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Synthesis, antiproliferative activity and genotoxicity of novel anthracene-containing aminophosphonates and a new anthracene-derived Schiff base

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ABSTRACT

A new Schiff base, 9-anthrylidene-furfurylamine and three novel anthracene-containing α-aminophosphonates, [*N*-methyl(dimethoxyphosphonyl)-1-(9-anthryl)]-*p*-toluidine, [*N*-methyl(diethoxyphosphonyl)-1-(9-anthryl)]-*p*-toluidine and [*N*-methyl(diethoxyphosphonyl)-1-(9-anthryl)]furfurylamine were synthesized. The compounds have been characterized by elemental analysis, TLC, IR, NMR and fluorescent spectra. The aminophosphonates and their synthetic precursors were tested for in vitro antitumor activity on a panel of seven human epithelial cancer cell lines. Safety testing was performed both in vitro (3T3 NRU test) and in vivo on ICR mice for genotoxicity and antiproliferative activity. 9-Anthrylidene-furfurylamine and [*N*-methyl(diethoxyphosphonyl)-1-(9-anthryl)]furfurylamine were most potent cytotoxic agents towards colon carcinoma cell line HT-29. The latter compound exhibited also antiproliferative activity to HBL-100, MDA-MB-231 and 647-V cells. The aminophosphonate [*N*-methyl(dimethoxyphosphonyl)-1-(9-anthryl)]-*p*-toluidine and its synthetic precursor 9-anthrylidene-*p*-toluidine were found to be cytotoxic to HBL-100 and HT-29 tumor cell lines, respectively. Moderate genotoxic and antiproliferative activity in vivo and low toxicity to Balb/c 3T3 (clone 31) mouse embryo cells were observed for all tested compounds. The subcellular distribution of two tested compounds in a tumor cell culture system was also studied.

1. Introduction

Aminophosphonic acid derivatives have been found to posses various important biological activities that enable wide range applications of these compounds in the pharmacological and agrochemical fields.[1–4] α-Aminophosphonates are quite attractive compounds in the development of potential drugs against several metabolic disorders because of their structural similarity to the natural α-aminocarboxylic acids.[2,3] Thus, due to the tetrahedral configuration at phosphorus, aminophosphonates can act as analogues of the transition state in enzymatic reaction and therefore they are widely used in the design of the enzyme inhibitors.[5,6] Aminophosphonates can suppress bacterial and viral growth and enhance transport through cellular membranes.[7,8] Bisaminophosphonates inhibit osteoclast-mediated bone resorption, delay the progression of bone metastases and exert marked apoptotic and antiproliferative effect on tumor cells.[9] Numerous of these compounds are used as haptens for the generation of catalytic antibodies, antibiotics, bone seeking radiopharmaceuticals and therapeuticals.[10–12] Some authors suggest that the biological activity of the aminophosphonates is correlated to their lipophilicity.[13,14]

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 for the treatment of human cancers.[15,16] Highly active antimitotic anthracene-based agents, inhibit tubulin polymerization.[17–19] For some of these anthracene-containing substances have been reported to display strong antiproliferative activity against several tumor cell lines, including multidrug resistant phenotypes.[20] In addition, fluorescent anthracenoyl crown ethers are used as a sensitive probe for the solid phase transitions of phosphatidylcholines.[21] Therefore,

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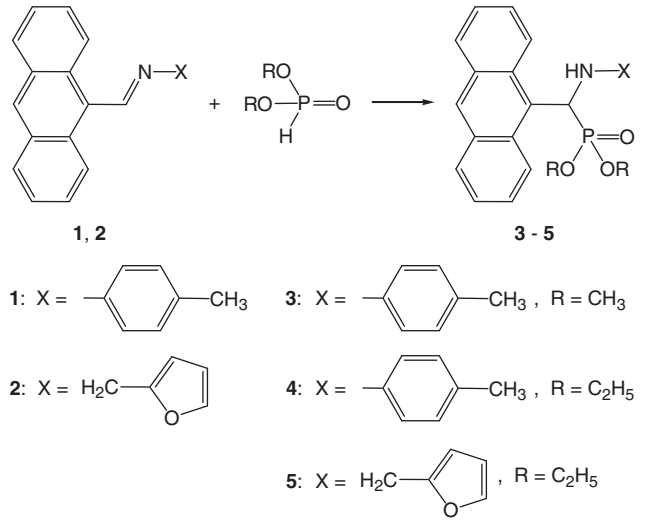
the fluorescent properties of anthracene-based aminophosphonates could find valuable bioanalytical application in studying the subcellular distribution and binding in normal and tumor cells.

To the best of our knowledge, only a few examples of anthracene-derived aminophosphonates are described in the literature.[22–24] The addition of dialkyl (or diaryl) phosphites to Schiff bases is a convenient method for the preparation of aminophosphonate derivatives.[22,24–26] In this work we report on the synthesis, in vitro antitumor activity and in vitro and in vivo safety evaluation of new anthracene-derived Schiff base and three novel anthracene-containing aminophosphonates. Data about the cytotoxic potential of an earlier described anthracene-containing Schiff base[27] are also presented. In addition, we report data about subcellular distribution of two of the tested compounds in a tumor cell culture system.

2. Results and discussion

2.1. Chemistry

The Schiff bases, 9-anthrylidene-*p*-toluidine (**1**) and 9-anthrylidene-furfurylamine (**2**) were prepared by condensation of 9-anthracenecarboxaldehyde with *p*-toluidine and furfurylamine, respectively. Three novel α-aminophosphonic acid diesters, bearing anthracene ring, were synthesized: [*N*-methyl(dimethoxyphosphonyl)-1-(9-anthryl)]-*p*-toluidine (**3**), [*N*-methyl(diethoxyphosphonyl)-1-(9-anthryl)]-*p*-toluidine (**4**) and [*N*-methyl(diethoxyphosphonyl)-1-(9-anthryl)]furfurylamine (**5**). The aminophosphonates were obtained through addition of dimethyl phosphite and diethyl phosphite to the Schiff bases **1** and **2** according to the Scheme 1. The reaction of dialkyl phosphites to the Schiff base **1** was carried out without catalyst. The products **3** and **4** were obtained as yellow fluorescing crystalline solids. The addition of diethyl phosphite to the imine **2** was performed using CdI_2 as a catalyst. The compound **5** was prepared as an oil and purified by column chromatography on silica gel. Thin layer chromatography (TLC) gave one spot for each of the compounds **1**–**5**. The formation of the aminophosphonate structures was confirmed by IR and NMR spectroscopy. The ^1H NMR spectra of the aminophosphonates exhibit a doublet signal at 6.37 (**3**), 6.35 (**4**) and 5.73 (**5**) ppm, which can be assigned for the CHP proton. The NH proton signal of **3**–**5** is observed as a broad singlet. The data from



Scheme 1. Synthesis of α-aminophosphonic acid diesters **3**–**5**. Reagents and conditions: **3**, 9-anthrylidene-*p*-toluidine, dimethyl phosphite, diethyl ether, rt, 13 h; **4**, 9-anthrylidene-*p*-toluidine, diethyl phosphite, benzene, reflux, 14 h; **5**, 9-anthrylidene-furfurylamine, diethyl phosphite, benzene, CdI_2, reflux, 6 h.

$^{13}C\{^1H\}$ NMR spectra of the aminophosphonates also confirm the P–C bond formation. The doublets with large coupling constants 154.1 (**3**), 153.2 (**4**) and 158.4 (**5**) Hz have to be assigned to the carbon atom connected to the phosphorus. The assignment of the anthracene proton and carbon signals of the compounds **1**–**5** is based on the analysis of their 2D spectra and literature data.[23,28,29] The $^{31}P\{^1H\}$ NMR spectra of the aminophosphonates reveal one singlet at 27.69 (**3**), 25.25 (**4**) and 25.80 (**5**) ppm. The fluorescent emission of the compounds **1**–**5** was registered and the maxima appear in the region 318–442 nm of the spectra.

2.2. In vitro antitumor activity

The Schiff bases **1** and **2** and the aminophosphonates **3–5** were tested for cytotoxicity against a panel of seven cancer cell lines representative of some important types of human tumors. All compounds exerted concentration-dependent antiproliferative effects after 24 h exposure which enabled the construction of concentration–response curves (not shown) and the calculation of the corresponding IC_{50} values summarized in Table 1. As evident from the cytotoxicity data the Schiff base **2** and the corresponding aminophosphonate **5** proved to be the most potent cytotoxic agents towards colon carcinoma cell line HT-29, which implies that the presence of both anthracene ring and furan ring is an important prerequisite for optimal activity for these compounds. This is further supported by the fact that compound **5** showed two times higher activity to HBL-100 cells than the referent anticancer drug Doxorubicin. In addition, the cytotoxic potential of the same compound was comparable to that of the positive control substance, used in the experiments, when tested on cell lines MDA-MB-231 (highly metastatic carcinoma of the breast) and 647-V (bladder carcinoma). The other Schiff base **1** and its aminophosphonate derivative **3** were found to be cytotoxic to colon carcinoma cells (HT-29 line) and HBL-100 cell line, respectively. All compounds were generally less active as compared to the referent anticancer drug Doxorubicin after testing on cell cultures from the cancer cell lines MCF-7, HepG2 and HeLa (Table 1).

The results obtained imply that compounds **2** and **5** could be considered as promising leads for further development of agents active in chemotherapy of malignant breast and colon disease.

2.3. 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 compounds **1–5** (Fig. 1). The aminophosphonate **4** and the starting Schiff base **1** appeared to be less toxic than other tested compounds with lowest toxic doses of 1.36 and 0.15 mg/ml, respectively. The Schiff base **2** and the corresponding aminophosphonate **5** showed statistically significant (p <0.001) cytotoxicity in a wide concentration range (1–0.07 mg/ml) to mouse embryo fibroblastic cells, compared to untreated control cell cultures. The aminophosphonate **3** also induced significant cytotoxic effect (p <0.001) within the concentration range tested. Compared to the cytotoxic effect on Balb/c 3T3 cells of the positive control substance sodium dodecyl sulphate, however, the cytotoxicity of the tested compounds was comparable (**2** and **5**), two-fold lower (**1** and **3**) and over 10-fold (**4**) lower (Fig. 1).

2.4. Clastogenic and antiproliferative activity

The results obtained about the induced frequency of chromosome aberrations in bone marrow cells of ICR mice after treatment with the Schiff bases **1** and **2** and the α-aminophosphonates **3–5** are presented in Tables 2 and 3. The Schiff base **1** exhibited a moderate clastogenic effect, varying from 5.25 ± 0.53% to 6.57 ± 1.04%.

Table 1
Comparative cytotoxic activity of compounds **1**–**5** versus referent substance Doxorubicin in a panel of human tumor cell lines after 24 h treatment (MTT-dye reduction assay)

Tumor cell lines	Mean IC_{50} values (mg/ml)[a]					
	1	3	4	2	5	Doxorubicin
MCF-7	0.45 ± 0.017	0.45 ± 0.024	>2	0.26 ± 0.014	0.10 ± 0.003	<0.068
MDA-MB-231	0.12 ± 0.007	0.88 ± 0.023	>2	0.45 ± 0.142	0.07 ± 0.001	<0.068
HBL-100	0.31 ± 0.027	0.02 ± 0.006	0.73 ± 0.023	0.46 ± 0.018	0.07 ± 0.001	0.14 ± 0.011
HepG2	0.18 ± 0.011	0.83 ± 0.025	>2	0.12 ± 0.005	0.11 ± 0.002	<0.068
HT-29	0.08 ± 0.003	>2	>2	0.20 ± 0.012	0.11 ± 0.001	0.58 ± 0.013
647-V	0.13 ± 0.004	0.60 ± 0.021	>2	0.08 ± 0.002	0.07 ± 0.001	<0.068
HeLa	0.17 ± 0.004	>2	1.94 ± 0.06	0.36 ± 0.008	0.12 ± 0.001	<0.068

[a] Values are means ± standard deviation from three consecutive experiments.

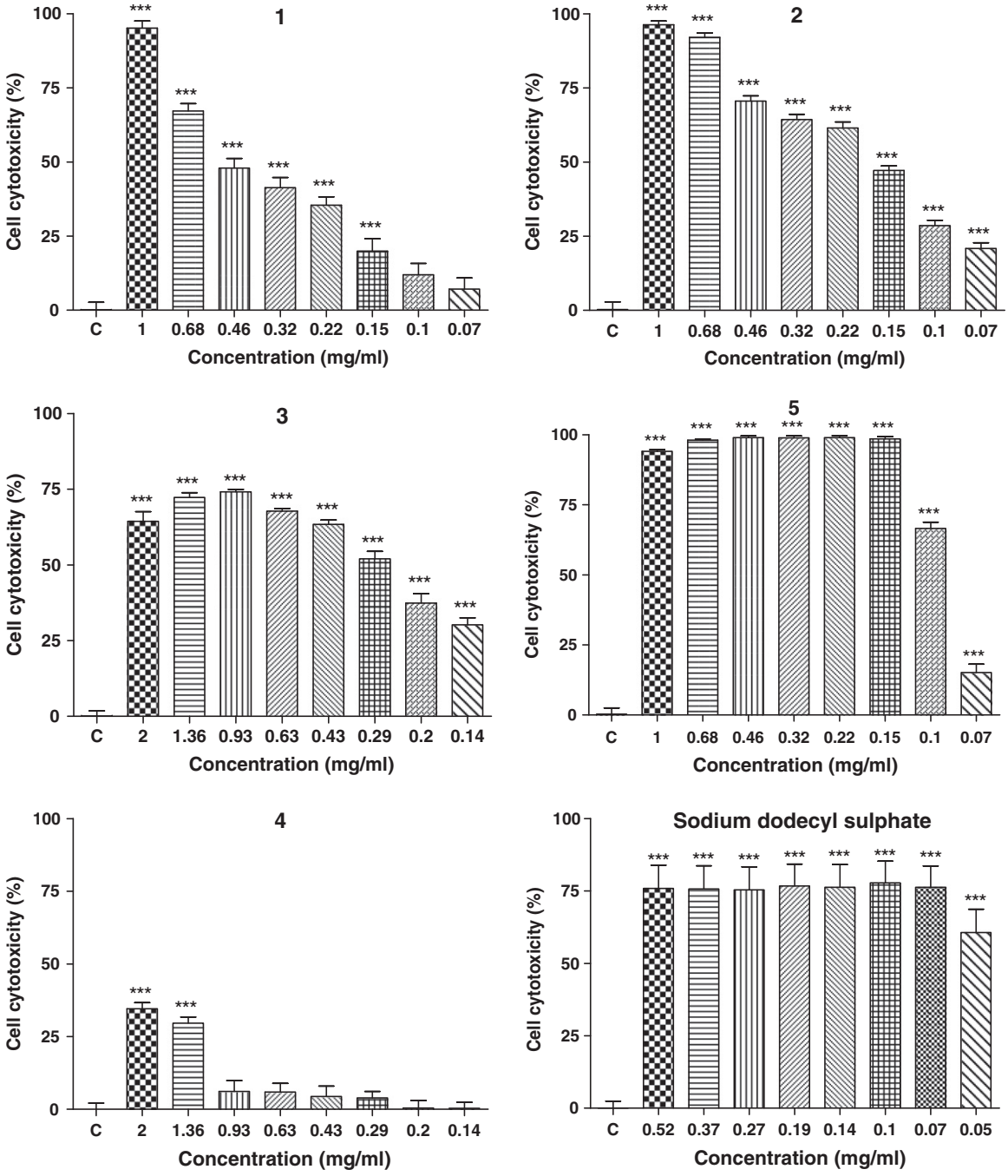


Figure 1. In vitro cytotoxicity of compounds **1**–**5** on cultures from cell line Balb/c 3T3, clone 31 (3T3 NRU test). C, vehicle treated cell cultures (negative control); sodium dodecyl sulphate, positive control substance; ***p <0.001, compared to negative control.

Table 2
Clastogenic effect and proliferative activity of bone marrow cells of ICR line laboratory mice after ip treatment with Schiff base **1** and the corresponding aminophosphonates **3** and **4**

Compounds and doses	Time after treatment (h)	Number of metaphases scored	Type of chromosome aberrations					Percentage of cells with aberrations (X ± m)	Statistical significance				Mitotic index (‰) (X ± m)	Statistical significance			
			Breaks	Fragments	Rearrangements				a	b	c	d		a	b	c	d
					c/c	t/t	c/t										
(**1**) 10 mg/kg	24	400	6	5	9	1	0	5.25 ± 0.53	***	***			12.63 ± 1.02	***	***		
	48	400	3	5	10	2	0	5.00 ± 0.38	***	***			8.45 ± 1.82		***		
(**1**) 100 mg/kg	24	350	5	8	9	1	0	6.57 ± 1.04	***	***			10.66 ± 1.42	***	***		
	48	400	7	7	11	1	0	6.5 ± 0.73	***	***			11.69 ± 1.98	*	*		
(**3**) 10 mg/kg	24	400	2	1	13	0	0	4.0 ± 0.38	***	***			12.60 ± 0.77	***	***		
	48	400	6	3	12	0	0	4.75 ± 0.37	***	***			15.64 ± 1.38	***			**
(**3**) 100 mg/kg	24	400	6	2	12	2	0	5.5 ± 0.5	***	***			11.97 ± 1.,08	***	***		
	48	400	6	4	14	0	0	6.0 ± 0.84	***	***			14.11 ± 1.64	***			
(**4**) 10 mg/kg	24	400	5	3	10	1	0	4.75 ± 0.65	***	***	**		13.41 ± 1.14	***	**		
	48	400	9	4	10	1	0	6.00 ± 0.75	***	***			11.86 ± 1.24	**	**		
(**4**) 100 mg/kg	24	400	7	11	14	2	0	8.5 ± 0.73	***	***			16.08 ± 1.16	***	*		**
	48	400	8	14	9	1	0	8.00 ± 0.53	***	***			11.78 ± 1.28	**	**		
Mit. C 3.5 mg/kg	24	200	17	30	7	1	0	30.5 ± 2.36		***			5.49 ± 0.19		***		
	48	400	17	24	20	0	0	15.8 ± 0.81		***			7.29 ± 0.34		***		
Control 0.9% NaCl	24	700	4	0	4	0	0	1.14 ± 0.34	***				20.06 ± 1.38	***			
	48	500	0	0	3	0	0	0.6 ± 0.3	***				16.88 ± 0.56	***			

c/c, Centromeric/centromeric fusion; t/t, telomere/telomeric fusion; c/t, centromere/telomeric fusion.
a, Compared to Mitomycin C; b, compared to control; c, compared to dose 100 mg/kg; d, compared to 1.
Statistics: Student *t*-test.
* p <0.05.
** p <0.01.
*** p <0.001.

Dose-dependent effect was not detected. The values for the aberrant metaphases in the samples obtained after treatment with Schiff base **1** at doses 10 and 100 mg/kg body weight did not significantly differ (p >0.05). The corresponding aminophosphonate **3** had lower clastogenic effect than the Schiff base **1**, but the differences did not reach statistical significance. Main types of structural alterations in chromosomes that have been observed in slides analyzed were as follows: breaks and fragments—35.6 ± 5.63% and centromere/centromeric fusions—62.17 ± 5.72%. The quantity of metaphases with chromosome breaks and fragments in the bone marrow cells of the experimental animals treated with the aminophosphonate **3** were lower than those in animals treated with the Schiff base **1** (Table 2). The compound **4** at a dose100 mg/kg after 24 h exposure induced significantly higher percentage of chromosome aberrations (p <0.01), compared to the lower concentration applied (10 mg/kg) and the quantity of bone marrow cells with damaged chromosomes reached 8.5 ± 0.73%. Significant differences in the number of aberrant mitoses were not detected in samples obtained at 24th and 48th hour after ip application of **4**. Both doses of this aminophosphonate ensured enough amounts of active molecules to provoke the same percentage of aberrations at 48th hour post inoculation (p.i.). The compound **2** showed clastogenic effects, ranging from 4.43% to 5.5%. These values were significantly lower (p <0.001) compared with the values calculated after treatment of experimental animals with the alkylating agent Mitomycin C. An increase in the yield of chromosomal aberrations in samples examined after injection into laboratory mice of the higher dose of the compound **2** (100 mg/kg) was not found (p >0.05). The clastogenic effect of the aminophosphonate **5** was similar to that of the parent compound **2**. The values ranged from 4.75% metaphases with chromosomal aberrations in samples obtained 24 h after treatment of ICR mice with a dose 10 mg/kg to 6.25% in 48th hour p.i. samples. The difference was statistically insignificant (p >0.05). The main type of chromosome aberrations provoked were centromere/centromeric fusions (c/c). This type of aberrations represented 68.76 ± 3.13% of all reported aberrations in the analyzed slides. For comparison, treatment with Mitomycin C had resulted only in 24.9% damaged metaphases with c/c fusions, while metaphase plates with breaks and fragments were 75.1% (Table 3).

The data reflecting the antiproliferative activity of test substances, expressed through changes in mitotic index values in bone marrow cell population are presented in Tables 2 and 3. All substances applied in the experiments showed lower antiproliferative effect on the normal bone marrow cells than the positive control Mitomycin C with a level of statistical significance ranging from p <0.01 to 0.001. Mitotic index values ranged from 8.45‰, observed in experimental animals treated with the Schiff base **1** (10 mg/kg, 48 h after treatment) to 16.08‰ in the experimental group injected with the corresponding aminophosphonate **4** (Table 2). It was also found that the compounds **2** and **3** 48 h after ip administration did not inhibited cell division in the bone marrow cell population.

In summary, the compounds did not induce clear expressed 'dose-effect' clastogenic activities, in contrast to the alkylating agent Mitomycin C. The low percentage of bone marrow metaphases with chromosome aberrations and the lack of aberrant metaphase plates with disintegrated chromosomes and dispersed chromatin is an evidence of the moderate clastogenic effect of the tested substances. The main types of chromosome structure alterations in the ICR mice bone marrow cells were c/c fusions, breaks and fragments, which suggests that the tested compounds affect the centromeric chromosome regions, which allow centromere/centromeric recombinations between non-homologous chromosomes. Along with the moderate clastogenic activity of the compounds an inhibition of cell division was observed, but the antiproliferative effects were fairly lower than that of the alkylating anticancer drug Mitomycin C, indicative of a lesser damage to proliferating bone marrow cell population.

The data from in vivo experiments are in a good correlation with the data on the cytotoxic effect of tested Schiff bases and their aminophosphonate derivatives on mouse embryo fibroblastic cell line Balb/c 3T3 (clone 31). It can be speculated that these compounds have lower toxic effect on normal cells as compared to anticancer and cytotoxic agents.

Table 3
Clastogenic effect and proliferative activity of ICR mice bone marrow cells after ip application of the compounds **2** and **5**

Compounds and doses	Time after treatment (h)	Number of metaphases scored	Type of chromosome aberrations					Percentage of cells with aberrations (X ± m)	Statistical significance				Mitotic index (‰) (X ± m)	Statistical significance			
			Breaks	Fragments	Rearrangements				a	b	c	d		a	b	c	d
					c/c	t/t	c/t										
(**2**) 10 mg/kg	24	400	1	3	15	0	0	4.75 ± 0.84	***	***			11.26 ± 1.04	***	***		
	48	400	3	1	12	0	0	4.43 ± 0.70	***	***			10.18 ± 0.67	**	***	***	
(**2**) 100 mg/kg	24	400	4	4	13	1	0	5.50 ± 0.5	***	***			12.43 ± 1.31	***	***		
	48	400	1	3	17	1	0	5.5 ± 0.5	***	***			15.17 ± 0.89	***			
(**5**) 10 mg/kg	24	400	4	4	17	0	0	6.25 ± 0.80	***	***			15.09 ± 1.19	***	**		*
	48	400	7	2	12	0	0	4.75 ± 0.37	***	***			10.07 ± 0.99	**	***		
Mit. C 3.5 mg/kg	24	200	17	30	7	1	0	30.5 ± 2.36		***			5.49 ± 0.19		***		
	48	400	17	24	20	0	0	15.8 ± 0.81		***			7.29 ± 0.34		***		
Control 0.9%	24	700	4	0	4	0	0	1.14 ± 0.34	***				20.06 ± 1.38	***			
NaCl	48	500	0	0	3	0	0	0.6 ± 0.3	***				16.88 ± 0.56	***			

c/c, Centromeric/centromeric fusion; t/t, telomere/telomeric fusion; c/t, centromere/telomeric fusion.
a, Compared to Mitomycin C; b, compared to control; c, compared to dose 100 mg/kg; d, 5 compared to 2.
Statistics: Student *t*-test.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

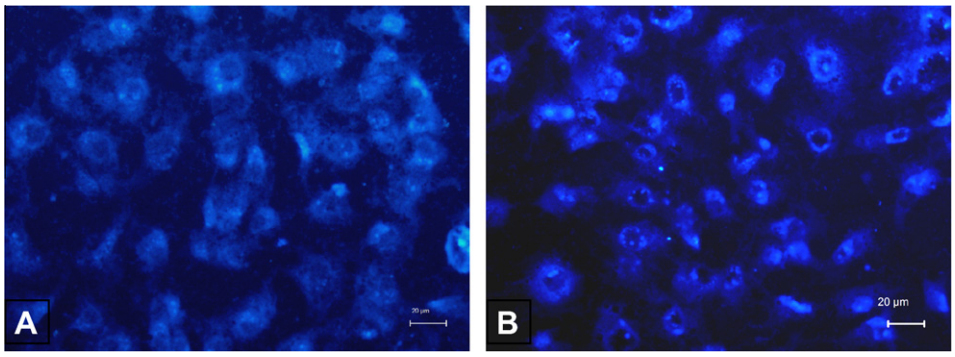


Figure 2. Fluorescence microscopy of cytoplasmic distribution of Schiff base **1** (A) and nuclear accumulation of aminophosphonate **4** (B) in HBL-100 cells.

2.5. Fluorescent studies

The fluorescent properties of the anthracene-containing compounds **1** and **4** were used to study their subcellular distribution in HBL-100 model cell culture system after 24 h exposure to non-toxic concentrations of the substances. The fluorescent signal of Schiff base **1** was observed mainly in the cytoplasm of tumor cells. In contrast, the most intensive fluorescence after application of aminophosphonate **4** was found in the nuclei and nuclear membranes of HBL-100 cells (Fig. 2).

3. Conclusion

A new anthracene-derived Schiff base 9-anthrylidene-furfurylamine **2** and three novel α-aminophosphonic acid diesters, bearing anthracene moiety—[*N*-methyl(dimethoxyphosphonyl)-1-(9-anthryl)]-*p*-toluidine **3**, [*N*-methyl(diethoxyphosphonyl)-1-(9-anthryl)]-*p*-toluidine **4** and [*N*-methyl(diethoxyphosphonyl)-1-(9-anthryl)]furfurylamine **5**, were synthesized and characterized. The aminophosphonates and their precursors were evaluated for in vitro antitumor activity on a panel of seven human epithelial cancer cell lines. Two of the compounds (**2** and **5**), combining in their molecules an anthracene residue and a furan ring, showed optimal antiproliferative activity to human tumor cells from colon carcinoma and from malignant tumors of the breast and urinary bladder. Moreover, the aminophosphonate **5** exhibited higher activity against all tested cancer cell lines, than its synthetic precursor—Schiff base **2**. The Schiff base **1**, bearing only an anthracene ring and its aminophosphonate derivative **3** were found to be cytotoxic to colon carcinoma cells (HT-29 line) and HBL-100 cell line, respectively. The other aminophosphonate **4**, obtained from the same Schiff base (**1**) and diethyl phosphite, was less active against all tested cancer cell lines, as compared to the referent anticancer drug Doxorubicin. The results obtained underline the importance of the simultaneous presence of three pharmacophoric fragments in the same molecule—an anthracene moiety, a furan ring and an aminophosphonate group, which is an important prerequisite for higher antitumor activity.

In vitro and in vivo safety testing revealed that the compounds exert lower toxicity to normal cells, as compared to well known anticancer and cytotoxic agents. Therefore, the novel substances are promising for future work on the development of agents active in chemotherapy of malignant breast and colon disease. Moreover, 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.

4. Experimental

Dimethyl phosphite (Sigma Aldrich Chemie GmbH, Steinheim, Germany) and diethyl phosphite (Fluka Chemie Ag, Buchs, Switzerland) were purified by vacuum distillation. 9-Anthracenecarboxaldehyde, *p*-toluidine and furfurylamine were purchased from Fluka. All solvents were freshly distilled prior to use. The melting points

of the compounds were determined on a Kofler microscope and are uncorrected. IR spectra were taken on a IRAffinity-1 spectrophotometer. ^{1}H, ^{13}C{^{1}H} and 2D NMR spectra in $CDCl_3$ (compounds **1**–**4**) and ^{1}H NMR spectrum in CD_3OD (compound **2**) were recorded on a Bruker DRX-250 spectrometer at rt and tetramethylsilane (TMS) as an internal standard. ^{1}H, ^{13}C and 2D NMR spectra in $CDCl_3$ of compound **5** were recorded on an Avans 600 MHz spectrometer at rt and TMS as an internal standard. ^{31}P{^{1}H} NMR spectra in $CDCl_3$ were taken on a Bruker DRX-250 spectrometer using 85% H_3PO_4 as an external standard. The thin layer chromatography was performed on Merk Silica gel 60 F_{254} at room temperature. The samples were applied as methanolic solutions and the chromatograms were developed ascendingly in the eluting system diethyl ether:hexane = 4:1. The spots were detected under UV light. Column chromatography: For purification of single product molecules, the crude reaction mixture was separated by normal pressure liquid chromatography in a 2 cm × 35 cm column, containing 50 g silica gel 60 particle size 0.063–0.2 mm (70–230 mesh, Fluka), with mobile phase diethyl ether: 1,4-dioxane (4:1 (v/v). Fractions of 2 ml were collected and analyzed by thin layer chromatography (TLC). The desired products were separately pooled and the solvent evaporated under reduced pressure. Fluorescent spectra were recorded on a Jasko fluorimeter 6600.

4.1. Synthesis

4.1.1. Schiff bases (1 and 2)

4.1.1.1. 9-Anthrylidene-*p*-toluidine (1). It was prepared from 9-anthracenecarboxaldehyde and *p*-toluidine according to Prot,[27] using diethyl ether as solvent, instead of benzene, conducting the reaction at room temperature and recrystallized from ethyl alcohol. Yield: 82%; mp 114–115 °C (literature mp 106–107 °C); R_f = 0.90. IR (neat), ν (cm^{-1}): 1622 ($\nu_{C=N}$); 1610, 1587, 1520, 1440 ($\nu_{C=C}$). Fluorescent spectrum: Ex 306 nm—λ_{max} = 348 nm. ^{1}H NMR ($CDCl_3$), δ (ppm): 9.68 (s, 1H, CH=N), 8.75 (m, 2H, AnthH), 8.53 (s, 1H, AnthH-10), 8.03 (m, 2H, AnthH), 7.55 (m, 4H, AnthH), 7.36 (m, 4H, ArH); 2.47 (s, 3H, ArCH_3). ^{13}C{^{1}H} NMR ($CDCl_3$), δ (ppm): 158.94 (CH=N), 130.38 (AnthC-10), 129.87 (ArC), 128.95, 127.10, 125.31,124.77 (AnthC), 120.94 (ArC), 21.03 (ArCH_3).

4.1.1.2. 9-Anthrylidene-furfurylamine (2). 9-Anthracenecarboxaldehyde (2.20 g, 10.7 mmol) dissolved in diethyl ether (150 ml) and furfurylamine (1.04 g, 10.7 mmol) were mixed and stirred at an ambient temperature for 15 h. Then diethyl ether was removed in vacuum and the crude product was recrystallized from petroleum ether. Yield: 2.19 g (72%); mp 84–85 °C; R_f = 0.87. Anal. calcd for $C_{20}H_{15}NO$: C, 84.21; H, 5.26; N, 4.91. Found: C, 83.91; H, 5.16; N, 5.06. IR (neat) ν (cm^{-1}): 1627 ($\nu_{C=N}$); 1558, 1519, 1440 ($\nu_{C=C}$); 1016 (ν_{COC}). Fluorescent spectrum: Ex 365 nm—λ_{max} = 426 and 442 nm. ^{1}H NMR ($CDCl_3$), δ (ppm), J_{HH} (Hz): 9.48 (t, ^4J = 1.4, 1H, CH=N), 8.51 (m, 2H, AnthH), 8.48 (s, 1H, AnthH-10), 8.07 (m, 2H, AnthH), 7.51 (m, 5H, AnthH, FurH-5), 6.44 (2 pseudo-s, 2H, FurH-3,4), 5.11 (d, ^4J = 1.4, 2H, CH_2Fur). ^{1}H NMR (CD_3OD), δ (ppm), J_{HH} (Hz): 9.43 (t, ^4J = 1.2, 1H, CH=N), 8.52 (s, 1H, AnthH-10), 8.28 (m, 2H, AnthH), 8.00 (m, 2H, AnthH), 7.58 (dd, ^3J = 1.8, ^4J = 1.0, 1H, FurH-5), 7.49 (m, 4H, AnthH), 6.47 and 6.46 (2 pseudo-s, 2H, FurH-3,4); 5.07 (d, ^4J = 1.3, 2H, CH_2Fur). ^{13}C{^{1}H} NMR ($CDCl_3$), δ (ppm): 162.43 (CH=N), 152.18 (FurC-2), 142.32 (FurC-5), 129.47 (AnthC-10), 128.78, 126.70, 125.20, 124.72 (AnthC), 110.49 (FurC-4), 107.82 (FurC-3), 58.36 (CH_2Fur).

4.1.2. Aminophosphonates (3–5)

4.1.2.1. [*N*-Methyl(dimethoxyphosphonyl)-1-(9-anthryl)]-*p*-toluidine (3). 9-Anthrylidene-*p*-toluidine (**1**) (1.51 g, 5.1 mmol) was dissolved in diethyl ether (40 ml) and dimethyl phosphite (0.56 g, 5.1 mmol) was added to the solution. The reaction mixture was stirred at ambient temperature for 13 h. The yellow precipitate obtained was recrystallized from methanol. Yield: 1.53 g (74%); mp 179 - 180 °C; R_f = 0.51. Anal. calcd for $C_{24}H_{24}NO_3P$: C, 71.11; H, 5.93; N, 3.46; P, 7.65. Found: C, 70.88; H, 5.67; N, 3.39; P, 7.51. IR (neat), ν (cm^{-1}): 3327 (ν_{NH}); 1232 ($\nu_{P=O}$); 1161, 1020 (ν_{POMe}). Fluorescent spectrum: Ex 295 nm—λ_{max} = 326 and 345 nm; Ex 325 nm—λ_{max} = 403 and 421 nm. ^{1}H NMR ($CDCl_3$), δ (ppm), J_{HH} (Hz), J_{PH} (Hz): 9.06 (d, ^3J = 9.0, 1H, AnthH-α), 8.47 (d, ^3J = 8.4, 1H, AnthH-α), 8.46 (d, ^6J = 2.8, 1H, AnthH-10), 8.08 (d, ^3J = 8.4, 1H, AnthH-α), 8.00 (d, ^3J = 8.9, 1H, AnthH-α), 7.57 (m, 4H, AnthH-β), 6.79 (m, 2H, ArH-3′,5′), 6.46 (m, 2H, ArH-2′,6′), 6.37 (d, ^2J = 27.0, 1H, CHP), 4.68 (br s, 1H, NH), 3.90 and 3.21 (2d, ^3J = 10.8 and 10.6, 6H, OCH_3), 2.11(s, 3H, ArCH_3). ^{13}C{^{1}H} NMR ($CDCl_3$), δ (ppm), J_{PC} (Hz): 144.58 (d, ^3J = 13.2, ArC-1′), 129.88 (AnthC-α), 129.62 (ArC-3′,5′), 129.24 (AnthC-α), 129.06 (d, ^5J = 4.5, AnthC-10), 127.51 (ArC-4′), 127.03 (AnthC-β), 126.30 (AnthC-α), 126.09 (AnthC-β), 125.14 (AnthC-β), 124.72 (AnthC-β), 122.53 (AnthC-α), 113.61 (ArC-2′,6′), 53.68 and 53.54 (2d, ^2J = 6.9 and 6.9, OCH_3), 52.91 (d, ^1J = 154.1, CHP), 20.23 (ArCH_3). ^{31}P{^{1}H} NMR ($CDCl_3$), δ (ppm): 27.69.

4.1.2.2. [*N*-Methyl(diethoxyphosphonyl)-1-(9-anthryl)]-*p*-toluidine (4). 9-Anthrylidene-*p*-toluidine (**1**) (2.16 g, 7.3 mmol) and diethyl phosphite (1.01 g, 7.3 mmol) were dissolved in benzene (15 ml) and placed in a flask equipped with magnetic stirrer and a reflux condenser. The reaction mixture was refluxed for 14 h with stirring. After removal of the benzene in vacuum, the crude product was recrystallized from ethyl alcohol. Yield: 2.16 g (68%) ; mp 133–134 °C; R_f = 0.36. Anal. calcd for $C_{26}H_{28}NO_3P$: C, 72.06; H, 6.47; N, 3.23; P, 7.16. Found: C, 71.79; H, 6.72; N, 3.21; P, 6.88. IR (neat), ν (cm^{-1}): 3315 (ν_{NH}); 1238 ($\nu_{P=O}$); 1156, 1026 (ν_{POEt}). Fluorescent spectrum: Ex 290 nm—λ_{max} = 318, 348, 362, and 403 nm. ^{1}H NMR ($CDCl_3$), δ (ppm), J_{HH} (Hz), J_{PH} (Hz): 9.09 (d, ^3J = 9.0, 1H, AnthH-α), 8.50 (d, ^3J = 9.0, 1H, AnthH-α), 8.45 (d, ^6J = 2.9, 1H, AnthH-10), 8.07 (d, ^3J = 8.4, 1H, AnthH-α), 7.98 (d, ^3J = 8.5, 1H, AnthH-α), 7.56 (m, 4H, AnthH-β), 6.79 (m, 2H, ArH-3′,5′), 6.45 (m, 2H, ArH-2′,6′), 6.35 (d, ^2J = 27.1, 1H, CHP), 4.53 (br s, 1H, NH), 4.27, 3.76 and 3.32 (3 m, 4H, OCH_2), 2.11 (s, 3H, ArCH_3), 1.38 and 0.69 (2td, ^3J = 7.1 and 7.1, ^4J = 0.3 and 0.6, 6H, CH_3). ^{13}C{^{1}H} NMR ($CDCl_3$), δ (ppm), J_{PC} (Hz): 144.76 (d, ^3J = 13.1, ArC-1′), 129.74 (AnthC-α), 129.51 (ArC-3′,5′), 129.09 (AnthC-α), 128.84 (d, ^5J = 4.5, AnthC-10), 127.25 (ArC-4′), 126.75 (AnthC-β), 126.47 (d, J = 1.9, AnthC-α), 125.83 (AnthC-β), 125.03 (AnthC-β), 124.62 (AnthC-β), 122.74 (AnthC-α), 113.50 (ArC-2′,6′), 63.21 and 62.96 (2d, ^2J = 7.1 and 7.0, OCH_2), 53.23 (d, ^1J = 153.2, CHP), 20.16 (ArCH_3), 16.48 and 15.71 (2d, ^3J = 5.8 and 5.8, CH_3). ^{31}P{^{1}H} NMR ($CDCl_3$), δ (ppm): 25.25.

4.1.2.3. [*N*-Methyl(diethoxyphosphonyl)-1-(9-anthryl)]furfurylamine (5). Diethyl phosphite (1.67 g, 12.1 mmol) dissolved in dry benzene (15 ml) and CdI_2 (0.09 g, 0.25 mmol) were placed in a flask, equipped with magnetic stirrer, a thermometer, an inlet for inert gas and reflux condenser. After stirring for a half an hour at rt for dissolving the catalyst to the reaction mixture was added 9-anthrylidene-furfurylamine (**2**) (3.45 g, 12.1 mmol), dissolved in dry benzene (10 ml). The mixture was refluxed for 6 h with stirring. Then benzene was removed in vacuum and the residue was purified on Silica gel 60 (230–400 mesh) using diethyl ether–hexane = 4:1 and adding 10–50% methanol step by step to give a pure product. Yield: 3.38 g (66%); oil; R_f = 0.27. Anal. calcd for $C_{24}H_{26}NO_4P$: C, 68.09; H, 6.15; N, 3.31; P, 7.33. Found: C, 67.88; H, 6.01; N, 3.22; P, 7.07. IR (neat), ν (cm^{-1}): 3302 (ν_{NH}); 1230 ($\nu_{P=O}$); 1163, 1008 ($\nu_{POEt,COC}$). Fluorescent spectrum: Ex 242 nm—λ_{max} = 396, 418, and 441 nm. ^{1}H NMR ($CDCl_3$), δ (ppm), J_{HH} (Hz), J_{PH} (Hz): 9.36 (d, ^3J = 9.0, 1H, AnthH-α), 8.44 (d, ^6J = 2.6, 1H, AnthH-10), 8.12 (d, ^3J = 8.9, 1H, AnthH-α), 8.00 (d, ^3J = 7.8, 1H, Ant-

hH-α), 7.99 (d, ^3J = 8.3, 1H, AnthH-α), 7.49 (m, 4H, AnthH-β), 7.24 (dd, ^3J = 1.9, ^4J = 0.8, 1H, FurH-5), 6.20 (dd, ^3J = 3.2 and 1.9, 1H, FurH-4), 5.89 (dd, ^3J = 3.2, ^4J = 0.7, 1H, FurH-3), 5.73 (d, ^2J = 23.9, 1H, CHP), 4.07 (m, 2H, OCH_2), 3.77 (m, 2H, OCH_2, CH_2Fur), 3.50 (m, 2H, OCH_2, CH_2Fur), 3.21 (br s, 1H, NH), 1.26 and 0.80 (2td, ^3J = 7.1 and 7.1, ^4J = 0.4 and 0.5, 6H, CH_3). $^{13}C\{^1H\}$ NMR ($CDCl_3$), δ (ppm), J_{PC} (Hz): 152.82 (FurC-2), 141.91 (FurC-5), 129.30 (AnthC-α), 128.83 (d, ^5J = 4.5, AnthC-10), 128.79 (AnthC-α), 127.48 (AnthC-α), 126.09 (AnthC-β), 125.40 (AnthC-β), 125.20 (AnthC-β), 124.56 (AnthC-β), 123.59 (AnthC-α), 109.95 (FurC-4), 107.96 (FurC-3), 62.69 and 62.64 (2d, ^2J = 7.4 and 7.6, OCH_2), 54.80 (d, ^1J = 158.4, CHP), 44.27 (d, ^3J = 15.9, $FurCH_2$), 16.38 and 15.91 (2d, ^3J = 6.1 and 5.7, CH_3). $^{31}P\{^1H\}$ NMR ($CDCl_3$), δ (ppm): 25.80.

4.2. Biological assays

4.2.1. 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.[30] 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 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 either RPMI 1640 medium (ELTA 90, Ltd) (MCF-7 and HBL-100 lines) or high-glucose (4.5‰) Dulbecco's modified minimal essential medium (DMEM) (ELTA 90, Ltd), supplemented in both cases with 10% fetal calf serum (Sigma) and antibiotics in usual concentrations. Cultures were maintained at 37.5 °C in a humidified atmosphere and 5% CO_2. Cells were plated at a density of 1×10^4 cells in 100 ml 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 h. 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.

4.2.2. Cytotoxicity testing (3T3 NRU test)

The cytotoxicity testing was performed as described by Borenfreund and Puerner[31] and the latest modification[32] of the validated Balb/c 3T3 (clone 31) Neutral Red Uptake Assay (3T3 NRU test)[33] 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 10% 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 1×10^4 cells in 100 ml culture medium in each well of 96-well flat-bottomed microplates and allowed to adhere for 24 h before treatment with test compounds, dissolved in DMSO (ELTA 90, Ltd) and further diluted in PBS. A wide concentration rage was applied (from 2 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 fixative (Ethanol/Acetic acid) the absorption was measured on a TECAN microplate reader at 540 nm.

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.

4.2.3. Cytogenetical method

The cytogenetical investigation was conducted as described by Preston et al.[34] Male and female ICR mice, weighing 20 ± 1.5 g were kept at standard conditions −20 °C, 12 h light/dark cycle; food and water were available ad libitum. All the compounds investigated were administered ip at doses of 10 and 100 mg/kg. Mitomycin C (Kyowa) 3.5 mg/kg was used as a positive control substance. Group of animals injected with 0.9% saline were used as a negative control.

Bone marrow chromosome aberration assay was performed on seven groups of animals. The experimental groups consisted of four males and four females treated with the studied compounds and control groups consisted of 10 animals each. The experimental and control groups of animals were injected ip with colchicine at a dose of 0.4 mg/kg, 24 and 48 h after the administration of the applied chemicals and 1 h prior isolation of the bone marrow cells. All mice were euthanized by deep anaesthesia with diethyl ether. Bone marrow cells were flushed from the femur and hypotonized in 0.075 M KCl at 37 °C for 20 min. Thereafter the cells were fixed in methanol–acetic acid (3:1), dropped on cold slides, air dried and stained with 5% Giemsa solution (Sigma Diagnostic). At least 50 well-spread metaphases were analyzed per experimental animal at random. Mitotic indices were determined 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 for each group was calculated. Student's *t*-test was applied for statistical analysis. Statistical significance was expressed as $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$; $p > 0.05$—not significant).

4.2.4. Fluorescence studies

HBL-100 cells (1×10^5/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 Schiff base **1** and the aminophosphonate **4** for 24 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.

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