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ORIGINAL PAPER

Anti-mitogenic and apoptotic effects of 5-HT1B receptor antagonist on HT29 colorectal cancer cell line

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Abstract

Purpose There is lack of evidence about impact of 5-HT receptors on colorectal cancers. The current study was designed to investigate the role of serotonin and its receptors in colorectal cancer cell line and tissues.

Methods In cell cultures, we investigated the effects of 5-HT and 5-$HT_{1A,1B,1D}$ agonists and antagonists on proliferation of HT29 cells. We also tested apoptosis for the receptor antagonists. The expression of 5-$HT1_{A,B,D}$ receptor subtypes was examined by immunohistochemistry and western blotting.

Results Our data indicated that 5-HT_{1B} receptor was fully expressed in HT29 cell line and tumor tissues. MTT proliferation assay also revealed that serotonin and CP93129 dihydrochloride, a selective 5-HT_{1B} receptor agonist, stimulated growth of HT29 cells. Further, SB224289 hydrochloride (that is a selective 5-HT_{1B} receptor antagonist) had anti-proliferative and apoptotic effects on HT29 cells.

Conclusions The findings of this study provide evidence for the potential role of 5-HT_{1B} receptor in colorectal cancer. Further investigation is required to explore the effect of receptor antagonists on the prevention, prognosis and treatment of patients with colorectal cancer.

Keywords Colon cancer · Serotonin · 5-HT receptor · Apoptosis · Protein expression

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Introduction

Colorectal cancer is the most common cause of cancer-related deaths in the world (Midgley and Kerr 1999). During the last decade, significant advances have been made in the treatment of this malignancy and new chemotherapeutic agents have been introduced. As a result, the survival rates of patients with metastatic colorectal cancer being treated with new therapeutics have improved (El-Salhy et al. 2003). Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter that mediates a wide variety of physiological effects including peripheral and central action through the binding of multiple receptor subtypes (Wilkinson and Dourish 1991). Although the mitogenic effects of serotonin receptors, especially 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D} and 5-HT_{2A}, in breast cancer, prostate cancer and bladder cancer have been investigated (Dizeyi et al. 2004; Siddiqui et al. 2006; Sonier et al. 2005, 2006), there is a lack of evidence about whether these receptors play a significant role in colorectal cancer.

5-HT receptors are found in both the central and peripheral nervous systems, in intestinal enterochromaffin cells and blood platelets. They are important for a variety of physiological functions, including platelet aggregation, smooth muscle contraction, appetite, cognition, perception, mood, and other central nervous system functions (El-Salhy et al. 1998; Hoyer et al. 1994; Roth 1994;

Yue and Liu 2005). These diverse physiological functions are mediated by large number of 5-HT receptor classes and subtypes that are encoded by distinct genes. The 5-HT_1 and 5-HT_5 classes of receptors interact negatively with adenylate cyclase, while the 5-HT_2 and 5-HT_4 are coupled to the activation of phospholipase C-β. The 5-HT_3 receptor is a ligand-gated ion channel receptor, while 5-HT_6 and 5-HT_7 receptors activate adenylate cyclase (Banes et al. 2005; Ishizuka et al. 1992; Wilkinson and Dourish 1991). It has been suggested that the mitogenic activity of 5-HT might be due to the stimulation of phospholipase C-β/protein kinase C, for which in vitro expression has been documented. The stimulation of 5-HT receptors also triggers the mitogen-activated protein kinase and extracellular signal-regulated kinase cascade. It activates the cell division rate that is a common reaction to many extracellular stimuli, including growth factors, hormones and neurotransmitters in mammalian cells, which leads to transcription factor phosphorylation and cell division (Ishizuka et al. 1992; Launay et al. 1996).

Selective serotonin reuptake inhibitors (SSRIs) that increase serotonin concentration in central and peripheral nerve ends have been suggested for the use in the prevention of colorectal cancer (Pantaleo et al. 2006; Xu et al. 2006). It has been shown that fluoxetine, an SSRI drug, in concentrations of (10–100 μM), reduced the growth of cancerous cells in vitro due to the ability to increase serotonin levels in peripheral nerve endings in the gastrointestinal tract (Yue and Liu 2005). Triple therapy with serotonin, octreotide and galanin (central and peripheral neuropeptides) also reduced the size and blood vessel density and increased apoptosis in rat colon carcinoma (El-Salhy et al. 1998). Although there are studies suggesting a preventive role for SSRIs, other reports suggest that SSRIs induce mammary tumors in animals and humans (Hilakivi-Clarke et al. 1993; Sharpe et al. 2002).

Previously, it was indicated that neuroendocrine peptides and amines play a significant role in regulating the proliferation and growth of gastrointestinal epithelial and mesenchymal cells (Hill 1991). These bioactive substances are also involved in regulating the body's immune defense (Weigent and Blalock 1995). The cell proliferation and local immune defense of the gut are important in the development of colorectal cancer. Thus, an abnormality in the neuroendocrine system in the colon of patients with colon carcinoma might initiate or promote the development of the disease (El-Salhy et al. 1998). Notwithstanding wide distribution of 5-HT_1 and 5-HT_2 group receptors in enteric nervous system and peripheral smooth muscle of alimentary tract (Oosterbosch et al. 1993), there is a lack of evidence about the impact of 5-HT receptors in colon cancers. Having considered the role of 5-HT_1 receptors, especially 5-$HT_{1A,B,D}$ subtypes in some malignancies, this study aims to investigate the role of 5-$HT_{1A,B,D}$ receptor subtypes in colon cancer.

Materials and methods

In this study, we used HT29 cell line and tumor biopsy tissues of patients diagnosed with colorectal cancer for mitogenicity and protein expression and immunohistochemical studies. Thirty (14 females and 16 males) human colorectal cancer paraffin blocks tissues were obtained from patients in the age range of 30–65 years whose cancers (grades II or III) had already been identified in Pathology Department of Queensland Institute of Medical Research (QIMR) in Brisbane, Australia (under ethics approval no: 13442). HT29 human colon cancer cell line, LS174 human colon normal cell line (negative control) and A172 human brain normal cell line (positive control) were obtained from cell bank of QIMR (under ethics approval no: 13440).

For the purpose of this study, MTT assay was used to investigate proliferation effects of 5-HT receptor agonists and antagonists. Cell cycle kinetics was examined using propidium iodide method and TUNEL apoptosis assay was utilized for apoptosis studies. For investigating 5-HT receptor expressions, we used an immunohistochemistry (IHC) and immunoblotting procedure.

RPMI 1640 medium, MTT reagent, Bradford reagent, trypsin, 5-HT, polyacrylamide absorbent, *NN*-ethylene-bisacrlamide, *NNNN*-tetramethylen diamide, ammonium sulfate, sodium dodecyl sulfate (SDS), Triton X-100 and propidium iodide were all purchased from Sigma Co. (Saint Louis, Missouri, USA). Fetal calf serum, L-glutamine, 3,3 diaminobenzidine (DAB), avidin–biotin peroxidase kit, TUNEL, in situ fluorescein detection kit, Na–deoxycholate powder were purchased from Roche Company, Germany. BP554 maleate, CP93129 dihydrochloride, L-694247 hydrochloride, BRL15572 hydrochloride, SB224289 hydrochloride, Nan190 hydrobromide were purchased from Tocris Company, UK. Primary polyclonal antibodies (anti-5-$HT_{1A,1B,1D}$ receptors goat IgG) and secondary antibodies (donkey anti goat IgG, HRP and biotinylated conjugates) all were purchased from Santacruz Company (USA).

MTT proliferation assay

HT29 cells maintained in 25 or 75 cm^2 flasks and then washed with PBS and harvested with a 0.5% trypsin solution at 50–60% confluence. Cells were then added to wells at a density of 10^4 cells/well in a 96-well plate in a final volume of 100 μl/well. After 24, 48 and 72 h of incubation at 37°C in 5% CO_2 atmosphere, 5-HT hydrochloride and 5-HT receptors agonists (BP554 maleate for 5-HT_{1A}, CP93129

dihydrochloride for 5-HT_{1B} and L-694247 hydrochloride for 5-HT_{1D}) and antagonists (Nan190 hydrobromide for 5-HT_{1A}, SB224289 hydrochloride for 5-HT_{1B} and BRL15572 hydrochloride for 5-HT_{1D}) diluted at concentrations of 3.125, 6.25, 12.5, 25 and 50 µM in final volume of 100 µL/well were added to the cells. It is noted that these concentrations and optimum incubation times were determined after pilot experiments and with regard to cell growth curve considered in other studies (Ishizuka et al. 1992). Cells cultured with media only served as a negative control. After 48-h incubation of cells at 37°C in 5% CO_2 atmosphere, the culture medium was removed and 8 µl MTT reagent (diluted in PBS at a concentration of 4 mg/mL) was added to 50 µl of fresh culture medium at a final concentration of 0.55 mg/mL (final volume of 100 µl/well).

After returning the plates for 2–4-h incubation at 37°C in 5% CO_2 atmosphere, MTT crystals were dissolved with DMSO and the absorbance was measured by spectrophotometer at wavelength of 570 nm (with a reference wavelength of 690 nm). For each assay, we used blank wells, containing only culture medium without cells. Mitogenic effect of 5-HT and 5-HT receptors agonists and antagonists on HT29 cells (proliferation index) was determined with the following formula:

Proliferation index = [mean absorbance in (570–690 nm) of each treated wells/mean absorbance in (570–690 nm) of control wells) × 100].

For each concentration of both receptor agonists and antagonists, we examined the proliferation of cells in three wells (triplicate, repeated three times) separately. Similar tests were carried out for the negative control. Finally, median and standard deviations of proliferation index for treated and control groups were determined and compared by one-way ANOVA. A $P < 0.05$ was used as the cut off. Dennett's multiple comparison test was used for the post hoc comparisons.

Cell cycle kinetics

HT29 cells (5×10^4) were treated with a single 5 µM dose of 5-$HT_{1A,1B,1D}$ antagonist for 4, 24 and 48 h. At the end of the treatment period, cells were collected, washed twice with PBS and treated with ribonuclease, Triton X-100 and propidium iodide. The percentage of cells in the $G_{1,}S$ and G_2/M phases of the cell cycle were determined using a FACScan flow cytometer (Becton–Dickinson, FACSort) and Multifit cell cycle analysis software (Pourgholami et al. 2005).

Western blotting analysis

For protein extraction from cells (HT29 as tumor cells, LS 174 human colon normal cells as negative control cells and A172 human brain cells as positive control cells), we used standard protocols according to other studies (Ishizuka et al. 1992; Launay et al. 1996; Pantaleo et al. 2006; Xu et al. 2006). After homogenization on ice seeds, cells were lysed in 1 mL lysis buffer (with 0.1% SDS and 0.5% sodium deoxycholate). The supernatants of lysis extracts of cells containing proteins were analyzed by the Bradford method for proteins assay (Bradford 1976). Then, aliquots of the extraction containing 5–10 mg/mL of protein were inserted into polyacrylamide SDS gel (10%) for electrophoresis. Electrophoresis was performed at 200 V (constant) with electrophoresis apparatus (Iran Pajooh Co.). Proteins of gel were then transferred onto a PVDF membrane during blotting process (constant 14 V, 3 h) through blotting instruments (Pharmacia Co.).

After blotting, nonspecific binding proteins on PVDF sheets was blocked with BSA1% in TBST overnight at a temperature of 4°C. Subsequently, the membranes were divided into three groups, and each group was incubated for 1 h with one of three kinds of primary antibodies (anti 5-HT_{1A} IgG, anti 5-HT_{1B} IgG and anti 5-HT_{1D} IgG at a dilution of 1:1,000 in 0.5% blocking solution); then the membranes were washed three times for 15 min each time with 50 mL of tris-buffered saline [50 mM tris base 150 mM NaCl containing 0.05% Tween 20 (TBS-Tween)]. They were incubated with the secondary antibody (donkey anti goat IgG-HRP 1:10,000) for 1 h which was common for all three primary antibodies. Finally, the membranes were washed four times for 15–20 min in 50 mL of TBST. Immunodetection was performed using luminol solution (Santacruz Co.) under chemiluminescence reaction on graph sheets (Amarsham Co.).

For loading positive control, we used homogenate proteins of human brain cells (A172), in which proteins of all 5-HT receptors subtypes express clearly. For negative control, we used normal human colon normal cell line (LS 174). This test was repeated three times.

Immunohistochemistry

We prepared 12 slides from each of 30 patients; 9 slides from tumor tissue and 3 slides from normal marginal tissue of the same patient for negative control. First, we prepared and adhered 3-µm paraffin sections on slides, de-paraffinized with xylol and dehydrated with graded alcohol, then prepared for antigen retrieval. For this purpose, they were incubated with citrate buffer (10 mM, pH 6.0) and heated twice in a microwave oven at 750 W for 5 min. This was followed by incubating the sections with primary polyclonal goat IgG (ant 5-$HT_{1A,1B,1D}$) at 1:250 dilution for 24 h at 4°C. For each patient, three slides were incubated with 5-HT_{1A} receptor primary antibody, three slides were

incubated with 5-HT$_{1B}$ primary antibody and another three slides were incubated with 5-HT$_{1D}$ primary antibody. The slides were then washed in TBST (for 3 times) and incubated with secondary antibody (donkey anti goat IgG-biotinylated conjugate at 1:300 dilution) for 1 h at 37°C. After washing, they were incubated with avidin–biotin peroxidase complex for 45 min. Subsequently, we washed the tissues again and added DAB for 10 min. The sections were finally counterstained with hematoxylin, dehydrated, cleared and mounted. Immunoreactivity index for each receptor was determined using a semiquantitative method as follows:

Scanning and scoring of the slides were conducted using Aprio Slide Scanner and Software (Germany). For each slide, 1 μm^2 zone has been scored with 10 × 10 and 10 × 40 magnifications. Positive results were detected by brown DAB precipitates in membranes and cytoplasms of epithelial cells of the tissues. The percentage of cells with their cytoplasmic membranes or cytoplasms colored with DAB was determined as an index for each slide. After calculating immunoreactivity index of the receptors for each patient, we calculated mean and SD of 30 patients. Data analysis was carried out using Student's *t* test (Bradford 1976; Dizeyi et al. 2004; Siddiqui et al. 2006).

Apoptosis assay

TUNEL (TdT-mediated dUTP-biotin nick end labeling) tests were used to measure cell apoptosis after exposure to 5-HT receptor antagonists. For the purpose of this study, the TdT-FragEL DNA fragmentation (fluorescein labeling) detection kit (Roach Co., Germany) was utilized. HT29 cells in quantity of 5 × 10^5 per well were incubated with receptor antagonists at three concentrations (1, 2, 5 μM/well) (3 wells for each concentration) for 24 and 48 h in 37°C incubator with 5% CO_2. Three wells were not administered receptor antagonist and were considered negative controls. After the incubation period, the cells were examined for apoptosis with TUNEL staining method.

After labeling, we used fluorescence microscope to count apoptotic cells (identified by fluorescent green color absorption) in microscopic zone of 1 mm^2. For detecting apoptotic cells and determining apoptotic index, 250 μL of cells supernatant from both treated and control groups were spread on the slides and viewed with a fluorescence microscope at 10 × 10 and 10 × 40 magnifications. At least, 500 tumor cells were counted for each sample and the apoptotic index was determined by the percentage of apoptotic cells in total cells. This apoptosis study was repeated three times.

Results

MTT proliferation assay

MTT assay showed a proliferative effect of 5-HT, 5-HT$_{1A}$ and 5-HT$_{1B}$ receptor agonists (BP554 maleate and CP93129 dihydrochloride, respectively) in a dose–response relationship (Table 1; Fig. 1a). Within a concentration between 3,125 and 12,500 μM, there was a significant increase in HT29 cell proliferation after a 48-h incubation period when compared with the control group. The data also showed that, while 5-HT$_{1B}$ and 5-HT$_{1A}$ receptor agonists showed significant effect on HT29 proliferation, the effect of 5-HT$_{1D}$ was not significant. In addition, there was a

Table 1 One-way ANOVA plus Dennett's multiple comparison tests for 5-HT$_1$ receptors' agonists and antagonists compared with controls (0 μM)

Comparison	Concentrations (μM)	Mean difference	*F*	*P* value
Control versus antagonist 5-HT$_{1A}$	12.5	15.000	4.896	**
Control versus antagonist 5-HT$_{1A}$	6.25	4.667	1.523	NS
Control versus antagonist 5-HT$_{1B}$	12.5	66.000	30.466	***
Control versus antagonist 5-HT$_{1B}$	6.25	48.000	22.157	***
Control versus antagonist 5-HT$_{1D}$	12.5	2.456	0.9150	NS
Control versus antagonist 5-HT$_{1D}$	6.25	1.867	0.4575	NS
Control versus serotonin	12.5	−25.000	7.485	**
Control versus serotonin	6.25	−12.000	4.491	*
Control versus agonist 5-HT$_{1A}$	6.25	−13.000	5.988	*
Control versus agonist 5-HT$_{1A}$	3.125	−12.000	4.491	*
Control versus agonist 5-HT$_{1B}$	6.25	−48.000	14.371	**
Control versus agonist 5-HT$_{1B}$	3.125	−25.000	7.784	*
Control versus agonist 5-HT$_{1D}$	12.5	−9.000	2.695	NS
Control versus agonist 5-HT$_{1D}$	6.25	−1.333	0.3992	NS
Control versus agonist 5-HT$_{1D}$	3.125	−1.212	0.384	NS

NS non-significant

Mean difference is defined as the difference in cell proliferation between treated and normal cells for each concentration

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

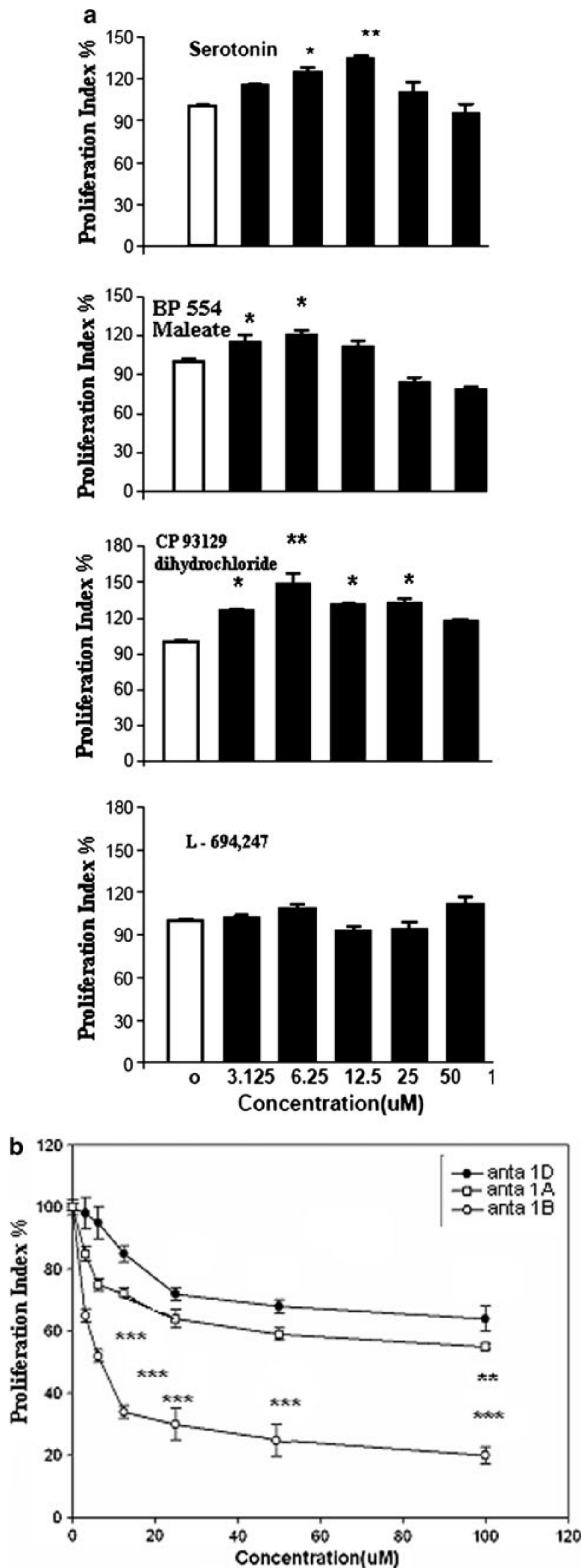

Fig. 1 **a** MTT assay results effect of different concentrations of 5-HT (serotonin) and 5-HT receptor agonists on proliferation index of HT29 cells after 48-h incubation period (BP554 maleate = selective 5-HT_{1A} agonist, CP93129 dihydrochloride = selective 5-HT_{1B} agonist, L-694,247 = selective 5-HT_{1D}agonist.), $*P < 0.05$; $**P < 0.01$. **b** Dose–response curve for the effect of different concentrations of 5-$HT_{1A,\ 1B,\ 1D}$ receptors antagonists (*anta 1A*, *1B*, *1D*) on proliferation index of HT29 cells after 48-h incubation period; $**P < 0.01$; $***P < 0.001$. **c** Cell cycle kinetics by flowcytometry for HT29 cells incubated (for 48 h) with 5-$HT_{1B,\ 1A,\ 1D}$ receptor selective antagonists (5 μM). Cells were processed by FACS analysis to determine the cell cycle phase kinetics (*A* G_0; *B–D* cells in G_1, S and G_2/M phases, respectively); *I* flowcytometry of HT29, cells incubated with 5-HT_{1B} antagonist, *II* flowcytometry of HT29, cells incubated with 5-HT_{1D} antagonist, *III* flowcytometry of HT29 cells incubated with 5-HT_{1A} antagonist

dose-dependent inhibitory effect for selective antagonists of 5-HT_{1A} (Nan190 hydrobromide) and 5-HT_{1B} (SB224289 hydrochloride) on proliferation of HT29 cells in a 48-h period. The most striking finding was for 5-HT_{1B} antagonist, while 5-HT_{1D} antagonist showed no significant anti-proliferative effect (Table 1; Fig. 1b).

Cell cycle kinetics

Flowcytometric analysis of 5-HT_1 receptor antagonists in association with HT29 cells proliferation revealed cell cycle arrest at G_2/M phase especially for 5-HT_{1B} (Table 2; Fig. 1c). FACScan analysis showed that during the 48 h after treating the cells with 5-HT_{1B} the percentage of cells in G_0/G_1 phase significantly declined from 81.1% (SE = 0.2) to 32.5% (SE = 0.1). However, during the same period the proportion of cells in G_2/M phase increased from 1.3% (SE = 0.2) to 40.3% (SE = 0.4). Concomitantly, there was a reduction in the percentage of cells in the S phase. Cell cycle arrest for 5-HT_{1A} receptor antagonist was much less than 5-HT_{1B} and there was no significant effect related to 5-HT_{1D} (Table 2).

Immunohistochemistry

We used IHC assay to identify 5-HT receptors and their distribution semiquantitatively. IHC staining of colon cancer tissues of 30 patients in grades II and III colon or rectum adenocarcinoma indicated numerous 5-HT_{1B} receptors in tumor biopsies (immunoreactivity index = 78%). However, for 5-HT_{1A} and 5-HT_{1D}, there was low or insignificant immunoreactivity, respectively; and nil or very low immunoreactivity in the negative control group (normal colon tissue). Both cancerous and control tissues were administered primary and secondary antibodies to detect 5-HT_1 receptor and its sub types (Fig. 2). Positive immunoreactivity was

Fig. 1 continued

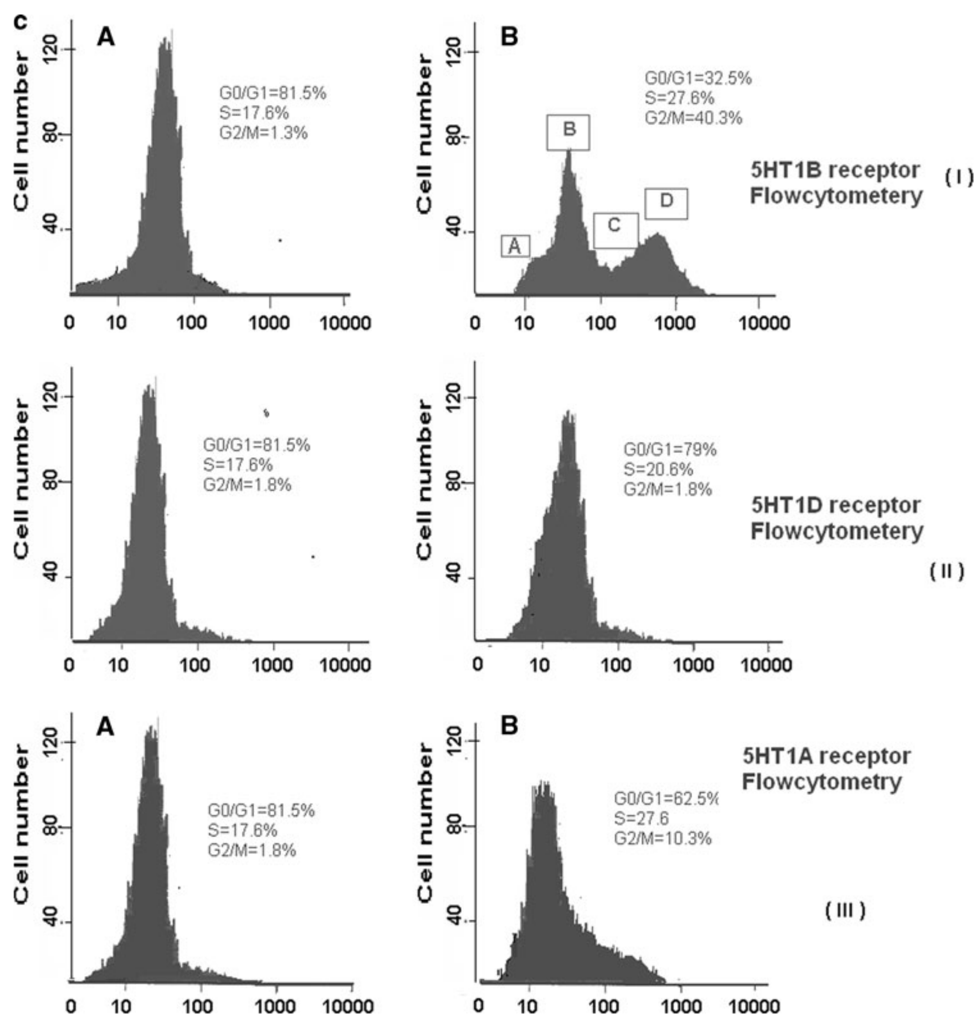

Table 2 Induction of cell cycle arrest in HT29 cells after 48-h incubation with Nan190 hydrobromide (5-HT_{1A} antagonist), SB224289 hydrochloride (5-H_{1B} antagonist), and BRL15572 hydrochloride (5-H_{1D} antagonist) with dosage of 5 μM

	Cell cycle phase		
	G_0/G_1	S	G_2/M
Control	81.1% ± 0.2	17.6% ± 0.1	1.3% ± 0.2
Nan190 hydrobromide	56.1% ± 0.2	37.6% ± 0.3	16.3% ± 0.2
SB224289 hydrochloride	32.5% ± 0.1	7.6% ± 0.2	40.3% ± 0.4
BRL15572 hydrochloride	71% ± 0.1	22.7% ± 0.1	6.3% ± 0.3

demonstrated by brown-colored areas in cytoplasmic membranes of colon or rectal epithelial cells. These analyses demonstrated extensive distribution of membranous 5-HT_{1B} receptors on cancerous tissues (Fig. 2).

Western blotting analysis

Western blot analysis of HT29 cells showed high expression of 5-HT_{1B} receptor proteins in HT29 cells. Protein expressions for 5-HT_{1A} and 5-HT_{1D} were mild and non-significant, respectively (Fig. 3). Polyclonal anti-5-HT_{1B} IgGs recognized potent single bands of 46 kDa in HT29 cell line and polyclonal anti-5-HT_{1A} IgG revealed single bands of 43 kDa for 5-HT_{1D} receptors. For positive protein control, we used homogenate of protein extracted from human brain cells (A172 cell line) in which all 5-HT receptors expressed clearly. For negative control, we used normal human colon normal cells (LS174 cell line) (Fig. 3).

Apoptosis assay

As primary analyses indicated antiproliferative effect for 5-HT_1 receptors antagonists, we limited our apoptosis study to receptor antagonists. This assay revealed that incubation of HT29 cells at a cell density of 5×10^5 cells/mL in the presence of 5-HT_{1B} antagonist (5 μM/mL, 24 h at 37°C, 5% CO_2, 90% humidity) led to apoptosis which was analyzed by fluorescence microscopy. For 5-HT_{1A} and 5-HT_{1D} antagonist, on the other hand, showed low or insignificant apoptosis (Fig. 4). Fluorescence microscopy was used to detect apoptotic cells as measured by green fluorescence

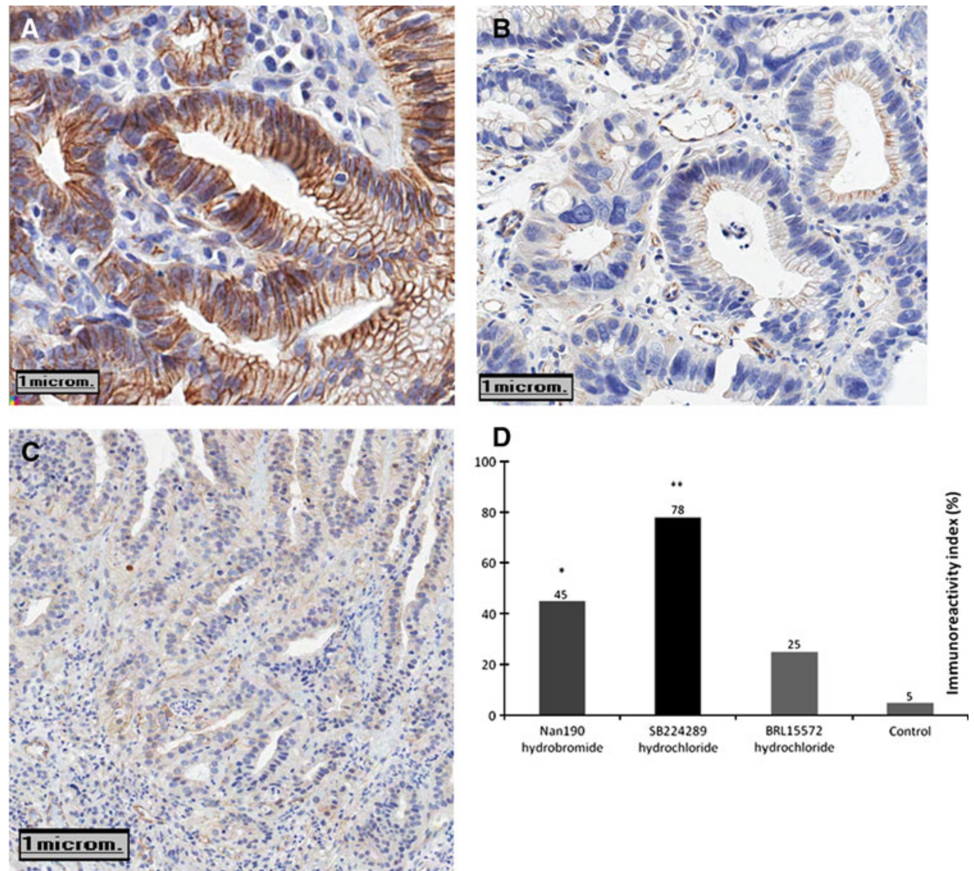

Fig. 2 Immunohistochemistry (IHC) (avidin–biotin peroxidase staining procedure) of tumor tissues from a 45 years old patient with grade II colorectal cancer; positivity is illustrated as *brown* areas resulted from precipitation of DAB-Biotinylated conjugate complex in cell membrane. **a** High positive IHC result for 5-HT1B receptor of tumor tissue, **b** moderate positive IHC result for 5-HT_{1A} receptor, **c** mild positive IHC result for 5-HT_{1D} receptor, **d** comparison of IHC immunoreactivity between patients with three kinds of receptors (5-$HT_{1A,\ 1B,\ 1D}$) and negative control; *$P < 0.05$; **$P < 0.01$

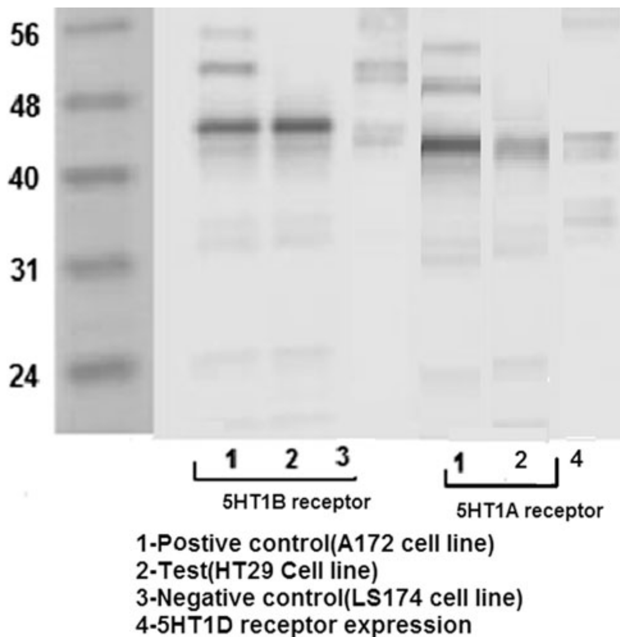

Fig. 3 Western blotting analysis of HT29 cells with 5-HT receptor antibodies; positive control was homogenate proteins of human brain cell line (A172) and negative control was proteins of normal human colon cell line (LS174). Weight ranges of protein bands were determined by comparing the size markers of proteins

coloring, taken during TUNEL process, which was detectable in the range of 515–565 nm (green light) (Fig. 4). By counting the number of these apoptotic cells and dividing by total cells (at least 500 tumor cells in each slide) an apoptotic index for each study intervention was calculated; negative control was the group of cells which were not administered any intervention, and had shown some degree of apoptosis after a similar stain process. Apoptosis index of test groups was compared against controls using *t* test (Fig. 4). As expected, apoptosis index was highest (56%) for 5-HT_{1B} antagonist; for 5-HT_{1A} and 5-HT_{1D} the index was 32 and 18%, respectively. The latter two were not statistically and significantly different compared with control group (15%).

Discussion

In the present study, we found that 5-HT_1 serotonergic receptors are expressed in human colon cancer cell line (HT29) and in the tumor tissues. Among three subtypes of 5-HT_1, the most expression was observed for 5-HT_{1B}. MTT proliferation and apoptotic assays indicated the substantial role of this receptor subtype in the growth of tumor cells.

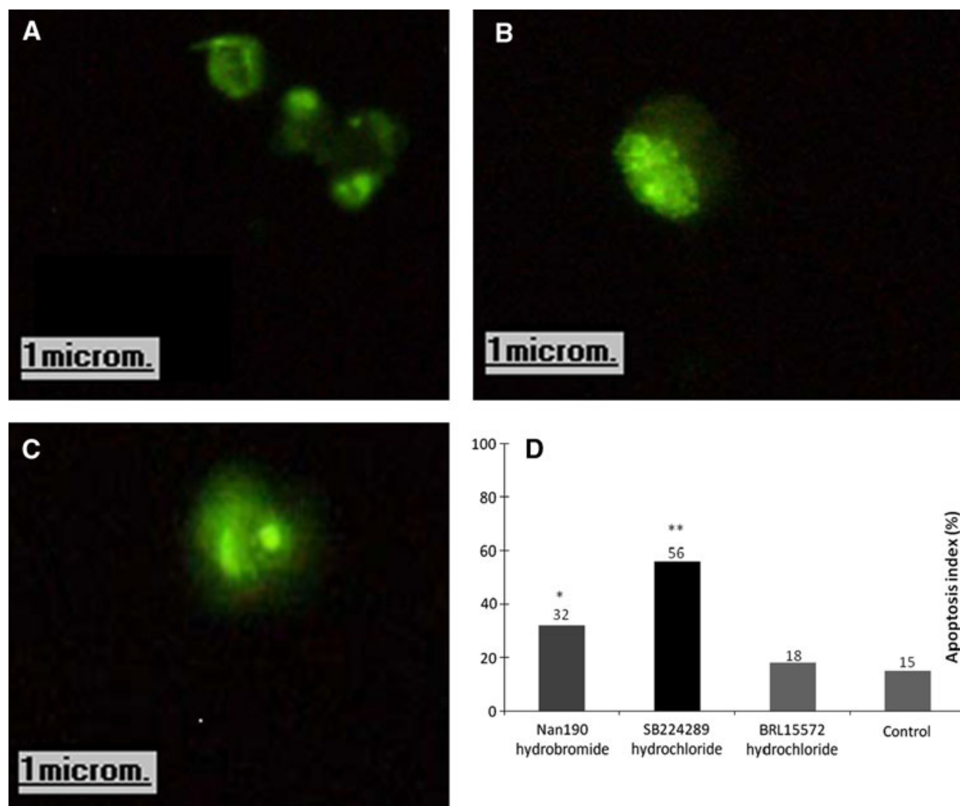

Fig. 4 Apoptosis assay for HT29 cells with TUNEL procedure after 48-h incubation of cells with 5-HT receptor antagonists (5 μM). Apoptosis was detected by fluorescence microscopy (excitation wavelength in the range of 450–500 nm, detection in the range of 515–565 nm). **a** Apoptotic HT29 cell after incubation with 5-HT_{1D} antagonist (5 μM) for 48 h (green fluorescence of cell nucleus). **b** Apoptotic HT29 cell after incubation with 5-HT_{1A} antagonist (5 μM) for 48 h. **c** Apoptotic HT29 cell after incubation with 5-HT_{1B} antagonist (5 μM) for 48 h. **d** Comparison of apoptotic index between three test groups and negative control; $*P < 0.05$; $**P < 0.01$

When compared with control cells, this study also showed that 5-HT can stimulate HT29 cell proliferation (preferably in a concentration between 3.125 and 12.5 μM) in the presence of 10% FBS. The mitogenic effect of 5-HT on HT29 cells may be dependent on the presence of serum and its interaction with other hormones or growth factors (Sharpe et al. 2002; Sonier et al. 2006). In support of our study, previous investigations have suggested that 5-HT increases the growth rate of HT1376 bladder cancer cells (Siddiqui et al. 2006) and human prostate cancer cells (Dizeyi et al. 2004). Another study has shown that the proliferative properties of 5-HT may occur due to its binding to different 5-HT receptor subtypes which are associated with cascades of mitogenic signaling pathways (Launay et al. 1996). There are also some reports indicating that 5-HT may be involved in the autocrine loops of growth factors contributing to cell proliferation in some aggressive tumors (Cattaneo et al. 1993, 1995; El-Salhy 2005; El-Salhy et al. 2005; Tjomsland and El-Salhy 2005).

In addition, our data show proliferative effects of BP554 maleate and CP93129 dihydrochloride (selective 5-HT_{1A} and 5-HT_{1B} receptors agonists, respectively) on HT29 cells (in 3.125–6.25 μM concentrations). Although we have seen proliferative effects for serotonin and 5-HT_{1A} and 5-HT_{1B} receptors' agonists in low to moderate concentrations, their antiproliferative effects at high concentrations may be associated with their chemical toxicity on cells (Menegola et al. 2004; Siddiqui et al. 2005). The antagonists of these receptors were found to have antiproliferative effects and to reduce cell growth. This antimitogenic effect was particularly notable for SB224289 hydrochloride.

Our study revealed one major potent protein band of 46 kDa for 5-HT_{1B} receptor and a mild protein band for 5-HT_{1A} receptor (43 kDa) in HT29 cell line comparable with that of normal human brain cells in positive controls. Previously, it was indicated that the 5-HT_{1A} and 5-HT_{1B} receptors are extensively expressed in the human prostate and bladder cancer cell lines which could explain the mitogenic effects of 5-HT_{1B} and 5-HT_{1A} receptor agonists in prostate and bladder cancers (Dizeyi et al. 2004; Siddiqui et al. 2006; Sonier et al. 2005, 2006).

This study found the most apoptotic effect induced by SB224289 hydrochloride. Previous research has suggested that apoptosis is impaired in colon adenocarcinoma and one important goal in colorectal cancer is to find some agents that increases the apoptosis process (Oosterbosch et al. 1993). Our findings suggest that 5-HT_{1A} and 5-HT_{1B} receptors can be involved in pathophysiology of colorectal cancer and the role of 5-HT_{1B} receptor is of major importance. Accordingly, it is suggested that serotonin might most likely induce its mitogenic effect via 5-HT_{1B} receptors.

To date, the role of central serotonergic systems in the etiology of depression is well documented (Pezzella et al. 2001). There is evidence that the incidence of depression

increases in patients with cancer, comprising 25% of this sub-population (Huerta et al. 2006; Pezzella et al. 2001). It seems plausible that introducing phytochemicals which have the most interactions with specific monoamine receptors (expressed highly in colorectal cancer tissues) may initiate new approaches in control of this malignancy (Mastrangelo et al. 2008). There is ongoing debate about whether antidepressant medications may increase or decrease the risk of colon cancer. There is a need for further research to investigate their role in prevention, treatment or prognosis of patients with colon cancer, and to study other selective 5-HT receptors subtypes that may play a significant role in the pathophysiology of this disease.

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