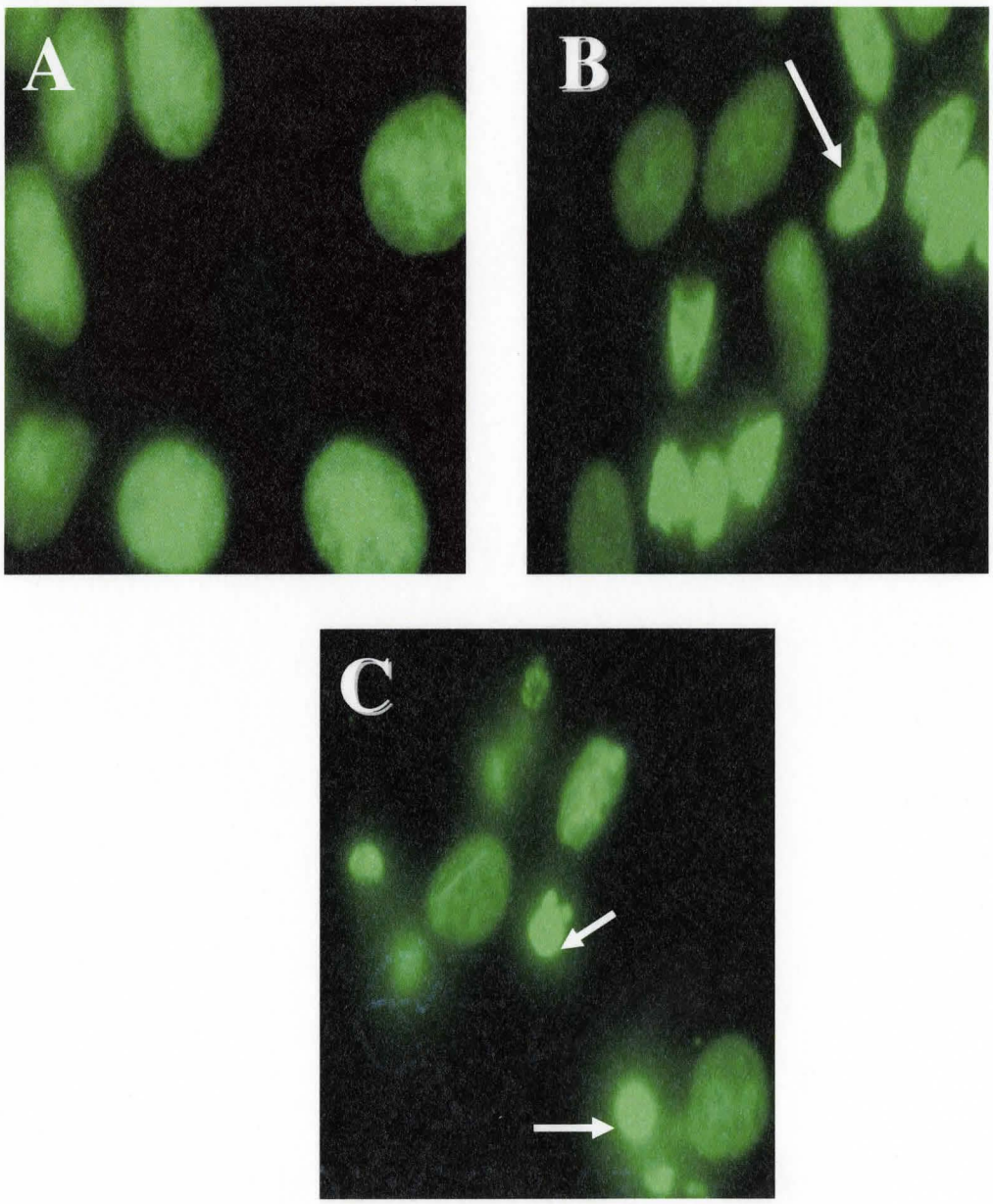

A
Absorbance (%)
100
75
50
25
0
*
VPA (mM) 0.1 0.5 1.0 3.0 5.0 - -
MEL (nM) - - - - - 1.0 10
B
Absorbance (%)
80
70
60
50
40
30
20
10
0
*
*
**
**
**
**
**
**
VPA (mM) 0.1 0.1 0.5 0.5 1.0 1.0 3.0 3.0 5.0 5.0
MEL (nM) 1.0 10 1.0 10 1.0 10 1.0 10 1.0 10

Figure 4: Hoescht Staining on MCF-7B cells. (**A-B**) Images of Hoescht-stained controls and cells treated with VPA (1.0 mM) for 24h. Cell treated with VPA (1.0 mM) and melatonin (1 nM). Nuclear condensations are indicated by arrow heads.

Fig. 4



A: Control
B: 1mM VPA
C: 1mM VPA ± 1nm melatonin

Figure 5: Dose-dependent effects of 24h VPA treatment on melatonin MT_1 receptor mRNA expression in MCF-7B cells grown and treated in 1% FBS. (**A**) Data shown are for percentage (%) values of MT_1/GAPDH optical density (OD) ratios for a single experiment. (**B**) Data shown are means ± S.E.M. (n = 3) for % values of MT_1/GAPDH OD ratios. **P<0.05 vs. control (ANOVA and Newman Keuls). (**A-B**) Representative gel images of RT-PCR detection of MT_1 (397 bp) and GAPDH (237 bp) mRNA. Lanes 1-6: Control, 0.1, 0.5, 1.0, 3.0, and 5.0 mM VPA.

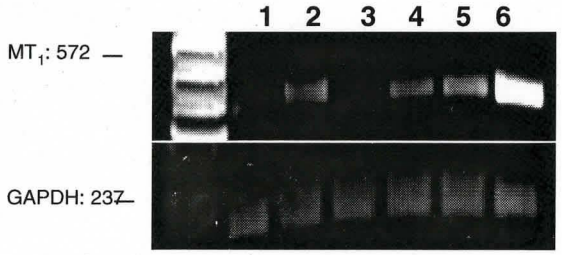
Fig. 5 **VPA Treatment on MCF-7B**

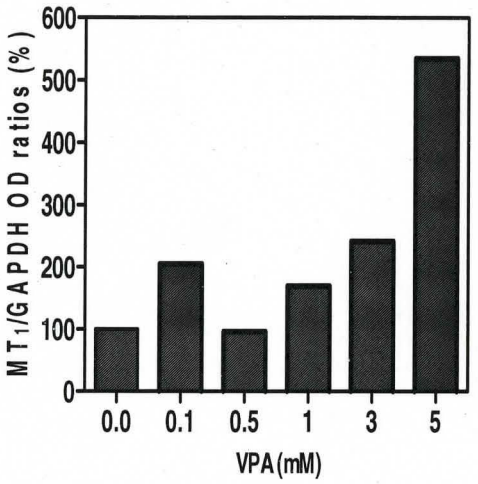
MT_1

***Media Type*: 1%FBS/DMEM**

24h

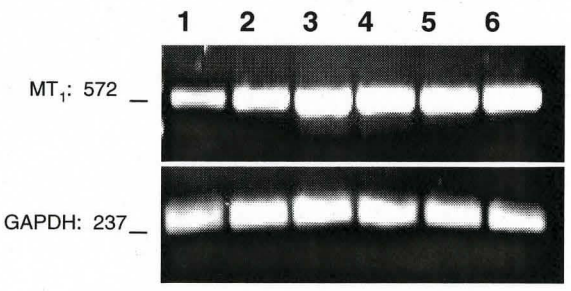
A





72h

B



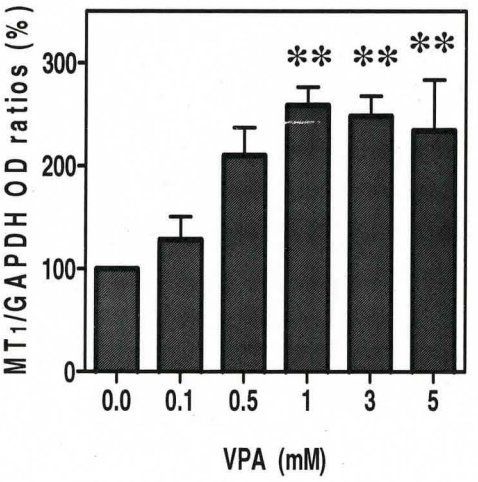


Figure 6: Dose-dependent effects of 6h or 24h VPA treatment on melatonin MT_1 receptor mRNA expression in MCF-7B cells grown and treated in 10% FBS. (**A**) Data shown are means ± S.E.M. (n = 2) for percentage (%) values of MT_1/GAPDH optical density (OD) ratios. (**B**) Data shown are means ± S.E.M. (n = 4) for percentage (%) values of MT_1/GAPDH optical density (OD) ratios. ***$p<0.05$ vs. control (Dunnett's Multiple Comparison Test). (**C**) Data shown are means ± S.E.M. (n = 3) for % values of MT_1/GAPDH OD ratios. ***$p<0.01$ vs. control (ANOVA and Neuman Keuls). (**A-C**) Representative gel images of RT-PCR detection of MT_1 (397 bp) and GAPDH (237 bp) mRNA. Lanes 1-6: Control, 0.1, 0.5, 1.0, 3.0 and 5.0 mM VPA.

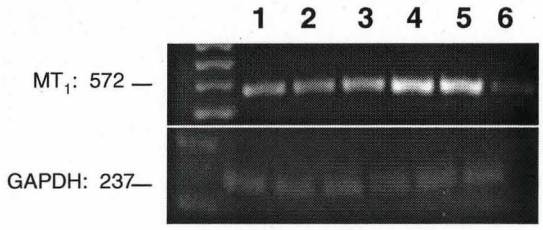
Fig. 6 **VPA Treatment on MCF-7B**

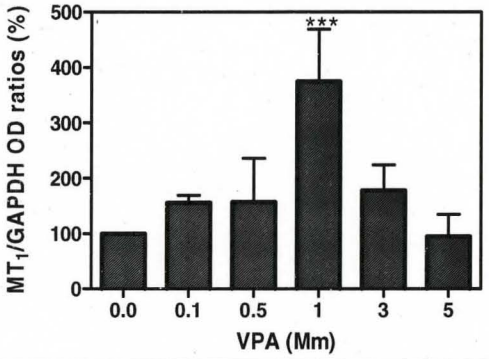
MT_1

***Media Type*: 10%FBS/DMEM**

6h

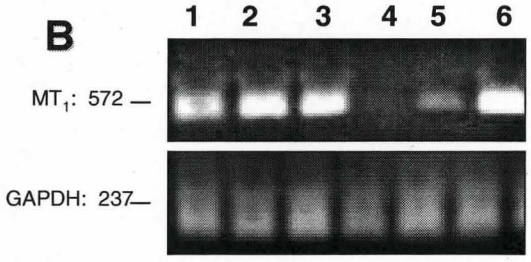
A

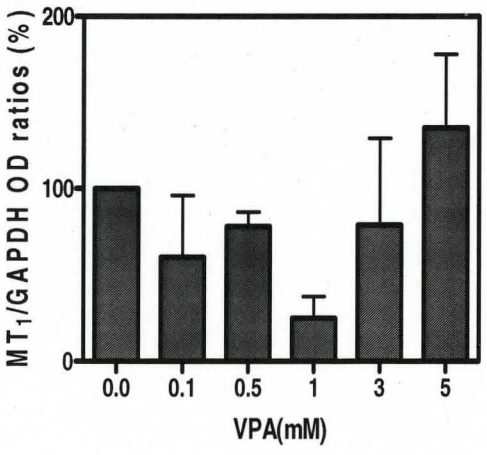




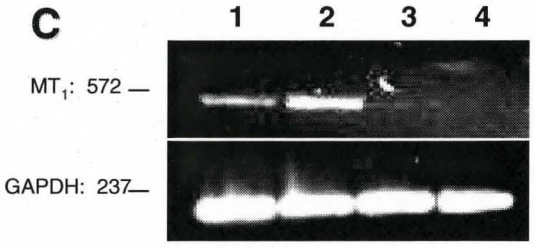
24h

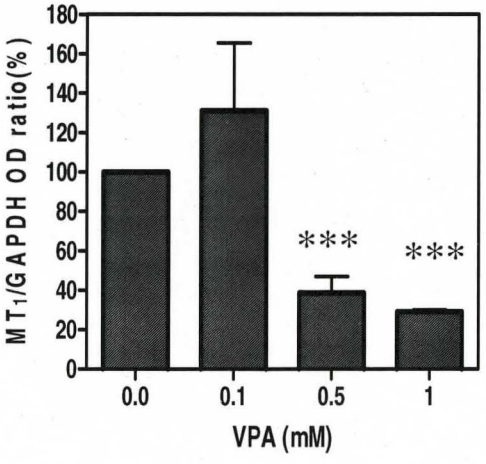
B





C





3.3: Effects of VPA Treatment on MT_1 protein in MCF-7B cells maintained in 10%FBS.

Treatment of MCF-7B cells with VPA for 24h caused a concentration-dependent increase of the melatonin MT_1 (37 kDa) receptor as shown in figure 7b, or a U- shaped dose response with induction at lower doses and inhibition at higher doses at 6h (Figure 7A).

3.4: Effects of VPA and/or Melatonin Treatment on p53 mRNA expression in MCF-7B cells.

3.4.1: Cells grown and treated in 1%FBS

Treatment of MCF-7B cells for 24h with doses of VPA (0.1-5.0 mM) suggested a decrease in p53 levels (Figure 8A). Additional replications of this single experiment are needed in order to carry out statistical analysis.

3.4.2: Cells grown and treated in 10%FBS

Treatment of MCF-7B cells with VPA (0.1, 0.5, 1.0, 3.0 and 5.0 mM) indicated that melatonin and p53 mRNA expression was decreased significantly ($F=15.54$, $p<0.001$) in cells exposed to 0.5 or 1.0 mM VPA for 24h (Figure 8C). After treatment for 6h, p53 mRNA appeared to be higher, but one-way ANOVA did not indicate any significant effects due to variance and low n value (Figure 8B). As seen in figure 9b, treatment of these cells for 24h with physiological doses of melatonin (1-10 nM) resulted in reduction of p53 mRNA levels. On the contrary, post hoc analysis by a Neuman-Keuls test indicated that p53 mRNA expression was increased significantly ($F=10.71$,

$p<0.0129$) in cells exposed for 6h to 10nM melatonin, when compared to control ($p<0.01$) or 1, 100 nM melatonin ($p<0.05$) (Figure 9A).

In addition, when both VPA and melatonin were added to cultures simultaneously at the start of treatment for 6h, there appeared to be marked increase of p53 mRNA expression for some combinations as shown in figure 10. This finding will be confirmed by replications of mRNA experiments and western blotting.

Figure 7: Regulation of the 37 kDa melatonin MT_1 receptor protein in MCF-7B cells by VPA after 6h or 24h. (**A**) Data shown are percentage (%) values of MT_1/ β-actin optical density (OD) ratios for a single experiment. Representative immunoblots of MT_1 (37 kDa) and β-actin (40 kDa) proteins. Lanes 1-6: Control, 0.1, 0.5, 1.0, 3.0 and 5.0 mM VPA. (**B**) Data shown are percentage (%) values of MT_1/ β-actin optical density (OD) ratios for a single experiment. Representative immunoblots of MT_1 (37 kD) and β-actin (40 kDa) proteins. Lanes 1-4: Control, 0.1, 0.5, and 1.0 mM VPA

Fig. 7

Melatonin Treatment on MCF-7B

MT_1

Media Type: 10%FBS/DMEM

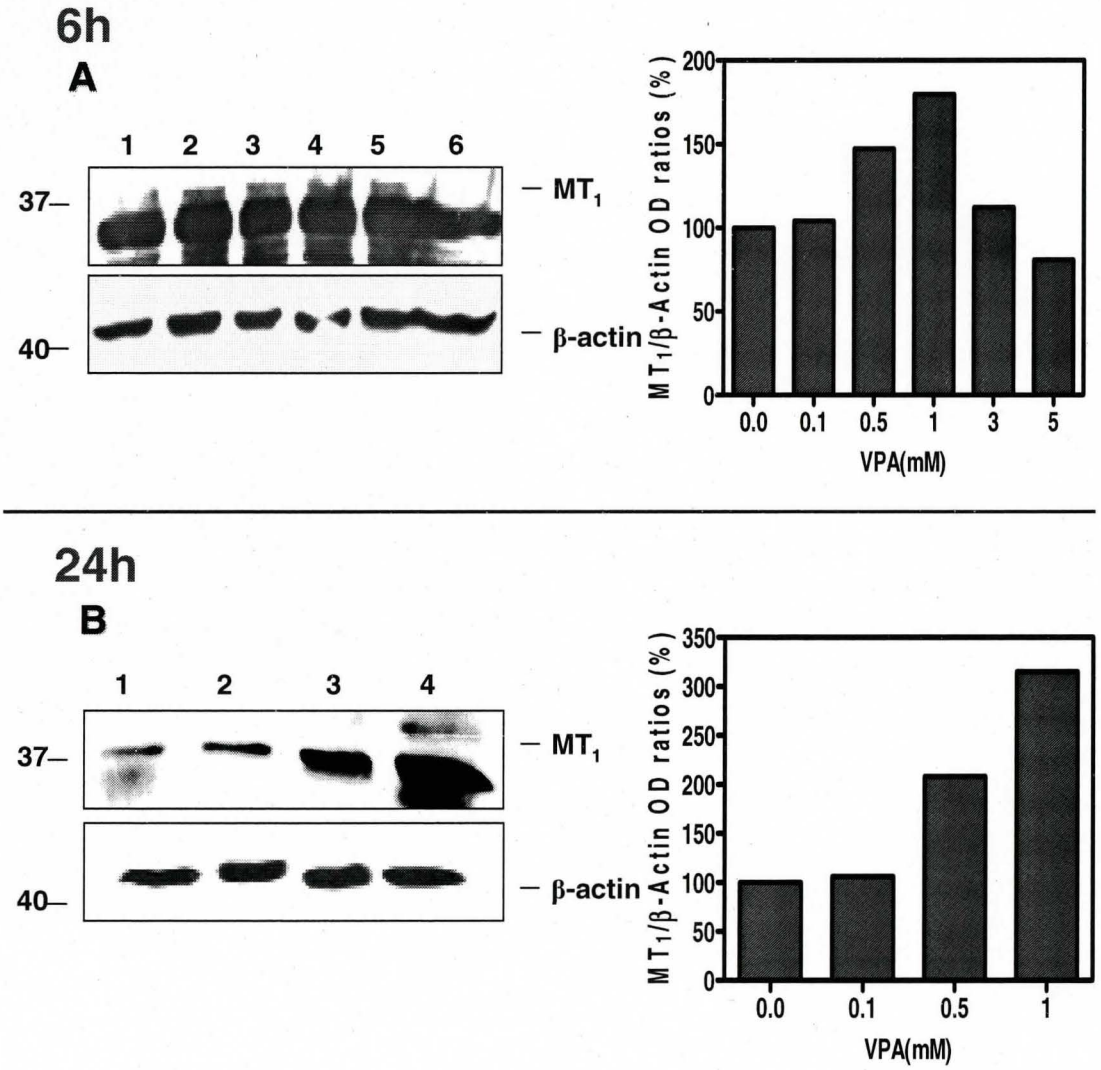


Figure 8: Regulation of p53 mRNA after VPA treatment for 6 or 24h. **(A)** Cell were grown in 1%FBS/DMEM. Data shown are for percentage (%) values of p53/GAPDH optical density (OD) ratios in a single experiment. (**B**) Cells were grown in 10%FBS/DMEM. Data shown are means ± S.E.M. (n = 2) for percentage (%) values of p53/GAPDH optical density (OD) ratios (**C**) Data shown are means ± S.E.M. (n = 3) for % values of p53/GAPDH OD ratios. ***$p<0.01$ vs. control (ANOVA and Neuman Keuls). (**A-C**) Representative gel images of RT-PCR detection of p53 (426 bp) and GAPDH (237 bp) mRNA. Lanes 1-6: Control, 0.1, 0.5, 1.0, 3.0 and 5.0 mM VPA.

Fig. 8 **VPA Treatment on MCF-7B**

p53

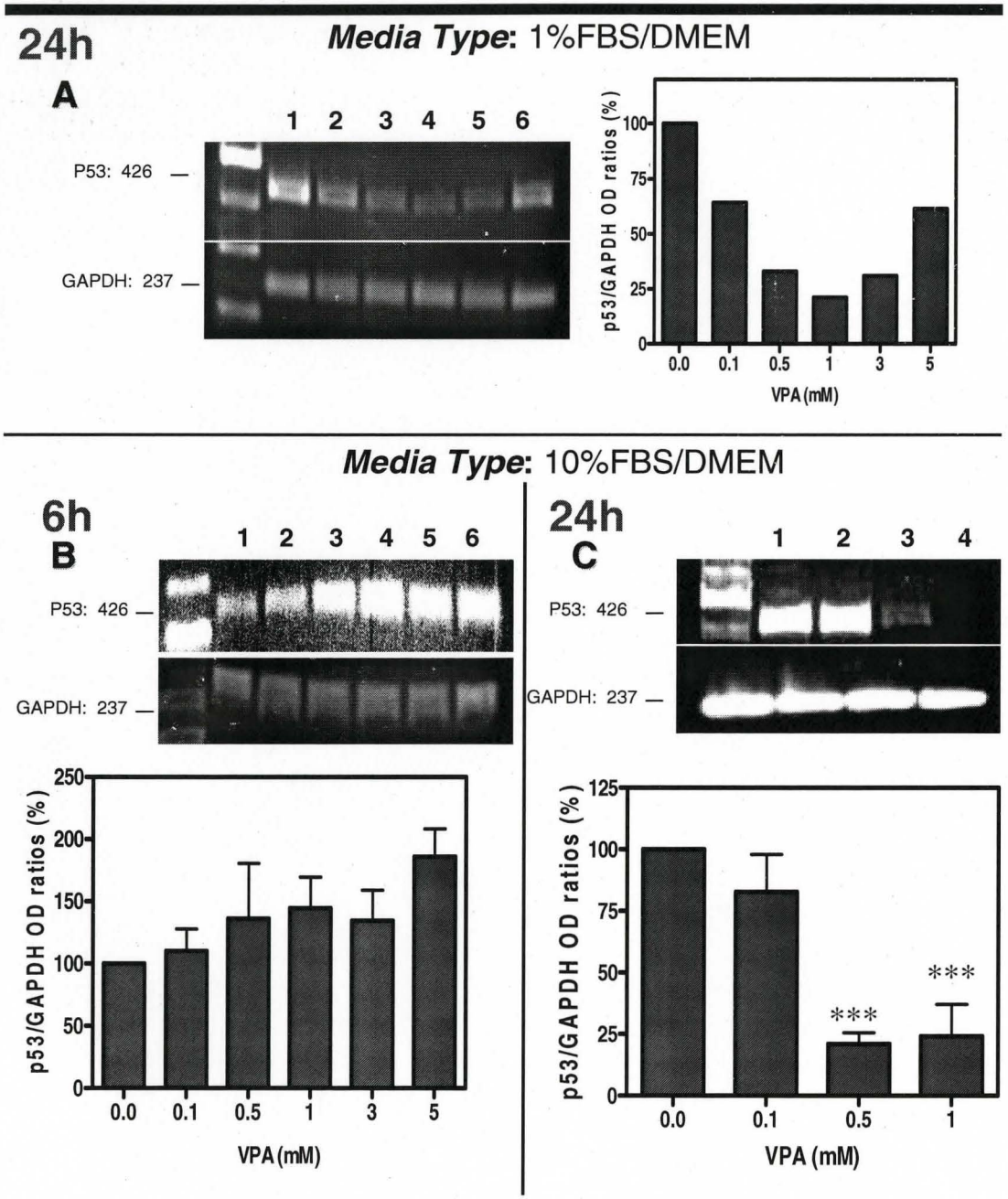


Figure 9: Modulation of p53 mRNA after melatonin treatment for 6 or 24h in 10%FBS/DMEM. (**A**) Data shown are means ± S.E.M. (n = 2) for percentage (%) values of p53/GAPDH optical density (OD) ratios. **p<0.01 vs. control; #p<0.05 vs. 1, 100 nM melatonin (**B**) Data shown are for percentage (%) values of p53/GAPDH optical density (OD) ratios in a single experiment. (**A-B**) Representative gel images of RT-PCR detection of p53 (426 bp) and GAPDH (237 bp) mRNA. Lanes 1-5: Control (DMSO), 0.1, 1, 10, and 100 nM melatonin.

Fig. 9

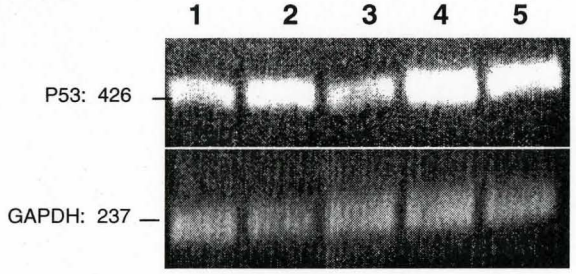
Melatonin Treatment on MCF-7B

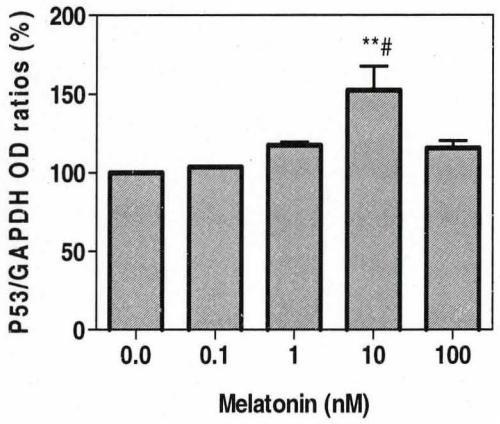
p53

***Media Type*: 10%FBS/DMEM**

6h

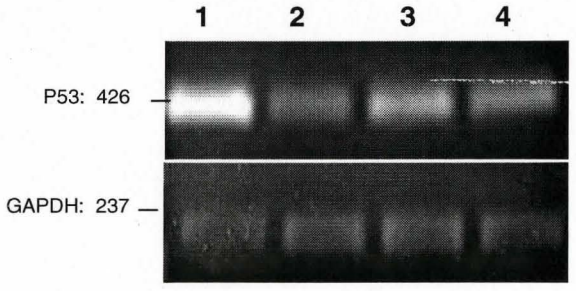
A





24h

B



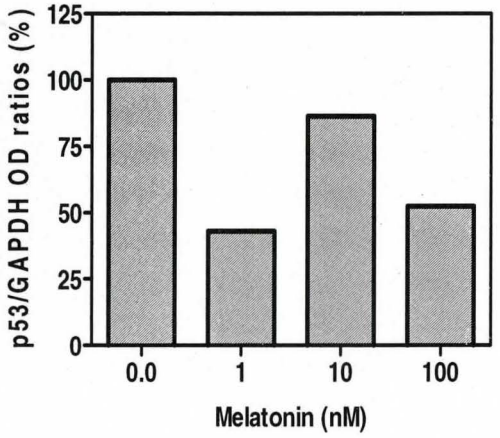


Figure 10: Effects of VPA + Melatonin on p53 mRNA expression after 6h of treatment in 10%FBS/DMEM. Cells were grown and treated in 10%FBS. Each combination was corrected with their appropriate DMSO/DMEM control. (**A**) Representative gel images of RT-PCR detection of p53 (426 bp) and GAPDH (237 bp) mRNA. (**B**) Data shown are for percentage (%) values of p53/GAPDH optical density (OD) ratios of a single experiment. Lanes 1-9: 0.5mM VPA+1nM melatonin, 0.5mM VPA+10nM melatonin, 0.5mM VPA+100nM melatonin, 1.0 mM VPA+1nM melatonin, 1.0 mM VPA+10nM melatonin,1.0 mM VPA+100nM melatonin, 3.0 mM VPA+1nM melatonin, 3.0 mM VPA+10nM melatonin, and 3.0 mM VPA+ 100nM melatonin.

Fig. 10

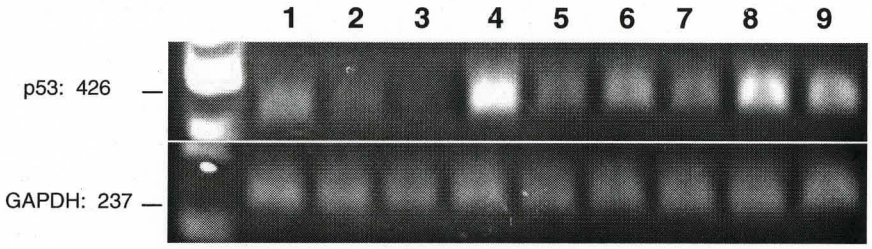
VPA + Melatonin Treatment on MCF-7B

p53

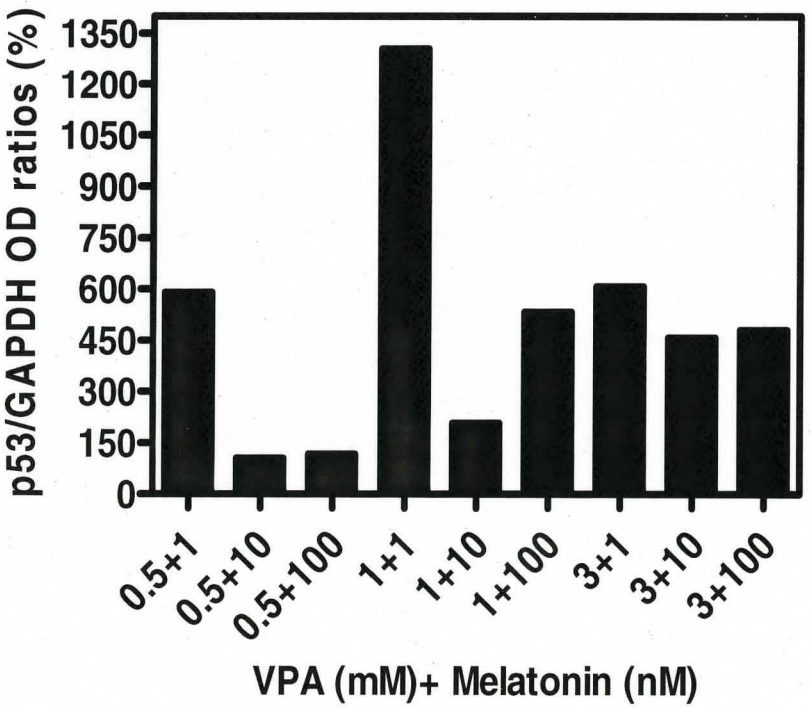
***Media Type*: 10%FBS/DMEM**

6h

A



B



3.5: Effects of VPA and/or Melatonin Treatment on ER-alpha mRNA expression in MCF-7B cells

3.5.1: Cells grown and treated in 1%FBS

Treatment of MCF-7B cells for 24h with doses of VPA (0.1-1 mM) suggested downregulation of ER-alpha mRNA at lower doses while at higher doses ER-alpha appears to show an increase (Figure 11A).

3.5.2: Cells grown and treated in 10%FBS

Post hoc analysis by a Neuman-Keuls test indicated that ER-alpha mRNA expression was decreased significantly ($F=23.17$, $p<0.0003$, $n=3$) in cells exposed to 0.5 mM VPA for 24h, when compared to control or 0.1 mM VPA ($p<0.001$). As seen in figure 11C, cells exposed to 1 mM VPA suggested significant downregulation of ER-alpha mRNA, when compared to control ($p<0.001$) or 0.1 mM VPA ($p<0.01$). In addition, one way ANOVA indicated significant effects after 6h of VPA treatment ($F=7.317$, $p<0.0074$, $n=2$). As seen in figure 11B, Newman-Keuls multiple test comparison indicated that the relative levels of ER-alpha mRNA was decreased significantly in cells exposed to 1 and 5 mM VPA for 6h, when compared to 0.5 mM VPA ($p<0.05$); 3 mM VPA treatment also resulted in significantly low levels of ER-alpha mRNA, when compared to 0.5, 1 ($p<0.05$) mM VPA.

On the contrary, when these cells were treated with physiological doses of melatonin (1-100 nM) for 24h, a possible increase of ER-alpha mRNA was observed (Figure 12B). Treatment of MCF-7B cells with 100 nM melatonin for 6h caused a

significant increase of ER-alpha mRNA expression, (ANOVA, $F=13.76$, $p<0.0042$, $n=3$). As seen in figure 12A, the relative levels of ER-alpha mRNA were significantly higher in cells treated with 100nM melatonin, compared to control and all other concentrations (Newman-Keuls, $p<0.01$).

As shown in figure 13, when both VPA and melatonin were added to cultures simultaneously at the start of treatment for 6h, there was a significant reduction of ER-alpha mRNA expression. Post hoc analysis showed significant effects (ANOVA, $p<0.0001$, $F=20.04$, $n=3$) with VPA (mM)/ melatonin (nM) combinations of 0.5/1 ($p<0.05$) or 0.5/10, 0.5/100, and 1/10 ($p<0$ 0.001) or 1/100 ($p<0.01$).

Figure 11: Regulation of ER-alpha mRNA after VPA treatment for 6 or 24h. (**A**) Cells were grown and treated in 1%FBS/DMEM for 24h. Data shown are for percentage (%) values of ER-alpha/GAPDH optical density (OD) ratios in a single experiment. (**B**) Cells were grown and treated in 10%FBS/DMEM for 6h. Data shown are means ± S.E.M. (n = 3) for percentage (%) values of ER-alpha/GAPDH optical density (OD) ratios. **p<0.05 vs. control, 0.1, 0.5 mM VPA; #p<0.001 vs. 0.5mM VPA; ***p<0.01 vs. 0.5mM VPA (ANOVA and Neuman Keuls). (**C**) Cells were grown and treated in 10%FBS/DMEM for 24h. Data shown are means ± S.E.M. (n = 3) for % values of ER-alpha/GAPDH OD ratios. ***p<0.001 vs. control, 0.1mM VPA; #p<0.01 vs. 0.1. (ANOVA and Neuman Keuls). (**A-C**)Representative gel images of RT-PCR detection of ER-alpha (629 bp) and GAPDH (237 bp) mRNA. Lanes 1-6: Control, 0.1, 0.5, 1.0, 3.0 and 5.0 mM VPA.

Fig. 11 **VPA Treatment on MCF-7B**

ER-alpha

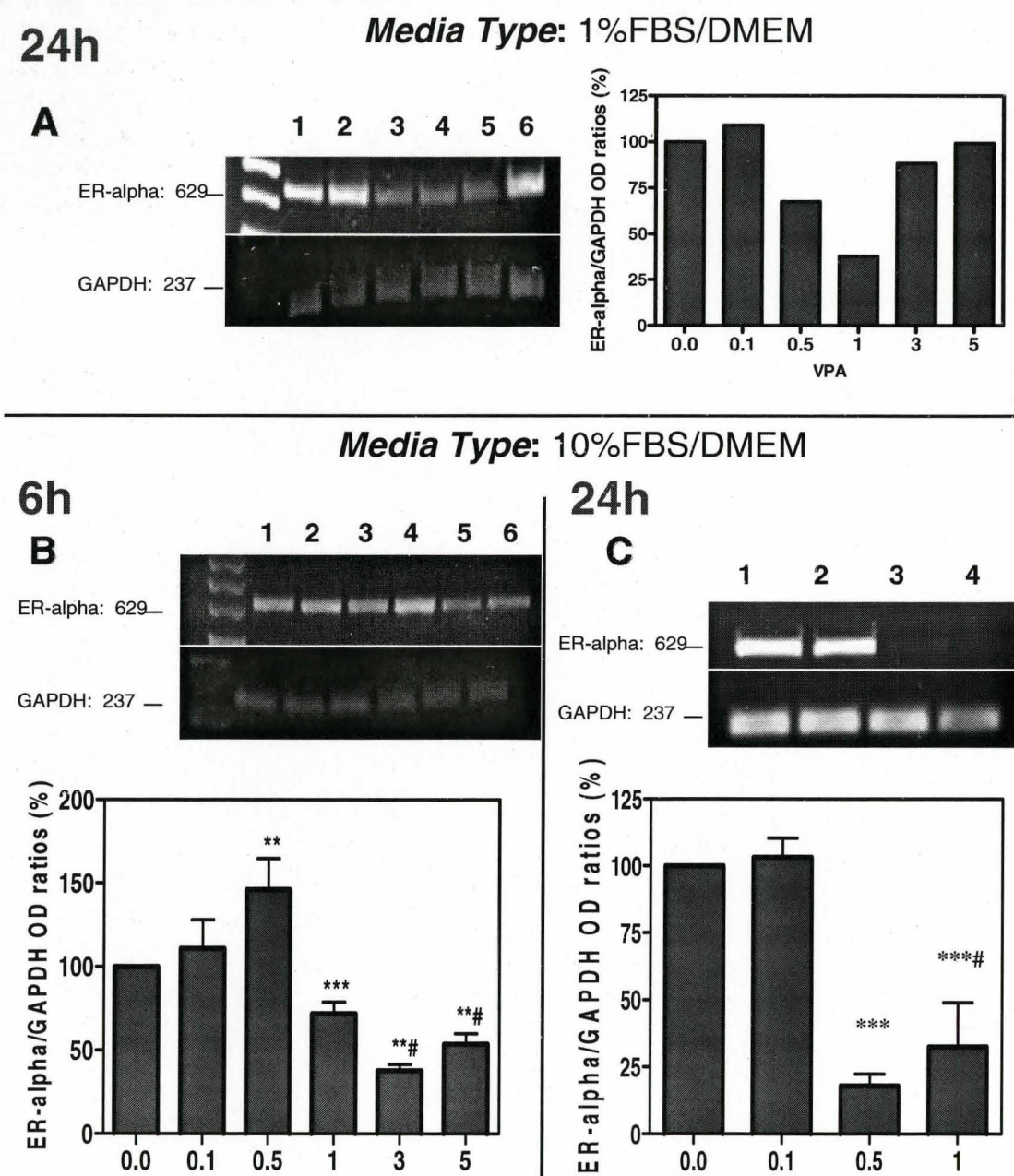


Figure 12: Modulation of ER-alpha mRNA after melatonin treatment for 6 or 24h. (**A**) Cells were grown and treated in 10%FBS/DMEM for 6h. Data shown are means ± S.E.M. (n = 3) for percentage (%) values of ER-alpha/GAPDH optical density (OD) ratios. ***$p<0.05$ vs. control, 1, 10 nM melatonin; # $p<0.01$ vs. 10 nM melatonin (ANOVA and Neuman Keuls). (**B**) Cells were grown and treated in 10%FBS/DMEM for 24h. Data shown are for percentage (%) values of ER-alpha/GAPDH optical density (OD) ratios in a single experiment. Lanes 1-4: Control (DMSO), 1, 10, and 100 nM melatonin.

Fig. 12

Melatonin Treatment on MCF-7B

ER-alpha

***Media Type*: 10%FBS/DMEM**

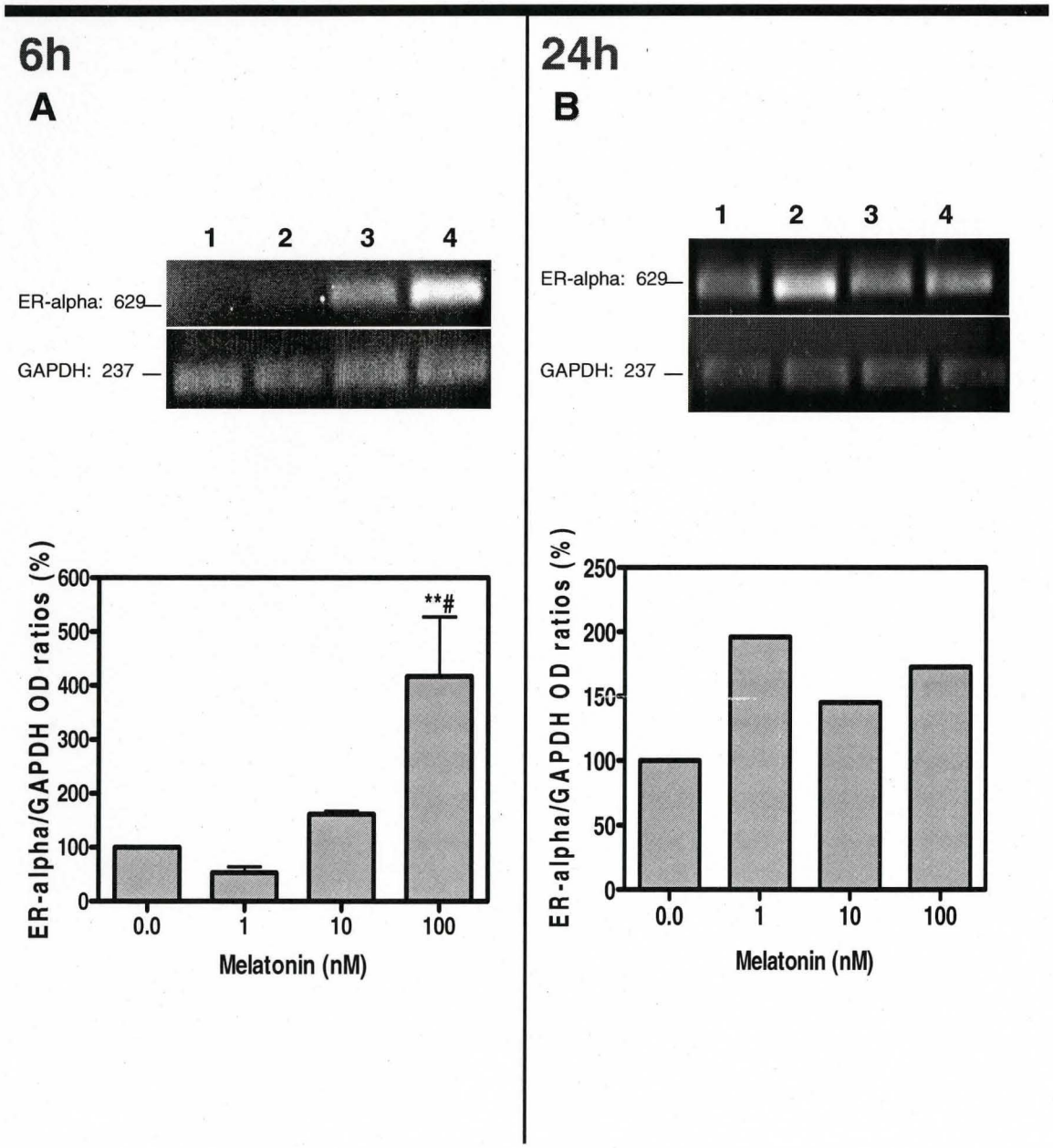


Figure 13: Effects of VPA + Melatonin on ER-alpha mRNA expression after 6h of treatment in 10%FBS/DMEM. Cells were grown and treated in 10%FBS/DMEM. Each combination was corrected with its appropriate DMSO/DMEM control. (**A**) Representative gel images of RT-PCR detection of ER-alpha (629bp) and GAPDH (237bp) mRNA. (**B**) Data shown are means ± S.E.M. (n = 3) for percentage (%) values of ER-alpha/GAPDH optical density (OD) ratios *p<0.05 vs. control; **p<0.01 vs. control; ***p<0.001 vs. control (ANOVA and Neuman Keuls). Lanes 1-9: 0.5 mM VPA+1 nM melatonin, 0.5 mM VPA+10 nM melatonin, 0.5 mM VPA+100 nM melatonin, 1.0 mM VPA+1 nM melatonin, 1 mM VPA+10 nM melatonin,1 mM VPA+100 nM melatonin, 3 mM VPA+1 nM melatonin, 3 mM VPA+10 nM melatonin, and 3 mM VPA+ 100 nM melatonin.

Fig. 13

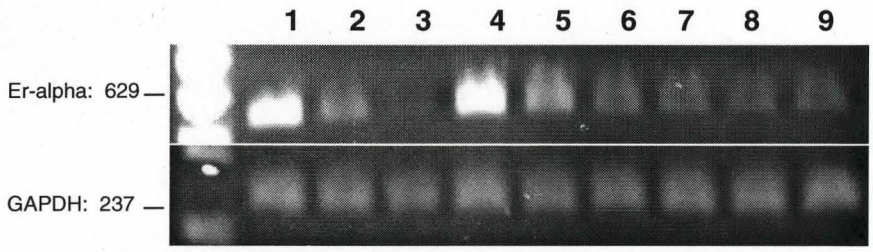
VPA + Melatonin Treatment on MCF-7B

ER-alpha

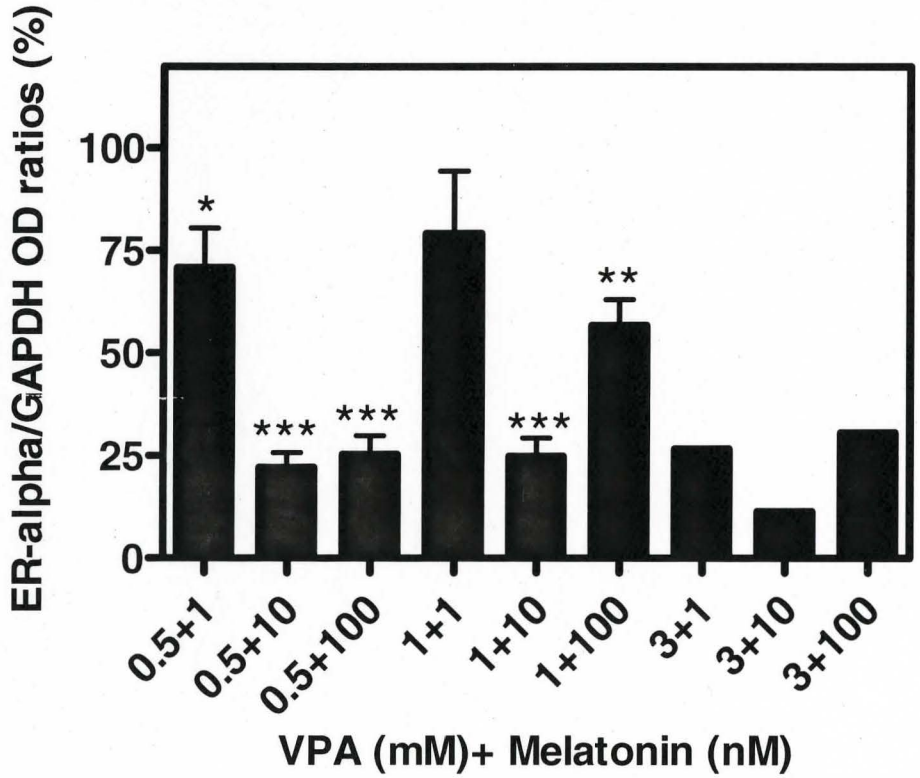
***Media Type*: 10%FBS/DMEM**

6h

A



B



Sections 4: DISCUSSION AND CONCLUSIONS

4.1: VPA enhances melatonin receptor MT_1 receptor expression in MCF-7B cells

An interaction between cellular estrogen response and melatonin signaling mediated by G-protein coupled receptors is present in breast cancer cells. A major mechanism through which melatonin reduces the development of breast cancer is based on its anti-estrogenic actions by interfering at different levels with the estrogen-signaling pathways. Melatonin inhibits both aromatase activity and expression in *vitro* (MCF-7 cells) as well as in *vivo*, thus behaving as a selective estrogen enzyme modulator. In recent studies where MT_1 was transfected into MCF-7cells, melatonin treatment inhibited aromatase mRNA expression and 1nM melatonin induced a higher and significant down-regulation of aromatase mRNA expression than in vector-transfected cells (Gonzalez et al., 2007). These findings suggest that the MT_1 receptor signaling pathway is a major mediator of the oncostatic effect of melatonin in breast cancer.

We have reported that valproic acid upregulates melatonin MT_1 receptor expression in rat C6 glioma cells (Castro et al., 2005). In addition to its anticonvulsant and mood stabilizing properties, valproic acid can also inhibit the growth of cancer cells (Jawed et al., 2007; Blaheta et al., 2002). Since the melatonin MT_1 receptor has been implicated in the oncostatic action of melatonin on human MCF-7 breast cancer cells, the effect of valproic acid on its expression was examined in this cell line. Findings suggest that VPA for 24h caused a concentration-dependent upregulation of melatonin MT_1 receptor mRNA expression in MCF-7A cells cultured in 1%FBS/DMEM as seen by nested RT-PCR (Jawed et al., 2007). MCF-7B cells express higher basal levels of the

melatonin MT_1 receptor, as seen from the standard RT-PCR. Therefore, it was expected that VPA would also modulate the expression of this receptor subtype in these human breast cancer cells cultured in 1%FBS/DMEM. This view is supported by the VPA concentration-dependent increase in melatonin MT_1 receptor mRNA at 24h and 72h as seen in figure 5. In contrast, when the same sub-line was cultured in 10%FBS/DMEM, we observed a significant inhibition of melatonin MT_1 receptor mRNA at 0.5 and 1mM VPA as shown in figure 6 (B-C).

The opposite effects of 24h treatment with VPA on MT_1 gene expression in 10% FBS as compared with 1%FBS may involve the effects of various hormones and growth factors such as EGF, FGF that are present in the enriched 10%FBS medium. The ERKs are strongly activated by mitogenic and growth factors and by physical stress, whereas p38-MAPK can be activated by various cell stresses, such as metabolic stress or protein synthesis.

Recent findings suggest that treatment of C6 glioma cells with VPA significantly upregulated the melatonin MT_1 receptor. This effect of VPA was enhanced with the addition of PD98059, suggesting a role for the MAPK/ERK pathway in the negative regulation of the melatonin MT_1 receptor (Castro et al., 2005). Since MAPK/ERK pathway is known to be involved in cell proliferation in response to various growth factors and VPA activates the MAPK/ERK pathway, which is implicated in diverse cellular functions (Yuan et al., 2001; Hao et al., 2004), we examined the potential involvement of this signaling cascade in the regulation of the melatonin MT_1 levels. In the one experiment that was conducted, PD98059 appeared to not reverse the effect of

VPA in MCF-7 cells after a 24h treatment (Data not shown). However, more experimental replicates and time-dependant studies need to be done in order to conclude about the involvement of MAPK/ERK pathway in modulation of the melatonin MT_1 receptor by VPA. Future studies will utilize the MAPK/ERK inhibitor, PD98059, to investigate its effects on the melatonin MT_1 mRNA and/or protein.

There is now substantial evidence that VPA can modify gene expression via its ability to inhibit histone deacetylation and DNA methylation (Phiel et al., 2001; Detich et al., 2003; Li et al., 2005). We have reported that VPA, at concentrations which upregulate the melatonin MT_1 receptor in C6 cells, alters HDAC mRNA expression in a manner which is consistent with the inhibition of HDAC enzyme activity (Castro et al., 2005). The possibility that this epigenetic regulation of gene transcription is involved in modulation of the melatonin MT_1 receptor, as observed with human MCF-7 breast cancer cells, awaits further study.

Earlier findings suggest that after 24h of VPA treatment of MCF-7A cells in 1%FBS culture medium, there is a significant dose-dependent induction of MT_1 receptor (Jawed et al., 2007). As seen in figure 6B, VPA treatment for 24h in 10% FBS culture medium results in a pronounced downregulation of MT_1 mRNA at clinical doses and a sudden increase at higher doses. These findings suggest that the enriched cell culture condition used may have changed the time-dependent effects of VPA. Therefore, we decided to study modulation of MT_1 mRNA after treatment with VPA for 6h. Findings suggest that there is a significant induction of MT_1 mRNA when cells were exposed to 1mM VPA, the same dose that caused a significant inhibition of MT_1 mRNA at the 24h

time point. We postulated that the MT_1 protein levels after treatment with clinical doses of VPA maybe still elevated at 24h while the mRNA levels are declining. This view is supported by western blot findings which suggest that 24h of VPA treatment in 10%FBS results in elevated levels of MT_1 protein. Taken together, we have for the first time shown that VPA, an HDAC inhibitor, modulates MT_1 melatonin receptor expression in the MCF-7 breast cancer cell line.

4.2: Combined exposure of MCF-7 Cells to VPA and melatonin is associated with synergistic inhibition

Oncogenic events are marked by uncontrolled cell proliferation. Today's major focus of cancer research is to identify treatments that reduce or inhibit cell growth. Over the years, various compounds, both naturally occurring and chemically synthesized, have been used to inhibit invasive cell proliferation. Three such oncostatic agents, melatonin, VPA, and retinoic acid (RA), have been shown to suppress the growth of hormone-responsive breast cancer. Recent findings suggest that a sequential treatment approach with melatonin and retinoic acid results in the complete cessation of cell growth of MCF-7 cells (Eck et al., 1998). In another study, combined use of the HDAC inhibitor m-carboxycinnamic acid bis-hydroxamide (CBHA) with RA produced a promising synergistically inhibitory effect on the growth of human neuroblastomas xenografts in *vivo* (Coffey et al., 2001).

In the present study, we observed the inhibition of MCF-7 cell growth by VPA which is consistent with increasing evidence that this drug is a potent oncostatic agent (Xue et al., 2005). Although melatonin has been reported to inhibit the proliferation of

MCF-7 cells (Ram et al., 2002), it was only modestly effective in these breast cancer cells, as determined by MTT analysis. However, a combination of VPA and melatonin caused a significant inhibition of cell proliferation, which was greater than that seen with either VPA or melatonin alone (Figure 3). Various MCF-7 cell stocks have been reported to exhibit significant differences in their sensitivity to the antiproliferative effect of melatonin, which may be correlated with the degree of estrogen-responsiveness (Ram, 2000) or the ERα/ERβ ratio (del Rio et al., 2004). Although, the inhibition of cell proliferation by melatonin in this MCF-7 stock, maintained and treated in 10%FBS/DMEM medium condition, was relatively weak, the modest sensitivity of these cells to melatonin appears to have been sufficient to allow the synergistic antiproliferative effect seen with this hormone in combination with VPA.

It is known that when growth factors bind to their transmembrane receptors, there is autophosphorylation of the receptor. A sequence of events occurs which results in the activation of Ras followed by MAPK pathway activation with its components Raf and MEK and the activation of the PI-3 kinase pathway with its downstream components: serine/threonine protein kinase Akt, and mammalian target of rapamycin (mTOR). At the same time, estradiol binds to the estrogen receptor (ER) and initiates transcription in the nucleus and as a result there is increased proliferation of breast cancer cells (Santen et al., 2005). On the contrary, MAPKs, such as p38, are phosphorylated by MAPK kinases (MAPKKS). The MAPKKs are themselves activated and phosphorylated by the MAPKK kinases (MAPKKKs). Finally, the MAPKKKs are regulated by cell surface receptors or other external stimulus. The substrates of this p38 kinase include transcription regulators:

ATF2, MEF2C, and MAX, cell cycle regulator CDC25B, and tumor suppressor p53, which suggest the roles of this kinase in cell cycle (Caiazza et al., 2007).

Currently, there have been no findings on the effects of p38 pathway on modulation of gene expression by VPA in MCF-7 cells. Recent findings suggest that melatonin's effect on breast cancer cell invasion is mediated, at least in part, through the G-protein-coupled MT_1 melatonin receptor. Moreover, melatonin appears to exert its anti-invasive actions through down-regulation of the p38 MAPK signaling pathway (Mao et al., 2007). Studies awaits to investigate the molecular mechanisms responsible for this enhanced inhibition, as a consequence of combinatorial treatment with VPA and melatonin, which maybe finely tuned, controlled, and exerted by multiple signals on different molecular events by both oncostatic agents.

4.3: Sequential exposure of MCF-7B Cells to VPA and melatonin results in enhanced induction of p53 and a pronounced downregulation of ER-α

Since we see an inhibition of MCF7-B cells after VPA and melatonin treatment alone and in combination, we decided to explore the mechanisms behind such inhibitory effects. Studies have shown that the antiproliferative effects of VPA on various tumour cell lines involves apoptosis and interfering with the estrogen signaling pathway (Kawagoe et al., 2002; Takai et al., 2004; Reid et al., 2005). Therefore, our goal was to study the effects of VPA and melatonin alone and in combination on expression of oncostatic genes such as the tumour suppressor p53.

The p53 protein plays a key role in the G1/S checkpoint in response to DNA damage as a regulator of cell cycle progression and a mediator of apoptosis in many cell

lines. In response to various types of DNA damage, the cell cycle checkpoints, and cell death signals are activated to stop cell growth and to eliminate multiplication of the genetically-altered cells. Damaged cells stop DNA replication at the G1 or G2 phase, presumably allowing the repair systems to function before the next cell cycle. Apoptosis is also triggered in response to various DNA damage. The activation of the apoptotic cell death pathway is a safeguard in removing irreparably damaged cells. Several cellular effector molecules, including p53, are involved in arresting damaged cells at these checkpoints and inducing apoptosis.

As seen in figure 8 (A and C) , we see a decline in p53 gene expression after 24h of VPA treatment regardless of the culture medium used. However, as seen in figure 4, treatment of MCF-7 cells with VPA (1.0 mM) for 24h, resulted in morphological changes, such as nuclear condensation, which are indicative of apoptosis. It is possible that: (1) the genes responsible for cell death such as p53 were elevated and active prior to 24h since we have observed a significant dose-dependent inhibition of MCF-7 cell growth after the combinatorial treatment at 24h in 10%FBS/DMEM. (2) There may be cell cycle transcriptional inhibition of other genes (e.g. cyclin D, ER-alpha) at 24h that is responsible for the antiproliferative effects of melatonin and VPA (Cini et al., 2005; Kaiser et al., 2006). Consistent with this, preliminary results with 6h of VPA treatment suggests a dose dependent up-regulation of p53 gene that might plausibly contribute to marked increase in apoptosis (Figure 8b).

As seen in figure 9a, we show that physiological doses of melatonin result in a significant upregulation of p53 mRNA expression after 6h. Studies suggest that 48h of

melatonin treatment causes a significant increase in the expression of p53 as well as p21WAF1 proteins (Mediavilla et al., 1999). While the literature suggests an upregulation of p53 protein after VPA or melatonin treatment alone, we have the evidence for the first time that the p53 mRNA is significantly upregulated after VPA or melatonin treatment alone at 6h. As seen in figure 10, an induction of p53 mRNA is suggested, but replicates are needed to conclude the effects of combinatorial approach on p53 mRNA levels. These results suggest that the combined exposure might have played a role in the strong effects of this combination. For example, combined exposure of myeloma cells to proteosome inhibitor bortezomib/HDAC inhibitors induced activation of the stress-related kinase JNK, which has been associated with promotion of mitochondrial injury and apoptosis (Pei et al., 2004).

To test a second possibility for the observed synergistic inhibition of these cancer cells, we studied ER-alpha. Consistent with earlier findings, we demonstrate that VPA significantly suppresses expression of the mRNA encoding ER-alpha (Figure 11C) in a dose-dependent manner. It is interesting to note while the earlier findings suggest a significant fall off for ER-alpha protein at 6-10mM VPA in MCF-7 cells by 24h, we have observed a significant decline of ER-alpha mRNA at clinical doses of VPA (0.5-1mM) after 24h of treatment in MCF-7 cells. In addition, we see ER-alpha mRNA significantly declining after 1-5mM of VPA treatment for 6h (Figure 11B). Loss of ER-alpha is not restricted to MCF-7 cells as 16h of treatment with 6mM VPA also clears ER-alpha from the breast carcinoma cell lines: T47D and ZR75 and from the ovarian cell line PEO4 (Srinivasan et.al., 2007; Molis et al., 1994; Molis et al., 1995; Rato et al., 1999). Studies

suggest that this decrease is not dependent upon the presence of estrogen since similar decreases in steady state ER mRNA levels were seen in MCF-7 cells cultured in both complete and estrogen-depleted media (Molis et al., 1994).

Findings suggest that simultaneous addition of the proteasome inhibitor MG132 (10 mM) prevented VPA and TSA induced loss of ER-alpha, indicating that the observed degradation of ER-alpha protein is dependent on the activity of the proteasome (Molis et al., 1994). Quantitative RT–PCR demonstrated that VPA rapidly reduced the steady-state level of ER-alpha mRNA in MCF-7 cells to approximately 15% of control values. Presumably, proteasome mediated turnover then remove existing ER- alpha (Molis et al., 1994).

In contrast, melatonin (100nM) treatment resulted in upregulation of ER-alpha mRNA after 6h (Figure 12A). This finding is not consistent with published findings where melatonin (1-10 nM) is reported to reduce to levels of ER- alpha mRNA after 24h. It is possible that this upregulation is due to melatonin antagonizing the calcium dependent activity of calmodulin. As a consequence of this blockade, there is a conformational change in calmodulin that results in destabilizing the E2/ER/calmodulin complex and inhibition of ER-mediated transcription. Calmodulin is known to stabilize the ER receptor against proteolysis (Figure 14). Therefore, by inhibiting calmodulin, there is possibly more proteolysis of the ER. Taken together, we postulate that the observed increase of ER-alpha mRNA might be a compensatory mechanism used by the cells to make up for the decreasing levels of ER proteins. Future studies will check levels of ER protein to confirm this. It is noteworthy to mention that differential regulation of

ER-alpha by melatonin is mediated via different G proteins (Kiefer et al., 2005). However, when the combinatorial approach was used to test melatonin's effect after 6h, several combinations suggested induced inhibition of ER- alpha mRNA (Figure 13).

Recent data suggest that the main mechanism of TSA–induced cell death in androgen receptor (AR)-positive prostate cancer is inhibition of AR gene expression. The synergistic effect of simultaneous treatment with TSA and doxorubicin, a chemotherapeutic agent that targets DNA topoisomerase II enzyme activity and causes DNA breaks, is mediated via inhibition of AR expression, induction of protease activity and proteolysis of p21 (Rokhni et al., 2006). It is interesting to note that trichostatin A and doxorubicin when used separately induced p21 expression, but under simultaneous treatment TSA and doxorubic inhibited the expression of p21, and this effect was caspase independent (Rokhni et al., 2006).

Recent studies from our lab suggest that melatonin, like VPA, acts as a HDAC inhibitor by causing hyperacetylation of H3 (Sharma et. al., 2007). The present pronounced interaction between these HDAC inhibitors, VPA and melatonin, provide further support for the notion that combination treatments using these regimens may trigger cell death through multiple interacting mechanisms, including increasing p53 expression and disabling of the estrogen signaling pathway.

Figure 14: Potential direct and indirect actions of melatonin. (**Direct Actions**) Melatonin can act directly on Protein Kinase C (PKC) to activate Ras/ Raf/ MAPK/ ERK pathway. This hormone can directly act on calcium binding calmodulin and antagonize its effects. (**Indirect Actions**) The physiological effects of melatonin are mediated by high affinity G_i protein coupled receptors MT_1 and MT_2. In MCF-7 cells, it is thought that oncostatic effects of melatonin are mediated by MT_1 receptor. $G_{i\alpha}$ protein is coupled to inhibition of adenylyl cyclase (AC) and decrease in 3'5' cyclic monophosphate (cAMP). $G_{q\alpha}$ activate phospholipase C (PLC). PLC hydrolyses phosphatidylinositol bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP_3), leading to changes in intracellular Ca^{2+} levels.

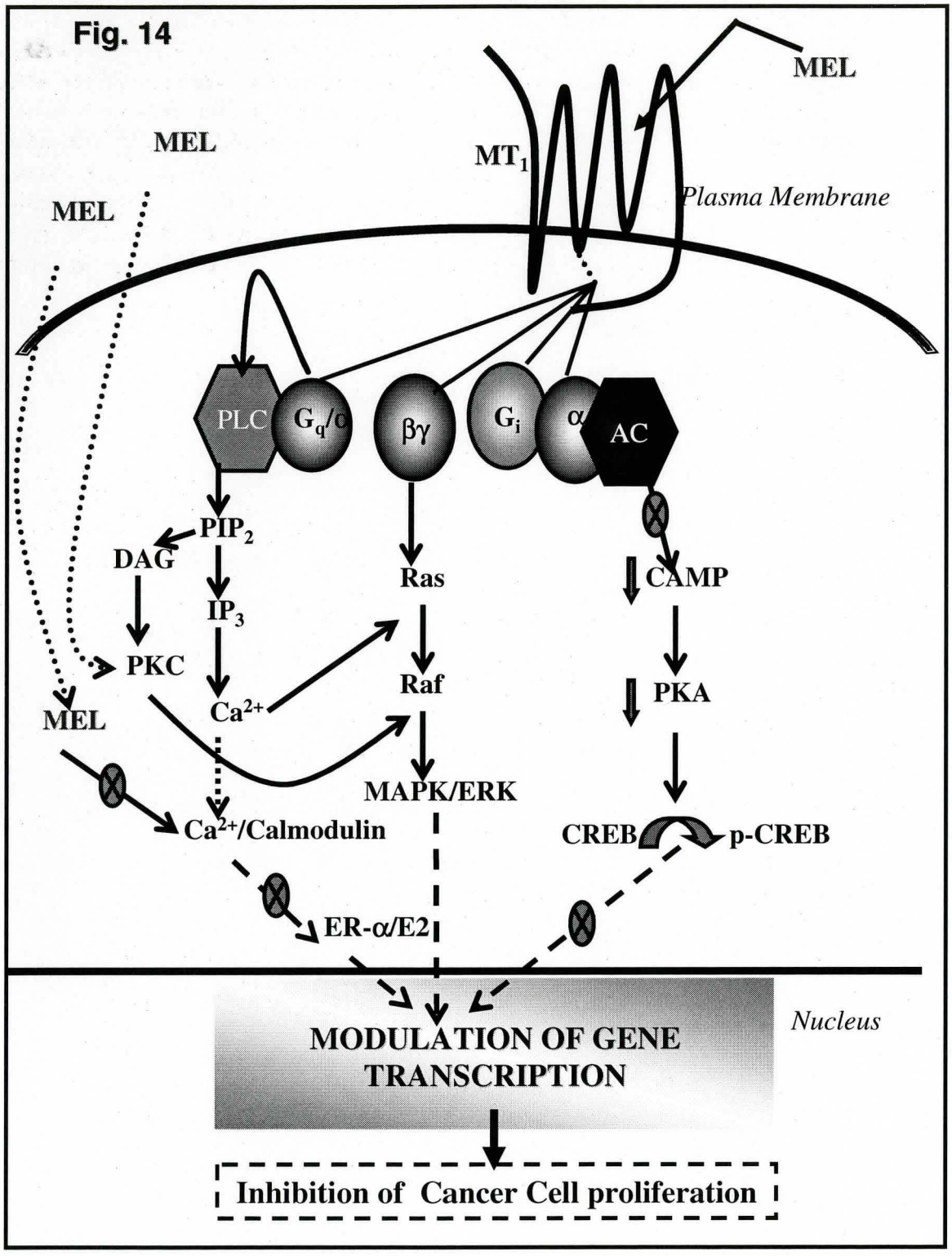


Fig. 14
MEL
MEL
MEL
MEL
MT1
Plasma Membrane
PLC
Gq/G
βγ
Gi
α
AC
PIP2
DAG
IP3
Ras
cAMP
PKC
Raf
PKA
Ca2+
MEL
MAPK/ERK
Ca2+/Calmodulin
CREB
p-CREB
ER-α/E2
Nucleus
MODULATION OF GENE TRANSCRIPTION
Inhibition of Cancer Cell proliferation

4.4: Future Directions and Concluding Remarks

Since MCF-7 cells express MT_1 but not MT_2, luzindole, a non-selective blocker for melatonin receptors, will be utilized in the future to determine whether the MT_1 receptor is involved in the antiproliferative effects of melatonin. Recent studies suggest that melatonin acts via the MT_1 to inhibit aromatase (P450) activity and as a result causes MCF-7 cell growth inhibition. In addition, VPA, when combined with aromatase inhibitor, letrozole, enhances the effects seen by either agent alone. Therefore, P450 expression will also be examined following combinational treatment with VPA and melatonin. Two different TSA induced pathways may result in AR protein degradation: at the protein level (destabilization/proteolysis) or at the gene level (direct or indirect inhibition of the AR gene expression) (Rokhlin et al., 2006). Since we have seen inhibition of ER-alpha mRNA, future studies will focus on studying protein levels using western blotting.

Although the precise mechanisms by which apoptosis is triggered in these breast cells remain elusive, it is believed that healthy cells constitutively express the molecules required for their own destruction and that the function of cell death genes is to activate these molecules. It is possible that cell death genes which are normally silenced in hypoacetylated form, where, histone deacetylase activity is high, and these areas may be rendered more accessible to transcription factors by the suppression of deacetylation (Weil et al., 1996). It is therefore likely that histone hyperacetylation facilitates apoptosis by increasing the expression of one or more cell death genes. Since both VPA and melatonin can inhibit HDAC activity, future experiments focusing on HDAC activity and

histone acetylation will help clarify the mechanisms involved in the striking effects seen by the combination approach. Findings suggest that HDAC inhibitors, butyrate and TSA, are capable of inducing growth factor unresponsiveness in colon cancer suggesting that strategies targeting tumor cells with hyperacetylating agents could provide a means for inhibiting carcinogenesis (Wu et al., 2001).

One of the major pathways for caspase activation involves the participation of mitochondria. Bcl-2 inhibits the apoptotic process and promotes cell survival, and Bax acts in the mitochondria to cause the release of cytochrome c, leading to the activation of caspase-9, and the subsequent activation of caspase-3. Moreover, Bax expression is regulated by p53 and the protein products of the target genes of p53, including Bcl-2, are involved in this process. More recently, it has been discovered that in response to apoptotic stimuli, mitochondria can also release caspase independent cell death effectors such as apoptosis inducing factor (AIF). AIF is known to recruit or activate an endonuclease to facilitate DNA fragmentation and chromatin condensation.

It is possible that inhibition of MCF-7 cells from the combinatorial approach maybe associated with multiple perturbations in cell cycle and apoptotic regulatory proteins, including degradation and/ or cleavage of Bid, Bcl-2, $p21^{WAF1/CIP1}$, $p27^{KIP1}$, cyclin D1, and acetylation of nonhistone proteins (e.g., Hsp90) resulting in interruption of survival signal transduction pathways. Further studies focusing on effects of VPA and/or melatonin on the expression of these proteins will allow a better understanding of the multiple pathways that maybe involved in the enhanced effects seen by the combinatorial approach.

In this study we have demonstrated that VPA inhibits cell growth and appeared to induce apoptosis in MCF-7 cells. We also demonstrated that modest sensitivity of melatonin towards these cells is enough to cause synergistic cell death when this hormone is combined with VPA. These results clearly define the need for additional in depth studies to dissect the cellular events leading to VPA-melatonin induced growth inhibition in breast tumor cells. As seen in figure 15, we have proposed a model that suggests involvement of cellular/molecular events such as activation of the p53 tumor suppressor gene apoptotic pathway and interference with the ER-alpha receptor signaling pathway.

Figure 15: Potential signaling pathways of VPA and/or melatonin. The physiological effects of melatonin are mediated by high affinity G_i protein coupled receptors MT_1 and MT_2. In MCF-7 cells, it is thought that oncostatic effects of melatonin are mediated by MT_1 receptor. Although mechanisms underlying the upregulation of MT_1 receptor awaits further study, the possible mechanisms we would like to investigate in the future includes the activation of MAPK ERK pathway and the inhibition of histone deacetylase activity. G protein is coupled to inhibition of adenylyl cyclase (AC) and decrease in 3'5' cyclic monophosphate (cAMP) and also modulation of phospholipase C (PLC). PLC hydrolyses phosphatidylinositol bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP_3), and lead to changes in intracellular Ca^{2+} levels. Both VPA and melatonin are known oncostatic agents that may work via multiple signaling pathways to cause the synergistic inhibition of estrogen positive breast cancer cells.

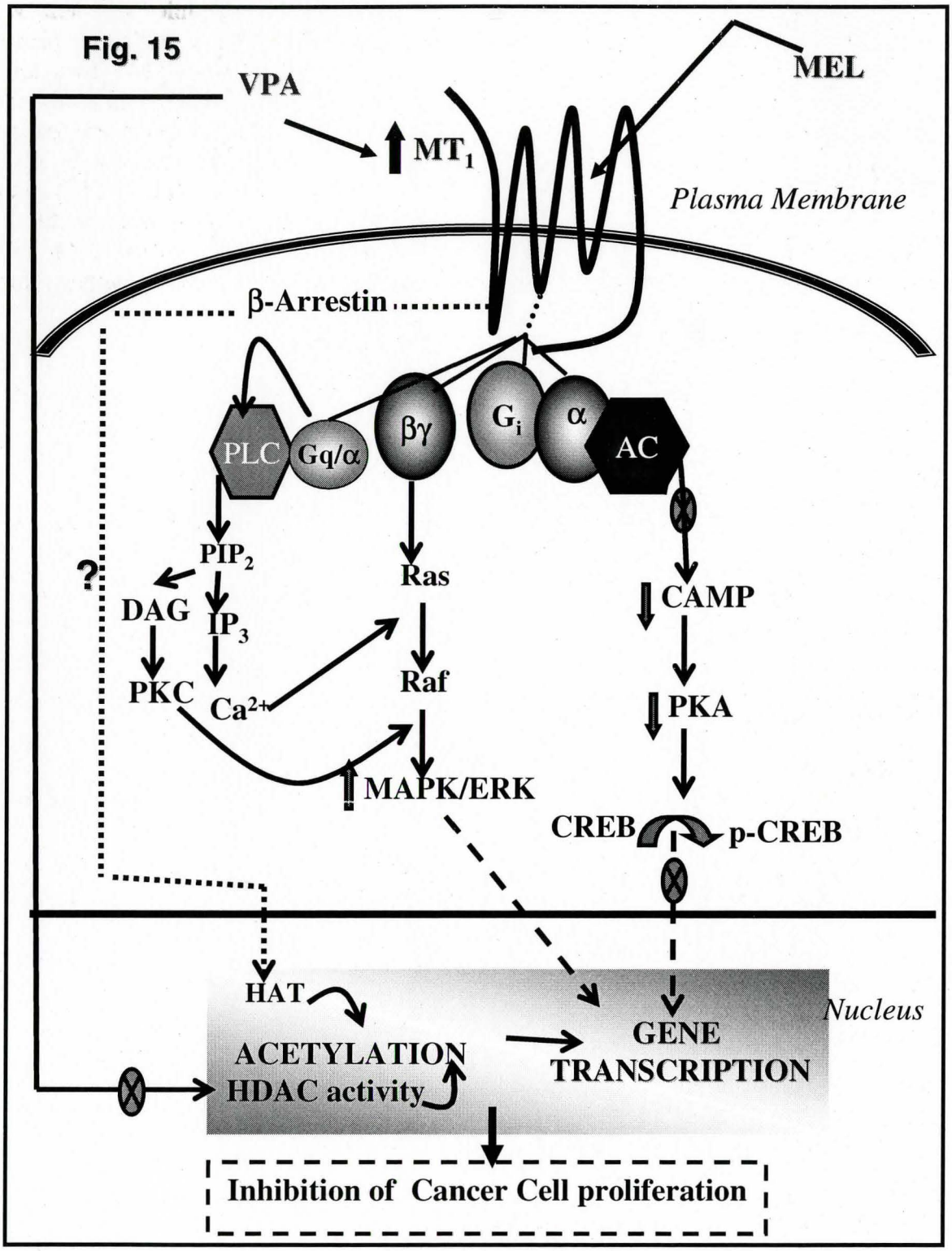


Fig. 15
VPA
MEL
MT1
Plasma Membrane
β-Arrestin
PLC
Gq/α
βγ
Gi
α
AC
PIP2
?
DAG
IP3
PKC
Ca2+
Ras
Raf
MAPK/ERK
CAMP
PKA
CREB
p-CREB
HAT
ACETYLATION
HDAC activity
GENE
TRANSCRIPTION
Nucleus
Inhibition of Cancer Cell proliferation

Section 5: REFERENCES

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