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SPANDIDOS PUBLICATIONS

Effect of phytic acid and inositol on the proliferation and apoptosis of cells derived from colorectal carcinoma

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Abstract. We characterized the effect of phytic acid (inositol hexaphosphate, IP6) as a potential adjuvant in treatment of colorectal carcinoma and evaluated the optimal concentration and treatment time to produe the maximal therapeutic effect. There is some evidence that myoinositol (Ins) can potentiate anti-cancer effects of IP6. Therefore, we tested both IP6 and Ins individually and in combination on human cell lines HT-29, SW-480 and SW-620 derived from colorectal carcinoma in different stages of malignancy. The effect of tested chemicals on the cells was measured using metabolic activity assay (WST-1), DNA synthesis assay (BrdU), protein synthesis assay (Brilliant Blue) and apoptosis (caspase-3 activity). We tested IP6 and Ins at three concentrations: 0.2, 1 and 5 mM for 24, 48 and 72 h. The concentrations and incubation periods were chosen according to low toxicity of the tested substance that was observed in a long-term clinical study. We found that all employed concentrations of IP6 or IP6/Ins decreased proliferation of the cell lines, with the maximum decrease being observed in HT-29 cells. Metabolic activity of treated cells differed in response to IP6 and IP6/Ins treatment; in HT-29 and SW-620 significant decrease was observed only at the highest concentration, whereas in SW-480 cells metabolic activity was lower at each concentration except 0.2 and 1 mM IP6 or IP6/Ins in 24-h incubation. The results from protein content assay corresponded to the results obtained from WST assay. In addition, we found maximum increase in caspase-3 activity at concentration 5 mM IP6 or IP6/Ins in HT-29 cells and with IP6 at concentration of 0.2 mM or IP6/Ins in SW-480 cells with clear indication of Ins enhancing the proapoptotic effect of IP6 in all the cell lines studied.

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Introduction

Phytic acid also known as a inositol hexaphosphate (IP6) is a promising anti-cancer agent. IP6 has been found in whole grains, cereals, nuts and leguments. IP6 and its chemical variants with fewer phosphate groups are also found in mammalian cells, where they are necessary for regulating vital cellular function, particularly cell division and differentiation (1-3). Anti-cancer potential of IP6 has been studied both in *in vivo* (4,5) and *in vitro* (6,7) experiments, with demonstrated effects in colon (8,9), liver (10,11), prostate (5), breast (12) rhabdomyosarcoma (13), pancreas (14) and skin cell lines (15). Despite available data on IP6 anti-neoplastic effects, the exact mechanism of its cytostatic activity is not completely explained. Among proposed mechanisms of action are anti-oxidant activities (16), increase in nature killer cells (8), decreased cell division by arresting cells in G0/G1 phase (17), and increased expression of the tumor suppression gene p53 (18). *In vivo* animal and human studies have shown that IP6 is very safe and without unwanted toxic effects (19). For example, the dose of 8.8 g/day administered to 35 patients was reported to be without side effects upon long-term observation.

There is some evidence that myoinositol (Ins) - vitamin B can potentiate anti-cancer effects of IP6. Ins and IP6 in equimolar ratio can yield inositoltriphosphate, the key regulator of cell growth. This theory was investigated in colon cancer and mammary cancer (20,21) and it has been suggested that combined administration provides significantly better results than treatment with either agent alone.

Incidence of colorectal carcinoma is increasing in the Czech Republic and the disease does not respond well to cytostatic treatment. Therefore it is necessary to broaden the spectrum of chemoprevention and therapeutic possibilities. We characterized the effect of IP6 and Ins as a potential adjuvant in cytostatic treatment and compared this effect in colorectal cells with different malignant potential. Moreover, we tested the ability of Ins to enhance anti-cancer effect of IP6.

Materials and methods

Chemicals. 7-amino-4-methylcoumarin (AMC, Cat. No. A9891), Ac-DEVD-AMC (caspase-3 substrate Cat. No. A1086), Ac-DEVD-CHO (caspase-3 inhibitor Cat. No.

A0835), Bicinchoninic acid kit for protein determination (Cat. No. BCA-1KT), Myoinositol (Cat. No. I7508) and Phytic acid dipotassium salt (Cat. No. P 5681) were purchased from Sigma-Aldrich (Prague, Czech Republic).

4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate (WST-1), anti-bromodeoxyuridine-POD, Fab fragments (Cat. No. 1 585 860), Blocking reagent for ELISA (Cat. No. 1 112 589) and BM Chemiluminescence ELISA Substrate (POD) (Cat. No. 1 582 950) were from Roche (Mannheim, Germany).

5-bromo-2'-deoxyuridine (BrdU) (Cat. No. 16 880-1G) was obtained from Fluka (Prague, Czech Republic). Brilliant Blue G (Cat. No. 20,139-1) was purchased in Aldrich, Prague, Czech republic). All other chemicals were of highest analytical grade.

Cell culture and treatment. The continuous cell lines HT29 (Cat. No. HTB-38, ATCC, USA), SW480 (Cat. No. CCL-228 ATCC), SW620 (Cat. No. CCL-227, ATCC) were cultivated in a humidified 5% CO_2 atmosphere at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Sevapharma, Prague, Czech Republic) with 10% fetal bovine serum (ZVOS Hustopece, Uhercice, Czech Republic), penicillin G (100 U/ml) and streptomycin (100 μg/ml). For experiments, the cells were seeded into cultivation flasks (Nunclon, Roskilde, Denmark) at the density of $1x10^5$ cells/ml (in 20 ml total volume) or 96-well plates (Nunclon, Roskilde, Denmark; Greiner bio-one, Germany) at concentration of 6,000 cells/well, always with the first column of wells without cells (blank) and allowed to grow overnight. After 24 h, the medium was aspirated and cells were exposed to the freshly prepared medium containing IP6, Ins or their combination for 24, 48 or 72 h.

Stock solutions of IP6 and Ins were prepared in PBS without ions and stored in a refrigerator until use. Before each experiment, stocks were diluted to the final concentration in Nutrient Mixture F-12 Ham (N-4888) with 1% FBS and 1% L-glutamine (Cat. No. G7513, Sigma, Prague, Czech Republic). Experiments were repeated at least three times.

Cell metabolic assay WST-1. At the end of each incubation, the medium was removed; cells were washed twice and exposed to medium with 100 μl of WST-1. After 2 h, the changes in absorbance were recorded by a photometer SPEKTRAFluor Plus, Tecan (Salzburg, Austria) at 450 nm with 650 nm of reference wavelength. In all cases the absorbance of the tested substance in medium alone was recorded to determine whether it interfered with the assay.

DNA synthesis assay - BrdU. BrdU was added 4 h prior to the end of cultivation in final concentration 100 μM. At the end of experiment, the medium was removed; cells were washed twice, fixed with 70% methanol and 2.3 M HCl and exposed to blocking reagent for 15 min. Three times washed cells were incubated with anti-BrdU-POD (0.2 U/ml) for 2 h under room temperature. Cells were washed three times and BM chemiluminescence ELISA substrate was added into each well. Chemiluminiscence was recorded by a photometer SPEKTRAFluor Plus, Tecan at 3 min after the start of reaction.

Protein content - Brilliant Blue assay. At the end of each incubation, the medium was removed; cells were washed twice and fixed by a two-step procedure with methanol and fixation solution (50% ethanol, 49% H_2O, 1% glacial acetic acid). Cells were exposed to Brilliant Blue stain solution in concentration 0.4 mg/ml. After 1 h the blue product formation was dissolved (98 mg potassium acetate in 700 ml ethanol, 300 ml H_2O) and the absorbance was recorded by the photometer at 620 nm with 450 nm of reference wavelength. In all cases the absorbance of the tested substance in medium alone was recorded to determine whether it interfered with the assay.

Apoptosis assay - caspase-3 activity determination. At 24, 48 and 72 h cells were harvested by centrifugation (600 x g, 5 min) and lysed on ice for 20 min in a lysis buffer containing 50 mM HEPES, 5 mM CHAPS and 5 mM DTT. The lysates were centrifuged at 14,000 x g, 10 min, 4°C, the supernatants were collected and stored at -80°C. The enzyme activity was measured in a 96-well microplate using a kinetic fluorometric method based on the hydrolysis of the peptide substrate Ac-DEVD-AMC by caspase-3, resulting in the release of the fluorescent 7-amino-4-methylcoumarin (AMC) moiety. Ac-DEVD-CHO, a specific inhibitor of caspase-3, was used to confirm the specificity of the cleavage for caspase-3. Fluorescence was recorded by the photometer at λ_{ex} 360 nm and λ_{em} 465 nm. The concentration of the AMC released was calculated from a standard curve constructed with known concentrations of AMC. Caspase-3 activity was expressed as nmol AMC/min/mg protein. Protein was determined spectrophotometrically using bicinchoninic acid with bovine serum albumin as a standard.

Statistics. Statistical analysis was carried out with Student's t-test. Results were compared with control samples and means were considered significant at $P<0.05$. All experiments were performed in triplicate.

Results

Metabolic activity - WST-1. IP6 decreased the metabolic activity in HT-29 and SW-620 cells only at the highest employed concentrations while in SW-480 cells this decrease was visible at all concentrations except 0.2 mM and 1 mM IP6 for 24-h of treatment (Fig. 1). We observed no changes in metabolic activity between IP6 and IP6+Ins incubations (data not shown). Inositol did not statistically change metabolic activity in comparison to control (data not shown).

Cell proliferation. All concentrations of IP6 inhibited proliferation in each of the cell lines in a concentration-dependent manner. The maximum decrease was observed in HT-29 cells (Fig. 2A). Inositol with IP6 at concentration of 1 mM slightly potentiated the inhibition of cell proliferation of both SW-480 and SW-620 cell lines at all incubation times (Fig. 2B). Inositol as well as IP6 statistically significantly decreased proliferation of each cell line in a concentration-dependent manner. We observed the maximum decrease at 5 mM concentration of Ins (43-87% of control according to the cell line and incubation period, Fig. 2C). IP6 as well as

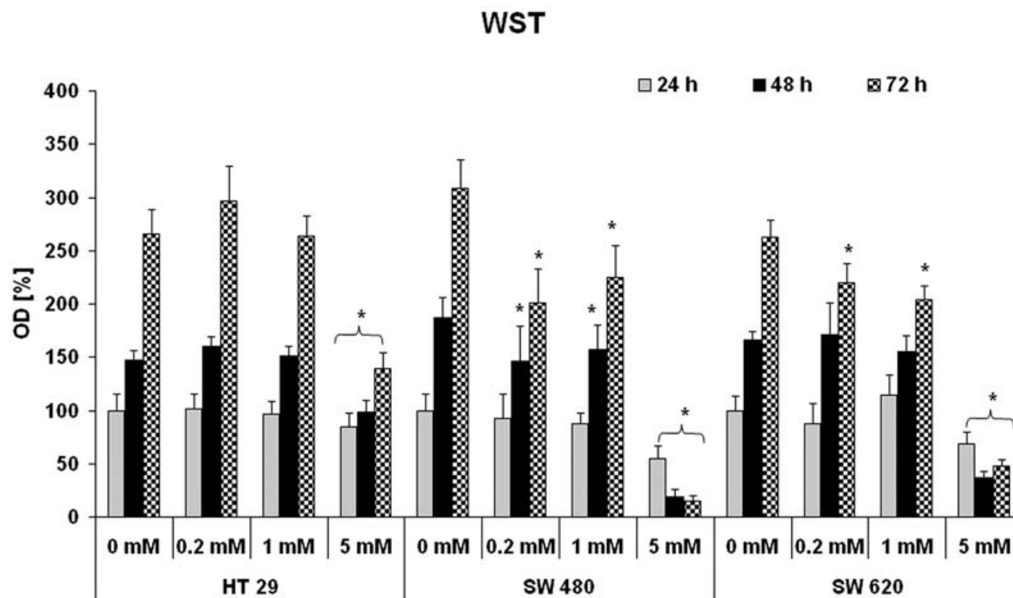

Figure 1. Effect of inositol hexaphosphate (IP6) at concentrations of 0.2, 1 and 5 mM on proliferation of HT29, SW480 and SW620 cells during 24, 28 and 72 h as measured by WST-1 assay. Data (optical density) represent the mean expressed as percentage of control (0 mM) at 24 h incubation ± SD of three different experiments. *P<0.05 with Student's t-test.

IP6+Ins at 5 mM concentration decreased cell proliferation to a range of 0-10% of control.

Protein content. IP6 decreased protein content in HT-29 cells only upon the highest concentrations. In SW-620 cells this decrease was observed at concentrations of 1 and 5 mM IP6 except 24-h incubation (1 mM IP6). SW-480 cell line was the most sensitive. IP6 decreased protein content at all employed concentrations except 0.2 and 1 mM IP6 at 24-h incubation (Fig. 3A). We observed no statistically significant changes in metabolic activity between IP6 and IP6+Ins except at 0.2 mM IP6+Ins in SW-620 cells for 72 h of treatment (Fig. 3B). Compared to controls, inositol did not statistically change metabolic activity (data not shown).

Caspase-3 activity

HT-29 (Fig. 4A). IP6 increased activity of caspase-3 in all concentrations except 0.2 and 1 mM IP6 for 24-h incubation. Ins supplemented with IP6 potentiated the pro-apoptotic effect of IP6 in all concentrations but not upon all incubation periods. Ins alone had no or only slight pro-apoptotic effect.

SW-480 (Fig. 4B). We observed a marked increase of caspase-3 activity with 0.2 mM IP6 at all incubation periods and with 5 mM IP6 for 24-h of treatment. At these concentrations and treatment times, inositol always potentiated the pro-apoptotic effect of IP6 while having no pro-apoptotic potential by itself.

SW-620 (Fig. 4C). IP6 at the concentration of 0.2 mM statically significantly increased the activity of caspase-3 for 48 and 72 h of incubation. In addition, we observed the marked increase in caspase-3 activity upon 5 mM concentration for 24 h of treatment. Inositol potentiated this pro-apoptotic effect with both employed concentrations of IP6 while having no pro-apoptotic effect by itself with exception of 0.2 mM concentration and 24 h incubation period.

The highest increase in caspase-3 activity was observed at concentrations of 5 mM IP6 or IP6+Ins in HT-29 cells and with 0.2 mM IP6 or IP6+Ins in SW-480 cells.

Discussion

It has been suggested that IP6 (phytic acid) is a potential novel anti-neoplastic agent (22,23). In addition, some studies reported that myoinositol can enhance anti-cancer effect of IP6 (20,21,24,25). Since both chemicals alone or in combination may easily be included in the human diet while showing convenient pharmacological properties in the digestive system, their chemopreventive potential warrants closer investigation, in particular considering the fact that very little is known about effects of these compounds on cells with varying malignant potential. Thus in this study we evaluated anti-proliferative and proapoptotic potential of IP6 alone or in combination with Ins in three colorectal cell lines: HT-29 (non-invasive, non-migrating), SW-480 (non-invasive, migrating), SW-620 (invasive, migrating) for 72 h.

The results of the present study showed that IP6 at doses 0.2-5 mM markedly inhibited the DNA synthesis of all tested cell lines, with the most sensitive to inhibition of DNA synthesis being HT-29 cells. This observation is in line with the evidence reported by other research groups and seems to have a more universal nature since IP6 has shown a similar inhibitory effect in other cell types including human breast cancer lines MCF-7 and MDA-MB231, prostate line PC-3, TRAMP-C1 and DU145 (17,26-29). In addition, in some cases IP6 treatment was associated with G0/G1 arrest which suggests more complex nature of DNA-IP6 interactions including possibly DNA damage (17,27,28).

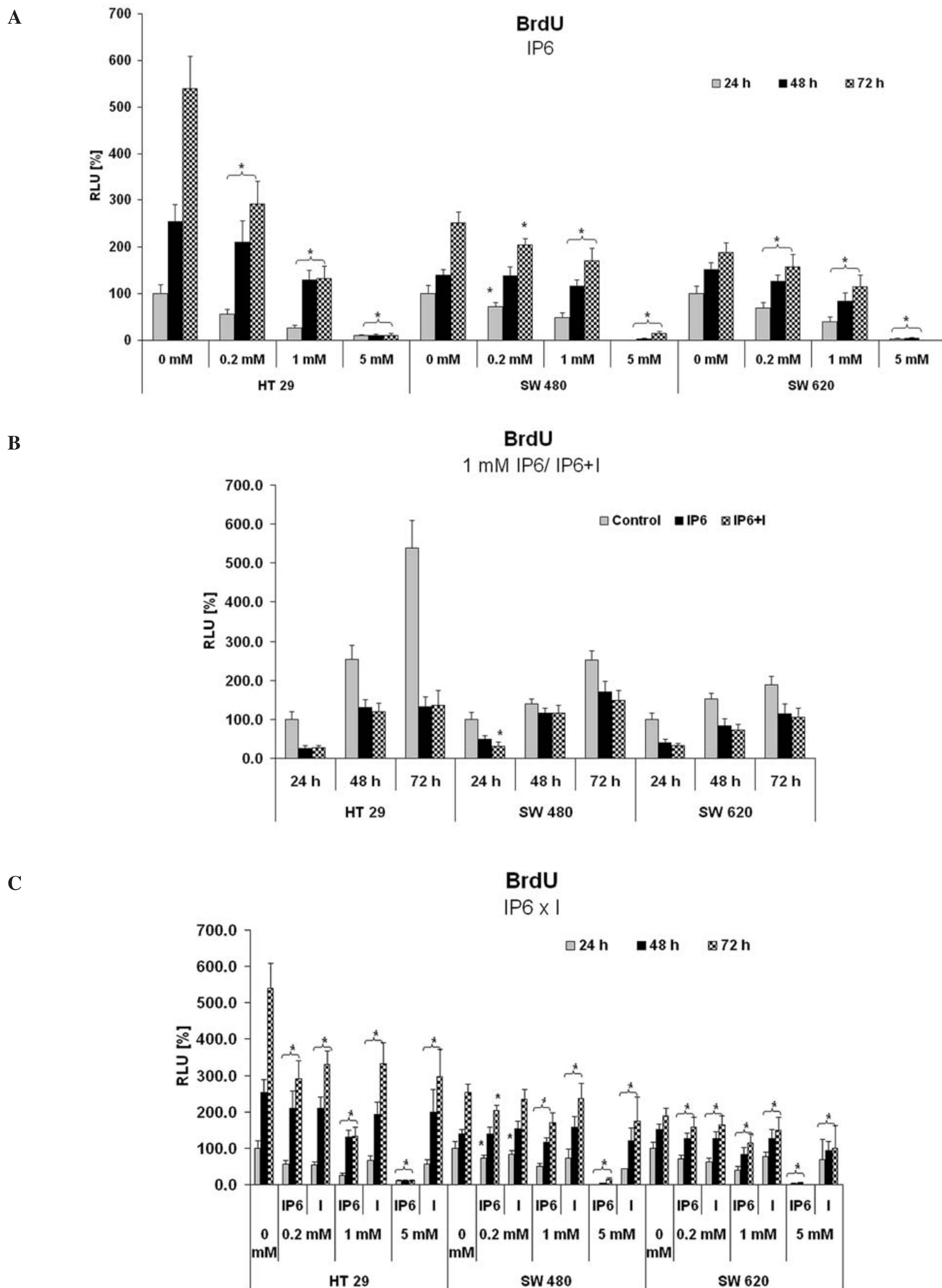

Figure 2. (A) Effect of inositol hexaphosphate (IP6) at concentrations of 0.2, 1 and 5 mM on DNA synthesis of HT-29, SW-480 and SW-620 cells for 24, 28 and 72 h as measured by BrdU assay. Data (relative light units) represent the mean expressed as percentage of control (0 mM) at 24 h incubation ± SD of three different experiments. *$P<0.05$ with Student's t-test. (B) Effect of IP6 and its combination with inositol at concentrations of 1 mM on DNA synthesis of HT-29, SW-480 and SW-620 cells for 24, 28 and 72 h as measured by BrdU assay. Data (relative light units) represent the mean expressed as percentage of control (0 mM) at 24 h incubation ± SD of three different experiments. Potentiation of pro-apoptotic effect *$P<0.05$ with Student's t-test. (C) Effect of inositol hexaphosphate and inositol (comparison of their effect) at concentrations of 0.2, 1 and 5 mM on DNA synthesis of HT-29, SW-480 and SW-620 cells for 24, 28 and 72 h as measured by BrdU assay. Data (relative light units) represent the mean expressed as percentage of control (0 mM) at 24 h incubation ± SD of three different experiments. *$P<0.05$ with Student's t-test.

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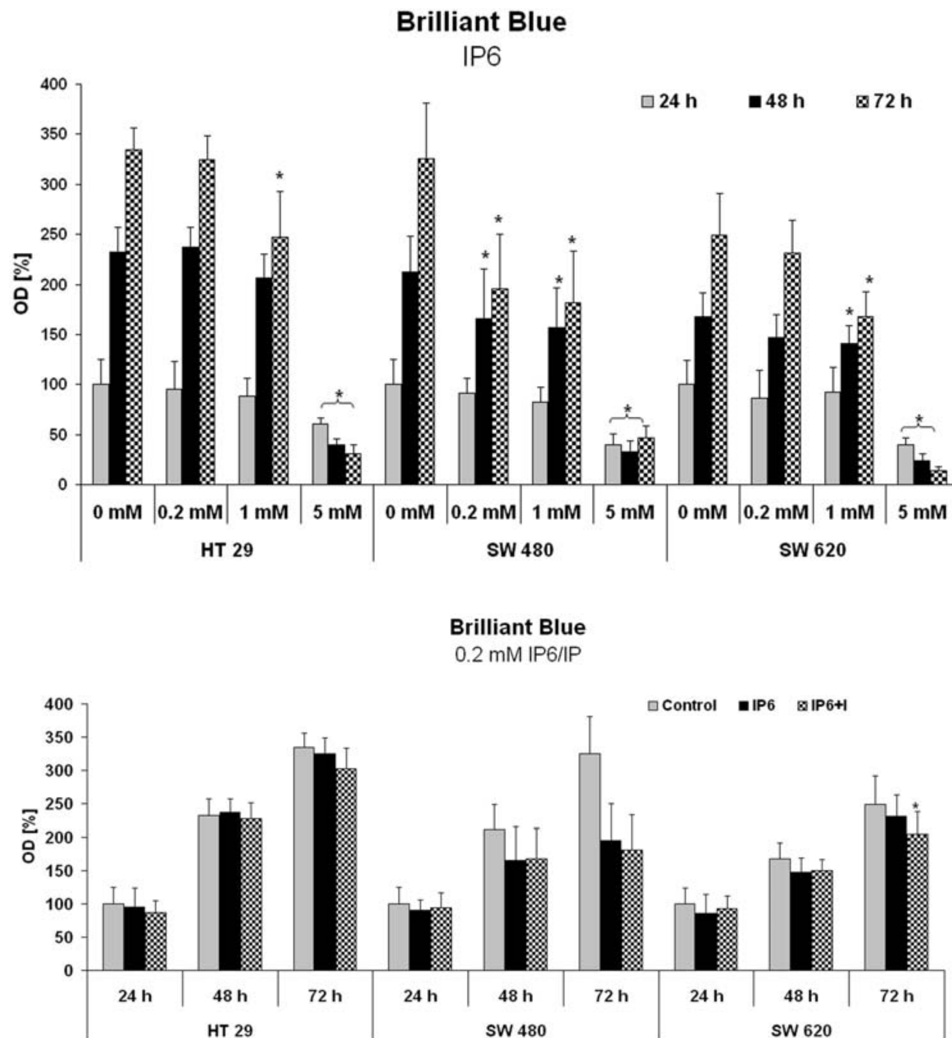

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Figure 3. (A) Effect of inositol hexaphosphate (IP6) at concentrations of 0.2, 1 and 5 mM on protein content of HT-29, SW-480 and SW-620 cells for 24, 28 and 72 h as measured by Brilliant Blue assay. Data (optical density) represent the mean expressed as percentage of control (0 mM) at 24 h incubation ± SD of three different experiments. *P<0.05 with Student's t-test. (B) Effect of IP6 and its combination with inositol at concentrations of 0.2 mM on DNA synthesis of HT-29, SW-480 and SW-620 cells for 24, 28 and 72 h as measured by BrdU assay. Data (relative light units) represent the mean expressed as percentage of control (0 mM) at 24 h incubation ± SD of three different experiments. Potentiation of pro-apoptotic effect *P<0.05 with Student's t-test.

On the other hand, combined treatment of IP6 with Ins did not enhance anti-proliferative activity of IP6 on tested cells which concurs with observations made *in vivo* (20,21,23,24).

Metabolic activity and protein synthesis in cells treated with IP6 decreased in a dose and time-dependent manner and were similar to the DNA-specific effects of this chemical. Still, compared to the inhibition of DNA synthesis, the obtained results clearly show a comparatively weaker suppressive effect of IP6, with again HT-29 cells being the most resistant. Besides other factors, here it is obviously important to acknowledge potentially oscillating sensitivity of diverse assays used for evaluation of cytostatic effects in individual study models as demonstrated by the use of MTT or neutral red assays in HT-29 cells (1,29). Despite certain variability in response of individual tumor cells to IP6, this chemical effectively inhibits proliferative activity of numerous cancer cell lines although sometimes at later treatment intervals (14,15,26,30-33). As before, Ins did not contribute to anti-proliferative activity of IP6 as measured by WST assay, however, we recorded a week potentiation effect seen as a decreasing protein content in treated cells.

Members of caspase (cystein aspartic acid-specific protease) family play key effector role in apoptosis in mammalian cells (34). Caspase-3 is one of the critical enzymes of apoptosis and is the most studied of mammalian caspases in many cases linking both extrinsic as well as intrinsic apoptotic pathways. In addition caspase-3 plays a central role in mediating nuclear apoptosis including chromatin condensation and DNA fragmentation as well as cell blebbing (35). Thus determination of its status reflects in most cases active apoptosis in studied models despite the fact that apoptosis may be executed in a caspase-independent way. In our experiments, caspase-3 was increased in all employed cell lines but not in time and dose-dependent manner with the exception HT-29 cells which are the most sensitive in terms of levels of activated caspase. The proapoptic potential of IP6 is generally known and has been documented in a wide range of tumor cell lines (7,14,15,32,33,36). Still, this is the first study on this activity in colon cancer cell lines with a differing malignant potential. Ins enhanced the proapototic effect of IP6 as measured by activity of caspase-3 which seems to contradict results from cytotoxicity assays, thereby pointing at potentially

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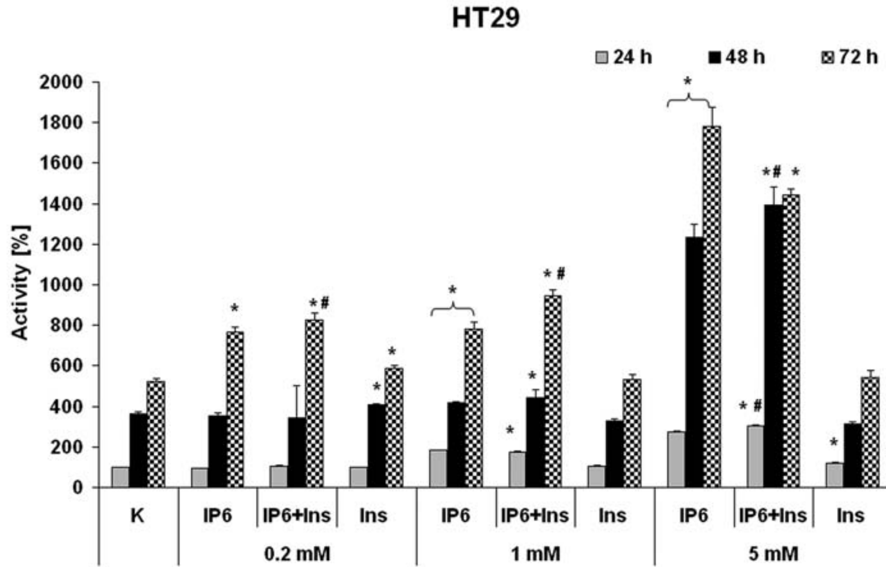

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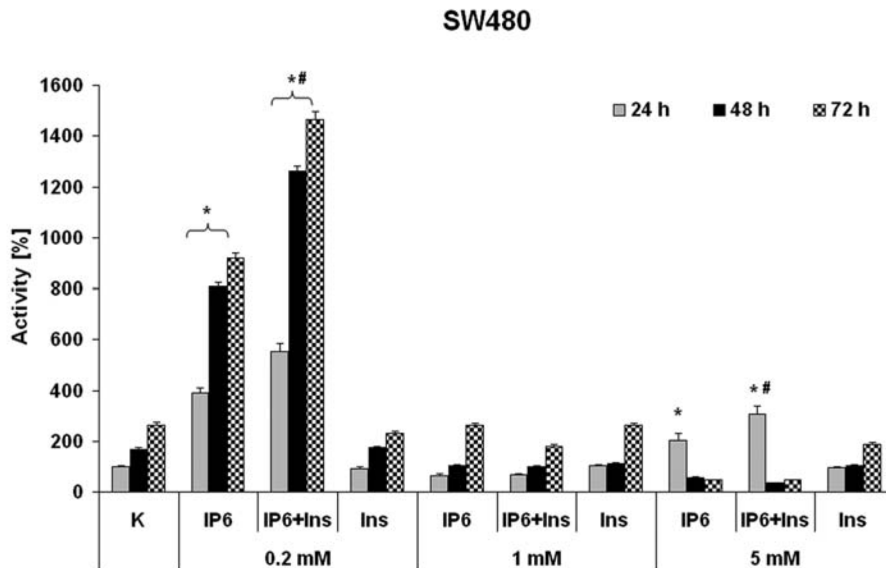

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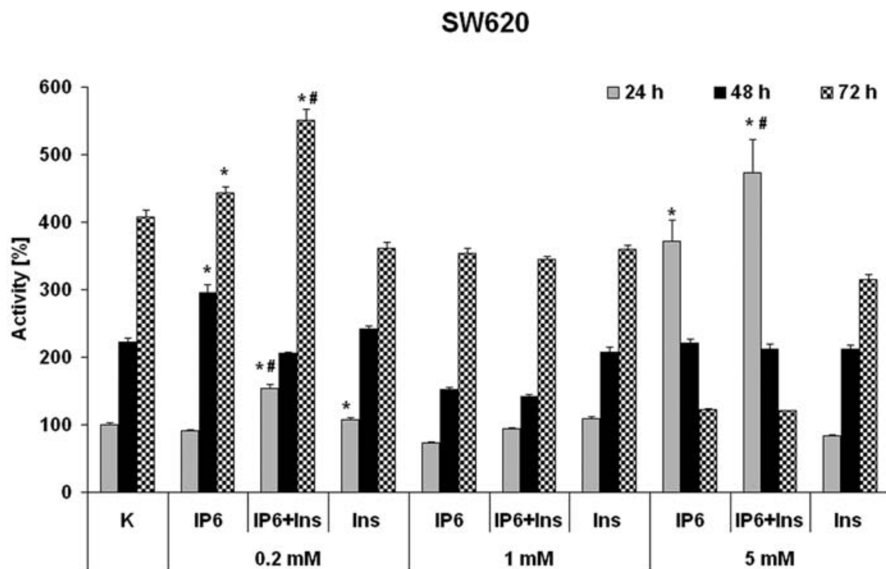

Figure 4. Effect of inositol hexaphosphate (IP6), inositol (Ins) and their combinations on apoptosis of HT-29 (A), SW-480 (B) and SW-620 (C) cells for 24, 28 and 72 h as measured by caspase-3 activity assay. Data (% of activity) represent the mean expressed as percentage of control at 24 h incubation ± SD of three different experiments. *P<0.05 with Student's t-test. Potentiation of pro-apoptotic effect #P<0.05 with Student's t-test.

mechanism of the studied IP6 and Ins combination may be preferentially proapoptic in our studied models.

In conclusion, afore-mentioned results indicate that IP6 is a potent anti-cancer agent which decreases proliferation and increases apoptosis in all tested colon cancer cell lines with different malignant potential including highly invasive and metastatic ones. On the other hand, as exemplified by the three used assays targeting different aspects of cell proliferation, individual cell lines show varying sensitivity; i.e. HT-29 cells are mostly sensitive to tested chemicals when measured by DNA synthesis suppression while SW-480 and SW-620 cell lines show this sensitivity in other two proliferation assays. Both HT-29 and SW-480 are sensitive to induction of apoptosis but at different concentrations of tested compound. The combination of IP6 and Ins did not prove to have a generally superior cytotoxic potential unlike its proaptotic activity which warrants further studies.

Acknowledgements

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