In vitro Antitumour Activity, Genotoxicity, and Antiproliferative Effects of Aminophosphonic Acid Diesters and their Synthetic Precursors

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The Schiff bases *N*-furfurylidene-*p*-toluidine and *N*-(4-dimethylaminobenzilidene)-*p*-toluidine, and the recently synthesized aminophosphonic acid diesters *p*-[*N*-methyl-(diethoxyphosphonyl)-(2-furyl)]toluidine and *p*-[*N*-methyl(diethoxyphosphonyl)-(4-dimethylaminophenyl)]toluidine were tested for *in vitro* antitumour activity on six human epithelial cancer cell lines. The genotoxicity and antiproliferative activity of these compounds were tested in mice. The aminophosphonates showed high *in vitro* antitumour activity towards the breast cancer-derived cell lines (MCF-7 and MDA-MB-231), the cervical carcinoma cell line (HeLa), and the human colon adenocarcinoma cell line (HT-29). In addition, the Schiff base *N*-furfurylidene-*p*-toluidine significantly inhibited the growth of bladder carcinoma cells (647-V) and the hepatocellular carcinoma line HepG2, and U-shaped dose-response curves were observed after treatment of 647-V and MCF-7 cells. All studied compounds had a moderate genotoxic and antiproliferative activity *in vivo*.

Key words: Aminophosphonates, Cancer Cell Lines, Genotoxicity

Introduction

The pharmacological importance of α-aminophosphonic acid derivatives is commonly known (Cherkasov and Galkin, 1998). Much attention and research efforts have concentrated on the study of the synthesis and the biological activities of these compounds, because they offer considerable advantages in the development of potential drugs against several metabolic disorders (Orsini *et al.*, 2010). Their negligible mammalian toxicity, the resistance to hydrolysis by proteolytic enzymes, and the structural similarity to the natural amino acids make them extremely attractive for use as antimetabolites (Kafarski and Lejczak, 2000). Thus, due to the tetrahedral configuration at the phosphorus atom, aminophosphonates serve as stable analogues of the unstable tetrahedral carbon intermediates formed in enzymatic processes and therefore act as enzyme inhibitors (Rassukana *et al.*, 2009). Numerous aminophosphonate derivatives are being used as haptens for catalytic antibodies, metabolic regulators, antibiotics, antihypertensive, antiviral and antibacterial agents (Orsini *et al.*, 2010; Palacios *et al.*, 2003). Moreover, some of them inhibit bone resorption, delay the progression of bone metastases, exert direct cytostatic effects on a variety of human tumour cell lines, and have found clinical application in the treatment of bone disorders and cancer (Green, 2000).

Among the numerous synthetic approaches to the aminophosphonates, the addition of dialkyl phosphites to Schiff bases is the most convenient procedure, which gives much opportunity for combining various pharmacophoric groups in aminophosphonate molecules (Kraicheva *et al.*, 2004). Their synthetic precursors – the Schiff bases – are also of interest as antimicrobial, tuberculostatic, anticonvulsant, and antitumour agents (Lipkin *et al.*, 1970; Trefilova and Postovskii, 1957; Verma *et al.*, 2004; Phatak *et al.*, 2000).

The synthetic pathway, and the structural and spectral characterization of the aminophosphonates *p*-[*N*-methyl(diethoxyphosphonyl)-(2-furyl)]toluidine (**M-5**) and *p*-[*N*-methyl(diethoxyphosphonyl)-(4-dimethylaminophenyl)]toluidine (**M-6**) have been reported (Kraicheva *et al.*, 2009). The cytotoxicity against a panel of leukaemic cell lines (HL-60 and its multidrug-resistant sublines HL-60/Dox, LAMA-84, and K-562) of these aminophosphonates and of their precursors, Schiff bases *N*-furfurylidene-*p*-toluidine (**M-2**) and *N*-(4-dimethylaminobenzilidene)-*p*-toluidine (**M-3**) have been also described (Kraicheva *et al.*, 2009).

The aim of this study was to investigate the *in vitro* antitumour activity of the above mentioned compounds (**M-2**, **M-3**, **M-5**, and **M-6**) towards a panel of epithelial human cancer cell lines, as well as to evaluate their safety *in vivo*.

Material and Methods

Chemicals

The Schiff bases *N*-furfurylidene-*p*-toluidine (**M-2**) and *N*-(4-dimethylaminobenzilidene)-*p*-toluidine (**M-3**) were prepared by condensation of *p*-toluidine with furfural and 4-(dimethylamino) benzaldehyde, respectively, following previously described procedures (Head and Jones, 1966; Császár, 1987). The aminophosphonates *p*-[*N*-methyl(diethoxyphosphonyl)-(2-furyl)]toluidine (**M-5**) and *p*-[*N*-methyl(diethoxyphosphonyl)-(4-dimethylaminophenyl)]toluidine (**M-6**) were synthesized through addition reaction of diethyl phosphite to the azomethine bond of the Schiff bases **M-2** and **M-3**, respectively (Kraicheva *et al.*, 2009). Diethyl phosphite, furfural, and 4-(dimethylamino)benzaldehyde were purchased from Fluka Chemie AG (Buchs, Switzerland). All solvents were freshly distilled prior to use.

In vitro investigations

Cell lines from ductal carcinoma of the breast (MCF-7 and MDA-MB-231 – with low and high metastatic potential, respectively), bladder carcinoma (647-V), hepatocellular carcinoma (HepG2), colon carcinoma (HT-29), and cervical carcinoma (HeLa) were used in all experiments. The cell lines were routinely grown as monolayers in 75-cm^2 tissue culture flasks (Corning Inc., Corning, NY, USA) in high-glucose (4.5‰) Dulbecco's modified minimal essential medium (DMEM), supplemented with 10% fetal calf serum (Sigma-Aldrich, Inc., St. Louis, MO, USA) and the antibiotics penicillin (100 UI/ml) and streptomycin (100 μg/ml). Cultures were maintained at 37.5 °C in a humidified atmosphere containing 5% CO_2.

The antitumour activity was tested with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay (Mosmann, 1983). Briefly, trypsinized tumour cells were adjusted to a density of $1 \cdot 10^5$ cells/ml culture medium and plated (100 μl/well) in 96-well flat-bottomed microplates (Orange Scientific, Braine-l'Alleud, Belgium). The cells were allowed to adhere for 24 h before treatment with the test compounds dissolved in dimethylsulfoxide (DMSO), further diluted in phosphate-buffered saline (PBS) to reach the desired test concentrations. A concentration range from 1 to 0.0681 mg/ml (six wells per concentration) was applied for 24 h. The DMSO content never exceeded 1% (v/v). The MTT solution (5 mg/ml in PBS) was added (100 μl/well), and plates were incubated for 3 h at 37.5 °C in a humidified atmosphere containing 5% CO_2. The MTT-formazan crystals were dissolved by adding 100 μl/well of an absolute ethanol/DMSO (1:1 v/v) solution, and the absorption was registered using a microplate reader (TECAN, Sunrise TM, Groedig/Salzburg, Austria) at 580 nm. There was good reproducibility between replicate wells with standard errors below ± 10%. All experiments were performed in triplicate. 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.

In vivo investigations

The cytogenetical investigation was conducted according to the procedure of Preston *et al.* (1987). Male and female ICR mice, weighing (20 ± 1.5) g were kept at 20 °C and a 12 h/12 h light/dark cycle. Food and water were available *ad libitum*. All compounds were administered intraperitoneally (i.p.) at doses of 10 mg/kg body weight (BW) and 100 mg/kg BW. Mitomycin C (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan) at 3.5 mg/kg BW 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 C-treated) control groups consisted of 4 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 BW colchicine (Fluka AG) at the 24th and 48th hour after the administration of the tested substances and 1 h prior to the isolation of bone marrow cells.

The slides for microscopic evaluation were prepared according to the previously described procedure (Topashka-Ancheva *et al.*, 2003).

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 (SEM) were calculated for each group.

For statistical analysis Student's t-test was applied. Statistical significance was expressed as $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$; $p > 0.05$ not significant.

Results and Discussion

Antitumour activity

In vitro antitumour activity of the four studied compounds, *i.e.* the aminophosphonates **M-5** and **M-6** and their precursors **M-2** and **M-3** (Fig. 1) was tested on a panel of six human tumour cell lines. The results show that the Schiff base **M-2** had *in vitro* antitumour activity in a concentration range from 1 mg/ml to 0.0681 mg/ml against cells derived from human hepatocellular carcinoma (line HepG2), highly metastatic ductal carcinoma of the breast (line MDA-MB-231), and the HeLa cell line (cervical carcinoma). A slightly weaker cytotoxic effect of **M-2** (0.1 mg/ml) was found for the cell line 647-V, derived from human bladder cancer, while the effect was noticeably weaker (up to 0.464 mg/ml) in cells from human colon carcinoma (line HT-29) and absent in cells from ductal breast carcinoma with low metastatic potential (line MCF-7). A U-shaped dose-response curve (hormesis) was observed for MCF-7 and 647-V tumour cells (Fig. 2A), which could be a consequence of a reduction of free **M-2** by complex formation with a component in the medium, the co-occurrence of low- and high-affinity receptors, down-regulation of a ligand-saturated receptor or up-regulation of a clearance mechanism (Tedjarati *et al.*, 2002). Such a bell-shaped dose-response curve of a drug can even have advantages in clinical applications (Doñate *et al.*, 2008).

The aminophosphonate **M-5**, derived from **M-2**, showed very high and statistically significant ($p < 0.001$ to a concentration of 0.1 mg/ml) *in vitro* antitumour activity towards the cell line MCF-7 (Fig. 2B). Amplification of the activity of the aminophosphonate, compared to the corresponding Schiff base, was also observed in the HT-29 cell line (Figs. 2A, B).

No marked differences in the *in vitro* antitumour activity of **M-2** and **M-5** were observed against the cell line MDA-MB-231. Surprisingly, lower activity of **M-5** was found in the tumour

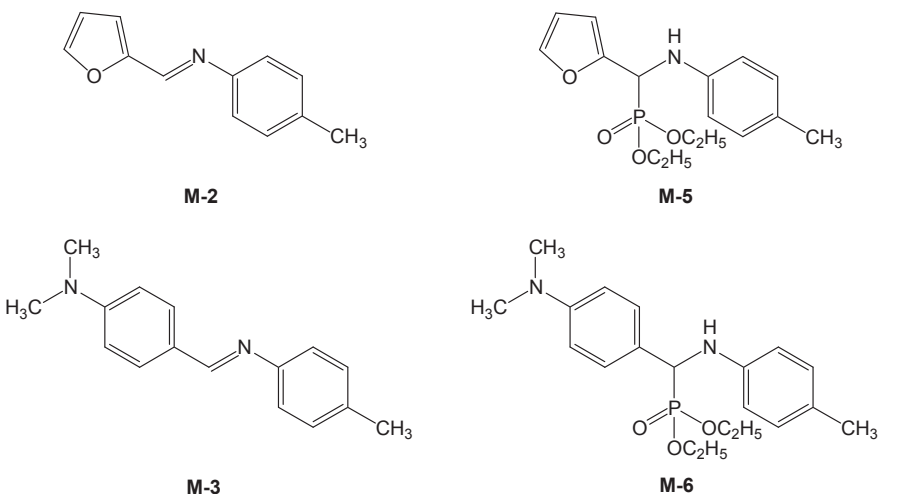

Fig. 1. Schiff bases (**M-2**, **M-3**) and corresponding aminophosphonates (**M-5**, **M-6**).

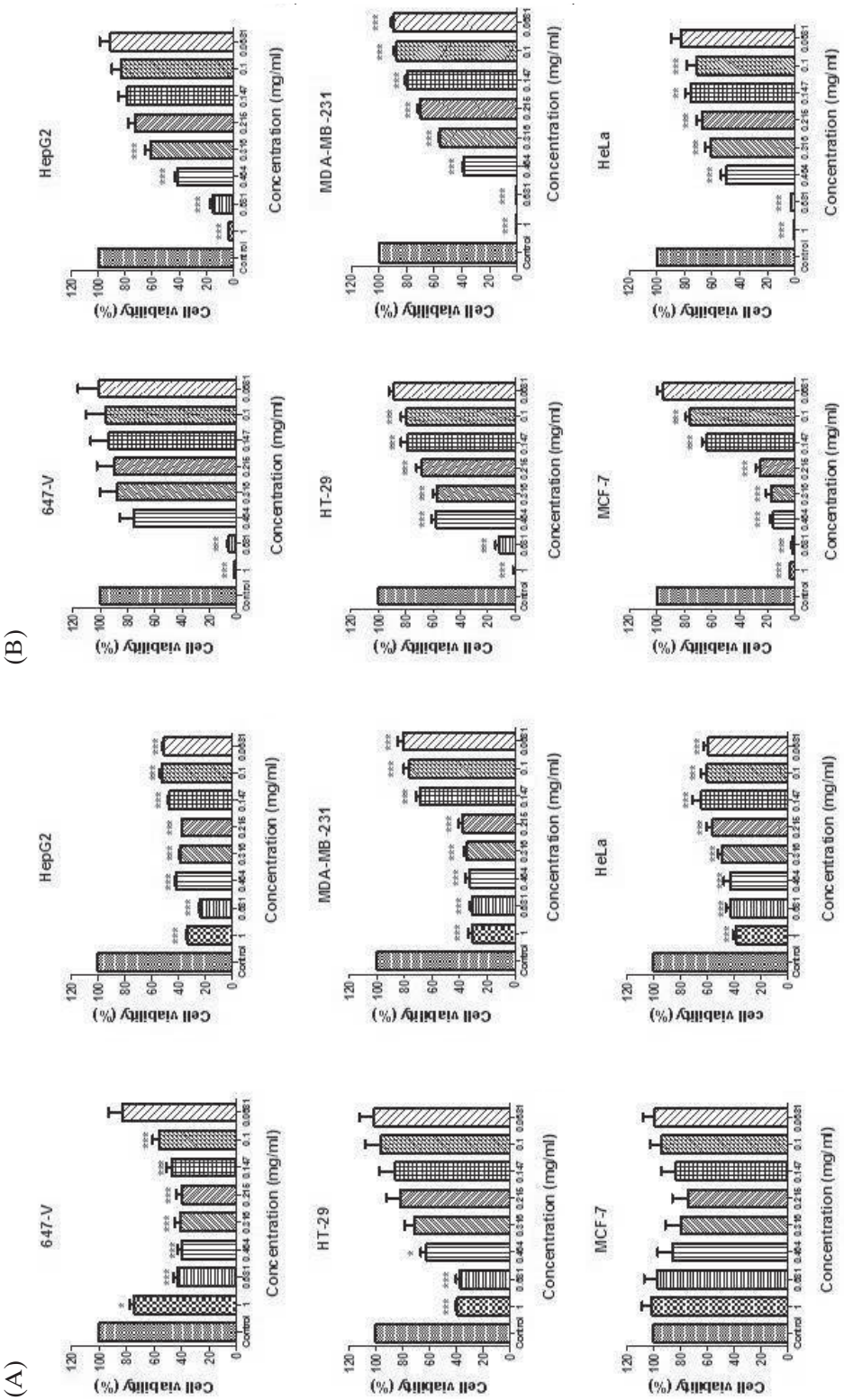

Fig. 2. *In vitro* antitumour activity (MTT test) of (A) the Schiff base **M-2** and (B) the aminophosphonate **M-5**. Control, untreated tumour cells (negative control).

cell lines 647-V and HepG2, and even to a lesser extent in line HeLa (Figs. 2A, B).

Of the second studied pair of compounds, the Schiff base **M-3** showed weak antitumour activity *in vitro*. Highest activity at a concentration of 0.316 mg/ml ($p < 0.05$) was found with cell line HepG2. An even weaker effect was found at a concentration of 0.681 mg/ml in the cell lines 647-V, MDA-MB-231 ($p < 0.01$), and MCF-7 ($p < 0.05$), and at 1 mg/ml in the cell line HeLa ($p < 0.01$), and no cytotoxicity was seen in the HT-29 line (Fig. 3A). The corresponding aminophosphonate **M-6** was also not toxic to this cell line (Fig. 3B), suggesting that these cells lack receptors for the two compounds (Surowiak *et al.*, 2006; Naydenova *et al.*, 2010). There was no difference in the actions of **M-3** and **M-6** on the HepG2 cell line. In contrast, **M-6** was found to be significantly more active in HeLa cells at a concentration of 0.1 mg/ml ($p < 0.01$), as well as in both ductal breast carcinoma cell lines ($p < 0.001$) in a wide concentration range from 1 mg/ml to 0.0681 mg/ml (Figs. 3A, B). It can be concluded that the aminophosphonates are more toxic than the corresponding Schiff bases in cell lines MCF-7 (**M-5**, **M-6**), HT-29 (**M-5**), MDA-MB-231 and HeLa (**M-6**).

Clastogenic and antiproliferative effects

The Schiff base **M-2** was weakly genotoxic 24 and 48 h after its application, with (4.25 ± 0.45)% and (3.71 ± 0.23)% of aberrant metaphases, respectively. The clastogenic effect of the corresponding aminophosphonate **M-5** did not significantly differ from that of the starting Schiff base at a dose of 10 mg/kg BW [(4.25 ± 0.45)% and (3.67 ± 0.55)%, respectively]. At a dose of 100 mg/kg BW, **M-5** increased the percentage of aberrant mitoses about twofold (Table I). The main type of structural chromosome aberrations in the metaphase plates were centromere/centromeric fusions (Robertsonian translocations), almost 70%. The cytogenetic effect of compounds **M-2** and **M-5** differed from that of the alkylating agent mitomycin C. The positive control substance provoked a strong clastogenic effect in (30.5 ± 2.36)% of all evaluated metaphase plates. Breaks and fragments were the predominating types of chromosomal aberration (85% of all studied metaphases with aberrant chromosomes). The Schiff base **M-2** at 10 mg/kg BW significantly ($p \leq 0.01$) suppressed proliferation of the bone marrow cells at both 24 and 48 h (mitotic indices of 9.03‰ and 9.13‰, respectively), compared to the untreated control group (17.3‰) (Table I). The aminophosphonate **M-5** at doses of 10 and 100 mg/kg BW inhibited proliferation more strongly than the Schiff base **M-2**. **M-5** was almost as active as the positive control mitomycin C.

The clastogenic effect of **M-3** was quite similar to that of **M-2**, *i.e.* (4.0 ± 0.38)% and (4.5 ± 0.91)% at a dose of 10 mg/kg BW after 24 and 48 h, respectively. The aminophosphonate **M-6** also had only a moderately harmful effect on the chromosomes. **M-6** also affected the centromeric region of the chromosomes causing Robertsonian translocations (78% of all types of aberrations scored). The mitotic indices of mouse bone marrow cells after **M-3** treatment appeared to be higher than those seen with **M-6**, but the differences did not reach statistical significance. The mitotic activity of the bone marrow cells of **M-6**-treated mice was lower ($p < 0.05$) than that of the untreated control animals. The two aminophosphonates (at 10 and 100 mg/kg BW) had a significantly lower antiproliferative potential ($p \leq 0.01$) than mitomycin C (Table II).

In summary, the clastogenic effects of the two Schiff bases **M-2** and **M-3** and their corresponding aminophosphonates **M-5** and **M-6** are significantly above the negative control level ($p < 0.001$), but nevertheless much lower than those of mitomycin C at a much lower concentration ($p < 0.001$). Similar results were previously obtained with another set of aminophosphonates (Naydenova *et al.*, 2007).

In conclusion, the aminophosphonates tested here are good candidates for evaluating their *in vivo* antitumour activity in animal models and for the development of drugs for treatment of breast and cervical cancer (**M-5** and **M-6**) and colon adenocarcinoma (**M-5**). In addition, the significant *in vitro* antitumour activity of the Schiff base **M-2** to bladder carcinoma and hepatocellular carcinoma cell lines and the apparently U-shaped dose-response curves of 647-V and MCF-7 cells exposed to **M-2** suggest that this compound is also promising for the development of drugs with antineoplastic activity.

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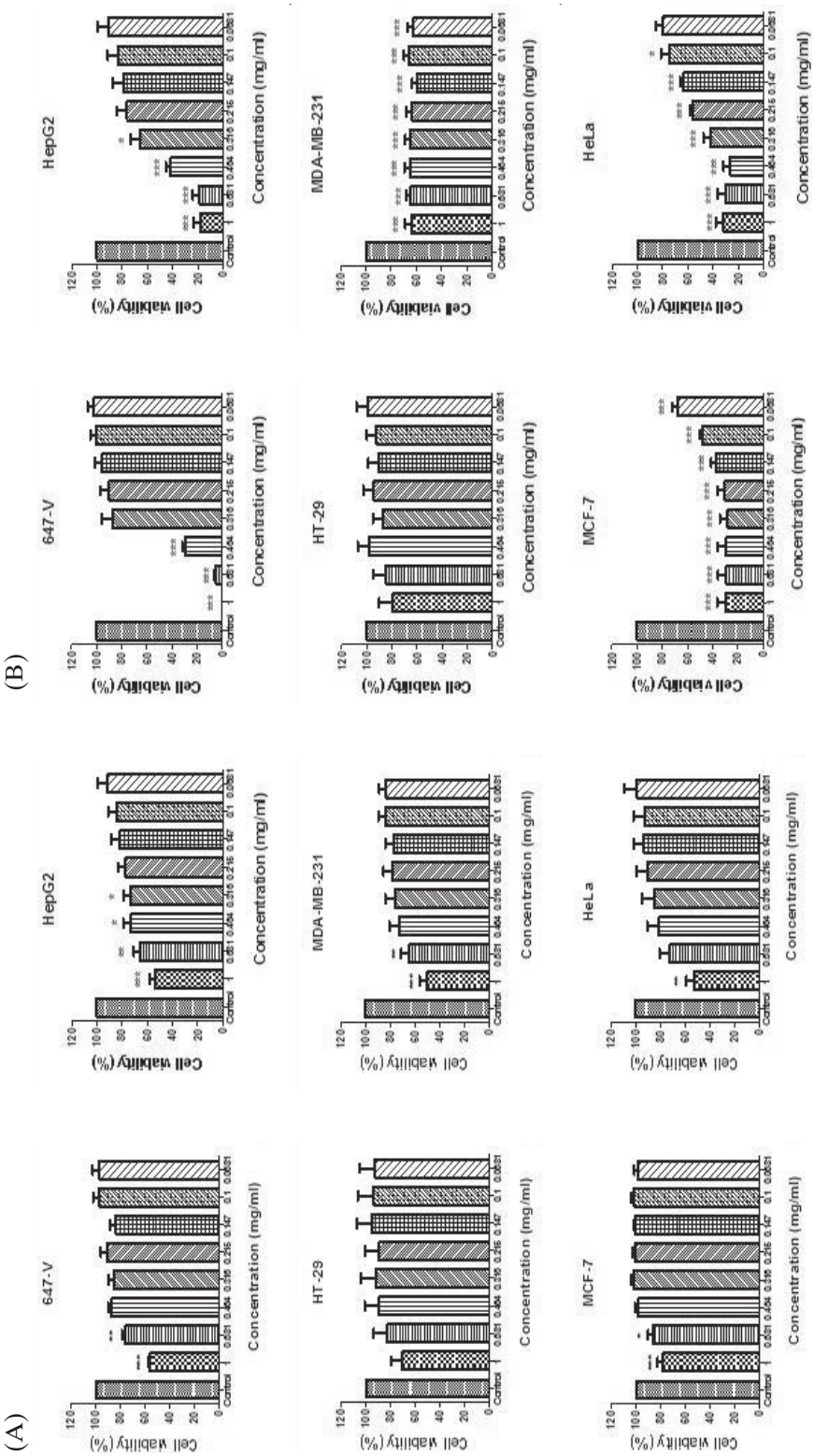

Fig. 3. *In vitro* antitumour activity (MTT test) of (A) the Schiff base **M-3** and (B) the aminophosphonate **M-6**. Control, untreated tumour cells (negative control).

Table I. Clastogenic effect and proliferative activity of ICR mice bone marrow cells after i.p. application of the compounds **M-2** and **M-5**.

Compound and dose	Time after treatment [h]	Number of metaphases scored	Type of chromosome aberrations					Percentage of cells with aberrations (± SEM)	Statistical significance[b]				Mitotic index (‰) (± SEM)	Statistical significance[b]			
			Breaks	Fragments	Rearrangements[a]												
					c/c	t/t	c/t		a	b	c	d		a	b	c	d
M-2	24	400	4	0	13	0	0	4.25 ± 0.45	***	***			9.03 ± 0.82	***	**		**
10 mg/kg	48	377	1	0	13	0	0	3.71 ± 0.23	***				9.13 ± 1.39		**		***
M-5	24	400	2	4	11	0	0	4.25 ± 0.45	***	***	***		5.59 ± 0.65		***		
10 mg/kg	48	291	3	2	4	1	0	3.67 ± 0.55	***		**		2.56 ± 0.40	***	***		
M-5	24	372	2	7	17	1	0	7.71 ± 0.46	***	***			5.76 ± 0.92		***		
100 mg/kg	48	340	5	7	11	1	0	7.02 ± 0.80	***				2.98 ± 0.64	***	***		
Mitomycin C	24	200	17	30	7	1	0	30.5 ± 2.36		***			5.49 ± 0.19		***		
3.5 mg/kg	48	400	17	24	20	0	0	15.8 ± 0.81					7.29 ± 0.34		***		
Control	24	500	3	0	2	0	0	1.0 ± 0.33	***				17.3 ± 2.49	***			
0.9% NaCl	48	500	0	0	3	0	0	0.6 ± 0.3	***				16.88 ± 0.56	***			

[a] c/c, centromere/centromeric fusion; t/t, telomere/telomeric fusion; c/t, centromere/telomeric fusion.
[b] Statistics: Student's t-test; $^{**}p < 0.01$; $^{***}p < 0.001$; a, compared to mitomycin C; b, compared to control; c, compared to the dose 100 mg/kg; d, **M-2** compared to **M-5** (10 mg/kg).

Table II. Clastogenic effect and proliferative activity of ICR mice bone marrow cells after i.p. application of the compounds **M-3** and **M-6**.

Compound and dose	Time after treatment [h]	Number of metaphases scored	Type of chromosome aberrations					Percentage of cells with aberrations (± SEM)	Statistical significance[b]				Mitotic index (‰) (± SEM)	Statistical significance[b]			
			Breaks	Fragments	Rearrangements[a]												
					c/c	t/t	c/t		a	b	c	d		a	b	c	d
M-3	24	400	5	0	11	0	0	4.0 ± 0.38	***	***	***		11.81 ± 1.58	***			
10 mg/kg	48	400	4	1	13	0	0	4.5 ± 0.91	***	***			10.27 ± 1.32				
M-3	24	400	6	2	18	0	0	6.5 ± 0.33	***	***			10.80 ± 1.84	**	*		
100 mg/kg	48	400	1	2	15	0	0	4.5 ± 0.33	***	***			9.70 ± 1.96				
M-6	24	350	0	3	15	0	0	5.14 ± 0.74	***	***			9.65 ± 1.18	***	**		
10 mg/kg	48	400	3	1	15	0	0	4.75 ± 0.84	***	***			9.54 ± 0.87	*			
M-6	24	396	6	4	16	0	0	6.82 ± 0.64	***	***			6.46 ± 1.20		***		
100 mg/kg	48	350	6	1	15	0	0	6.29 ± 1.10	***	***			9.21 ± 1.49				
Mitomycin C	24	200	17	30	7	1	0	30.5 ± 2.36		***			5.49 ± 0.19		***		
3.5 mg/kg	48	400	17	24	20	0	0	15.8 ± 0.81		***			7.29 ± 0.34				
Control	24	500	3	0	2	0	0	1.0 ± 0.33	***				17.3 ± 2.49	***			
0.9% NaCl	48	500	0	0	3	0	0	0.6 ± 0.3	***				16.88 ± 0.56	***			

[a] c/c, centromere/centromeric fusion; t/t, telomere/telomeric fusion; c/t, centromere/telomeric fusion.
[b] Statistics: Student's t-test; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; a, compared to mitomycin C; b, compared to control; c, compared to the dose 100 mg/kg; d, **M-3** compared to the respective doses of **M-6**.

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