

ANTI-PROLIFERATIVE EFFECTS OF INOSITOL HEXAPHOSPHATE AND VERAPAMIL ON HUMAN COLON CANCER CACO-2 AND HT-29 CELLS

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Inositol hexaphosphate (IP6, phytic acid), a natural dietary ingredient, is also found in mammalian cells at concentrations as high as 1 mM (1). It has shown a significant anti-cancer effects in various *in vivo* and *in vitro* models, including breast, prostate, liver and colon carcinomas (2-7). Concomitant to inhibition of cancer cell growth, cellular differentiation has also been reported in divergent cancer cell lines (8).

In addition to the basic importance of intracellular calcium ion concentration in the regulation of cell growth, recent observations on the reversal of multidrug resistance to chemotherapeutic agents by certain calcium channel blockers has aroused considerable interest. Several studies have suggested that calcium antagonists, such as verapamil (VP) have inhibitory influence on cell proliferation or may potentiate the effects of numerous chemotherapeutic drugs on malignant cells (9). VP and similar substances may retard active outward transport of drug, may interfere with ion exchange, calcium or/and sodium channel blockage or other cell membrane related mechanisms (10). Combination therapy has become a popular trend of cancer treatment in order to increase efficacy and decrease side effects of conventional chemotherapy.

In the present study, we investigated the growth of inhibitory activity of IP6 in combination with VP in comparison to that of IP6 alone and VP alone using two colon carcinoma-derived cell lines Caco-2 and HT-29. In combination treatment, IP6 and VP were added simultaneously and sequentially with VP added 2 h prior to IP6 to the cell cultures.

EXPERIMENTAL

Reagents

The RPMI 1640 culture medium and fetal bovine serum (FBS) were purchased from Sigma and Gibco, BRL, respectively. Inositol hexaphosphate, as dodeca-sodium salt from corn, was purchased from Sigma. Stock solution of 100 mM was prepared in deionized water, pH was adjusted to 7.4 and sterilization was made by filtration. This stock was then diluted to the final concentrations of 0.5, 1 and 5 mM in RPMI medium. Verapamil hydrochloride, Recordati, was obtained from POLPHARMA, Starogard Gdański, Poland and it was used at the concentrations of 0.1, 0.5, and 1 mM, which were prepared from 1 mM stock solution made in deionized apyrogenic water. The dilutions were made prior to the use and they were sterilized by filtration

through 15 mm syringe filter 0.020 m RC membrane (Corning) prior to addition to the cultures.

Cell lines and cell culture

The cell line Caco-2 was purchased from German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cell line HT-29 was kindly provided by Prof. S. Szala (Center of Oncology, Gliwice, Poland). The cells were grown in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 mg/mL) at 37°C in a humidified atmosphere with 5% CO₂. For cell proliferation assay, the RPMI 1640 modified medium containing 20 mM HEPES was used.

Cell culture treatment and the growth assay

For each assay, 5×10^3 cells in 200 mL culture medium were seeded in 96-well sterile polystyrene flat-bottom plates and incubated for 24 h at standard conditions to adhere to the plate. Then, the culture medium was removed and replaced with 200 mL of RPMI medium containing the appropriate concentrations of verapamil or IP6. Control cell wells were replaced with fresh cell culture media only. Cells were exposed to each of the compounds for 2 h, then washed with 0.01 M phosphate buffered saline (PBS) and placed in RPMI medium for 72 h at standard conditions. In combination treatment, IP6 and VP were added simultaneously and sequentially with VP added 2 h prior to IP6 to the cultures. Cells were cultivated for 72 h in RPMI medium.

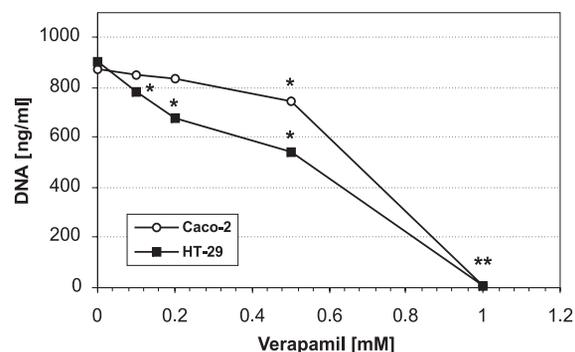


Figure 1. Comparison of HT-29 and Caco-2 cells proliferation in the presence of various concentrations of verapamil. Results are expressed as mean values of three experiments. *p < 0.05 vs. control.

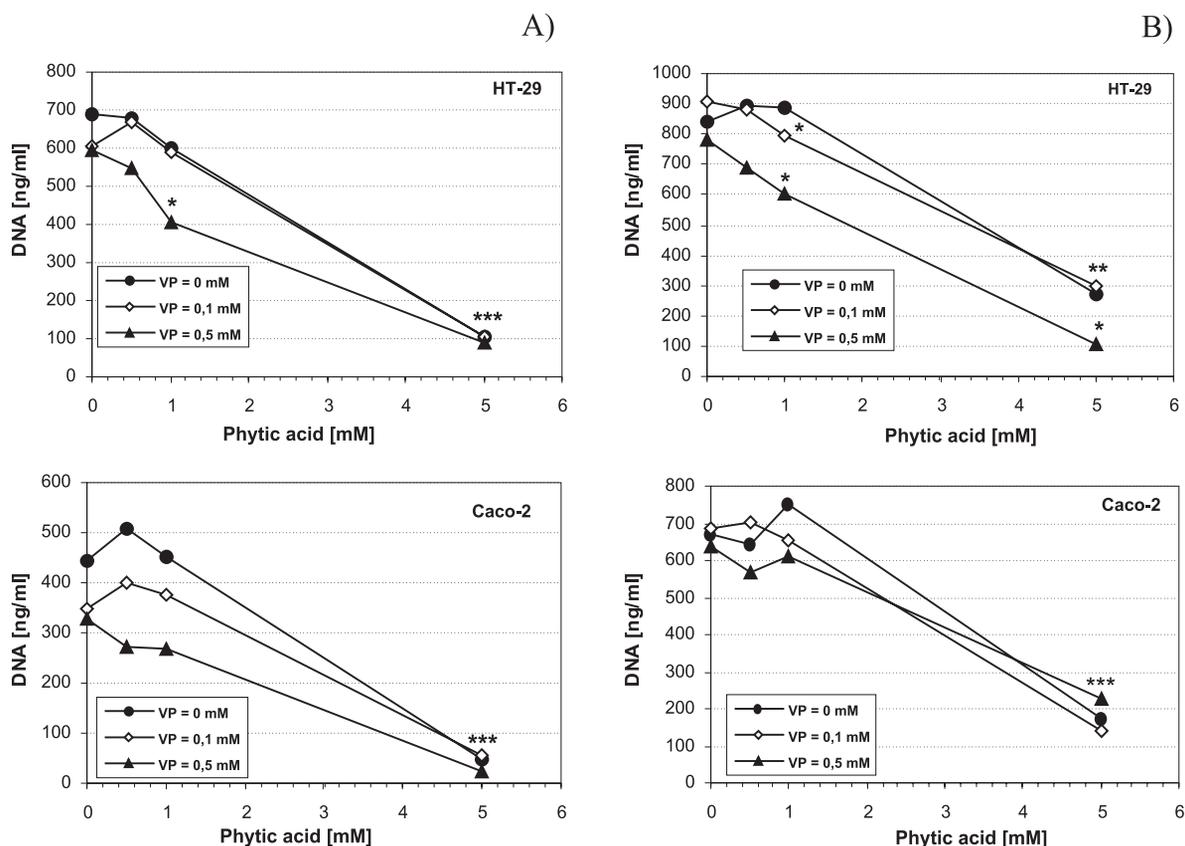


Figure 2. Comparison of DNA concentrations in HT-29 and Caco-2 cells exposed simultaneously (A) and sequentially (B) to verapamil (VP) and phytic acid. Results are expressed as mean values of three experiments. * $p < 0.05$ vs. control.

Following washing adherent cells with PBS, they were frozen at -70°C and unfrozen several times. Cellular growth was quantified with the use of CyQUANT Cell Proliferation Assay Kit (Molecular Probes) based on cellular DNA content determination. Briefly, 200 μL of CyQUANT GR dye, which exhibits strong fluorescence enhancement when bound to cellular nucleic acids, was added with cell-lysis buffer to each well and after vortexing, the fluorescence was measured at 535 nm using microplate Victor 2TM reader (Perkin-Elmer-Wallac). The DNA content was determined based on the standard curve prepared in the range of 0-800 ng/mL of bacteriophage λ DNA solutions included in the test used. All treatments were performed in six repetitions and the standard deviation was calculated.

Histochemical assessment of nuclear morphological features associated with apoptosis/necrosis was performed on the basis of staining characteristics of VP- and IP6-treated cells with hematoxylin/eosin and with trypan blue.

Statistics

The comparisons of cellular DNA concentrations were performed using one-way ANOVA. All the results are expressed as means \pm SD of three independent experiments and data were considered significant when $p < 0.05$. Statistical analysis was carried out by computer program Statistica PL version 6.0.

RESULTS AND DISCUSSION

As shown in Figure 1, VP alone reduced colon cancer cell growth and HT-29 cells appeared to be more susceptible to inhibition than Caco-2 cells. Statistically significant inhibition of Caco-2 cells proliferation was observed at VP doses ≥ 0.5 mM, whereas its growth inhibitory activity in regard to HT-29 cells was observed with ≥ 0.1 mM dose. The 1 mM VP was highly cytotoxic resulting in a reduction of cell growth by 100%. Figure 2 shows the results from the simultaneous (Figure 2A) and sequential (2B) administration of VP and IP6 to HT-29 and Caco-2 cell cultures compared to the effects revealed by IP6 alone. IP6 at 5 mM induced significant growth suppression of both HT-29 and Caco-2 cells and its lower doses (0.5, 1 mM) were ineffective. Joint administration of 0.5 mM VP and 1 mM IP6 enhanced growth inhibitory effect of 1 mM IP6 against HT-29 but not Caco-2 cells (Figure 2A). When 1 mM IP6 was given after 0.1 or 0.5 mM VP treatment, there was a significant improvement in growth inhibition of HT-29 cells compared to the action of 1 mM and 0.5 mM IP6 alone. (Figure 2B). However, the sequence mode of administration of VP and IP6 could not enhance the efficacy of IP6 against Caco-2 cells in terms of its lower concentrations used (Figure 2B).

Although some of nuclear changes associated with apoptosis such as chromatin condensation and nuclear

fragmentation appeared sporadically after cells treatment with 0.5 and 1 mM VP, and with 1 and 5 mM IP6, microscopic examination of trypan blue-stained cells treated with VP and IP6 revealed prevalence of morphological changes typical for necrosis (data not shown).

One of the major obstacles in cancer chemotherapy is the emergence of multidrug resistance which is frequently associated with the expression of P-glycoprotein encoded by the MDR1 through 6 genes in the human intestine and Caco-2 cells (11). Verapamil has been shown to reverse resistance of some malignant cells to chemotherapy by increasing the cytotoxicity effects of adriamycin, mitoxantrone and vincristine (12). VP in conjunction with 5-FU significantly reduced HT-29 cell count by 37% in comparison to 5-FU alone, and it did not enhance the growth inhibitory effect of 5-FU on SW-620 cell (9). Combined VP and hyperthermia showed a significant decrease in HT-29 and SW-620 cell counts by 61.5% and 77.6%, respectively, when compared to control (9). The death of both HT-29 and Sw-620 cells occurred through apoptosis. Combined administration of hyperthermia and VP resulted also in significant tumor growth delay of human colon cancer xenografts *in vivo* (13). The investigation of the growth inhibitory effect of IP6 alone and in combination with either adriamycin or tamoxifen on breast cancer cells showed that IP6 acted synergistically with both drugs, being particularly effective against estrogen receptor-negative cells and adriamycin-resistant cell lines (14). Growth suppression was markedly decreased when IP6 was administered prior to the addition of adriamycin. When cells were treated simultaneously for 96 h with IP6 and adriamycin, no statistically significant growth inhibition was observed. Growth inhibition of breast cancer cell lines was also enhanced when cells were treated with IP6 prior to addition of tamoxifen, producing an additive to synergistic effects.

The results of the present study showed that IP6 alone at 5 mM dose inhibited the growth of colon cancer HT-29 and Caco-2 cells and that in combination

treatment VP enhanced anti-proliferative activity of IP6 on HT-29 cells but not Caco-2 cells. This has been manifested by synergistic effects of 0.1 or 0.5 mM VP with 1 mM IP6 against HT-29 cells. The two different modes i.e., simultaneous and sequential, of VP and IP6 administration produced similar anti-proliferative effects.

REFERENCES

1. Szwergold B., Graham R., Brown T.: *Biochem. Biophys. Res. Commun.* 264, 874 (1987).
2. Sakamoto K., Venkatraman G., Shamsuddin A. M.: *Carcinogenesis* 14, 1815 (1993).
3. Ullah A., Shamsuddin A.M.: *Carcinogenesis* 11, 2219 (1990).
4. Vucenik I., Yang G., Shamsuddin A.M.: *Carcinogenesis* 16, 1055 (1995).
5. Shamsuddin A.M., Yang G.: *Carcinogenesis* 16, 1975 (1995).
6. Vucenik I., Tantivejkul K., Ramakrishna G., Anderson L., Ramljak D.: *Anticancer Res.* 18, 4083 (1998).
7. Shivapurkar N., Tang Z., Frost A., Alasbaster O.: *Cancer Lett.* 100, 169 (1996).
8. Shamsuddin A.M., Yang G., Vucenik I.: *Anticancer Res.* 16, 3287 (1996).
9. Shchepotin I.B., Soldatenkov V., Buras R.R., Nauta N.J., Shabahang M., Evans S.R.T.: *Anticancer Res.* 14, 1027 (1994).
10. Batra S., Alenfall J.: *Anticancer Res.* 11, 1221 (1991).
11. Prime-Chapman H.M., Fearn R.A., Cooper A.E., Moore V., Hirst B.H.: *J. Pharmacol. Exp. Ther.* 311, 476 (2004).
12. Sela S., Husain S.R., Pearson J.W., Longo D.L., Rahman A.: *J. Natl. Cancer Inst.* 87, 73 (1995).
13. Shchepotin I.B., McRae D.A., Shabahang M., Buras R.R., Evans S.R.T.: *Anticancer Res.* 17, 2213 (1997).
14. Tantivejkul K., Vucenik I., Eiseman J., Shamsuddin A.M.: *Breast Cancer Res. Treat.* 79, 301 (2003).