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In vitro antiproliferative activity and subcellular distribution of recently synthesized anthracene-derived Schiff base and anthracene-containing aminophosphonate

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Organophosphorus compounds have wide range of commercial applications ranging from agriculture to medicine owing to their unique physicochemical and biological properties. The α-aminophosphonates, as structural analogues of natural α-amino acids, constitute a valuable class of compounds with a wide spectrum of biological activities. A recently synthesized anthracene-derived Schiff base – 9-anthrylidene-furfurylamine and novel anthracene-containing aminophosphonate – [N-methyl(diethoxyphosphonyl)-1-(9-anthryl)]-p-toluidine were tested for *in vitro* antitumor activity on HT-29 cell line. *In vitro* safety testing of the compounds was performed by Balb/c 3T3 Neutral Red Uptake Assay. The aminophosphonate and the Schiff base showed high *in vitro* antitumor activity towards HT-29 cell line. Both tested compounds were found to exert moderate cytotoxicity on the Balb/c 3T3 cells. In addition, the fluorescent properties of these compounds allowed precise observation of their subcellular distribution in the normal and tumor cells.

Keywords: Aminophosphonic acid, Schiff base, Antiproliferative activity, Subcellular distribution

Introduction

The α-aminophosphonates are organophosphorus compounds, which have found a wide range of applications in the areas of industrial, agricultural and medicinal chemistry owing to their biological and physical properties as well as their utility as synthetic intermediates. As analogues of natural amino acid, α-aminophosphonates constitute an important class of compounds with diverse biological activities, including enzyme inhibitory, antibacterial, antifungal, antiviral and antitumor effects [3, 4, 8, 9]. Some aminophosphonate derivatives inhibit bone resorbtion, delay the progression of bone metastases, exert direct cytostatic effects on a variety of human tumour cells and have found clinical application in the treatment of bone disorders and cancer [2].

Anthracene-bearing α-aminophosphonates might be of particular interest in the design of new antitumor therapeuticals considering the fact that the DNA-intercalating anthracene-derived planar structure is the main pharmacophoric fragment of some cytostatic drugs, which have found clinical applications in the treatment of human cancers [5]. Some of these anthracene-containing substances have been reported to display strong antiproliferative activity against several tumor cell lines, including multidrug resistant phenotypes [7]. The fluorescent properties of anthracene-based aminophosphonates could find valuable bioanalytical application in studying of their subcellular distribution and binding in normal and tumor cells.

The aim of the present work was to assess the *in vitro* antiproliferative activity of recently synthesized [4] anthracene-containing aminophosphonate [N-methyl (diethoxyphosphonyl)-1-(9-anthryl)]-p-toluidine and its synthetic precursor – an anthracene-derived Schiff base 9-anthrylidene-furfurylamine on HT-29 cell line and on Balb/c 3T3 cell line. In addition, we report data about subcellular distribution of the tested compounds in normal and tumor cell culture systems.

Materials and methods

Cell lines and culture conditions

The cell lines Balb/c 3T3 (mouse embryo fibroblasts) and HT-29 (human colon carcinoma) were used for *in vitro* safety assessment and *in vitro* antitumor activity testing, respectively. All cells were grown as monolayers in DMEM (*Sigma*), supplemented with 10% fetal bovine serum (*Gibco*) and antibiotics in usual concentrations. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

Test chemicals preparation

The tested compounds were dissolved in DMSO and further diluted in complete culture medium to reach the desired test concentrations. A constant dilution factor ($\sqrt[6]{10}$) was used in each experiment for the preparation of eight test concentrations of both compounds.

Safety assessment

The cytotoxicity testing was performed by the Balb/c 3T3 Neutral Red Uptake (Balb/c 3T3 NRU) Assay [1]. Optical density was measured after 24 h of treatment by microplate reader at wave length 540 nm.

In vitro antitumor activity

The antitumor activity of the tested compounds was assessed by MTT-dye reduction assay [6] on the HT-29 cell line. Cultures treated with the referent antineoplastic drug Doxorubicin hydrochloride (*Lemery*) and non-treated cultures were used as positive and negative controls, respectively. The MTT-formazan absorption was measured at wave length 580 nm after 24 hour treatment. All experiments were performed in triplicate.

Statistical analysis

The relative cell cytotoxicity/viability, expressed as a percentage of the untreated negative controls, was calculated for each concentration. The statistical analysis included application of One-way ANOVA followed by Bonferroni's *post hoc* test. $p < 0.05$ was accepted as the lowest level of statistical significance. IC_{50} values were calculated using non-linear regression analysis (*GraphPad Prizm5 Software*).

Fluorescent studies

HT-29 and Balb/c 3T3 cells were seeded on sterile 12 ring diagnostic slides and treated with non-toxic concentrations of the Schiff base and the aminophosphonate for 24 h. After fixation in cold (20°C) acetone the slides were air-dried, covered and examined with fluorescent microscope.

Results and discussion

In vitro safety testing

The results from the Balb/c 3T3 NRU Assay revealed dose-dependent cytotoxic activity of both tested compounds (Fig. 1). The Schiff base and the aminophosphonate showed statistically significant ($p<0.001$) cytotoxic effect on Balb/c 3T3 cells in a wide concentration range (1–0.07 mg/ml), compared to untreated control cell cultures. The aminophosphonate appeared to be more toxic than its synthetic precursor and the mean IC_{50} values from three consecutive experiments were 0.095 ± 0.002 mg/ml and 0.16 ± 0.006 mg/ml, respectively. The results from these experiments indicate that tested compounds exert moderate cytotoxic activity on the Balb/c 3T3 cells.

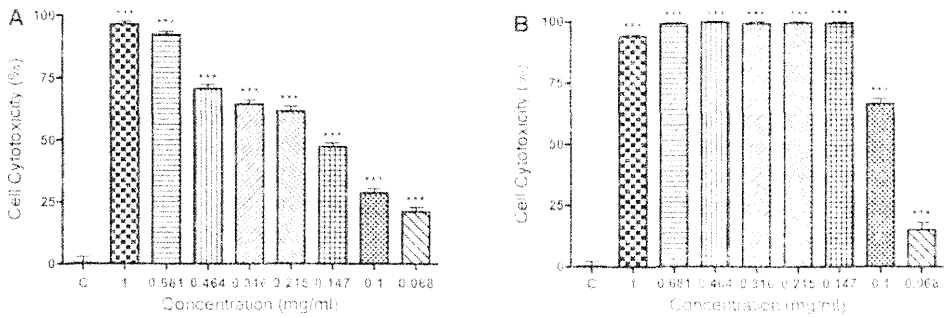

Fig. 1. Cytotoxicity of Schiff base (A) and aminophosphonate (B). Neutral Red Uptake assay. C – negative control; ***p <0.001, compared to C.

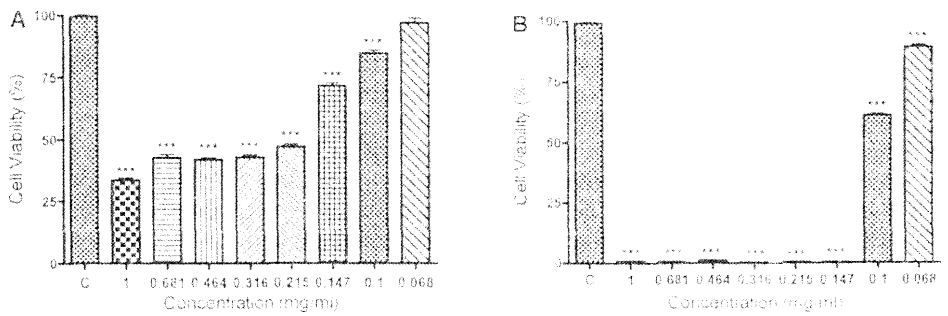

Fig. 2. Antiproliferative activity of Schiff base (A) and aminophosphonate (B) on HT-29 cell line. MTT-dye reduction assay. C – negative control; ***p <0.001, compared to C.

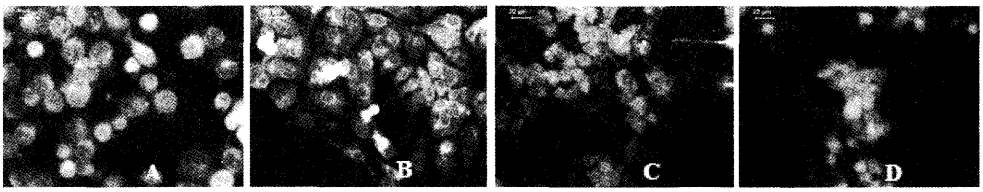

Fig. 3. Subcellular distribution of 9-anthrylidene-furfurylamine (A, C) and [N-methyl (diethoxyphosphonyl)-1-(9-anthryl)]-p-toluidine (B, D) in Balb/c 3T3 (A, B) and HT-29 (C, D) cells; Fluorescent microscopy.

In vitro antitumor activity

The Schiff base and the aminophosphonate exerted concentration-dependent antiproliferative effects after 24 h exposure (Fig. 2) and the mean IC_{50} values from three consecutive experiments were 0.2± 0.003 mg/ml and 0.11± 0.002 mg/ml, respectively. Both compounds were found to have significantly higher antiproliferative activity than the positive control substance Doxorubicin (mean IC_{50} = 0.58 ± 0.013 mg/ml). As evident from the cytotoxicity data, the tested compounds proved to be potent cytotoxic agents towards colon carcinoma cell line HT-29. The results obtained imply that these compounds could be considered as promising leads for further development of agents active in chemotherapy of malignant colon disease.

Fluorescent studies

The fluorescent signal of the Schiff base was observed in the nucleus, the perinuclear region and in the nucleoli of Balb/c 3T3 cells (Fig. 3 A), while the aminophosphonate was distributed mainly perinuclearly in the cytoplasm (Fig. 3 B). In contrast, the most intensive fluorescence was found in the cytoplasm and nucleoli of the HT-29 cells treated with the Schiff base (Fig. 3 C) and in the nuclei of aminophosphonate-treated tumor cells (Fig. 3 D). The latter observation confirms the fact that DNA-intercalating anthracene-derived planar structure is the main pharmacophoric fragment of the recently synthesized aminophosphonate [N-methyl (diethoxyphosphonyl)-1-(9-anthryl)]-p-toluidine.

Conclusion

In vitro safety assessment of the anthracene-derived Schiff base – 9-anthrylidene-furfurylamine and the aminophosphonate [N-methyl(diethoxyphosphonyl)-1-(9-anthryl)] furfurylamine revealed that the compounds exert moderate toxicity to normal mouse cells. Both compounds were found to be cytotoxic to HT-29 human colon carcinoma cells. The aminophosphonate exhibited higher antiproliferative activity than its synthetic precursor. Therefore, the novel substances are promising for future work on the development of agents active in chemotherapy of malignant colon disease. In addition, the fluorescent properties of anthracene ring allow adequate and precise studies on the cellular uptake and intracellular distribution of the novel compounds in malignant and normal cells.

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