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Human melatonin MT_1 receptor induction by valproic acid and its effects in combination with melatonin on MCF-7 breast cancer cell proliferation

Sana Jawed, Bora Kim, Thomas Ottenhof, Gregory M. Brown, Eva S. Werstiuk, Lennard P. Niles *

Department of Psychiatry and Behavioural Neurosciences, McMaster University, HSC-4N77, 1200 Main Street West, Hamilton, Canada ON L8N 3Z5

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Abstract

We have reported that valproic acid upregulates melatonin MT_1 receptor expression in rat C6 glioma cells. In addition to its anticonvulsant and mood stabilizing properties, valproic acid can also inhibit the growth of cancer cells. 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. Treatment of MCF-7 cells with valproic acid (0.5 or 1 mM) for 24 or 72 h caused a significant increase in melatonin MT_1 receptor mRNA or protein expression, as shown by reverse transcription-polymerase chain reaction (RT-PCR) analysis and western blotting, respectively. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays revealed a concentration-dependent inhibition of MCF-7 cell proliferation by valproic acid (0.5, 1.0 or 5 mM), but melatonin (1 or 10 nM) was ineffective alone or in combination with valproic acid, in the first (MCF-7A) subline examined. However, in subsequent experiments using a different (MCF-7B) subline, which expressed higher levels of MT_1 receptor mRNA and showed modest sensitivity to melatonin, a combination of this hormone with valproic acid produced a significant synergistic inhibition of cell proliferation. These findings indicate that clinically relevant concentrations of valproic acid upregulate melatonin MT_1 receptor expression in human breast cancer cells. Moreover, the enhanced antiproliferative effect observed with a combination of valproic acid and melatonin suggests that a similar therapeutic approach may be beneficial in breast cancer.

Keywords: Melatonin MT_1 receptor mRNA and protein; Reverse transcription-polymerase chain reaction (RT-PCR); Western blot; MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay; Antiproliferation; Apoptosis

1. Introduction

The physiological effects of the pineal hormone, melatonin, are mediated by two G_i protein coupled melatonin MT_1 and MT_2 receptors in mammals (Dubocovich and Markowska, 2005). Several studies have shown that melatonin inhibits the growth of various types of cancer cells (Sainz et al., 2005; Blask et al., 2005). The melatonin MT_1 receptor is present in human MCF-7 breast cancer cells and it is thought to mediate the antiproliferative effect of this hormone (Ram et al., 2002). Possible mechanisms involved in the anticancer effects of melatonin include suppression of fatty acid uptake and metabolism by tumour cells (Blask et al., 2005), induction of the tumour suppressor gene p53 (Mediavilla et al., 1999) and inhibition of telomerase activity in MCF-7 cells (Leon-Blanco et al., 2003).

Recently, several antiepileptic drugs have been associated with anti-cancer activity (Cinatl et al., 1997; Gottlicher et al., 2001; Olsen et al., 2004). One of these drugs is valproic acid (2-propylpentanoic acid), which can suppress tumour growth and metastasis in vitro and in vivo (Jung, 2001; Blaheta and Cinatl, 2002; Olsen et al., 2004). We have reported a significant concentration-dependent induction of melatonin MT_1 receptor mRNA and protein expression in rat C6 glioma cells, following treatment with valproic acid (0.1–5 mM) for 24 or 48 h (Castro et al., 2005). Since human MCF-7 cells express the melatonin MT_1 receptor, and both valproic acid and melatonin have been reported to have anticancer properties, the effect of valproic acid on melatonin MT_1 receptor expression, and the combinatorial effects of these agents on cell proliferation were examined in MCF-7 cells.

* Corresponding author. Tel.: +1 905 525 9140x22224; fax: +1 905 522 8804.
E-mail address: niles@mcmaster.ca (L.P. Niles).

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2. Materials and methods

2.1. Cell culture and drug treatment

An initial series of experiments was performed with late passages (55–65) of a subline of human MCF-7 breast cancer cells designated MCF-7A. In a subsequent set of experiments, a different subline (passages 13–22) designated MCF-7B was used. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% 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 DMEM/1% FBS for examination of MT_1 expression, or cells were maintained in DMEM/10% FBS for proliferation studies. When the cells reached 65–75% confluence, they were treated with valproic acid or melatonin as described. Sodium valproate and melatonin were purchased from Sigma-Aldrich (Oakville, ON).

2.2. Reverse transcription-polymerase chain reaction analysis

Following drug treatment, total RNA was extracted using TRIzol, as specified by the supplier (Invitrogen Canada Inc., Burlington, ON). After DNase treatment, 2 μg of RNA was reverse transcribed using an Omniscript RT kit (Qiagen) and oligo(dT) primers. MT_1 PCR was carried out using the HotStarTaq master mix kit (Qiagen Inc. Canada, Mississauga, ON), with 6.5 μl of cDNA for first round amplification, and 1 μl of the 10-fold diluted PCR product for nested PCR. After a hot start at 95 °C for 15 min, samples were amplified for 38 cycles (first round) or 30 cycles (nested), at 94 °C for 30 s, 55 °C (first round) or 59 °C (nested) for 30 s, and 72 °C for 1 min, followed by a final incubation at 72 °C for 10 min, as reported previously (Armstrong and Niles, 2002). The two sets of primers used for MT_1 PCR were as follows: MT_1 -forward-5′-agtcagtgggttcct-gatgg-3′ and reverse-5′-cattgaggcagctgttgaaa-3′; MT_1 (nested)-forward-5′-cagcagcaagaactccctct-3′ and reverse-5′-tggcaaagag-gacaaaaacc-3′. Primers for the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) internal control were: forward-5′-ttc accaccatggagaaggc-3′ and reverse-5′-ggcatggactgtggtcatga-3′.

2.3. Western analysis

MCF-7A membranes were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and transblotted overnight at 4 °C. 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 2 h, proteins were detected by enhanced chemiluminescent (ECL)-autoradiography, as reported previously (Castro et al., 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).

2.4. 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 triplicate on a 96-well plate at about 5000 cells per well for treatment, or in a range of about 750 to 30,000 cells per well for the standard curve, and allowed to attach overnight. MCF-7A cells were treated with valproic acid in doses ranging from 0.1 mM to 5 mM for 24–96 h. For combined treatments, cells were exposed to valproic acid (0.1–5 mM) for 24 h, and then retreated with valproic acid with or without melatonin (1 nM or 10 nM) for 72 h. In a second series of experiments using a different subline, MCF-7B cells were treated with valproic acid (0.1–5 mM), or melatonin (1 and 10 nM), or a combination of both agents simultaneously for 24 h. In the last 2 h of treatment 10 μL of a 5 mg/ml stock solution of MTT (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in phosphate buffered saline (PBS) was added to each well. Medium and MTT were then removed and cells were lysed using 100 μl of 20% SDS/50% formamide solution, as reported previously (Crocker and Niles, 1996). Plates were left to incubate overnight and read the following day using a 595 nm filter in a Titertek Multiskan Plus microplate reader.

2.5. Hoechst staining

In order to detect possible drug-induced apoptotic changes, MCF-7 cells (100,000) were seeded overnight on a 24-well

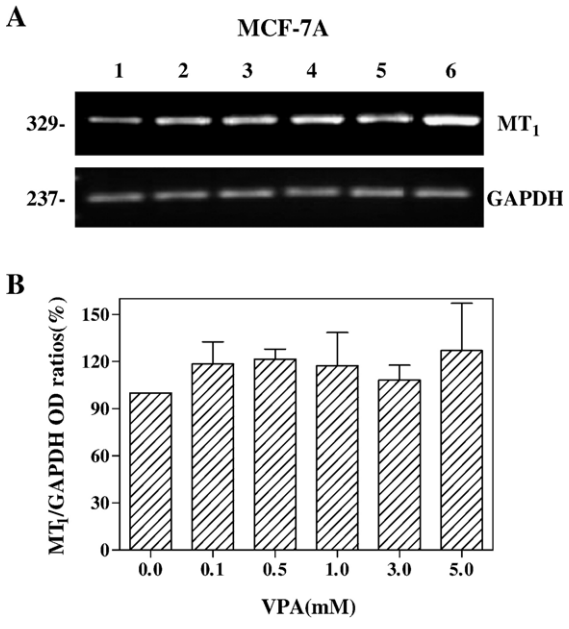

Fig. 1. Dose-dependent effects of valproic acid (VPA) on melatonin MT_1 receptor mRNA expression in MCF-7A cells after 24 h. (A) Representative gel images of nested RT-PCR detection of MT_1 (329 bp) and GAPDH (237 bp) mRNA. Lanes 1–6: Control, 0.1, 0.5, 1, 3 and 5 mM VPA. (B) Data shown are means ± S.E.M. (n = 3) for percentage (%) values of MT_1/GAPDH optical density (OD) ratios.

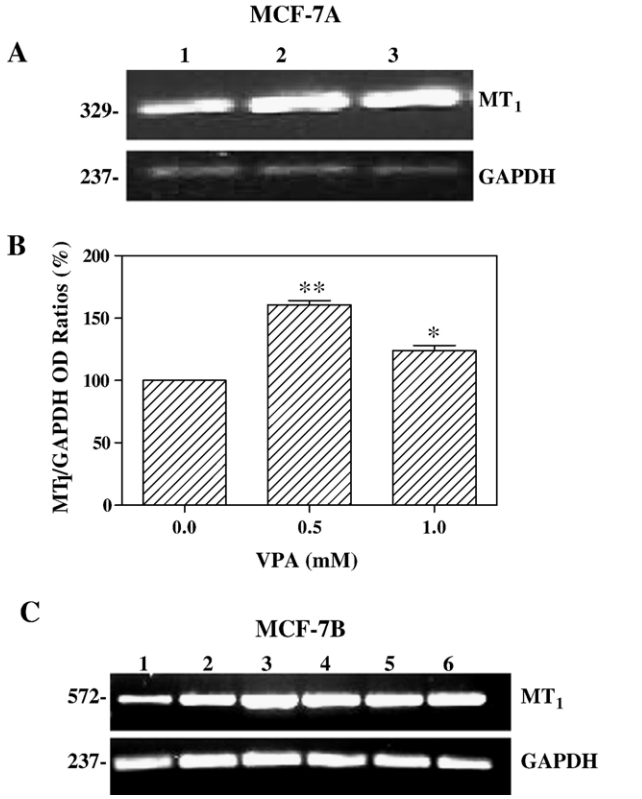

Fig. 2. Induction of melatonin MT_1 receptor mRNa expression in both MCF-7A and MCF-7B cells by VPA after 72 h. (A) Gel images of nested RT-PCR detection of MT_1 (329 bp) and GAPDH (237 bp) mRNA in MCF-7A cells. Lanes 1–3: Control, 0.5, and 1 mM VPA. (B) Data shown are means±S.E.M. ($n=3$) for % values of MT_1/GAPDH OD ratios. **$P<0.001$ vs. control and VPA (1 mM); *$P<0.01$ vs control (ANOVA and Neuman Keuls). (C) Gel images of RT-PCR detection of MT_1 (572 bp) and GAPDH (237 bp) mRNA in MCF-7B cells, for one of two separate experiments which gave similar results, are shown. Lanes 1–6: Control, 0.1, 0.5, 1, 3 and 5 mM VPA.

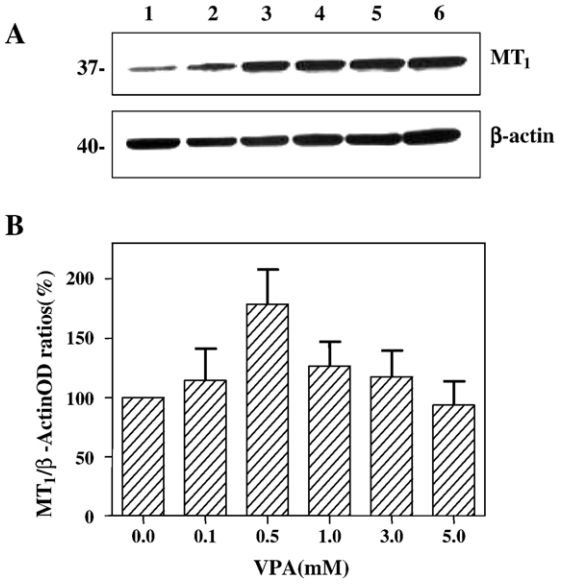

Fig. 3. Induction of the 37 kDa melatonin MT_1 receptor protein in MCF-7A cells by VPA after 24 h. (A) Representative immunoblots of MT_1 (37 kD) and β-actin (40 kDa) proteins. Lanes 1–6: Control, 0.1, 0.5, 1, 3 and 5 mM VPA. (B) Data shown are means±S.E.M. ($n=3$) for % values of MT_1/β-actin OD ratios.

plate in DMEM/10% FBS, and then treated with valproic acid and/or melatonin for 24 h. Cells were fixed in a 1% paraformaldehyde solution and washed with PBS, followed by staining with 0.2 mM of the DNA dye Hoechst 33258, for 15 min. Nuclear morphological changes were observed under a fluorescence microscope.

3. Results

3.1. Effects of valproic acid on melatonin MT_1 receptor mRNA expression

Treatment of MCF-7A cells with valproic acid (0.1, 0.5, 1.0, 3.0 and 5.0 mM) for 24 h appeared to cause a dose-dependent increase in melatonin MT_1 receptor mRNA expression as determined by nested RT-PCR, but one-way analysis of variance (ANOVA) did not indicate any significant effects (Fig. 1A,B). Longer treatment with valproic acid for 72 h produced a significant induction of melatonin MT_1 receptor mRNA ($F=155$, $P<0.0002$, $n=3$). Post hoc analysis by a Neuman–Keuls test indicated that melatonin MT_1 receptor mRNA expression, as indicated by the nested 329 bp PCR product, was increased significantly ($P<0.001$ or $P<0.01$) in cells exposed to 0.5 or 1.0 mM valproic acid for 72 h (Fig. 2A,B). Similar treatment of MCF-7B cells with valproic acid (0.1–5 mM) for 72 h, also induced MT_1 mRNA expression (Fig. 2C). Importantly, in contrast to MCF-7A cells which required nested RT-PCR for detection of MT_1 mRNA, this melatonin receptor transcript was detected in MCF-7B cells after standard RT-PCR, as shown by the 572 bp PCR product (Fig. 2C).

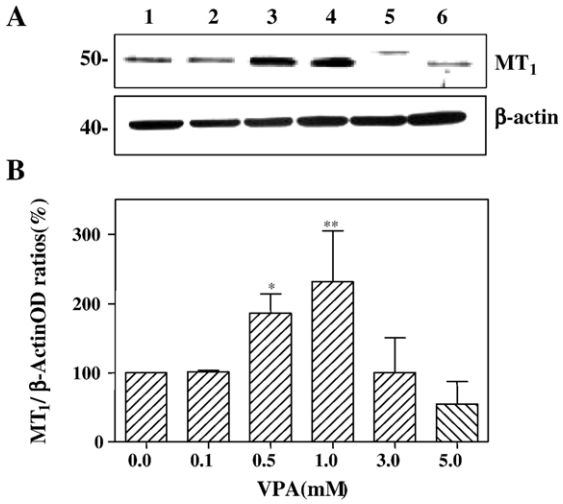

Fig. 4. Induction of the 50 kDa melatonin MT_1 receptor protein in MCF-7A cells by VPA after 24 h. (A) Immunoblots of MT_1 (50 kDa) and β-actin (40 kDa) proteins. Lanes 1–6: Control, 0.1, 0.5, 1, 3 and 5 mM VPA. (B) Data shown are means±S.E.M. ($n=3$) for % values of MT_1/β-actin OD ratios. *$P<0.05$ vs. 3 and 5 mM VPA; **$P<0.05$ vs. control, 0.1, 3, and. 5 mM VPA.

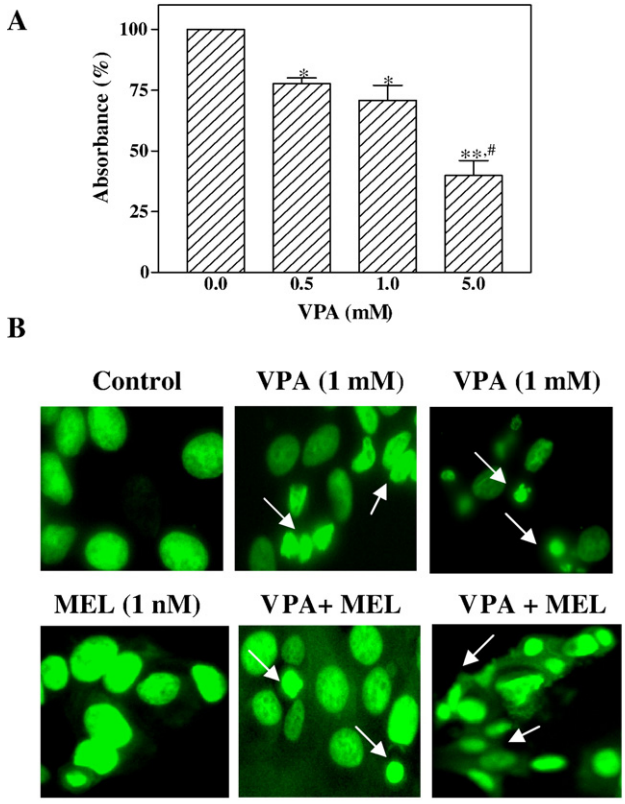

Fig. 5. Dose-dependent inhibition of MCF-7 cell growth by VPA. (A) MCF-7A cells were treated with the indicated doses of VPA for 96 h as described in the text. Data shown are means±S.E.M. (n=3) of % values obtained from MTT analysis of cell proliferation. *P<0.01 vs control; **P<0.001 vs control and 0.5 mM VPA; #P<0.01 vs 1 mM VPA. (B) Nuclear images of Hoechst-stained MCF-7B cells following treatment with VPA (1 mM) and/or melatonin (MEL, 1 nM) for 24 h. Morphological changes are indicated by arrows.

3.2. Effects of valproic acid on melatonin MT_1 receptor protein expression

Treatment of MCF-7A cells with valproic acid for 24 h caused either a concentration-dependent increase of the melatonin MT_1 (37 kDa) receptor as shown in Fig. 3A, or a biphasic response with induction at lower doses and inhibition at higher doses. Consequently, the pooled data from three experiments did not reveal any significant effects, although cells treated with 0.5 mM valproic acid exhibited a consistent increase in melatonin MT_1 receptor expression (Fig. 3B). This was confirmed by the significant (F=12.98, P<0.01) treatment effect observed when data were analyzed for controls versus lower doses of valproic acid (0.1–1 mM). In addition to the predicted 37 kDa protein, a putative glycosylated 50 kDa melatonin MT_1 receptor (Niles et al., 2004) was also upregulated after treatment for 24 h with valproic acid (0.5 mM and 1.0 mM; F=6.374, P<0.0085), as shown in Fig. 4. In preliminary experiments, the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) antagonist, PD98059 (25 μM), did not block melatonin MT_1 receptor induction by valproic acid, suggesting that this kinase pathway is not involved (data not shown).

3.3. Effects of valproic acid and melatonin on MCF-7 cell growth

Treatment of MCF-7A cells with valproic acid (0.5, 1 or 5 mM) for 96 h caused a significant inhibition of growth (F=30.56, P<0.0001), as revealed by MTT analysis (Fig. 5A). Treatment of these cells with valproic acid for 24 h or 48 h similarly caused a dose-dependant inhibition of growth (data not shown). Administration of melatonin at doses of 1 or 10 nM for 72 h did not affect the growth of MCF-7A cells. Similarly, treatment with valproic acid (0.5, 1 or 5 mM) for 24 h followed by a combination of fresh valproic acid (0.5, 1 or 5 mM) plus melatonin (1 or 10 nM) for an additional 72 h did not produce additive inhibition of MCF-7A cell proliferation (data not shown). In a second series of experiments, using MCF-7B cells, valproic acid treatment for 24 h again suppressed cell growth (Fig. 6A). In this second subline, treatment with melatonin (1 or 10 nM) for 24 h did not cause significant antiproliferative effects, although about a 25% decrease in cell growth was observed (Fig. 6A). However, when both valproic acid and melatonin were added to cultures simultaneously at the start of treatment, there was a significant inhibition of MCF-7B cell proliferation, which exceeded that seen with either agent alone, as shown in Fig. 6B.

3.4. Apoptotic effects of valproic acid

Hoechst 33258 staining revealed cell shrinkage and nuclear condensation in MCF-7B cells treated with valproic acid in the

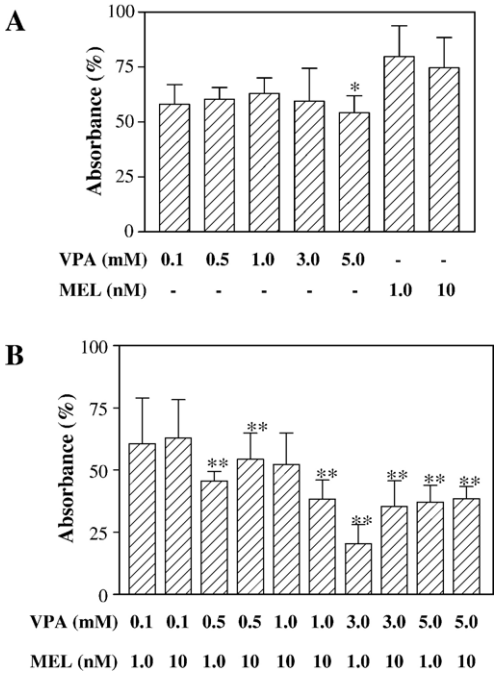

Fig. 6. Synergistic inhibition of MCF-7B cell growth by combined treatment with VPA and melatonin. (A,B) Cells were treated with the indicated concentrations of VPA and/or melatonin for 24 h. Data shown are means±S.E.M. (n=3) of % values obtained from MTT analysis of cell proliferation, as compared with 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.

absence or presence of melatonin for 24 h, suggesting induction of apoptosis (Fig. 5B). However, similar apoptotic changes were not seen in cells treated with just melatonin (1 or 10 nM) for 24 h (Fig. 5B).

4. Discussion

We have reported that treatment with valproic acid for 24 h causes a significant concentration-dependent upregulation of melatonin MT_1 receptor expression in rat C6 glioma cells (Castro et al., 2005). Since MCF-7 cells express the melatonin MT_1 receptor, it was expected that valproic acid would also modulate the expression of this receptor subtype in these human breast cancer cells. This view is supported by the significant increase in melatonin MT_1 receptor mRNA and protein expression observed following treatment with 0.5 or 1 mM valproic acid. In addition to the 37 kDa melatonin MT_1 receptor protein, a 50 kDa band, which presumably represents the glycosylated melatonin MT_1 receptor, as observed previously in C17.2 cells (Niles et al., 2004), was also enhanced by treatment with valproic acid. The clinical effects of valproic acid are seen at concentrations of about 0.4–1 mM (Gurvich and Klein, 2002; Yuan et al., 2001). Therefore, the induction of melatonin MT_1 receptor expression by valproic acid was detected at clinically relevant concentrations (0.5–1 mM) of this anticonvulsant, mood stabilizing and oncostatic agent. Earlier studies have shown that the antiproliferative effect of valproic acid on various tumour cell lines involves apoptosis (Kawagoe et al., 2002; Takai et al., 2004). Similarly in the present study, treatment of MCF-7 cells with VPA (1 mM) with or without melatonin (1 or 10 nM) for 24 h, resulted in morphological changes, such as cell shrinkage and nuclear condensation which are indicative of apoptosis. However, these changes were not seen when cells were treated with only melatonin, suggesting that short-term administration of physiological doses of this hormone may not induce apoptosis in MCF-7 cells.

The mechanism by which valproic acid upregulates the melatonin MT_1 receptor is unknown. Since valproic acid 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 induction of the melatonin MT_1 receptor. As noted earlier, PD98059 did not block the effect of valproic acid, which suggests that a MAPK/ERK-mediated mechanism is not involved in upregulation of the melatonin MT_1 receptor. This finding is similar to that observed in C6 glioma cells, where treatment with PD98059 was found to enhance the effect of valproic acid, suggesting a role for the MAPK/ERK pathway in the negative regulation of the melatonin MT_1 receptor (Castro et al., 2005).

There is now substantial evidence that valproic acid 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 valproic acid, at concentrations which upregulate the melatonin MT_1 receptor in C6 cells, alters histone deacetylase (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 upregulation of the melatonin MT_1 receptor, as observed with both rat C6 glioma and human MCF-7 breast cancer cells, awaits further study. The inhibition of MCF-7 cell growth by valproic acid is consistent with increasing evidence that this drug is a potent oncostatic agent (Li et al., 2005). Although melatonin has been reported to inhibit the proliferation of MCF-7 cells (Ram et al., 2002), it did not exhibit this effect in the first subline (MCF-7A) examined, while causing a modest nonsignificant decrease in the proliferation of a second (MCF-7B) subline, as determined by MTT analysis.

In keeping with the foregoing, a combination of valproic acid and melatonin was not effective in MCF-7A cells. However, this combinatorial approach caused a synergistic inhibition of MCF-7B cell proliferation, which was greater than the sum of the independent effects of valproic acid and melatonin. 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 et al., 2000) or the ERα/ERβ ratio (del Rio et al., 2004). Therefore, it appears that the MCF-7A subline used was insensitive to the antiproliferative effect of melatonin, and consequently no enhanced effect of valproic acid in combination with melatonin was observed with these cells.

Conversely, although the inhibition of cell proliferation by melatonin in the MCF-7B subline 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 valproic acid. Interestingly, while the MT_1 receptor has been implicated in the oncostatic effects of melatonin, pretreatment with valproic acid, which presumably upregulated this receptor in combinatorial experiments, did not induce sensitivity to melatonin in MCF-7A cells. This suggests that the MT_1 receptor may not be the mediator of the antiproliferative effect of melatonin. It has been suggested that the interaction of melatonin with the nuclear retinoid Z receptor/retinoid-related orphan receptor alpha (RZR/RORα) is involved in its antiproliferative effect on breast cancer (Girgert et al., 2003) and other tumour cell lines (Winczyk et al., 2002). However, an oncostatic role for the MT_1 receptor, at least in some cell lines, cannot be discounted at this time. The insensitivity of some MCF-7 sublines to melatonin may involve very low expression of the MT_1 receptor, as observed in MCF-7A cells, which required nested PCR for detection of this receptor. In contrast, the melatonin-sensitive MCF-7B subline expressed much higher levels of the MT_1 receptor, as shown by its detection in first round PCR, in both controls and VPA treated cells (see Fig. 2). Therefore, it is possible that higher basal and/or valproic acid-induced expression of the MT_1 receptor in MCF-7B cells is involved in the enhanced antiproliferative effect of combined valproic acid and melatonin treatment in this subline.

In conclusion, we present novel evidence that valproic acid upregulates melatonin MT_1 receptor expression in human MCF-7 breast cancer cells and that a combination of this agent and melatonin can synergistically inhibit the proliferation

of these tumour cells. Further studies are required to clarify the mechanisms underlying these findings in order to fully exploit the potential oncostatic benefits of combinatorial therapy with valproic acid and melatonin.

Acknowledgement

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