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Comparative assessment of *in vitro* and *in vivo* biological activity of some anthracene-derived aminophosphonates, bis-aminophosphonates and poly(aminophosphonate)s

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***Abstract*—Comparative assessment of in vitro and in vivo biological activity of anthracene-derived aminophosphonates, bis-aminophosphonates and poly(aminophosphonate)s, namely poly(oxyethylene aminophosphonate)s (4,5) and poly[oxyethylene aminophosphonate-co-H-phosphonate)]s, (6,7), were carried out in order to establish their value as potential drug candidates. The compounds were tested for in vitro cytotoxicity on a panel of seven human epithelial tumour cell lines. Safety testing was performed both in vitro (3T3 NRU test) and in vivo on ICR mice for genotoxicity and antiproliferative activity.**

The structure-activity relationship analysis showed that the anthracen-based aminophosphonates demonstrate high cytotoxic activity against tumour cell lines. Compounds APhA-t and APhA-f can be successful oral drugs according the rule of Lipinski. Bis-aminophosphonates tested exerted lower cytotoxicity towards human tumour cell lines than the aminophosphonates. The copolymer 7 showed excellent antiproliferative activity against cell lines HBL-100, MDA-MB-231, MCF-7 and HepG2. However, the in vitro safety testing revealed significant toxicity against Balb/c 3T3 mouse embryo cells. In contrast, the copolymer 6 showed complete absence of cytotoxicity against Balb/c 3T3 cells, but it inhibited the growth of breast cancer cells, cervical carcinoma cells (HeLa) and hepatocellular carcinoma cell cultures after prolonged (72 h) exposure.

The results from the fluorescent microscopic morphological studies correlate well with the data of the *in vitro* antitumor activity of the tested polymers and revealed apoptotic and necrotic alterations in breast cancer cells and in hepatocellular carcinoma cells after prolonged exposure, which point to the mechanisms of tumor cell death. The polymers (4-7) exhibited low (4, 6 and 7) to moderate (5) clastogenicity in vivo and slightly inhibited bone marrow cell division, compared to Mitomycin C. The tested polyphosphoesters are expected to act *in vivo* as prodrugs of aminophosphonates and could be valuable as a new class of biodegradable polymer drug carriers.

Keywords—aminophosphonates; Lipinski's rule, human cell cytotoxicity, genotoxicity

I. INTRODUCTION

α-Aminophosphonic acid derivatives constitute an important class of biologically active compounds, which have found wide range of applications in pharmacy and medicine [1]. They are quite promising compounds in the development of potential drugs against several metabolic disorders, because of their structural similarity to the natural α-aminocarboxylic acids [2,3]. Due to the tetrahedral configuration at phosphorus, aminophosphonates can mimic amino acids effectively and can act as analogues of the transition state in enzymatic reaction.

Therefore this class of compounds is widely used in the design of inhibitors for specific enzymes: synthase, HIV protease, rennin, trombin and PTPases [4,5]. It is known that diaryl esters of alpha-aminophosphonates as "bio-isosteric phosphorus analogues" of amino acids have a great affinity to serine proteases and exert enzyme inhibitory activity, without influence on cysteine or threonine proteases [3,6]. Aminophosphonates can suppress bacterial and viral growth and enhance transport through cellular membranes [7]. Bis-aminophosphonates inhibit osteoclast-mediated bone resorption, delay the progression of bone metastases and exert apoptotic and antiproliferative effect on tumor cells [8]. Numerous of these compounds serve as antibiotics, radiopharmaceuticals and therapeutics.

Aminophosphonate derivatives offer numerous opportunities for combining various pharmacophoric and chromophoric groups in their molecules. In addition, aminophosphonate moieties can be incorporated into biodegradable polymeric backbone. Lowmolecular and polymeric aminophosphonates, bearing anthracene ring, attract our attention in search for new compounds with potential anticancer activity. Anthracene-derived compounds play a major role as chemotherapeutic agents in cancer therapy [9]. Fluorescent properties of the anthracene ring could find useful bioanalytical application in our studies.

In this work we evaluate the molecular properties of anthracene-derived aminophosphonates and poly(aminophosphonate)s in relation to their *in vitro* cytotoxicity, antiproliferative activity and genotoxicity *in vivo* in order to establish their value as potential drug candidates.

II. MATERIALS AND METHODS

A. Compounds and Their Molecular Characteristics

The molecular properties of the antharacene-based α-aminophosphonate acid derivatives, important for drug pharmacokinetics in the human body, were evaluated with the Lipinski's rule of five (RO5) [10, 11] which states, that most "drug-like" molecules have logP ≤ 5, molecular weight ≤ 500, number of hydrogen bond acceptors ≤ 10 and number of hydrogen bond donors ≤ 5. Improved extensions of the RO5 related to the calculations of the Partition coefficient (logP range from −0.4 to +5.6) [12] were also applied.

The value of logP and the remaining parameters of drug similarity were calculated by site Molinspiration Cheminformatics (www.molinspiration.com). Method for logP prediction developed at Molinspiration (miLogP2.2 - November 2005) is based on group contributions. These have been obtained by fitting calculated logP with experimental logP for a training set more than twelve thousand, mostly drug-like molecules. The results were compared with the properties of Mitomycin C and Doxorubicin - drugs used in cancer chemotherapy and two approved bisphosphonate drugs - Alendronic acid and Pamidronic acid, used to treat osteoporosis and several other bone disorders [13, 14].

B. In vitro Antitumor Activity

The antitumor activity testing was performed on cell cultures from several human cancer cell lines using the standard MTT-dye reduction assay, described by Mosmann [15]. Cell lines from ductal carcinoma of the breast (MCF-7 and MDA-MB-231, with low and high metastatic potential, respectively), HBL-100 line (colostrum-derived mammary myoepithelial cells, expressing polyoma virus large T-antigen), bladder carcinoma (647-V), hepatocellular carcinoma (HepG2), colon carcinoma (HT-29) and the CL HeLa - cervical carcinoma were used in all experiments. The cell lines were routinely grown as monolayers in 75 cm^2 tissue culture flasks (Orange Scientific), in high-glucose (4.5‰) Dulbecco's modified Eagle's medium (DMEM) (ELTA 90, Ltd), supplemented with 10 % fetal calf serum (Sigma) and antibiotics in usual concentrations. Cultures were maintained at 37.5 $^{\circ}C$ in a humidified atmosphere and 5 % CO_2. Cells were plated at a density of 1 x 10^5 cells in 100 µl culture medium in each well of 96-well flat-bottomed microplates and allowed to adhere for 24 h before treatment with test compounds in DMSO solution, further diluted in phosphate-buffered saline (PBS) to reach the desired test concentrations. A concentration range from 1 to 0.0681 mg/ml (dilution factor of $6\sqrt{10} = 1.47$) was applied for 24 and 72 hours. The DMSO concentration never exceeded 1 % (v/v). The referent antineoplastic drug Doxorubicin hydrochloride (Lemery) was used as a commercially available sterile dosage form for clinical application and used after the appropriate dilution in phosphate-buffered saline. All experiments were performed in triplicate. The MTT-formazan absorption was registered using a microplate reader (TECAN, Sunrise TM, Groedig/Salzburg, Austria) at 580 nm. Cytotoxic activities were expressed as IC_{50} values (concentrations required for 50 % inhibition of cell growth), calculated using non-linear regression analysis (GraphPad Prizm5 Software). There was a good reproducibility between replicate wells with standard errors below ± 10 %. Student's t-test was applied and value of $p < 0.05$ was accepted as the lowest level of statistical significance.

C. Cytotoxicity Testing (3T3 NRU test)

The cytotoxicity testing was performed as described by Borenfreund and Puerner [16] and the latest modification [17] of the validated Balb/c 3T3 (clone 31) Neutral Red Uptake Assay (3T3 NRU test) [18] for cytotoxicity/phototoxicity testing. BALB/c 3T3, clone 31 mouse embryo cells were grown as monolayers in 75 cm^2 tissue culture flasks in low-glucose (1‰) DMEM (ELTA 90, Ltd), supplemented with 5 % fetal bovine serum and antibiotics. Cultures were maintained at 37.5 °C in a humidified atmosphere under 5 % CO_2. Cells were plated at a density of 1 x 10^4 cells in 100 µl culture medium in each well of 96-well flat-bottomed microplates and allowed to adhere for 24 h before treatment with the test compounds, dissolved in DMSO (ELTA 90, Ltd) and further diluted in PBS. A wide concentration range was applied (from 1 to 0.0681 mg/ml; dilution factor of $6\sqrt{10} = 1.47$) and after treatment with Neutral Red medium, washing and application of the Ethanol/Acetic acid (1:1, v/v) desorbing solution the absorption was measured on a TECAN microplate reader at 540 nm. The statistical analysis include application of One-way

ANOVA followed by Bonferroni's post hoc test and $p < 0.05$ was accepted as the lowest level of statistical significance.

D. *Cytogenetical Method*

The cytogenetical investigation was conducted according to the procedure of Preston et al. [19] Male and female ICR mice, weighing 20 ± 1.5 g were kept at 20 °C and a 12 h light/dark cycle. Food and water were available ad libitum. All the compounds were administered i.p. at doses of 10 mg/kg and 100 mg/kg. Mitomycin C (Kyowa Hakko Kogyo Co., Ltd. Tokyo, Japan) 3.5 mg/kg was used as a positive control substance. A group of animals injected with 0.9 % NaCl were used as a negative control. The bone marrow chromosome aberration assay was performed on six groups of animals. Each group consisted of 4 males and 4 females and was treated with the respective compound. The negative control groups consisted of 10 animals each, while positive (Mitomycin Ctreated) control groups consisted of four animals each. In order to obtain blocked metaphase plates the experimental and the control groups of animals were injected i.p. with 0.4 mg/kg colchicine (Fluka AG, Buchs S.G., Switzerland) at the 24-th and 48-th hour after the administration of the tested substances and 1 h prior the isolation of bone marrow cells. The slides for microscopic evaluation were prepared according to the previously described procedure [20]. Brakes, fragments and chromosomal rearrangements were separately scored. Mitotic indices were calculated by counting the number of dividing cells among 1500 cells per animal. The frequencies of abnormalities and the mitotic index were determined for each animal and then the mean ± standard error of mean was calculated for each group. Statistical analysis. Student's t-test was applied. Statistical significance is expressed as ***p<0.001; **p<0.01; *p<0.05; p>0.05 (not significant).

E. *Fluorescence Studies*

Balb/c 3T3 (clone 31) cells and HBL-100, Hep G2 and MDA-MB-231 tumor cells (1 x 10^5/ml) were seeded on sterile 12 ring diagnostic slides (Thermo Scientific), allowed to adhere overnight at 37.5 °C in a humidified atmosphere under 5 % CO_2 and treated with non-toxic concentrations of the polymer **6** for 24 h and 72 h. After fixation in cold (-20 °C) acetone the slides were air-dried, covered and examined with Leica DM 5000 B (Wetzlar, Germany) fluorescent microscope, equipped with a digital camera and the appropriate Leica Software. The same procedure was applied for studies on the subcellular distribution of **7** in Balb/c 3T3 (clone 31) cells and HT-29 colon carcinoma cells.

III. RESULTS AND DISCUSSION

All compounds used in the present work, namely anthracene-derived aminophosphonates (APhA-t, APhEA-t and APhA-f), bis-aminophosphonates and polyphosphoesters containing anthracene-derived aminophosphonate units-poly(oxyethylene aminophosphonate)s (**4,5**) and poly[oxyethylene(amino-phosphonate-co-H-phosphonate)]s (**6, 7**), were synthesized, characterized and described by Kraicheva et. al [21-23].

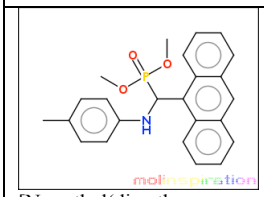
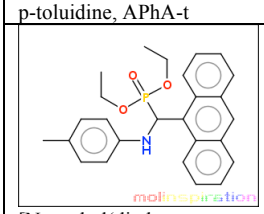
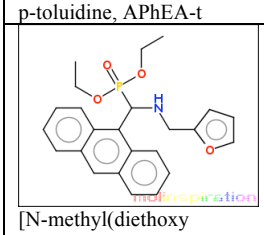
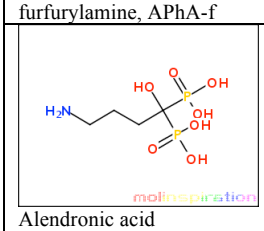
A. *Evaluation of Drug Likeness*

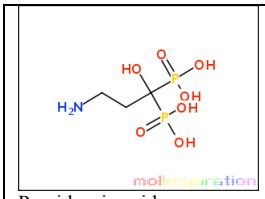
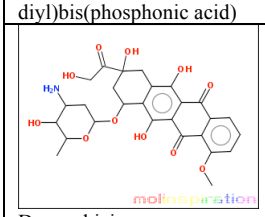
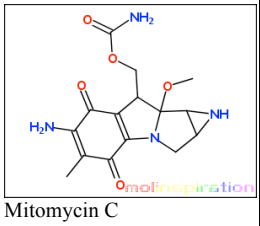
Lipinski's rule or simply the Rule of five (RO5) is a Rule of thumb for evaluation of drug likeness to similar compounds or for determination of probability for oral activity of a chemical compound with certain pharmacological or biological activity. The rule was formulated by Christopher A. Lipinski in 1997, based on the observation that most medication drugs are relatively small and lipophilic molecules.

The rule is not applicable to the molecules of bis- and poly-aminophosphonates as they are very big and their molecular weight exceeds too much the rule's limitations.

The results from molecular evaluations of anthracene-based α-aminophosphonate derivatives for drug similarity on the basis of Lipinski's rule of five are presented in Table I.

TABLE I. CHEMICAL STRUCTURE AND PARAMETERS OF EVALUATION OF ANTHRACENE-BASED AMINOPHOSPHONATE DERIVATIVES AND REFERENT DRUGS ACCORDING TO LIPINSKI'S RULE OF FIVE (RO5)

Compound*	LogP	M_w	O, N	OH, NH
 [N-methyl(dimethoxy phosphonyl)-1-(9-anthryl)]-p-toluidine, APhA-t	5.40	405.43	4	1
 [N-methyl(diethoxy phosphonyl)-1-(9-anthryl)]-p-toluidine, APhEA-t	6.15	433.49	4	1
 [N-methyl(diethoxy phosphonyl)-1-(9-anthryl)] furfurylamine, APhA-f	4.66	423.45	5	1
 Alendronic acid 4-amino-1-hydroxy-hydroxy-oxido-phosphoryl)-butyl phosphonic acid	-3.46	249.09	8	6

 Pamidronic acid (3-amino-1-hydroxypropane-1,1-diyl)bis(phosphonic acid)	-3.73	235.06	8	7
 Doxorubicin	0.57	543.52	12	7
 Mitomycin C [6-Amino-8a-methoxy-5-methyl-4,7-dioxo-1,1a,2,4,7,8,8a,8b-octahydroazireno[2',3':3,4]pyrrolo[1,2-*a*]indol-8-yl]methyl carbamate	-0.87	334.33	9	5

The calculations show that aminophosphonates and the referent drug Mitomycin C observed the boundary conditions of the Lipinski's rule of five. Incorporation of various substituents in the molecules affects the value of logP, i.e. modify the lipophilicity of the compounds. Compounds APhA-t and APhA-f did not violate any of the listed criteria. After substitution of methoxy groups in APhA-t with ethoxy groups in APhEA-t one violation of the rule appears (logP=6.154) which indicates a slightly decreased lipophilicity of the tested compound (Table I).

Although this rule provides a powerful and simple tool to find potential drugs, sometimes it could exclude the compound that would have proven the successful drug. For example, the molecules of two bisphosphonate drugs - Alendronic acid and Pamidronic acid have one violation of the Lipinski's rule. Moreover, the molecule of Doxorubicin in general do not follow the rule too (3 violations), but this compound is an anthracycline antibiotic used in cancer chemotherapy. However, the rule does not predict if a compound is pharmacologically active and we use it only as a preliminary screening.

B. Cytotoxicity and Genotoxicity

The data presented in Table II show that [N-methyl(diethoxyphosphonyl)-1-(9-anthryl)]furfurylamine (APhA-f) reaches 50 % decrease of the tumor cells at lower concentration in comparison with other two anthracene -

TABLE II. *IN VITRO* ANTITUMOR ACTIVITY OF ANTHARACENE-BASED A-AMINOPHOSPHONATES IN HUMAN TUMOR CELL LINES, ASSESSED BY THE MTT-DYE REDUCTION ASSAY AFTER 24 H TREATMENT*

Compound	MCF-7	MDA-MB-231	HepG2
APhA-t	0.45 ± 0.024	0.88 ± 0.023	0.83 ± 0.025
APhEA-t	> 2	> 2	> 2
APhA-f	0.10 ± 0.003	0.07 ± 0.001	0.11 ± 0.002
Doxorubicin	< 0.068	< 0.068	< 0.068

* Evaluation was made on the basis of IC_{50} values in mg/ml (mean±S.E.M.)

contained aminophosphonates APhA-t and APhEA-t. The IC_{50} values of the compounds on three cell lines are similar (between 0.07 and 0.11 mg/ml). The compound APhEA-t can be accepted as ineffective because does not give a 50 % inhibition at applied concentrations to 2 mg/ml.

Furthermore, in an attempt to evaluate antiproliferative activity of the tested compounds against the mice normal bone marrow cells, we provide in vivo test for determination of mitotic activity of the isolated bone marrow cells. In general, all compounds induce a moderate decrease of the proliferative activity of the normal bone marrow cells in comparison with untreated control (20.06 ‰ mitoses). Mitotic index ranged from 11.97 ± 1.08 ‰ observed in animals treated with APhA-t to 16.08 ‰ in the experimental group injected with high dose of 100 mg/kg APhEA-t. The results show that the compounds inhibit cell proliferation lower than the positive control Mitomycin C (Table III). It is possible to propose that the antiproliferative activity is due either to general structure of α-aminophosphonic acid derivatives, or is associated with anthracene ring.

The poly[oxyethylene aminophosphonate-co-H-phosphonate)]s **6** and **7** were tested for cytotoxicity on a panel of seven human cancer cell lines representative of important solid epithelial tumors of the breast, liver, colon, urinary bladder and cervix. The copolymers exerted concentration-dependent antiproliferative effects after 24 and 72 h exposure which enabled the construction of concentration–response curves (not shown) and the calculation of the corresponding IC_{50} values. The cytotoxicity data reveal that the polymer **7** proved to be more potent than the polymer **6** after 24 h treatment and appeared to be the most active cytotoxic agent towards breast myoepithelial tumor cell line HBL-100. In addition, the cytotoxic potential of the same polymer was comparable to that of the positive control substance Doxorubicin, used in the experiments, when tested on cell lines MDA-MB-231 (highly metastatic carcinoma of the breast) and HepG2 (hepatocellular carcinoma). In comparison to the activity of the positive control drug, the effect of **7** on tumor cell lines 647-V, MCF-7 and HeLa was two, three and four times lower, respectively. The HT-29 colon carcinoma cells were resistant to the antitumor activity of both tested polymers after 24 h exposure (Table IV).

The prolongation of the incubation time for 72 h resulted in an enhancement of the *in vitro* antitumor activity of both tested copolymers. The cytotoxicity of **6** towards cell lines MCF-7, MDA-MB-231 and HeLa was approximately two times higher and for the cell line HepG2 three times higher than values obtained after 24 h treatment. No alterations of IC_{50} values were noted after treatment of cell lines HBL-100, HT-29 and

647-V. The prolongation of the treatment with polymer **7** enhanced the in vitro antitumor activity to HBL-100 and 647-V cells (Fig. 1), reduced the viability of HT-29 cells (IC_{50} = 0.109 ± 0.003 mg/mL) and induced complete destruction of MCF-7 and MDA-MB-231 breast cancer cells and hepatocellular carcinoma (Hep G2) cell cultures.

TABLE III. ANTIPROLIFERATIVE EFFECT OF ANTHRACENE-BASED AMINOPHOSPHONATES AND BIS-AMINOPHOSPHONATES EVALUATED IN BONE MARROW CELL POPULATION 24 H AFTER TREATMENT OF ICR MICE

Compound/Dose (mg/kg)		Number of metaphases scored	Mitotic index (‰) Mean ± SD
APhA-t	10	400	12.60 ± 0.77 a *** b ***
	100	400	11.97 ± 1.08 a *** b ***
APhEA-t	10	400	13.41 ± 1.14 a *** b ***
	100	400	16.08 ± 1.16 a ***
Mitomycin C	3.5	200	5.49 ± 0.19
Control 0.9% NaCl		700	20.06 ± 1.38

Statistics: Student t-test; *p<0.05; **p<0.01; ***p<0.001; **a** - compared to Mitomycin C; **b** - compared to control;

TABLE IV. COMPARATIVE CYTOTOXIC ACTIVITY OF COPOLYMERS **6** AND **7** VERSUS REFERENT SUBSTANCE DOXORUBICIN IN A PANEL OF HUMAN TUMOR CELL LINES AFTER 24 H TREATMENT (MTT-DYE REDUCTION ASSAY)

Cell lines	Mean IC_{50} values (mg/ml)[a]		
	Compound 6	Compound 7	Doxorubicin
MCF-7	>1.000	0.199 ± 0.020	< 0.068
MDA-MB-231	>1.000	0.089 ± 0.001	< 0.068
HBL-100	>1.000	0.118 ± 0.005	0.14 ± 0.029
HepG2	>1.000	0.094 ± 0.003	< 0.068
HT-29	>1.000	> 1.000	0.58 ± 0.031
647-V	>1.000	0.114 ± 0.022	< 0.068
HeLa	>1.000	0.286 ± 0.003	< 0.068

Values are means ± standard deviation from three consecutive experiments.

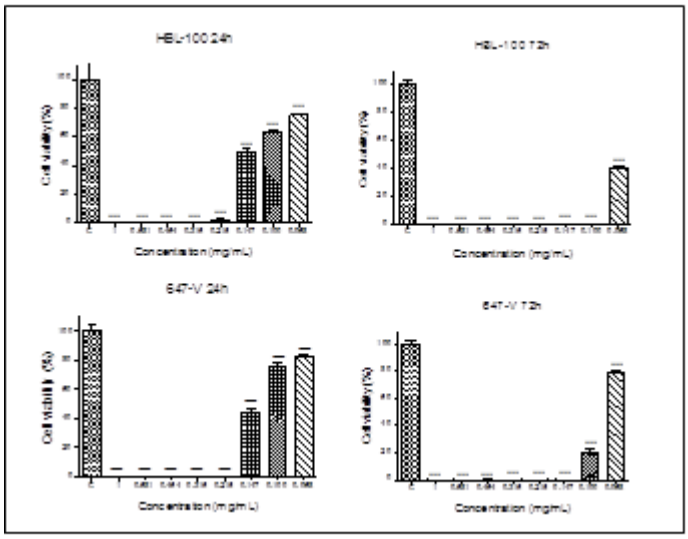


Fig. 1. Reduction of viability of HBL-100 and 647-V human tumor cells after treatment with polymer **7** for 24 and 72 hours. C – control (untreated) tumor cell cultures; ***p<0.001, compared to control

C. *In Vitro Safety Testing*

The results from the validated Balb/c 3T3 (clone 31) Neutral Red Uptake Assay (3T3 NRU test) revealed dose-dependent cytotoxic activity of copolymer **7**. Safety *in vitro* testing of **6** revealed complete absence of cytotoxicity to Balb/c 3T3 (clone 31) cell line, even after exposure for 72 h. In contrast, **7** induced significant reduction of viability of Balb/c 3T3 cells after 24 h treatment in a concentration range from 1 to 0.1 mg/mL. Enhancement of the cytotoxic activity has been observed after prolonged (72 h) exposure up to 0.068 mg/mL (Fig. 2). The cytotoxicity of **7** to mouse embryo fibroblasts, however, in the concentration range 0.318 – 0.068 mg/mL was comparable or much lower that the toxic activity of the positive control substance sodium dodecyl sulphate (data not shown).

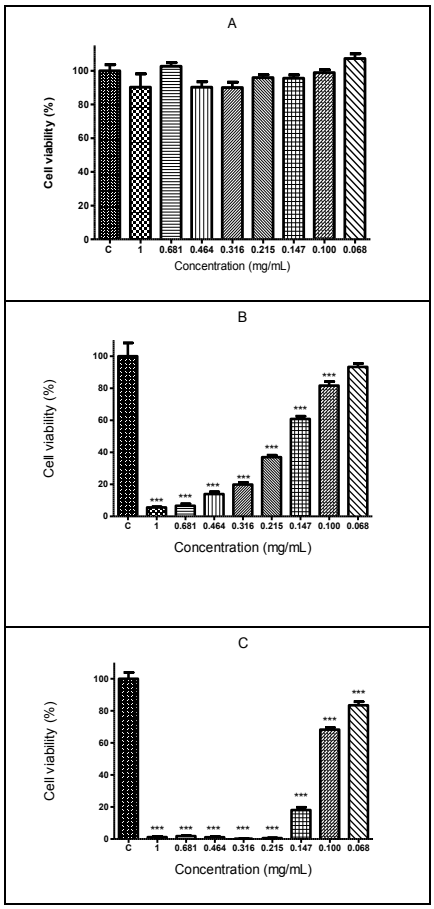


Fig. 2. In vitro cytotoxicity of compounds 6 and 7 on cultures from cell line Balb/c 3T3, clone 31 (3T3 NRU test) after 72 hours exposure to 6 (A) and 7 (C) and 24 hour treatment with 7 (B). C, vehicle-treated cell cultures (negative control); ***p < 0.001, compared to negative control.

D. *Fluorescent Studies*

The fluorescent properties of the anthracene-containing polymer **6** were used to study the subcellular distribution in model cell culture systems (Balb/c 3T3 (clone 31), HepG2, HBL-100 and MDAMB-231 cells) after exposure to nontoxic concentrations of the substance. The fluorescent signal of polymer **6** was observed mainly in the cytoplasm of Balb/c 3T3 (clone 31) non-tumorigenic mouse embryo cells (Fig. 3A). In

contrast, the most intensive fluorescence after application of **6** for 24 h was found in the nuclei and nuclear membranes of HBL-100 cells (Fig. 3C), but no morphological signs of cellular damage have been observed. After prolonged (72 h) treatment the fluorescence of **6** was also found mainly in the nuclei of HepG2 and MDA-MB-231 tumor cells. Chromatin margination and membrane blebbing, cell shrinkage and rounding-up of the cells were observed as signs of apoptotic and necrotic cell death, respectively (Figs. 3B and 3D).

Experiments have been also conducted in order to elucidate the intracellular distribution of **7** in model cell culture systems (Balb/c 3T3 (clone 31) and HT-29) by means of fluorescence microscopy. It has been observed (Fig. 4) that **7** readily penetrated to the nuclei of most Balb/c 3T3 cells on the 24-th hour after treatment (Fig. 4A) and induced necrosis of these cells 72 h treatment (Fig. 4B). In contrast, few HT-29 colon carcinoma cells showed fluorescent signal after 24 h incubation with the substance located in the nuclei. The most intensive diffuse fluorescence was found in the cytoplasm of most of these tumor cells (Fig. 4C). Moreover, after 72 h treatment the coarse granular fluorescence of **7** was observed in the cytoplasm and the cell membranes of HT-29 cells (Fig. 4D).

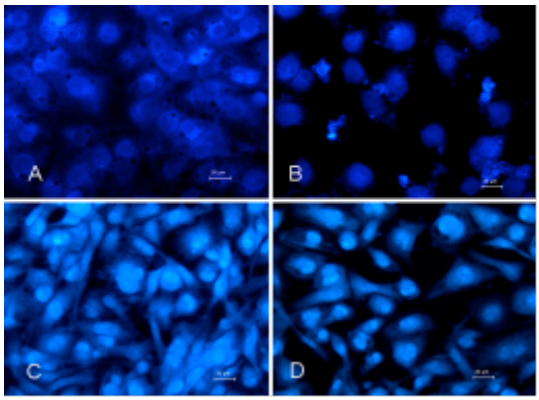


Fig. 3. Fluorescence microscopy of subcellular distribution of copolymer **6** in Balb/c 3T3 (clone 31) cells (A), HepG2 cells (B), HBL-100 cells (C) and MDA-MB-231 cells (D) after 24 h treatment (A, C) and 72 h treatment (B, D).

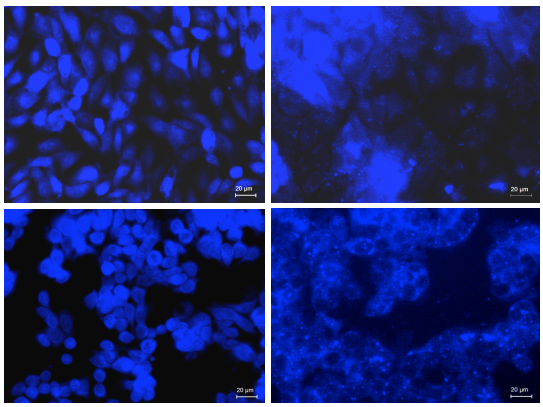


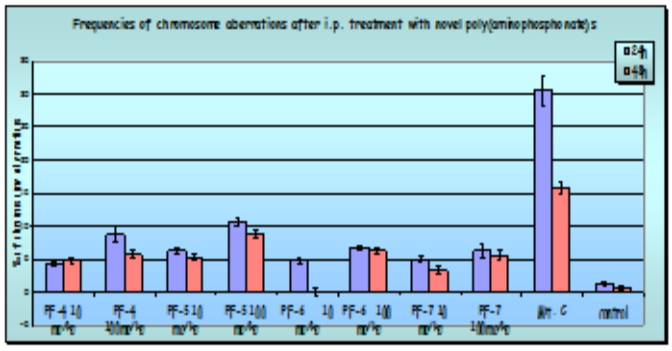
Fig. 4. Fluorescent microscopy of subcellular distribution of polymer **7** in Balb/c 3T3 (clone 31) cells (A, B) and HT-29 cells (C, D) after 24 h treatment (A, C) and 72 h treatment (B, D).

E. In Vivo Safety Testing

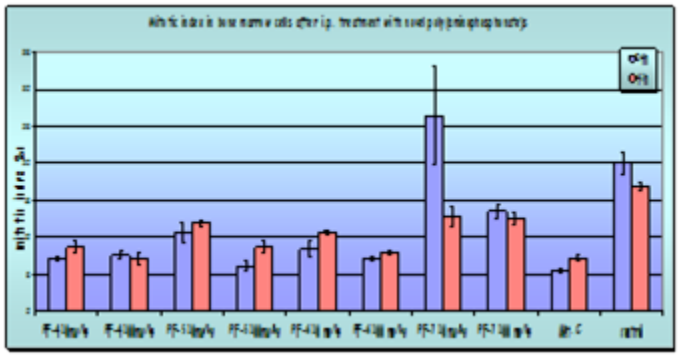
The genotoxicity of newly synthesized poly(amino-phosphonate)s **4-7** was determined by scoring the amount of bone marrow cells with chromosomal aberrations observed and the results are summarized on Graph 1 and 2.

The mean percentages of cells with aberrations were 4.29 ± 0.28 % at the 24-th hour and 4.75 ± 0.39 % at the 48-th hour in ICR mice injected with 10 mg/kg poly(oxyethylene aminophosphonate) **4**. In the group receiving a dose of 100 mg/kg significantly higher values were recorded at the 24-th hour – 8.75 ± 1.06 % ($p < 0.001$). At the 48-th hour the quantity of aberrant cells decreased considerably compared to the 24-th hour group. The polymer **5** showed the most pronounced clastogenic effect in the group treated with 100 mg/kg at the 24-th hour (10.67 ± 0.61 % of the metaphases with damaged chromosomes). In the group treated with 10 mg/kg of poly(oxyethylene aminophosphonate) 5, the clastogenic effect was significantly lower ($p < 0.001$). The poly [oxyethylene aminophosphonate-co-H-phosphonate)] **6** showed a chromosome damaging effect very similar to that of **4**.

The cytogenetic analysis of polymeric compound **7** demonstrated that the greatest impact on the structure of the chromosomes were observed in the experimental mice injected with 100 mg/kg dose 24 hours prior the chromosome slides preparations (6.25 ± 1.03 %). The effect of this polymer is maintained for 48 hours, although a slight decrease in the percentage of cells with aberrant chromosomes was evaluated (5.50 ± 0.73 %). As a whole the clastogenic effects of this compound can be reported as moderate.



Graph 1. Frequences of chromosome aberrations after i.p. treatment with novel poly(aminophosphonate)s.



Graph 2. Mitotic index in bone marrow cells after i.p. treatment with novel poly(aminophosphonate)s

In general, all four polymers exhibited from comparatively low (**6**) to moderate (**5, 7**) clastogenicity, but significantly lower than the effect of the alkylating agent Mitomycin C ($p<0.001$).

Studying the cytotoxicity of **4, 5** and **6** on the normal bone marrow cells, a significant suppression of mitotic processes was observed in the treated groups compared to the untreated control. The highest degree of inhibition was found in bone marrow cell populations of ICR mice treated with 100 mg/kg 5 at the 24-th hour after injection of the polymer (6.26 ± 0.63 ‰). In this experimental group higher antimitotic effect strongly correlated with the higher percentage of aberrant metaphases. However, the mitotic index values in the groups treated with 10 mg/kg of the same substance (11.93 ± 0.42 ‰) were rather high.

The three tested poly(aminophosphonate)s (**4, 5** and **6**) slightly suppressed the bone marrow cell division compared to Mitomycin C.

Particularly interesting are the results demonstrating that a low dose of 10 mg/kg poly[oxyethylene aminophosphonate-co-H-phosphonate)] **7** stimulated cell division in bone marrow. Such positive effect was not observed in the groups treated with 100mg/kg. All treated with **7** cell populations possessed relatively good proliferative activity in comparison with Mitomycin C.

In the samples treated with Mitomycin C the mitotic index was 5.49 ± 0.19‰, but in the samples treated with polymer 7 the lowest calculated value was 12.58 ± 0.83 ‰.

The analysis of the results from our study points out that the tested novel polymers bearing anthracene-derived aminophosphonate units 6 and 7 are active cytotoxic agents to all three tested breast cancer cell lines. The polymer 7 was more active than polymer 6 and in addition to its effect on breast cancer cells, induced also complete destruction of hepatocellular carcinoma (HepG2) cell cultures. However, this substance exerted toxicity to Balb/c 3T3 mouse embryo cells. The copolymer 6 has shown no signs of toxicity to normal mouse fibroblasts even after prolonged exposure, which is probably a consequence of its inability to pass the nuclear membrane. In tumor cells, however, the anthracene-containing polymer 6 readily penetrated to the nuclei where the most intensive fluorescence was observed. The results from the light microscopic morphological study correlate well with the data of the in vitro antitumor activity of the tested polymers and revealed apoptotic and necrotic alterations in breast cancer cells and in hepatocellular carcinoma cells after prolonged exposure, which point to the mechanisms of tumor cell death.

In general, investigated polymers showed moderately expressed genotoxic effects. The amount of proliferating bone marrow cells reduced without reaching a critical cytotoxic effect, as is observed after treatment with well-known cytotoxic and genotoxic agent Mitomycin C.

The data of present study points out that copolymer 7 is the most interesting compound for future investigation due to its high cytotoxic activity against human tumor cell lines and safety to normal bone marrow cell lines in vivo.

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