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ANTIPROLIFERATIVE EFFECT OF INOSITOL HEXAPHOSPHATE ON HUMAN SKIN MELANOMA CELLS *IN VITRO**

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Abstract: Human malignant melanoma is a highly metastatic tumor with poor prognosis. The majority of metastatic melanomas are resistant to diverse chemotherapeutic agents. Consequently, the search for novel antimelanoma agents continues. In recent years, the interest in plants and their biologically active constituents as a source of novel potential drugs significantly increased. Inositol hexaphosphate (IP6) is a naturally occurring compound that has been shown to inhibit the growth of a wide variety of tumor cells in multiple experimental model systems. The aim of this study was to evaluate the antiproliferative and cytotoxic influence of IP6 on melanotic melanoma cells *in vitro*. The A2058 cells used as a model of human skin *melanoma malignum* were exposed to different concentrations of IP6 (0.1-5 mM) for a various period of time and their growth was determined by sulforhodamine B assay after 24, 48 and 72 h. The cytotoxicity of IP6 was measured at 24 and 72 h by XTT assay. IP6 has been found to cause dose-dependent growth suppression of A2058 melanoma cells. At low concentrations (0.1 and 0.5 mM) it did not exert any influence on the cell proliferation as compared to control cultures. Higher concentrations of IP6 (from 1 to 5 mM) had a statistically significant, suppressive effect on cell proliferation after 24 h incubation. When the experimental time period was increased up to 72 h, statistically significant inhibition of cell proliferation was monitored at all IP6 concentrations used. Data obtained from XTT assay indicated that IP6 had dose- and time-dependent cytotoxic effect on melanoma cells. The results demonstrate the antiproliferative and cytotoxic properties of IP6 in a wide range of concentrations on human A2058 melanoma cells. Hence, it can be suggested that IP6 could have a promising therapeutic significance in treating cancer.

Keywords: inositol hexaphosphate, proliferation, A2058 cell line, *melanoma malignum*

Malignant melanoma (*melanoma malignum*) is one of the most lethal types of cancer and its rate of incidence has been rising for decades (1). It is an aggressive, highly metastatic tumor with poor prognosis arising from neoplastically transformed melanocytes. It is currently treated by local tumor surgery, radiotherapy and chemotherapy. The majority of metastatic melanomas are resistant to apoptosis and chemotherapeutic agents (2). Therefore, the development of a new, more effective therapy is justified.

Epidemiological studies suggest that diets rich in vegetables and fruits are associated with lower cancer risk (3). Much of the protective effect of these foods has been attributed to phytochemicals, which are naturally occurring, non-nutrient bioactive plant compounds that have disease preventive or therapeutic properties (4). In recent years, the interest in plants and their biologically active constituents as a source of novel potential anticancer drugs significantly increased. Among dietary agents inositol hexaphosphate (IP6), found in large amounts in cereal grain, nuts, legumes and oil seeds, has become a focus of interest in the latest years due to its chemopreventive properties against various types of cancer (5-7). Several studies documented the ability of IP6 not only to reduce the risk of developing cancer but also inhibit tumor progression in animal cancer models including colon, breast and prostate cancer (8). IP6 is supplied in daily human diet and rapidly absorbed from gastrointestinal tract (9). IP6 is also absorbed through the skin layers of humans and achieve important concentrations in tissues and biological fluids (10). *In vivo* studies have shown that IP6 is safe and devoid of toxicity (11).

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Little attention has been devoted, so far, to evaluation of the effects of IP6 on melanoma cells. Therefore, the aim of this study was to evaluate the antiproliferative and cytotoxic influence of IP6 in a wide range of concentrations on human melanotic melanoma A2058 cells *in vitro*.

EXPERIMENTAL

Cell culture

The human malignant melanoma cell line A2058 was obtained from LGC Promochem (Łomianki, Poland). The cells were routinely grown in

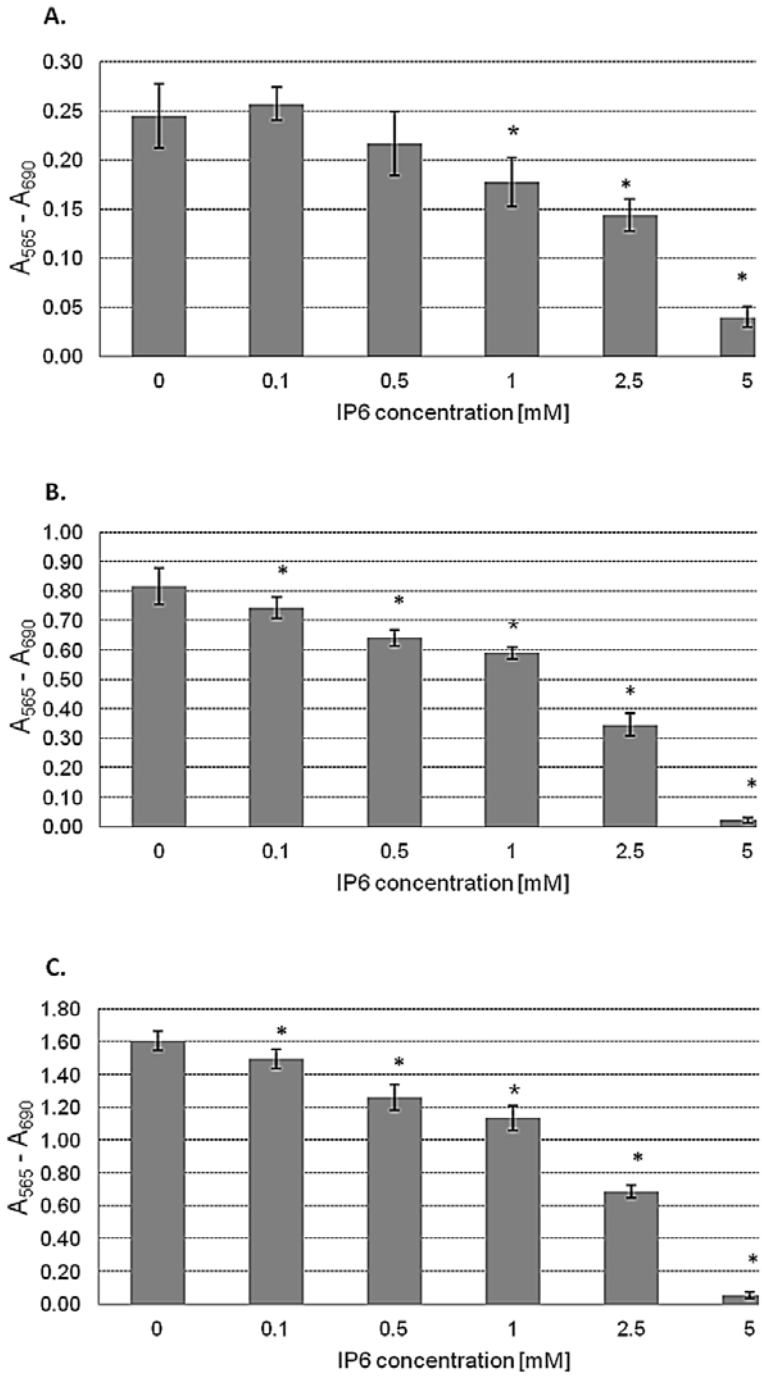

Figure 1. Growth of A2058 cells cultured in the presence of various concentrations of IP6 for 24 (A), 48 (B) and 72 (C) h. The results are expressed as the means ± SD; *$p < 0.05$ *vs.* control

RPMI 1640 medium (Sigma Aldrich), supplemented with 10% fetal bovine serum (FBS; PAA), 100 U/mL penicillin (Sigma Aldrich), 100 µg/mL streptomycin (Sigma Aldrich) and 10 mM HEPES (Sigma Aldrich). Cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. When the confluence of cells reached approximately 80%, cells were trypsinized and transferred into another cultivation flask.

Cell proliferation assay

IP6 as dipotassium salt was purchased from Sigma Aldrich. Stock solutions of IP6 were prepared in distilled water and adjusted to pH 7.4. Before each experiment, stocks were diluted to the final concentration in culture medium. Cells were plated at initial density of 1×10^3 cell/well in 200 µL of culture medium in 96-well culture plates and allowed to adhere for 24 h. The media were than replaced with the fresh ones containing IP6 (0.1; 0.5; 1; 2.5 and 5 mM) and the cells were cultured for 24, 48 or 72 h. After removal of culture media from the wells, the cells were washed with phosphate-buffered saline (PBS) and fixed in 10% trichloroacetic acid. Proliferation of the cells was quantitated using *In Vitro* Toxicology Assay Kit, Sulforhodamine B Based (Sigma Aldrich) according to the manufacturer's protocol. Colorimetric results were measured using the MRX Revelation plate reader (Dynex Technologies) at $\lambda = 570$ nm and $\lambda = 690$ nm (reference wavelength).

XTT cytotoxicity assay

The cytotoxic effect of IP6 towards human melanoma A2058 cells was determined by *In Vitro* Toxicology Assay Kit XTT Based (Sigma Aldrich), which measures the metabolic activity of viable cells. This method is based on the ability of mitochondrial dehydrogenases of cells to cleave tetrazolium ring of XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyanilide inner salt), yielding orange formazan crystals, which are soluble in aqueous solution. A2058 cells were seeded at a number of 1×10^4 cells in 200 µL culture medium in 96-well cultured plates and allowed to attach and grow for 24 h. The medium was then replaced with 200 µL of fresh media containing desired concentrations of IP6 (0.1 – 5 mM) and incubated for the periods of 24 and 72 h. At the end of the treatment, the freshly prepared XTT reagent was added to each well as specified by the manufacturer. After adding the reactive solution to all of the wells, microplates were incubated for 2 h at standard conditions, and then, the absorbance was measured at 450 nm using a MRX Revelation plate reader (Dynex Technologies) with a reference wavelength of 690 nm. The cytotoxic effect of IP6 was expressed as a percentage of cell viability compared to untreated cells (12). The toxicity of IP6 was determined by means of the formula:

Cell viability (%) = Absorbance of treated cells/Absorbance of untreated cells × 100.

Statistical analysis

Statistical analysis was performed with the use of Statistica PL ver. 9.0 Software (StatSoft). All the results were expressed as the mean values ± standard deviation (SD). Student's *t*-test was used to assess statistical significance of difference between two groups. For multiple comparisons one-way analysis of variance (ANOVA) was performed followed by *post-hoc* Tukey's test. Differences with a probability (p) value less than 0.05 were considered statistically significant.

RESULTS

The influence of IP6 on cell proliferation

The A2588 cell line was used as a model of skin *melanoma malignum* cells. Cells were cultured in the presence of various concentrations of IP6 (0.1, 0.5, 1, 2.5 and 5 mM) for 24, 48 and 72 h (Fig. 1). The effect of IP6 on A2058 cell proliferation after 24 h is shown in Figure 1A. IP6 concentrations up to 0.5 mM did not inhibit the cell growth. A substantial growth inhibition was observed in cultures incubated with higher concentrations of IP6 (= 1 mM). The maximum decrease of cellular growth *versus* corresponding control cultures (84.36 ± 3.05%) was observed after treatment with the highest dose of IP6 (5 mM). The impact of IP6 on the A2058 growth was markedly enhanced when the experimental time period was elongated up to 72 h. IP6 at all concentrations used caused statistically significant inhibition of cell proliferation after both 48 h (Fig. 1B) and 72 h (Fig. 1C). The results indicate that IP6 reduced cellular growth in a concentration-dependent manner. Incubation with 5 mM IP6 resulted in almost complete (> 98%) reduction of A2058 cell growth.

Cytotoxic effect of IP6 on A2058 cells

The cytotoxic effect of IP6 on A2058 cells was determined after 24 and 72 h of treatment by XTT assay (Fig. 2). After 24 h of treatment with IP6, a significant decrease in mitochondrial dehydrogenases based cell viability was observed only in cultures exposed to the highest concentration of

IP6 (5 mM) (Fig. 2A). At cultures exposed to IP6 at concentrations up to 2.5 mM no significant alteration in cell viability was observed after 24 h (Fig. 2A). The experimental data presented in Figure 2 indicate that the exposure of melanotic cells to IP6 for 72 h caused more potent, dose-dependent cytotoxic effects (Fig. 2B). At low concentration (0.1 and 0.5 mM) IP6 caused statistically significant, about 12% decrease in activity of mitochondrial dehydrogenases in relation to control. This suppressive effect tends to be more enhanced with increasing IP6 concentration. The maximum reduction of cell viability (59%) was observed in cells treated with 5 mM IP6.

DISCUSSION

The continuous increase in incidence and failure of conventional therapies against the advanced malignant melanoma warrants development of novel effective therapeutics designed to target this malignancy. Thus, naturally occurring phytochemicals may be very beneficial in the prevention and treatment of skin cancers including melanoma due to their ability to modulate a plethora of molecular targets (4). The promising dietary component with chemopreventive and chemotherapeutic potential is IP6 (13). IP6 has received much attention for its role in cancer prevention and control of tumor growth, progression and metastasis. Anticancer effects of IP6 have been observed in various cancer models, both *in vitro* and *in vivo* (8, 11, 14, 15). Its anticancer actions involve boosting immunity, antioxidant properties, reducing cell proliferation and inducing apoptosis. Several intracellular signaling pathways including PI3K, MAPK, PKC, AP-1 and NFκB could be affected by IP6. IP6 also was shown to reverse the transformed phenotype of cells to the normal one, as demonstrated in colon, pancreatic and breast cancer cell lines (8, 16).

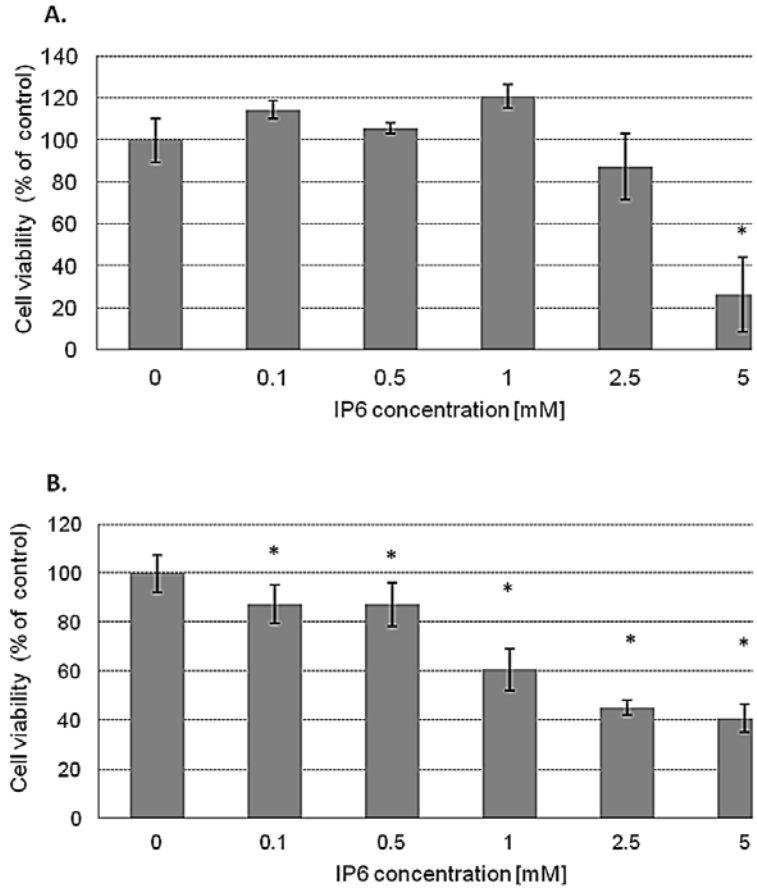

Figure 2. Cytotoxic effect of IP6 on human melanoma cells A2058 after 24 (A) and 72 (B) h. The results are expressed as the means ± SD; * - $p < 0.05$ statistically significant *vs.* control

One of the hallmarks of malignant cells is aggressive, uncontrolled proliferation. Numerous *in vitro* studies have demonstrated the ability of IP6 to inhibit growth of human leukemia, colon cancer, breast cancer, prostate cancer or hepatoma cells (11, 17). Cells of different origin presented different sensitivities to IP6 (8) and so, concentrations of IP6 capable of inhibiting cell proliferation varied depending on the cell line. For instance, 50% inhibition of growth of human rhabdomyosarcoma cells was achieved with IP6 at lower than 1 mM doses (18) and for human leukemia K-562 cells the IP6 concentration required to achieve IC_{50} (50% inhibition of cell number) was 7.5 mM (after 24 h) and 750 μM (after 120 h) (19).

Thus far, limited studies have investigated antiproliferative and antitumor effects of IP6 on skin cancer including melanoma. The prevention of skin cancer progression in a mouse model was supported by the observation of reduction in the number of DMBA-induced tumor formation following topical application of IP6 (20). Exposure to UVB radiation is believed to be responsible for most of the carcinogenic effects related to sun exposure and has been assumed to initiate mammalian melanoma (21, 22). IP6 exerted a protective effect against photoprecarcinogenesis as well as photocarcinogenesis. Recent studies indicated the effectiveness of oral administration of 2% IP6 in preventing preneoplastic or neoplastic skin lesions induced by UVB radiation in the SKH1 mouse model (23). Topical application of IP6 also decreased UVB-induced tumor incidence and multiplicity in mouse (24). Recent study showed that IP6 could prevent carcinogenesis by an impact on UVB-induced signal transduction pathways through inhibition of AP-1 and NFκB transcriptional activities (25).

Thus, in this study antiproliferative and cytotoxic potential of IP6 on human melanotic melanoma cell line A2058 was evaluated. The IP6 doses (0.1–5 mM) used in the experiment were based on earlier published studies, which in most cases used up to 5 mM IP6 concentration in cell culture treatments (26). The results of the present study show that exposure to IP6 decreases proliferation of A2058 melanoma cells in a concentration- and time-dependent manner. While IP6 in the doses up to 0.5 mM did not affect inhibition of cell growth after 24 h, a significant reduction in cell proliferative activity was manifested with higher concentrations of this compound. IP6 at all concentrations used caused statistically significant inhibition of cell proliferation when the experimental time period was elongated to 48 and 72 h. In this report, the cytotoxic property of IP6 on human melanoma cells has also been demonstrated. XTT assay showed dose- and time-related reduction of viability of A2058 cells with IP6 treatment and the observed effect enhanced with increasing IP6 concentration and prolongation of treatment time.

These data concur with the results published by Rizvi et al. (27), who demonstrated that IP6 (0.3-1 mM) inhibited growth of melanoma HTB68 cells in a dose-dependent manner after 72 h. Studies by Schneider et al. (28) showed that 1 mM IP6 significantly reduced the proliferation of MeWo cells derived from the lymphatic metastasis of a human cutaneous melanoma after 24, 48 and 72 h. Furthermore, treatment of MeWo cells with IP6 and pterostilbene, another phytochemical, produced a more profound extend of growth inhibition compared to treatment with either of them.

In conclusion, the present results show the antiproliferative and cytotoxic properties of IP6 against melanoma *in vitro*. Hence, it can be suggested that IP6 may have a promising therapeutic significance in treating melanoma.

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