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Serotonin and Melatonin Do Not Play a Prominent Role in the Growth of Prostate Cancer Cell Lines

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Key Words

Prostate cancer cell lines · Serotonin · Melatonin · Cellular viability · Apoptosis · Cell cycle

Abstract

Objectives: To investigate the effects of serotonin and melatonin (MLT) on the regulation of malignant growth and the activity of serotonin receptors (5HTR1a/-1b) in prostate cancer (PCa) cell lines. **Materials and Methods:** In four PCa cell lines (LNCaP, 22RV1, PC3, DU145) and two reference cell lines 5HTR1a and -1b, relative mRNA expression levels were assessed. Different serotonin and MLT receptor agonists and antagonists were used in stimulation and inhibition experiments. **Results:** mRNA expression of 5HTR1b was higher than that of 5HTR1a in all PCa cell lines. Serotonin showed a significant growth stimulatory effect in all PCa lines. The 5HTR1a and -1b agonists/antagonists did not significantly affect viability. MLT inhibited viability only in PC3 cells. Similarly, the 5HTR1a antagonist induced apoptotic changes in PC3 cells only at 10^{-4} M, while the 5HTR1b antagonist induced necrosis at 10^{-4} M in all cell lines. Cell cycle alterations were seen in PC3 and DU145 cells under the influence of the 5HTR1a antagonist. **Conclusions:** Serotonin receptor antagonists and agonists as well as MLT influence viability and apoptosis of PCa cell lines at supraphysiologic concentrations. In contrast to other reports, our results do not support a regulatory role of serotonin or MLT receptor activation or inhibition in PCa growth.

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Introduction

Hormone-refractory prostate cancer (PCa) represents the end stage of a progressive and fatal disease for which only palliative treatment options exist. Numerous concepts have been proposed to explain the biological progression of hormone-sensitive PCa to hormone-resistant PCa including stromal-epithelial interactions [1, 2], changes in androgen receptors [3], cell cycle machinery impairment [4], and abnormal signaling pathways [1, 5–8]. Currently, serotonin is considered to be of importance in the progression of PCa towards the hormone-refractory state [1, 9, 10]. The basis for this hypothesis is the reported expression of 5-HT receptors in LNCaP and PC3 cell lines [5, 6]. Serotonin-mediated mitogenic activity in vitro [11] and in vivo [12] as well as effects of 5-HT agonists/antagonists have been reported [5, 6, 13, 14]. 5-HT was thus described as a potent mitogen in vitro acting in the μ M concentration range and stimulating proliferation of PCa cell lines [11]. The serotonin receptor subtypes 5HTR1a, -1b, -2b and -4 were recently identified and characterized in PCa cell lines [5, 6]. These studies, involving the administration of 5-HT agonists/antagonists in vitro reported that stimulation at low concentrations (nM to μ M) leads to increased proliferative activity, while inhibition leads to decreased proliferation in a concentration-dependent manner [5, 6, 13]. For such experiments, hormone-dependent (LNCaP), hormone-independent (PC3, DU145) and androgen-responding (22RV1)

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human PCa cell lines are used as convenient in vitro models [15, 16].

The pineal hormone melatonin (MLT) may also be involved in the molecular interactions in different malignancies including PCa [2, 17–23]. Studies in LNCaP cell lines in vivo have shown an inhibition of tumor growth by MLT at nM concentrations with decreased expression of cell cycle markers [20]. It was concluded that the antiproliferative effects of MLT in LNCaP line were partly mediated by MT₁ receptors and influenced the cell cycle with the possible induction of apoptotic changes [20]. The data obtained from other studies [19] on androgen-deprived LNCaP lines also showed a direct antiproliferative effect of MLT by impairment of the cell cycle (G₀/G₁ arrest) [21]. Results obtained from studies on MLT activity in DU145 cell lines have shown cell cycle arrest in the G₀/G₁ phase due to direct stimulation of nuclear MT receptors [23]. Membrane-associated receptors are suggested to be different sites of action of MLT in PC3 cell lines [22].

The aims of our work were to investigate the expression of two serotonin receptors (5HTR1a and 5HTR1b) and the effects of serotonin, several serotonin agonists and antagonists and MLT in the regulation of malignant growth in several PCa cell lines (LNCaP, PC3, DU145, 22RV1).

Materials and Methods

Cell Culture and Reagents

The human PCa cell lines LNCaP (hormone-dependent), 22RV1 (hormone-responding), PC3 and DU145 (hormone-independent) were used for in vitro experiments (ATCC). As reference, human fibroblasts and BPH-1 cell line were used in experiments (DSMZ). The cell lines were cultivated in RPMI 1640 medium supplemented with 10% FBS and 1% MEM (Invitrogen, Karlsruhe, Germany) without antibiotics in 75-cm² flasks (Greiner, Frickenhausen, Germany). Cellular growth density was evaluated as low (LD; <50% visual density) and high (HD; 50–75% visual density). Cell counting was performed after trypsinization by Cell Coulter Z² Particle Count & Size Analyzer (Beckman-Coulter, Krefeld, Germany).

Reagents used were: 5-HT, MLT (highly purified >99.5%), (±)-8-OH-DPAT (selective high-affinity 5-HT_{1a} receptor agonist), CGS-12066A maleate salt (5-HT_{1b} receptor agonist), NAN-190 hydrobromide (5-HT_{1a} receptor antagonist), and methiothepin mesylate salt (5-HT_{1b} receptor antagonist). All these reagents were obtained from Sigma-Aldrich, Germany.

RNA Isolation, cDNA Synthesis, Quantitative PCR

Total cellular RNA was isolated using Invisorb[®] Spin Cell RNA Mini Kit (Invitex GmbH, Berlin, Germany). The concentration of extracted RNA was measured by UV/Vis spectrometry on

an Ultraspec 3000 (Amersham Biosciences, Little Chalfont, UK). cDNA synthesis was performed using SuperScript[™] RNase H reverse transcriptase (Invitrogen, Karlsruhe, Germany). The mRNA expression levels of the serotonin receptors 5HTR1a/-1b were measured by pre-made quantitative PCR assays (primers: Hs00265286_s1, Hs00265014_s1) and referred to housekeeping genes (HPRT – hypoxanthine phosphoribosyltransferase) as internal control (Applied Biosystems, Foster City, Calif., USA) using a Light Cycler instrument (Roche, Mannheim, Germany). The RT-PCR two-step program used 45 cycles of initial denaturation at 95°C for 600 s; amplification 1 at 95°C for 15 s; amplification 2 at 60°C for 15 s; cooling at 40°C for 60 s. RT-PCR products were analyzed by 2% agarose gel electrophoresis and visualized under UV after staining with ethidium bromide.

Cellular Viability Test in vitro

Cells were seeded in 96-well plates with 3,000 cells/well in 100 µl RPMI 1640 with 10% FCS for 1 day in order to obtain optimal growth and density of no more than 75%. Then, the medium was changed to 100 µl RPMI 1640 with 10% charcoal-stripped, hormone-free FCS (Biocrom AG, Berlin, Germany). After 24 h of medium adaptation, the 5HTR1a- or -1b-specific agonists at concentrations from 10⁻¹⁰ to 10⁻⁶ M and antagonists (10⁻⁸ to 10⁻⁴ M) were added to the medium and incubated for 3 days. 5-HT was freshly prepared and stored in DMSO stock solutions (10⁻¹ M) and diluted in medium to concentrations of 10⁻⁸ to 10⁻⁴ M. MLT was also freshly prepared and diluted in DMSO to 1.0 M stock solution, then in medium in concentration ranges of 10⁻¹¹ to 10⁻³ M and was administered daily during 4 days of incubation. Cellular viability was studied from 24 to 72 h after incubation with agonists/antagonists by cell proliferation reagent WST-1 (Roche, Penzberg, Germany) comparing control cell lines incubated with DMSO. For this, 10 µl/well WST-1 was added, followed by 1–1.5 h of incubation (37°C, 5% CO₂). Cell viability was determined by absorbance measurement at 450 nm (reference wavelength 620 nm) using a microplate reader (Anthos Labtec, Krefeld, Germany). Viability after incubation with agonists/antagonists was expressed relative to control (incubated with DMSO).

Cell Cycle, Apoptosis Analysis by Flow Cytometry

Changes in cell cycle of 2 × 10⁴ nuclei were analyzed using Cycle Test[™] Plus DNA Reagent Kit (Becton Dickinson, Heidelberg, Germany) and FACScan (Becton, Dickinson). The early (annexin+/propidium iodide-) and late (annexin+/propidium iodide+) apoptosis was studied by Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions.

Results

Basal mRNA Expression Levels of 5HTR1a and 5HTR1b in PCa Cell Lines

The results of the quantification of the relative mRNA expression levels of the 5HTR receptor subtypes HTR1a and HTR1b corresponding to the growth density are shown in figure 1. Transcripts were detectable in all cell

Fig. 1. Relative mRNA expression of 5HTR1a (a) and 5HTR1b (b) normalized to the reference gene HPRT in PCa cell lines corresponding to the growth density of the cells.

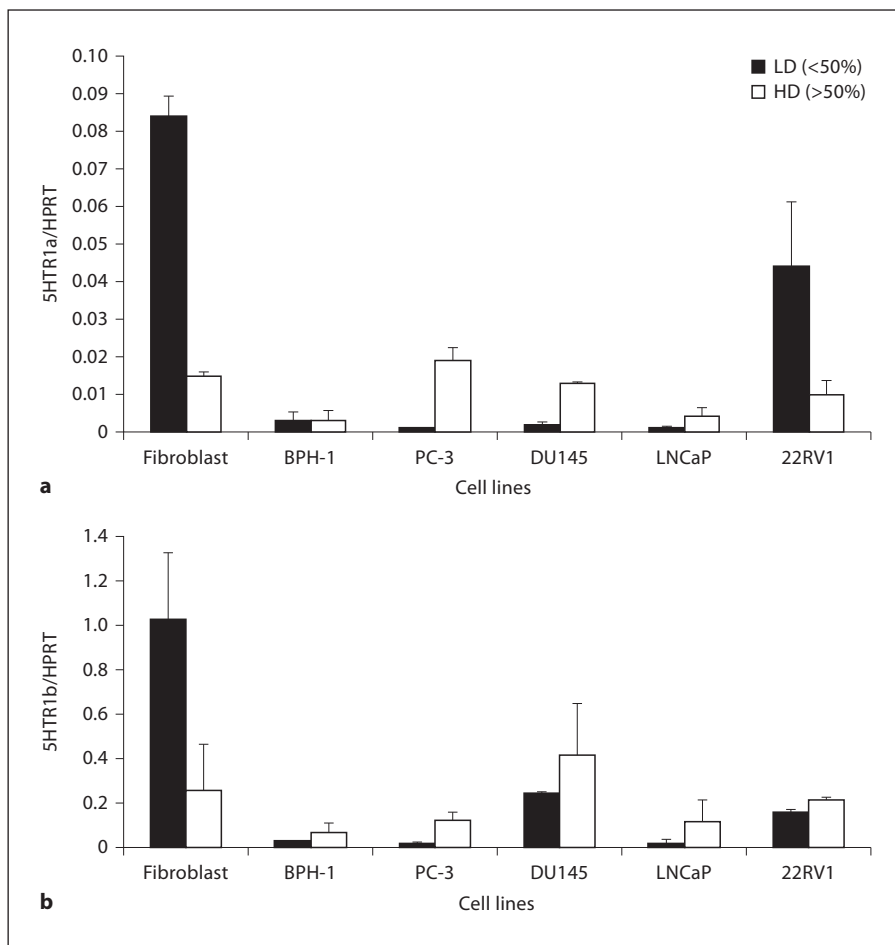
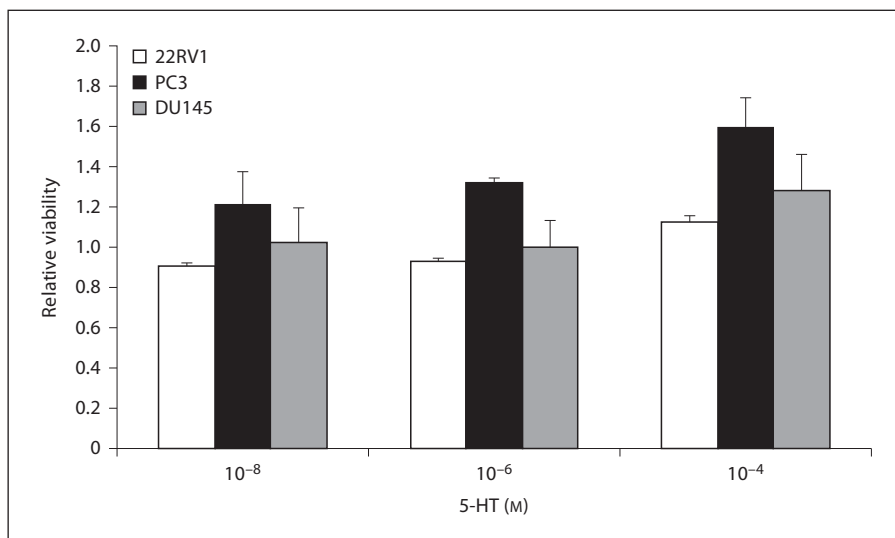


Fig. 2. Relative viability of 22RV1, PC3 and DU145 cell lines (normalized to control incubated with DMSO) 24 h after incubation with 5-HT.



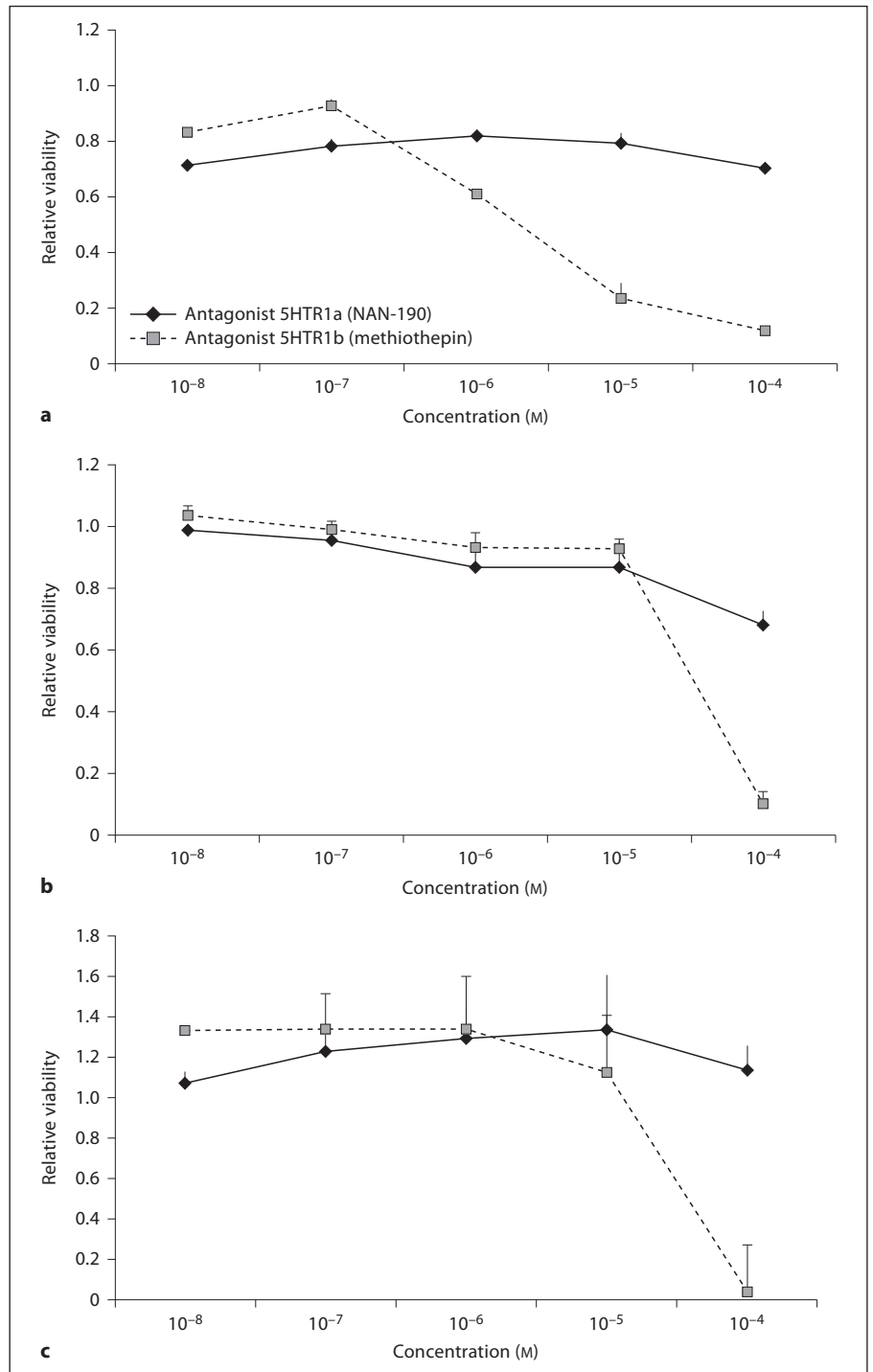


Fig. 3. Cellular viability in LNCaP (a), PC3 (b) and DU145 (c) after 48-hour incubation with NAN-190 hydrobromide and methiothepin mesylate salt in a concentration range of 10⁻⁸ to 10⁻⁴ M.

lines (data not shown). The comparison with growth density showed a higher expression of both 5HTR receptor subtypes in HD (50–75%) compared to LD (<50%) in all cell lines (fig. 1), with the exception of 5HTR1a expression in 22RV1 and human fibroblasts.

Effects of 5-HT and 5HTR1a/-1b Agonists and Antagonists on Cellular Viability

The cellular viability of 22RV1, PC3 and DU145 cells after 24 h of incubation with 5-HT (10⁻⁸ to 10⁻⁴ M) is shown in figure 2. The pronounced stimulatory effect

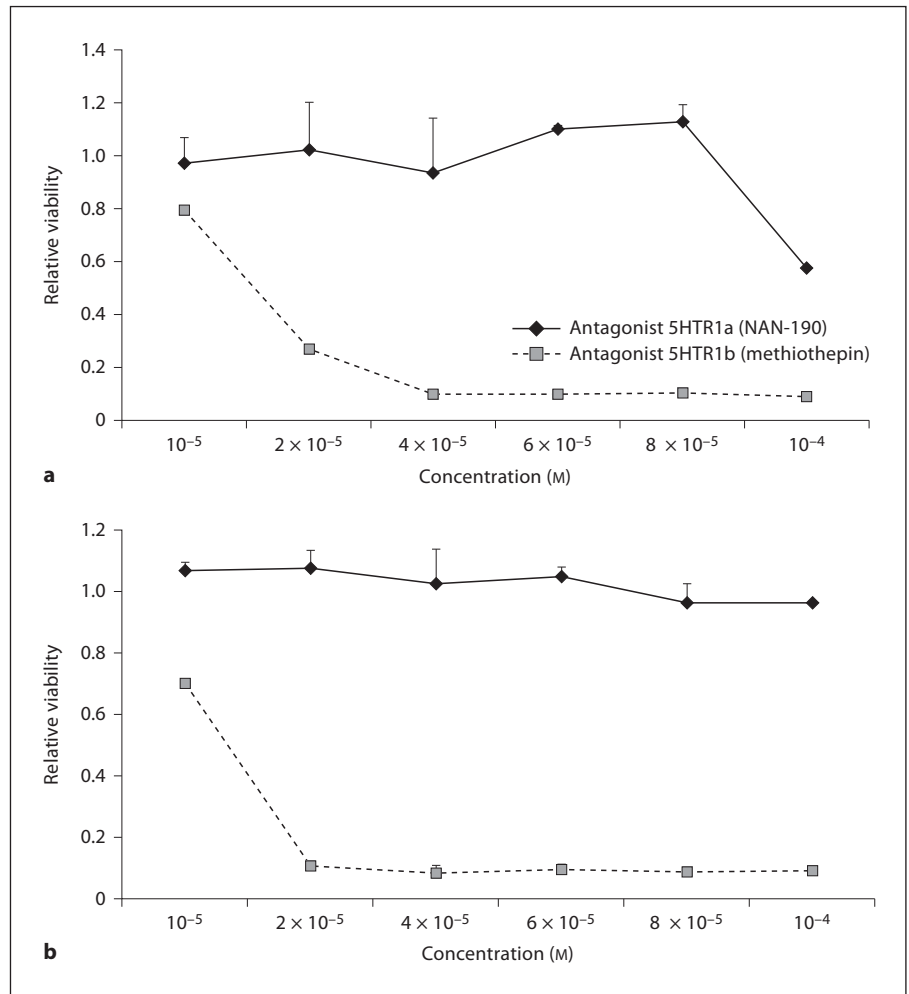


Fig. 4. Cellular viability test (WST-1) in PC3 (a) and DU145 (b) after 24-hour incubation with NAN-190 hydrobromide and methiothepin mesylate.

with increased cellular growth was observed in PC3 cells (+21.2, +31.8 and +59.5% at 10^{-8} , 10^{-6} and 10^{-4} M, respectively). In DU145 cells, a slight stimulatory effect was observed only at 10^{-4} M (+28.2%). LNCaP and 22RV1 cells did not respond at all to 5-HT stimulation (data not shown). Figure 3 gives the results of cell viability assessment without 5-HT stimulation in the presence of the 5-HT1a antagonist NAN-190 hydrobromide or the 5-HT1b antagonist methiothepin mesylate. After 48 h of incubation, cellular viability was not affected by the antagonist in the concentration range of 10^{-8} to 10^{-5} M. At a concentration of 10^{-4} M, no changes were observed after 5HT1a inhibition. A concentration of 10^{-4} M of methiothepin mesylate was cytotoxic and caused total necrosis after 12 h of incubation. A 24-hour incubation of the cell lines PC3 and DU145 after 24 h with NAN-190 and methiothepine mesylate in a constricted concentration range between 10^{-5} and 10^{-4} M confirmed these results (fig. 4). Methiothepine

mesylate at a concentration of $>10^{-5}$ M induced dramatic cell death. In the same experiments using the 5HT1a and -1b agonists (\pm)-8-OH-DPAT and CGS-12066A, maleate salt did not affect cellular viability (data not shown).

Effects of 5HT1a/-1b Antagonists on Apoptosis

In DU145 cells, significant apoptotic changes were observed after 24-hour incubation with NAN-190 at a concentration of 10^{-4} M. The changes were significant for early and late apoptosis. Incubation with NAN-190 did not induce apoptosis, but necrosis in PC3 cell culture was seen at a concentration of 10^{-4} M (fig. 5).

Changes in Cell Cycle Phases after Incubation with 5HT1a/-1b Antagonists

Figure 6 summarizes the effects seen on cell cycle. In PC3 and DU145 cell lines, the highest proportion of cells which underwent G_0 - G_1 phase arrest were detected after

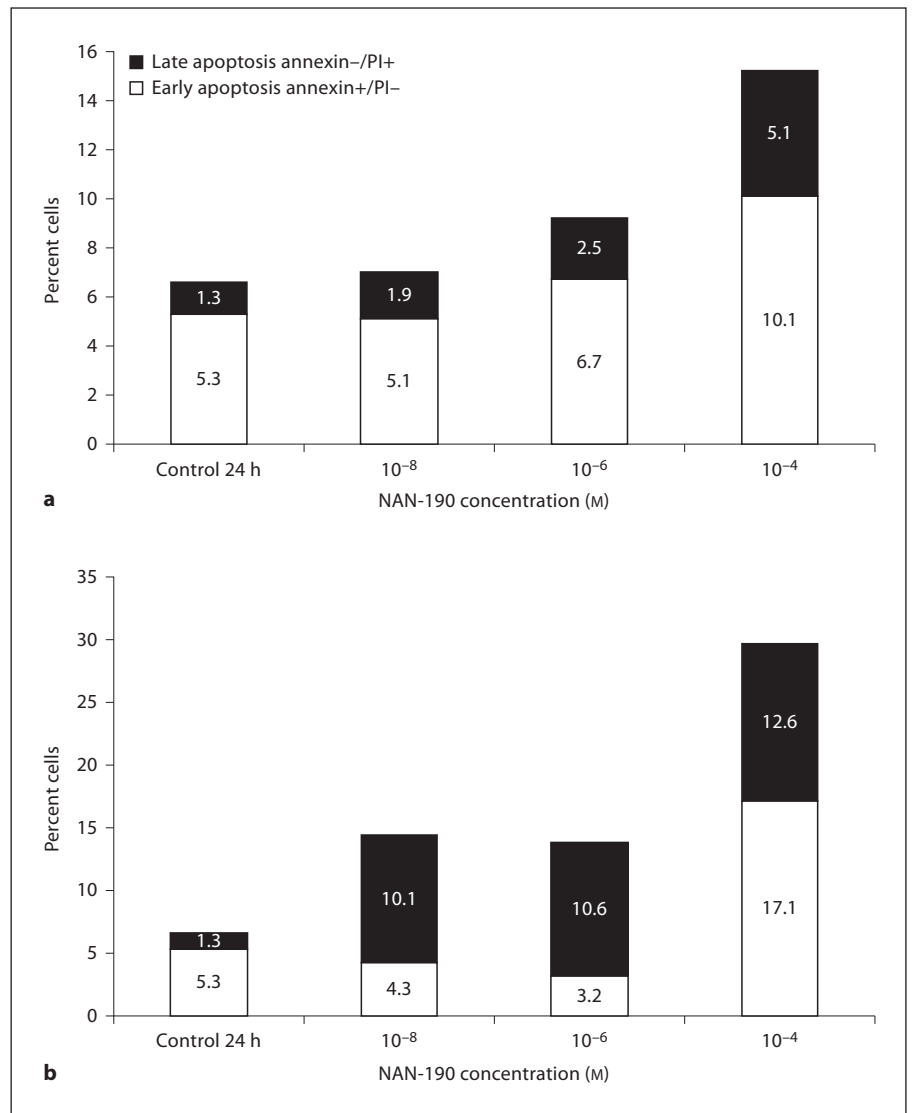


Fig. 5. Effects of NAN-190 on apoptosis after 24-hour incubation in DU145 (a) and PC3 (b).

24 h of incubation with the 5HTR1a antagonist (NAN-190) at a concentration of 10⁻⁴ M (fig. 6). Methiothepin caused no significant changes in cell cycle phases (data not shown).

Effects of MLT on Cellular Viability

Treatment of PC3, DU145 and 22RV1 cells with MLT showed time- and dose-dependent effects on cellular viability in supraphysiologic concentrations only (fig. 7). After 72 h of incubation with MLT in the concentration range 10⁻⁸ to 10⁻³ M, no inhibitory effects were observed (data not shown), while at supraphysiologic concentrations of MLT (10⁻³ to 10⁻² M) a marked concentration-dependent decrease in cellular viability was seen (fig. 7).

Discussion

PCa progression remains a complex process which still evades complete understanding. Many biologic factors capable of influencing or regulating cell growth have been implicated and probably are involved [24, 25]. MLT has been shown to have an influence on cancer cell growth in several cancer entities including PCa cells growing in vitro [26, 27]. There may even be potential therapeutic implications for MLT [28]. All these reasons make MLT a substance of interest in PCa research, and we therefore undertook these experiments on MLT receptors in PCa cell lines. We found an expression of both 5HTR subtypes 5HTR1a and 5HTR1b in the four PCa cell lines used (PC3, DU145,

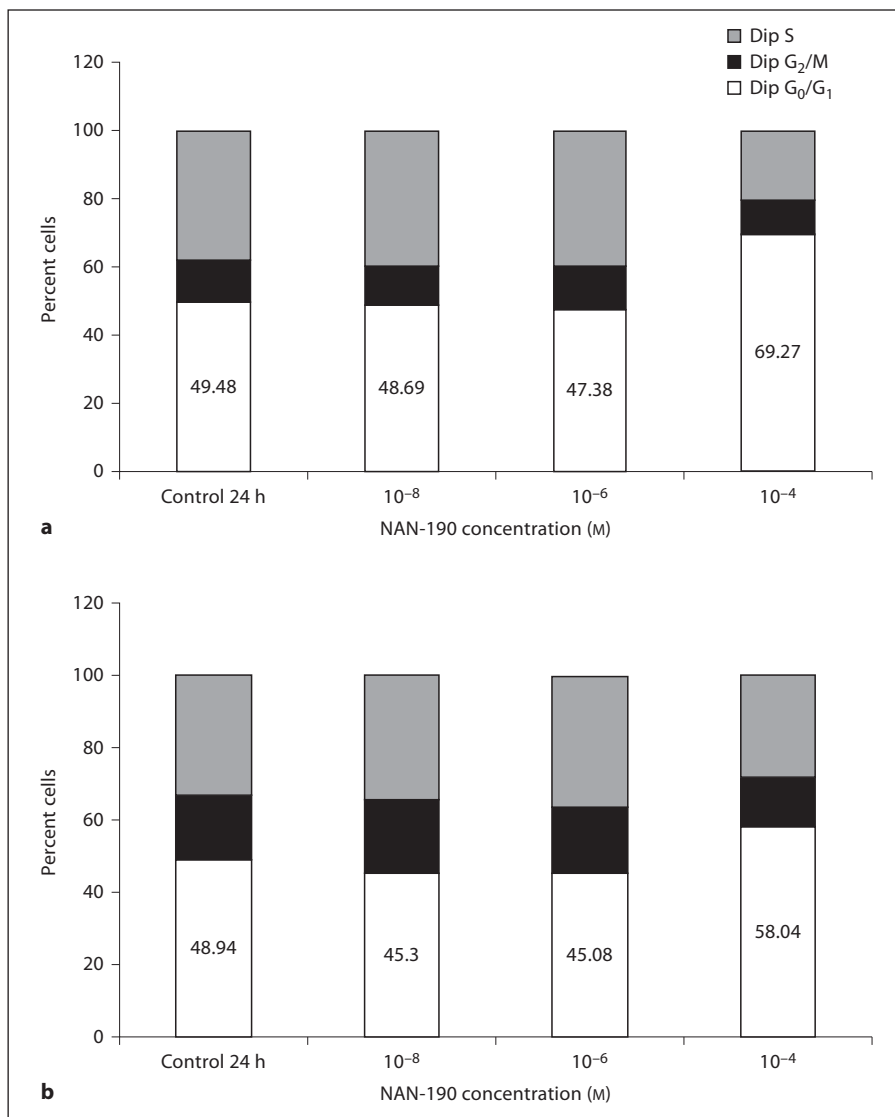


Fig. 6. Cell cycle arrest in G₀/G₁ in DU145 (a) and PC3 (b) cells after 24-hour incubation with NAN-190. Percentages of cells in different phases of the cell cycle (G₀/G₁, S, G₂/M) are shown. Control refers to incubation with DMSO.

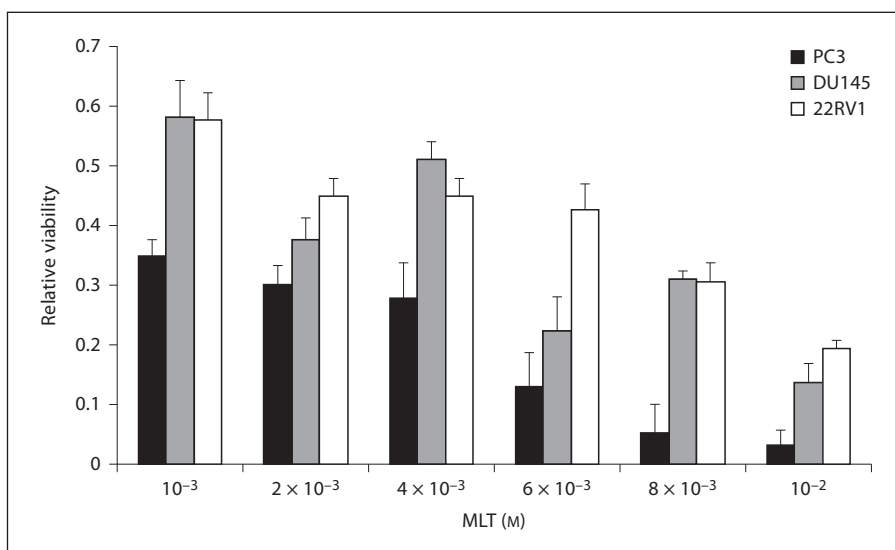


Fig. 7. Cellular viability test in PC3, DU145 and 22RV1 after 72-hour incubation with MLT in a concentration range of 10⁻³ to 10⁻² M.

LNCaP and 22RV1) as well as in the BPH-1 line and human fibroblasts. The relatively low expression of 5HT_{1a} and 5HT_{1b} receptors observed, however, may signify that in nonstimulated PCa cells the 5HT_{1a}/-1b expression at mRNA level does not play a significant role in regulation of growth. Reports in the literature [5] described the qualitative (detected by PCR) and immunohistochemical features of 5HT_{1a}/-1b, but their functional activity may well depend on the quantitative properties.

In experiments with 5-HT stimulation, we found that the stimulatory effect was dose-dependent at 10^{-8} to 10^{-4} M during the first 24 h and highest at 10^{-4} M. The data from the Malmö group described a stimulatory effect of 5-HT on proliferation in DU145 and PC3 cells at 10^{-8} M with no significant effect on LNCaP cells [5]. These and other authors describe 5-HT as a mitogen *in vitro* [5, 11, 12]. It is possible that with low 5HT_{1a}/-1b expression and high concentrations of 5-HT, the viability and proliferation effects may be mediated by other types of serotonin receptors as other types of serotonin receptors in PCa cell lines have been identified (5HT_{2b}, 5HT₄) with a putative role in proliferation and differentiation [6].

Our results with 5HT_{1a}/-1b agonists did not show any effect of selective 5HT_{1a}/-1b stimulation on physiological concentrations. This fact may also support the low functional activity of 5HT_{1a}/-1b receptor subtypes. The inhibition experiments showed physiological effects on cellular viability in the concentration range 10^{-8} to 10^{-4} M, and only cytotoxic effects of methiothepin at 10^{-4} M. Our data suggest that inhibition of 5HT_{1a}/-1b in physiologic concentrations does not affect cellular viability. In contrast to our findings, other authors [5] have reported that 5HT_{1a} inhibition in DU145, PC3 and LNCaP cells was concentration-dependent and maximal at 10^{-6} M. However, neither those nor our results support a prominent functional role of 5HT_{1a}/-1b or 5-HT stimulation in cell viability in PCa cell lines.

In agreement with others, we did find a time- and dose-dependent effect of MLT on cellular viability in PC3, DU145 and 22RV1 [22, 23]. In contrast to a hypothesis reported in the literature, this effect in our observation did not seem to be related to hormone dependency of the cell lines [19, 20, 26]. Reported maximum MLT inhibitory activity was at high concentrations of 10^{-3} to 10^{-2} M after 72 h of continuous incubation [26], making MLT a potent inhibitor *in vitro* only in mM concentrations with effects only after prolonged incubation. Our data correspond to these reported findings concerning concentration range and time effect as well as more effective inhibition in moderate cellular density (3,000

cells/well) [26]. These findings thus somewhat characterize MLT as an antiproliferative agent in PCa cell lines, but may cast doubt on the hypothesis that this effect is highly specific and of great biological significance in PCa.

Similarly, while a putative role of MLT in apoptosis is still controversial, we have observed an apoptotic effect of MLT in DU145 and PC3 only at concentrations of 10^{-4} M. Since MLT as a pineal hormone exerts its biological effect *in vivo* at nM concentrations [17, 18], the observed apoptotic effect in cell culture is highly unlikely to be specific.

Changes in cell cycle due to G₀/G₁ phase arrest were described as one of several possible mechanisms in the regulation of proliferation [4]. We observed a G₀/G₁ phase arrest induced by the 5HT_{1a} receptor antagonist NAN-190 after 24 h of incubation at 10^{-4} M in PC3 and DU145 cells. Again, the concentration at which this effect was observed does not support a biological significance.

Conclusions

Our results do not support the hypothesis that activation or inhibition of the serotonin receptor subtypes 5HT_{1a} or 5HT_{1b} plays a prominent role in the control of growth and viability of PCa cell lines. Inhibitory effects of MLT in PCa cell lines *in vitro* occur at supraphysiological concentrations only. Thus, neither MLT nor the serotonin receptor subtypes 5HT_{1a} and 5HT_{1b} seem to have an important role in the *in vitro* growth regulation of human PCa cell lines.

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