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Original Article

The Serotonin 5-HT2A Receptor Antagonist Ritanserin Induces Apoptosis in Human Colorectal Cancer and Acts in Synergy with Curcumin

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Curcumin exhibits both cancer-preventive activity and growth inhibitory effects on several neoplastic cells including human colon cancer. Serotonin and its receptors have also been implicated in tumor development. This study investigated the effect of ritanserin, a selective serotonin $5HT_{2A}$ receptor antagonist, alone and in combination with curcumin on colorectal cancer cell lines. Results show that the expression of the serotonin $5HT_{2A}$ receptor is higher in tumor than in normal colorectal tissues. Ritanserin, reduced cell viability in a dose-dependent manner, and increased apoptosis in HT29, SW480 and SW742 colorectal cancer cell lines as analyzed by MTT and TUNEL assays, respectively. Moreover, combined with curcumin, ritanserin synergistically increased the number of hypodiploid cells and DNA fragmentation and decreased cell viability in all colorectal cancer cell lines. This study demonstrates that curcumin and ritanserin have a synergic anti-mitogenic and apoptotic effect on colorectal cancer cell lines. These results suggest a potential use of serotonin $5HT_{2A}$ receptor antagonist in co- treatment with curcumin in colon cancer therapy.

Keywords: Colon cancer, serotonin $5HT_{2A}$ receptor, cell growth, ritanserin, TUNEL assay

Colorectal cancer affects worldwide one million people every year and is the second most common cause of cancer death in men and women (17). Over the last decade, significant progress has been made in the treatment of this tumor and new chemotherapeutic agents have been introduced such as 5- Fluorouracil- leucovorin (thymidylate synthase inhibitor), Irinothecan (topoisomerase inhibitor), Oxaliplatin (DNA replication inhibitor), Bevacizum -ab (anti- angiogenic), and Cetoximab (monoclonal antibody targeting the EGFR) (17).

However, chemotherapy is still fraught with

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side effects and long- term treatment failure.

Curcumin, a phenolic derived from the rhizome of the Curcuma longa linn (Zingiberaceae), has been recognized as a promising anti-cancer drug due to its multiple properties, including anti- inflammatory (8), antioxidant (10, 19) and anti-angiogenic (14, 28). In populations where curcumin is a part of the staple diet, lower incidence of urothelial malignancies and colorectal cancer has been observed (5, 11, 21). In rodents, oral administration of curcumin inhibit polyp formation and increased cell death in existing colon cancer lesions (30). Among the potential pathways involved in this anti- carcinogenic properties, inhibition of nitric oxide synthase, activation of receptor tyrosine kinase and protein kinase C (PKC), alteration of the transcriptional factors c-jun/AP-1 and p53 as well as inhibition of arachidonic acid metabolism, lipoxygenase and cycloxygenase activity have been described (6, 19, 24). Combined to other anti- cancer chemicals such as Notch1 and NFκ-B or green tea, curcumin induces a strong inhibitory effect in oral cancer and non- small cell lung cancer (13, 25). Based on these observations, curcumin may be a potential cancer chemo preventive agent.

Interestingly, a high intake of selective serotonin reuptake inhibitors (SSRIs) correlating with a reduced incidence of colorectal cancer was shown (27, 29). *In vitro*, fluoxetine reduced the growth of COLO320 DM colon cancer cells which was attributed to an increase in serotonin levels in gastrointestinal tract (29). Serotonin is a hormone that mediates a wide variety of physiological effects including peripheral and central action through the binding of multiple receptor subtypes (18). The diverse effects of serotonin are due to its multiple 5-HT receptor subtypes and their pharmacological complexity. The serotonin 5-HT2 receptor (5-HT2R) family comprises three subtypes: $5HT_{2A}R$, $5HT_{2B}R$ and $5HT_{2C}R$. The $5HT_{2A}R$ is widely distributed in peripheral tissue, including the human gastrointestinal tract (4, 12) and acts as a mitogenic receptor in different cell types (4, 7, 12, 20, 22, 23). We have previously found a mitogenic effect for $5HT_{1B}R$, $5HT_{3A}R$ and $5HT_{3B}R$ in colorectal cancer cells and tissues (1-3). However, the effects of $5HT_{2A}R$ agonists and antagonists in colorectal cancer have never been studied. Thus, in this report we investigated the effect of ritanserin, a selective $5HT_{2A}R$ antagonist, alone or in combination with curcumin on colorectal cancer cell lines.

Materials and methods

Colorectal cell lines and tissues

Three colorectal cancer cell lines (HT29, SW480, SW742) were obtained from the cell bank of Pasteur Institute of Iran (Teheran, Iran). Paraffin blocks belonging to 100 patients with grade II and III of colon or rectum adenocarcinoma were obtained from the department of pathology of Bou Ali hospital, Sari, Iran, between years 2012 and 2014. Patients were between 30- 70 years old and have not received any chemotherapy or radiotherapy treatment. Human colon cancer and normal marginal tissue samples were obtained after surgery from informed consenting patients. The study was approved by the ethical committee of Mazandaran University of Medical Sciences.

MTT assay

Colon cancer cell lines were plated at a density of 1×10^4 cells/well in a 96-well plate in a final volume of 100 µl/well in the presence of RPMI 1640 supplemented with L-glutamine and 10% fetal calf serum, (Sigma-Aldrich, St- Louis, MI, USA). After 24 h at 37 ºC in 5% CO_2 atmosphere, cells were treated with (±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI), serotonin or curcumin (Sigma-Aldrich, St- Louis, MI, USA) at 0, 3.125,

6.25, 12.5, 25, 50 and 100 µM with or without 60 min pre-treatment with ritanserin (Tocris, Bristol, UK) 0.1 µM or 10 µM. Cell cultures with medium only served as negative control. After 48 h of incubation at 37 ºC in 5% CO_2 atmosphere, the culture medium was removed and 8 µl MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) reagent (Sigma-Aldrich, St-Louis, MI, USA) 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 100 µl/well). Cells were then incubated for 4 h, the resulting MTT formazan crystals were dissolved with DMSO (50 µl/ well) by pipetting up and down 30 times, and the absorbance was spectrophotometrically measured at 570 nm (with a reference wavelength of 690 nm) using a microplate reader. Each assay included blank (culture medium without cells) and vehicle control (cells exposed to culture media only). Cell viability (% of control) was determined using the following formula: ($A_{(570–690nm)}$ of each treated wells/ $A_{(570–690nm)}$ average of control wells) × 100.

Western blot analysis

To detect the presence of $5HT_{2A}R$ proteins in colon cancer tissues and colorectal cell lines, total proteins were extracted by homogenization in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% SDS, 5% sodium deoxycholate, 1 tablet of complete proteases inhibitor (Sigma-Aldrich, St- Louis, MI, USA)). Homogenized extracts were agitated at 4 ºC for 1 h and then centrifuged at 1200 x g for 20 min. Total proteins were stored at -80 ºC for future analysis. Protein concentrations were measured using Bradford protein assay with bovine serum albumin (BSA) as standard. Fifteen mg of total protein extracts were denatured at 95 ºC for 10 min and fractionated by SDS- 10% polyacrylamide gel electrophoresis, followed by transfer to PVDF membrane (Millipore, Schwalbach/Ts, Germany). The membranes were blocked in TBS-T blocking solution (50 mM Tris-Base, 150 mM NaCl and 0.05% (v/v) Tween-20 (TBS-Tween)) containing 1% BSA, overnight at 4 ºC. Membranes were incubated for 1 h with polyclonal antibody against $5HT_{2A}R$ (Santa Cruz Biotechnology, CA, US) diluted 1:500 in TBS-T containing 1% BSA and washed three times with TBS-T for 15 min. and then were incubated with the horse radish peroxydase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, CA, USA) at room temperature for 1 h and then washed four times with TBS-T for 15 min. Immunodetection was performed using Amersham ECL™ Western Blotting System and autoradiography was performed using Photosensit -ive film (Hyperfilm-ECL, Amersham).

Immunohistochemical analysis (IHC)

IHC was performed to determine the localization of $5HT_{2A}R$ proteins in colorectal tissues. Tissue sections (3 µm) from paraffin blocks were deparaffinized and hydrated. For antigen retrieval, sections were incubated with citrate buffer (10 mM, pH 6.0), heated twice in microwave at 750 w for 5 min. After a cool recycling period, sections were washed three times in TBS and incubated with normal goat IgG (10%) for 20 min. To quench endogenous peroxidase activity, sections were treated with 0.05% hydrogen peroxide for 15 min. Non-specific antigen binding was blocked by incubation in blocking buffer (TBS containing 10 % goat serum, and 0.1% Triton X-100) for 20 min at room temperature. The sections were incubated 24 h at 4 ºC with the specific immunohistochemical primary antibody (anti-$5HT_{2A}R$, 1:250) diluted in TBS containing 0.1% Triton X-100. The sections were washed with TBS and incubated with the secondary antibody (Donkey anti goat IgG-Biotinylated Conjugate) for 2 h at 37 ºC followed by

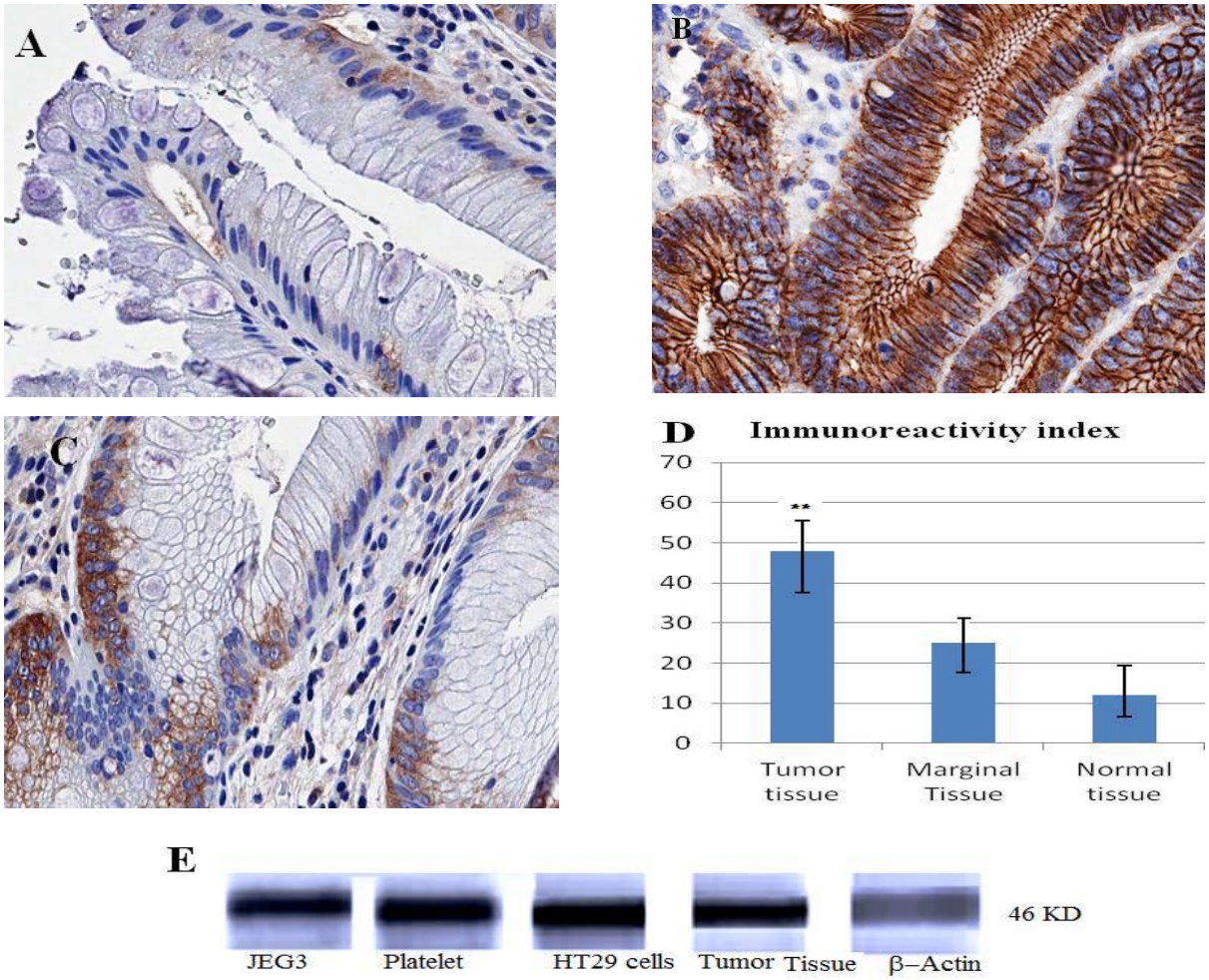

Figure 1. Expression of the $5HT_{2A}$ serotonin receptor in human colon cancer tissues and cell lines. (A) Negative control, marginal tissue incubated without primary antibody but with secondary antibody; (B) tumor tissue with pathological grade III, and (C) normal, marginal tissue incubated with anti-$5HT_{2A}$. Pictures are representative of at least 10 independent experiments from 100 different colon tumors at 100X magnification. (D) Comparison of immunoreactivity index for $5HT_{2A}R$ between 100 tumor tissues (patients) and 100 normal marginal tissues (** P< 0.01). (E) Western blot analysis of $5HT_{2A}R$ protein expression in the human colon tumor tissue and colon cancer cell lines. JEG-3 cells and platelet membrane proteins were used as positive controls. Representative immunoblots from three independent experiments are shown.

an incubation with avidin- biotin peroxidase (1:20000 in TBST) for 45 min. Sections were then incubated with the immunodetection solution (DAB + H_2O_2, 0.05%) for 10 min, washed with tap water, and counterstained with heamatoxylin. To exclude non-specific staining, parallel sections were processed as above but specific primary antibodies were omitted. Sections were viewed with a light microscope and photographed. For all immunolabeling studies, assessment of levels of immunoreactivity (immunoreactivity index) was performed using a semi-quantitative method. For each slide a total of five zones of 1 μm^2 were analyzed to estimate the average number of immunolabeled cells per unit area (μm^2). The immunoreactivity index (mean± SD) was determined for 10 patients (10 sections) and then estimated for 100 patients.

TUNEL assay

Apoptosis was evaluated using a commercially available TUNEL kit (Roche Diagnostics, Penzberg, Germany). as previously described (3, 7). Briefly colon cancer cells were seeded at a density of 1×10^4 cells/ well (final volume 100 µl/well) in 96-well plate. After 24 h, cells were treated with 25 µM of ritanserin or curcumin, with or without 10 µM

ritanserin, for 10 h at 37 ºC in 5% CO_2 atmosphere, and then were assayed for apoptosis with TUNEL apoptotic assay. After fixation and prelabeling, cells were washed twice with PBS (200 µl/well) and then suspended in 50 µl/ well TUNEL reaction mixture (50 µl of enzyme solution in 450 µl label solution); for the negative control, only 50 µl of label solution was added to the wells. Then cells were incubated for 60 min at 37 ºC in a humidified atmosphere in dark. After this labeling period, the number of TUNEL-positive cells was analyzed by microscope. Apoptosis index was determined as the percentage of TUNEL-positive cells to total cells in a suspension with at least 500 tumor cells. Under microscopic area, necrotic cells were differentiating from apoptotic cells according to their cellular morphologic changes and loss of nuclear membrane integrity.

Statistical analysis

For MTT assay, immunohistochemistry and TUNEL experiments, data analysis was performed by Student t-test analysis.

Results

Colorectal cancer tissues and cell lines express the $5HT_{2A}R$

Immunohistochemistry analysis performed on human colorectal tissues revealed that the $5HT_{2A}R$ is highly expressed in tumor grade III tissues Fig. 1B compared to normal marginal tissues Fig. 1C. The $5HT_{2A}R$ immunoreactivity was mainly detected in epithelial cells. The immunoreactivity index for 100 tumor biopsies was 48% which was significant compared to 100 normal biopsies 25% Fig. 1D. Immunoreactivity was observed in cytoplasmic membranes and cytoplasm of epithelial cells which showed a large distribution of the $5HT_{2A}R$ in colon tissues. To assess the selectivity of the primary antibody, a negative control was performed with secondary antibody only Fig. 1A.

Western blot analysis was used to confirm the expression of the $5HT_{2A}R$ in human colorectal tissues and to determine its expression in SW480, SW742 and HT29 human colorectal cell lines. Human platelet and JEG-3 choriocarcinoma cell line known to express serotonin $5HT_{2A}R$ were used as positive controls (22, 23). Immunoblotting using the goat polyclonal antibody against $5HT_{2A}R$ revealed, as expected, a single band of 46 kDa in examined cell lines and tissues as observed in positive control s Fig. 1E.

Serotonin increases colorectal cancer cell lines viability throughout the activation of its $5HT_{2A}R$

Figure 2 shows that serotonin and DOI increase SW480, SW742 and HT29 colorectal cancer cells growth and viability in a dose-dependent manner (ranging from 3.125- 50 µM) compared to control. DOI-induced colorectal cancer cells growth and viability is completely reversed by 10 µM of ritanserin in all three cell lines. These results indicate that $5HT_{2A}R$ plays an important role in the regulation of colorectal cancer cell growth and viability.

Ritanserin in synergy with curcumin decreased colorectal cancer cell lines viability

The effect of curcumin and ritanserin individually on HT29, SW480 and SW742 colorectal cancer cell lines viability was first analyzed by MTT assay. Curcumin alone at 25 to 100 µ M reduced cell viability in all cell lines, and this effect is higher in SW480 (40- 45%) compared to HT29 and SW742 (10- 15%) Figure.3. The same profile of reduction in viability was observed with ritanserin even slightly more pronounced than with

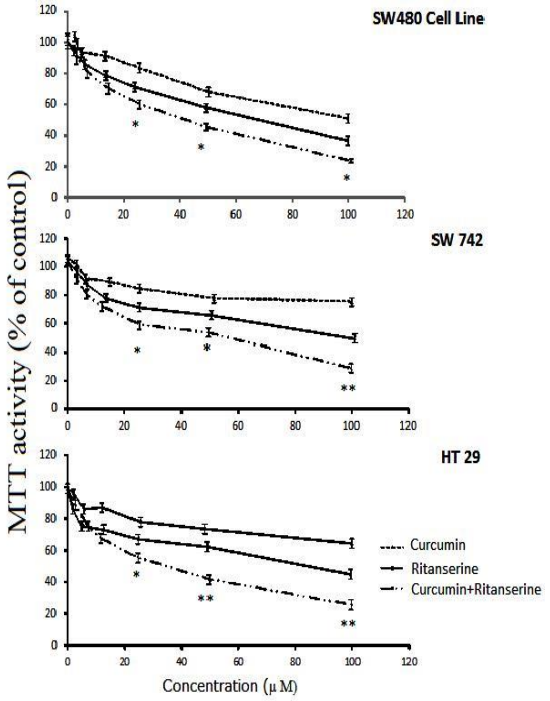

Figure 2. Effect of serotonin and $5HT_{2A}$ receptor agonist (DOI hydrochloride) on colon cancer cell lines viability.

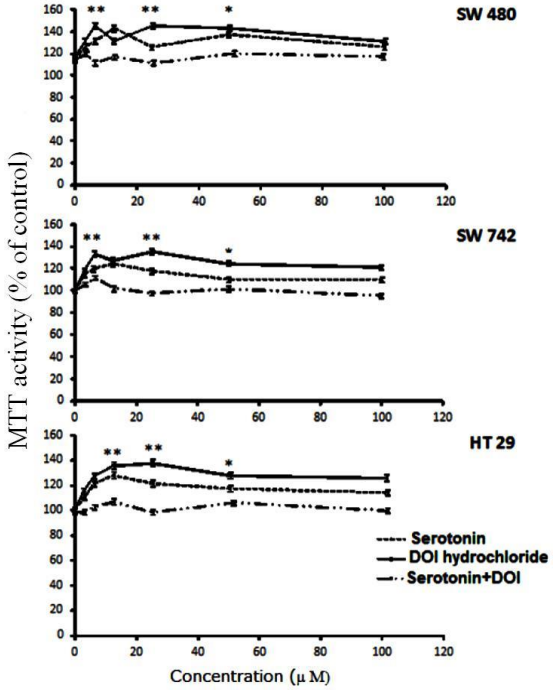

Figure 3. Effect of ritanserin and curcumin on colon cancer cell lines viability. The results represent the mean (± SEM) values obtained from three different assays (with three different cell passages). The coefficient of variation is less than 10%. * P< 0.05 and ** P< 0.01 vs. control.

curcumin. The combination of curcumin and ritanserin, at 25, 50 and 100 µM for both drugs, produced a significant synergic reduction of cell growth and viability in all three cell lines.

Ritanserin in synergy with curcumin induced colorectal cancer cell lines apoptosis

To determine if the effect of ritanserin on cell viability was due to apoptosis, colorectal cancer cells lines were treated with 25 µM ritanserin alone and in combination with 25 µM curcumin and analyzed by the TUNEL assay and fluorescence microscopy. Figure 4 shows that ritanserin induced SW480, SW742 and HT29 colorectal cancer cell apoptosis. At concentration less than 25 µM we observed low grade of apoptosis and at higher concentration the cells had gone toward necrosis. DOI had no significant effect on apoptosis compared to control in all cell lines. Interestingly, ritanserin combined to curcumin markedly enhanced (about 2 fold) colorectal cancer cells lines death compared to the curcumin or ritanserin alone in all cell lines Fig. 4 A- C.

Discussion

The present study demonstrated that $5HT_{2A}$ serotoninergic receptor is expressed in human colecrectal cancer cell lines and tissues. Serotonin and DOI, specific $5HT_{2A}R$ agonists, increased whereas ritanserin, a specific $5HT_{2A}R$ antagonist, decreased colorectal cancer cell lines viability in a concentration dependent manner. Interestingly, the combination of ritanserin with the plant derived-curcumin synergistically induced both a reduction of cell viability and an increase of apoptosis in colorectal cancer cells lines.

Western blot and IHC analysis demonstrated clearly the expression of the $5HT_{2A}R$ in colon cancer cells and tissues, suggesting that these cells are capable of responding to serotonin and $5HT_{2A}R$ agonists and antagonists. This is consistent with

results in figure 2 that demonstrate the ability of both DOI and serotonin to induce growth and viability of all colorectal cancer cell lines analyzed and the ability of ritanserin to block the action of DOI. The presence of $5HT_{2A}R$ in human colorectal cancer tissues as revealed by IHC analysis is relevant to the biology of colorectal cancer since the gastro-intestinal tract is the source of 90% of serotonin produced in body and that serotonin has been implicated in colon cancer as pro-inflammatory and pro-mitogenic molecule.

The fact that ritanserin alone reduced the basal growth and viability of all colorectal cancer cell lines analyzed, suggest a constitutive activation of $5HT_{2A}R$ in those cells which is consistent with the IHC staining that demonstrated a strong cytoplasmic

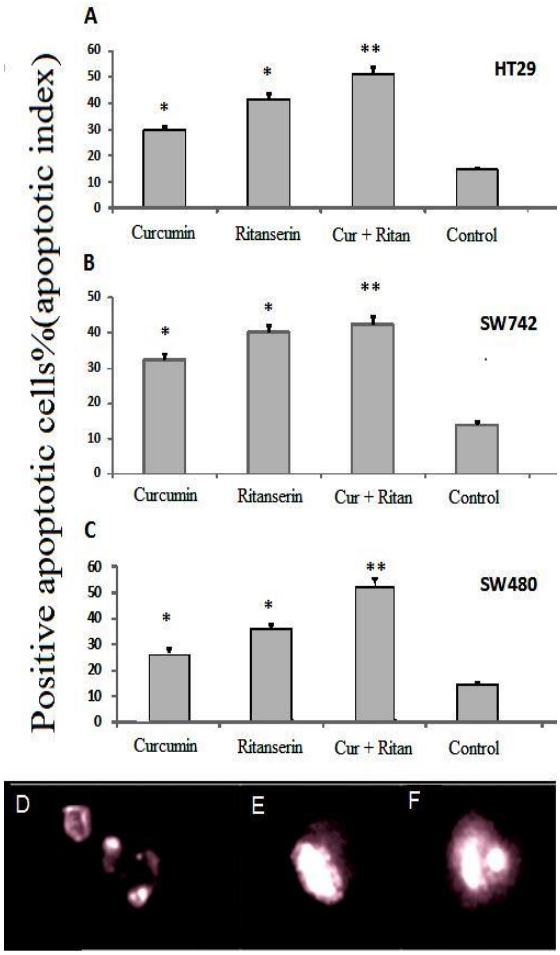

Figure 4. Effect of ritanserin and curcumin on colon cancer cell lines apoptosis. (A-C) apoptotic indexes of HT29, SW742 and SW480 cell lines respectively. (D-F) HT29, SW742 and SW480 apototic cells respectively. * P< 0.05 and ** P< 0.01 vs. control.

immunoreactivity of $5HT_{2A}R$ both in colorectal tumor biopsies and cancer cell lines. The immunofluorescence analysis shows that the $5HT_{2A}R$ is localized primarily in the cytoplasmic membrane of HT29 cell line and cytoplasm of epithelial cells. It has been proposed that this localization is typical of G-protein coupled receptors such as serotonin receptors (4, 9, 12, 20). The immunoreactive staining pattern that we observed at the cytoplasmic level, could be attributed to internalization of $5HT_{2A}$ serotonin. Accordingly, it's well defined that cytoplasmic distribution of $5HT_{2A}$ reflects its internalization from membrane compartment and subsequent initialization of some proliferative signaling pathways such as MAPK/ERK1/2 and JAK/STAT (4, 9, 12, 20).

Serotonin increases colorectal cancer cell lines growth and viability in a concentration dependent manner. This effect of serotonin is mimicked by DOI and inhibited by ritanserin suggesting that this effect is mediated by the activation of the $5HT_{2A}R$. The mitogenic effect of serotonin and its $5HT_{2A}R$ on cancer cells has been reported by several studies. Serotonin induced a dose-dependent increase in HT1376 bladder cancer cell growth (20) and promoted human breast and prostate cancer cell growth in vitro (7, 22, 23). Some reports indicate that $5HT_{2A}R$ may be involved in the autocrine loops of growth factors contributing to cell proliferation in some aggressive tumors (4, 12, 26). In human placental choriocarcinoma cell lines, activation of the $5HT_{2A}R$ enhances cell growth by promoting cell cycle progression (15, 16). Further studies are required to determine the mechanism by which the activation of $5HT_{2A}R$ induces colorectal cancer cell growth.

Ritanserin, a specific $5HT_{2A}R$ antagonist, alone decreased significantly the viability and increased apoptosis in colorectal cancer cell lines in a dose dependent manner. The same effect on cell

viability and apoptosis was observed with curcumin alone. Interestingly, ritanserin, in combination with curcumin, caused two fold cell growth reduction and death at concentrations that each drug alone is less effective.

Dietary constituents of food have been shown to provide protection against many diseases or reduce their severity (10, 11). It has been reported that plant derived curcumin, inhibit the clonogenic growth and induce apoptosis of several human and murine leukemia cell lines (5, 11). Indeed, curcumin is frequently used as anti-cancer drug and is known to sensitize cancer cells to chemical therapy. This drug induces its effects by blocking the cell cycle and up-regulating tumor suppressors such as P53 (5, 6). Here, we demonstrated that combination of curcumin with ritanserin reduced the growth and enhanced the apoptosis rate in colorectal cell lines. This is the first study to show a potentialization, by a $5HT_{2A}R$ antagonist, of the benefic effect of curcumin on colon cancer.

In summary, this study suggests that serotonin, via in part the $5HT_{2A}R$ may protect colorectal cell, by increasing cell viability and decreasing apoptosis. The combination of ritanserin and curcumin may be an alternative to the chemotherapy which often causes side effects, toxicity and long-term treatment failure. Hence, serotonin might represent a novel target for the prevention and treatment of colon cancer, especially as numerous safe and effective serotonin-targeting drugs are in clinical use today.

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Conflict of interests

The authors declared no conflict of interests.

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