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Original article

Novel *N*-(phosphonomethyl) glycine derivatives: Design, characterization and biological activity

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Abstract

A series of *C*α,α-disubstituted cyclic derivatives of *N*-(phosphonomethyl) glycine have been synthesized and characterized. They exhibited moderate clastogenicity, low antiproliferative activity on mice bone marrow cells and well expressed cytotoxicity against human tumor cell lines. The 8- and 12-membered cyclic analogs proved superior to the remaining compounds and were found to trigger apoptotic cell death in DOHH-2 cells. The latter compound caused 50% inhibition of the viability of hemobastose-derived cell lines at concentrations ranging from 20 to 67 μM.

Keywords: Aminophosphonic acids; *N*-(Phosphonomethyl) glycine derivatives; Chromosome aberrations; Cell proliferation; Clastogenic effects; Cytotoxic activity

1. Introduction

Aminophosphonic acids are considered to be an important class of amino acid mimetics. They have reached a position of eminence in the field of, or in the research works aimed at discovery, understanding, and modification of physiological processes in living organisms [1–3]. α-Aminophosphonic acids are found to compete effectively with their amino acid counterparts for binding to enzyme active centers or other cellular targets [4]. This, together with their low mammalian toxicity makes the α-aminophosphonic acids an important class of antimetabolites and a potential source of medicinal lead compounds [5]. The most important α-aminophosphonic acids are *N*-(phosphonomethyl) glycine and its derivatives, which have been found to exert prominent antineoplastic, antiviral and antibacterial effects [6–11]. Kabachnik and Medved [12], and Fields [13] have discovered the first method for the preparation of α-aminophosphonic acids. The impressive array of applications has recently stimulated considerable effort towards the synthesis of α-aminophosphonic acids and many methods are now available [14–20]. Novel α-aminophosphonic acids with moderate clastogenic effect were synthesized reacting 1,3-oxazolidin-2-one derivatives with formaldehyde and phosphorus trichloride [21]. One-pot reaction is used to prepare α-aminoalkylphosphonic acid in good yield via ethyl carbamate, aldehyde and dichlorophosphites [22]. Optically active aminophosphonic acids have found widespread application in medicinal chemistry and pharmaceutical science [23]. Highly effective solvent-free and catalyst-free microwave-assisted synthesis of α-aminophosphonates was shown [24,25]. The recent advance

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in synthesis, stereochemistry and biological activity of α-aminophosphonic acid and their esters is reported by Song and Jiang [26]. Cyclic or heterocyclic rings introduced into the molecular skeleton increase its rigidity and modify electronic effects. Thus in recent years, many cyclic α-aminophosphonic acids or aminophosphonates have been prepared [27]. Some cyclic *N*-(phoshonomethyl) glycine derivatives were prepared from cycloalkaneaminocarboxylic acids and their biological activity was studied [28–30].

Taking into account the fact that the aminocyclopentane-l-carboxylic acid, cycloleucin, exerts antineoplastic effects [31], we synthesized a series of new 1-(dimethoxyphosphonomethylamino) cycloalkanecarboxylic acids with 5-, 6-, 7-, 8- and 12-membered rings and studied their biological activity including the role of the ring size in the molecule.

As most of the antineoplastic agents interfere with cell division and are often genotoxic, the determination of their clastogenic and antiproliferative potential against normal cells is an inevitable component of the preliminary screening programs for identification of possible anticancer drugs. Thus we sought to evaluate the ability of the novel compounds to induce chromosomal aberrations and to inhibit the proliferation of murine bone marrow cells, following 24 or 48 h exposure. The cytotoxic activity of the compounds was evaluated in a panel of human tumor cell lines, whereby the clinically utilized drug cisplatin was used as positive control. The cytotoxicity determination was extended against the human embryonal kidney cell line 293T chosen as a model of normal, non-malignant cellular population.

2. Results and discussion

2.1. Chemistry

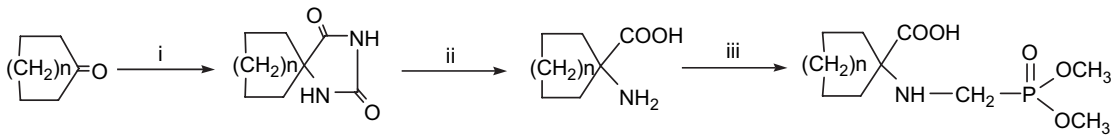
1-[(Dimethoxyphosphono)methylamino]cycloalkanecarboxylic acids with 5-, 6-, 7-, 8- and 12-membered rings **1c–5c** were prepared with good yields via Kabachnik–Fields reaction. The corresponding aminocycloalkanecarboxylic acid was treated with paraformaldehyde in the presence of triethylamine in methanol. When the reaction mixture became homogeneous, dimethyl hydrogen phosphonate was added. The *C*α,α-disubsituted amino acids Ac5c, Ac6c, Ac7c, Ac8c and Ac12c (**1b–5b**) were prepared following the procedure, which involves the formation of cycloalkanespiro-5-hydantoins (**la–5a**) from the corresponding cyclic ketones, followed by alkaline hydrolysis to the cycloaminoacid. The synthetic pathway followed to obtain the novel *N*-(phosphonomethyl) glycine derivatives **1c–5c** is reported in Scheme 1.

In the ^{1}H NMR spectra of all newly synthesized α-aminophosphonic acids doublets are present at: 2.51 ppm with $^{2}J_{P-H} = 14.7$ Hz for **1c**; 2.47 ppm with $^{2}J_{P-H} = 14.69$ Hz for **2c**; 2.50 ppm with $^{2}J_{P-H} = 15.1$ Hz for **3c**; 2.70 ppm with $^{2}J_{P-H} = 14.8$ Hz for **4c**; 3.16 ppm with $^{2}J_{P-H} = 13.22$ Hz for **5c** which can be assigned for the P–CH_2 protons (see Section 4). The doublets in the ^{13}C{^{1}H} NMR spectra at: 39.8 ppm with $^{1}J_{P-C} = 146.0$ Hz for **1c**; 42.0 ppm with $^{1}J_{P-C} = 144.2$ Hz for **2c**; 38.8 ppm with $^{1}J_{P-C} = 145.7$ Hz for **3c**; 39.3 ppm with $^{1}J_{P-C} = 144.7$ Hz for **4c**; 39.0 ppm with $^{1}J_{P-C} = 153.39$ Hz for **5c** can be assigned to the carbon atom connected to phosphorus (P–*C*H_2). The signals in the ^{31}P{^{1}H} NMR spectra are between 22.17 and 27.41 ppm which represent a triplet with $^{3}J_{P-H} = 12.3$ Hz, characteristic for the phosphorus atom of α-aminophosphonic acids. The spectral data including elemental analysis are reported in Section 4.

2.2. Biology

2.2.1. Clastogenic effect

Cytogenetical investigations were carried out on C57Bl murine bone marrow metaphase plates following i.p. treatment with the respective compounds in concentrations of 10 and 100 mg/kg body weight. Structural chromosome aberrations (breaks and fragments) and intrachromosome exchanges (centromere/centromeric and telomere/telomeric fusions) were reported. The results of this analysis are presented in Table 1. The data analysis showed that the highest percentage of metaphases with aberrations (10.0 ± 1.73) was scored in the cells of the experimental animals treated with 100 mg/kg **5c** at the 48th hour after administration. The slightest clastogenic effect was observed in **3**c. The differences between the experimental groups were within the statistical error limits ($p > 0.005$). All the other compounds showed moderate clastogenic effect. Dose depending effect was not detected. Differences were observed with respect to the types of aberrations. Compound **1c** provoked the highest amount of breaks and fragments (about half of the whole amount of the aberrations scored). Centromere/centromeric fusions were prevalent in the bone marrow cell slides of the other experimental groups' slides. These centromere/centromeric fusions are part of the interchromosome exchange groups known as Robetsonian



1 n = 0
2 n = 1
3 n = 2
4 n = 3
5 n = 7

comp. **a** comp. **b** comp. **c**

Scheme 1. General synthetic pathway. Reagents and conditions: (i) KCN, $(NH_4)_2CO_3$, NH_4OH, C_2H_5OH, H_2O, 6 h, then concentrated HCl; (ii) $Ba(OH)_2 \cdot 8H_2O$/H_2O, 3.5 h, then $(NH_4)_2CO_3$; (iii) HCHO, CH_3OH, $(C_2H_5)_3N$, 45 min, $(C_2H_5)_3N$, 3.5 h, then dimethyl hydrogen phosphonate, 3.5 h.

Table 1
Frequencies of chromosome aberrations in affected mouse bone marrow cells after i.p. treatment of α-aminophosphonic compounds

Sample	Interval (h)	Number of analyzed metaphases	Type of aberrations				Percentage of cells with aberrations ($\overline{X} \pm$ SE)	Mitotic index ($\overline{X} \pm$ SE) (%)
			br	fr	c/c	t/t		
1c								
10 mg/kg	24	350	7	1	9	0	4.85 ± 0.40	10.39 ± 1.13
	48	350	6	0	13	1	5.77 ± 0.63	10.66 ± 0.92
100 mg/kg	24	300	8	3	10	0	7.0 ± 0.92	10.97 ± 1.18
	48	250	6	0	8	2	6.40 ± 0.40	8.33 ± 0.72
2c								
10 mg/kg	24	300	4	0	11	0	5.33 ± 1.11	7.27 ± 0.98
	48	300	6	1	9	0	5.33 ± 0.42	6.71 ± 1.07
100 mg/kg	24	350	7	1	20	0	8.34 ± 0.61	7.15 ± 0.78
	48	350	14	0	12	0	7.42 ± 0.84	9.23 ± 1.2
3c								
10 mg/kg	24	300	3	0	7	0	2.85 ± 0.40	14.37 ± 1.14
	48	300	0	0	3	0	1.00 ± 0.68	9.58 ± 1.43
100 mg/kg	24	350	7	0	3	0	3.33 ± 1.12	13.24 ± 1.69
	48	273	4	0	3	0	2.72 ± 1.01	12.00 ± 0.41
4c								
10 mg/kg	24	350	2	1	10	0	3.71 ± 0.52	11.35 ± 0.85
	48	300	0	1	14	0	4.67 ± 0.67	13.95 ± 0.93
100 mg/kg	24	350	4	3	11	1	5.43 ± 0.37	13.06 ± 0.99
	48	300	3	0	12	0	5.00 ± 0.45	9.67 ± 1.09
5c								
10 mg/kg	24	300	4	2	11	0	5.57 ± 0.13	11.13 ± 0.58
	48	300	2	1	12	0	5.0 ± 0.85	8.6 ± 1.29
100 mg/kg	24	350	4	0	11	0	4.29 ± 0.73	11.82 ± 0.67
	48	250	6	6	11	2	10.0 ± 1.73	12.82 ± 0.48
Mitomycin C								
3.5 mg/kg	24	150	10	13	11	0	46.67 ± 3.03	5.49 ± 0.19
0.9% NaCl					2		1.0 ± 0.57	17.3 ± 2.49

br – breaks; fr – fragments; c/c – centromere/centromeric fusions; t/t – telomere/telomeric fusions.

translocations. These translocations change the groups of connecting, but do not drastically change the amount of the chromosome material in the affected cell. Comparing the results obtained under the influence of the newly synthesized aminophosphonic acids (Table 1) to the clastogenic effect of the positive control Mit. C, we find that the new compounds break the chromosomes' entity to a much smaller extent. After Mit. C administration 46% chromosome aberrations were encountered, whereby the prevalent types of aberrations were breaks, fragments, non-reciprocal translocations and pericentric inversions.

2.2.2. Antiproliferative effect

The proliferative activity of bone marrow cell populations was determined by evaluation of their mitotic index. The most prominent inhibition of the normal bone marrow cell proliferation was encountered with compound **2c**. It caused about 65% reduction in the number of mitoses compared to the untreated control. Compound **3c** possessed the weakest antiproliferative effect in vivo. Compounds **1c**, **4c** and **5c** showed similar antiproliferative effect – at a rate of about 36% suppression of cell division. Generally these data are in line with the results of our previous study of non-cyclic aminophosphonates. Moreover, compound 2-methyl-2-[(phosphonomethyl)amino]propanoic acid which could be considered as an analog of the herein described compounds, demonstrated similar low genotoxic effect, comparable to that of compound **3c**.

2.2.3. Cytotoxic effect

The cytotoxic activity of the tested aminophosphonates was evaluated by the MTT-dye reduction assay in a panel of six tumor cell lines of human origin. The IC_{50} values were determined as the concentration of tested agents producing 50% decrease of cell survival. Table 2 summarizes the cytotoxicity data derived following a 72 h continuous exposure.

As evident from the results obtained, the tested aminophosphonates demonstrated cytotoxic effects, whereby the leukemic cell lines HL-60, LAMA-84, K-562 and the non-Hodgkin lymphoma DOHH-2 were found to be more responsive than HD-MY-Z and EJ cells.

Throughout the spectrum of tumor models the 1-[(dimethoxyphosphono)methylamino]cyclododecanecarboxylic acid **5c** proved to be the most active cytotoxic agent amongst the newly synthesized aminophosphonates, causing 50% inhibition of cell viability at low micromolar concentrations. 1-[(Dimethoxyphosphono)methylamino]cyclooctanecarboxylic acid **4c** was characterized by somewhat lower activity, whereas

Table 2
Cytotoxic activity[a] of tested compounds in a panel of human tumor cell lines[b]

Tested compd.	IC_{50} (μM)[c]						
	HL-60	LAMA-84	K-562	DOHH-2	HD-MY-Z	EJ	293T
1c	157.4	162.2	>200	144.7	>200	>200	>200
2c	136.2	98.6	128.3	84.2	164.5	116.1	>200
3c	85.9	91.2	100.8	88.1	168.7	151.4	>200
4c	73.5	71.4	79.1	37.4	127.4	115.4	176.2
5c	41.4	67.3	47.1	20.1	67.3	110.7	159.2
Cisplatin	5.2	14.4	21.1	7.6	11.2	9.7	12.4

[a] Determined by MTT-dye reduction assay after 72 h exposure.
[b] Cell line origin: HL-60, acute promyelocyte leukemia; LAMA-84 and K-562, chronic myeloid leukemia; DOHH-2, non-Hodgkin lymphoma; HD-MY-Z, Hodgkin lymphoma; EJ, urinary bladder cancer; 293T, human embryonal kidney.
[c] Means from 8 separate measurements.

the further decrease in the molecular weight of 1-[(dimethoxyphosphono)methylamino]cycloalkanecarboxylic acid moiety was consistent with a progressive loss of activity. Conversely **2c** and **3c** were found to be practically equipotent with IC_{50} values being up to ca. 2–3 fold higher as compared to those of **5c**. Compound **1c** displayed the least pronounced cytotoxic effects, showing only marginal activity in K-562, HD-MY-Z and EJ cells.

The cytotoxicity determination was extended to the human embryonal kidney cell line 293T, chosen as representative for a normal, non-malignant cellular population. As evident from the IC_{50} values obtained the aminophosphonic acids were far less cytotoxic against 293T cells as compared to the tumor cell lines. Thus the most active compounds **4c** and **5c** caused 50% inhibition of 293T cells at substantially higher concentrations in comparison to their effects upon malignant cells, whereas the other aminophosphonates exerted only marginal cytotoxicity against the kidney cell line. In a dissimilar fashion the reference cytotoxic agent cisplatin exhibited significant cytotoxicity against 293T cells, comparable to its effects in the panel of human tumor cell lines.

2.2.4. Induction of apoptosis

In order to elucidate the mechanisms implicated in the cytotoxicity of tested aminophosphonates we evaluated the ability of the most active analogs **4c** and **5c** to induce apoptosis in DOHH-2 cells. To meet this objective we monitored the fragmentation of genomic DNA – a key hallmark feature of apoptosis, using a commercially available ELISA-kit. As evident from the data presented in Fig. 1 the 24 h treatment with both compounds led to significant increase of the levels of histone-associated oligonucleosomal DNA-fragments in DOHH-2 cell. At equipotent concentrations the more cytotoxic compound **5c** proved to be the superior inducer of apoptosis as well.

3. Conclusion

Novel 1-[(dimethoxyphosphono)methylamino]cycloalkanecarboxylic acids (**lc–5c**) representing *N*-(phosphonomethyl) glycine derivatives, were obtained via Kabachnik–Fields reaction. The results from this study unambiguously indicate that the newly synthesized aminophosphonates exert antineoplastic potential, combined with low clastogenicity. Current data show that the increase of lipophilicity and the steric bulk, consistent with the enlargement of the cycloalkane ring size in the molecule of the 1-[(dimethoxyphosphono)methylamino]cycloalkanecarboxylic acids appears to be a crucial prerequisite for optimal activity. The results obtained revealed that the increased number of methylene groups in the ring resulted in a marked augmentation of the cytotoxic activity. It should be pointed out that there was no correlation between the genotoxic effects of tested compounds against the bone marrow cells and their cytotoxic activity upon human tumor cell lines. Thus the most active cytotoxic agents **4c** and **5c** were found to be only moderately clastogenic. Moreover, these compounds as well as all the newly synthesized phosphonates exerted far less pronounced cytotoxicity against the 293T human embryonal kidney cells. These discrepancies between the cytotoxic/antiproliferative potential of **4c** and **5c** against tumor cells, on one hand, and embryonal kidney cells, on the other hand, could be largely attributed to their ability to trigger apoptotic cell death at low micromolar concentrations as evidenced by the established DNA-fragmentation in DOHH-2 cells. Moreover, aminophosphonic acids have been found to accumulate more intensively in malignant cells than in normal

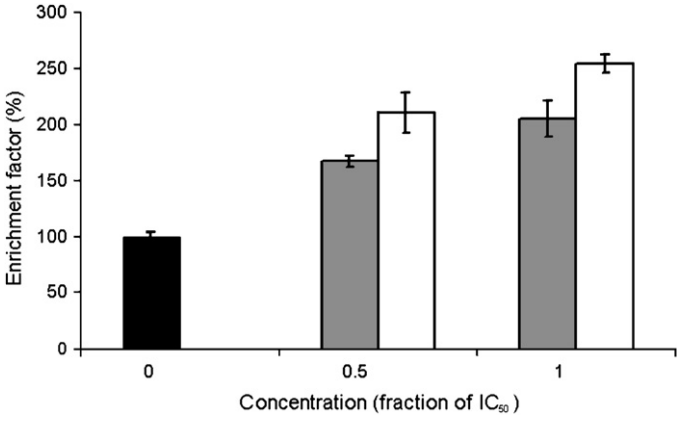


Fig. 1. Cytosolic enrichment of DOHH-2 with oligonucleosomal DNA-fragments, following 24 h exposure to equitoxic concentrations of **4c** (gray columns) or **5c** (white columns) versus the untreated control (black column). The results are arithmetic means (±SD) of three independent experiments.

cells, which could contribute to the established selective cytotoxicity [31].

The cytotoxicity of compounds **4c** and **5c** against tumor cells in low micromolar concentrations, added to their moderate genotoxic potential in vivo, gives us reason to consider both agents as potential lead compounds for further investigations.

4. Experimental

Cycloalkanone, dimethyl hydrogen phosphonate, paraformaldehyde, and solvents were purchased from Fluka and Merck, and used without further purification.

Melting points (mp) were determined on Koffler microscope and were uncorrected. The infrared (IR) spectra in KBr were recorded on a Perkin–Elmer Model 1600 Series FTIR instrument. The purity of the products was checked by TLC on pre-coated plates of Silica gel 60 F_{254} (Merck) using a mobile phase *n*-BuOH:AcOH:H_2O, 3:1:1. Spots on TLC chromatograms were detected by chlorine/*o*-tolidine reaction. Microanalyses were performed on Perkin–Elmer elemental analyzer. ^{1}H, ^{13}C and ^{31}P NMR spectra were determined by means of a Bruker DRX 250 spectrometer and referenced to the solvent. ^{13}C NMR spectra were fully decoupled. Chemical shifts are reported in δ values (ppm), and *J* values are reported in hertz (Hz).

Compounds **1a–5a** were prepared according to Bucherer–Lieb reaction [32,33].

4.1. Synthesis

4.1.1. General procedure for the preparation of 1-aminocycloalkanecarboxylic acids (*1b–4b*)

A suspension of 0.0227 mol cycloalkanespiro-5-hydantoins (**1a–4a**), 11.4 g (0.0665 mol) $Ba(OH)_2$ and 70 ml water was poured into a stainless steel autoclave equipped with a magnetic stirrer and a pressure gauge. The reaction mixture was heated at 170 °C for 3.5 h. The reaction solution was cooled to room temperature, diluted with distilled water and filtered to remove $BaCO_3$. $(NH_4)_2CO_3$ (2.6 g, 0.0270 mol) was added to the clear filtrate aiming at precipitating $BaCO_3$ and then filtered again. The clear filtrate was concentrated under reduced pressure and colorless crystal forms of the 1-aminocycloalkanecarboxylic acids (**1b–4b**) were obtained. The crude mixture was purified by crystallization from water.

4.1.2. 1-Aminocyclododecanecarboxylic acid (*5b*)

A suspension of 4.00 g 1,3-diazaspiro[4.11] hexadecane-2,4-dione (0.0158 mol) and 80 ml 1.25 N NaOH was poured into a stainless steel autoclave equipped with a magnetic stirrer and a pressure gauge. The reaction mixture was heated at 195 °C for 2.5 h. The reaction solution was cooled down to room temperature and diluted with distilled water and neutralized by concentrated HCl. The residue from 1-aminocyclododecanecarboxylic acid was filtered and washed with hot methanol.

Yield 90.27%, mp = 280 °C, R_f = 0.63; IR (KBr, cm^{-1}): 3467 (NH), 2933–2848 (C–H), 1654, 1473 (NH_3^+), 1526, 1405 (COO^-). ^{1}H NMR (250.13 MHz, D_2O), δ in ppm: 1.39–1.63 (m, 22H, CH_2). ^{13}C{^{1}H} NMR (62.90 MHz, D_2O), δ in ppm: 21.0 $^{3,11}CH_2$; 23.5 $^{7}CH_2$; 27.7 $^{4,5,6,8,9,10}CH_2$; 34.3 $^{2,12}CH_2$; 61.6 (–C–); 184.2 C=O.

4.1.3. General procedure for the preparation of 1-[(dimethoxyphosphono)methylamino]-cycloalkanecarboxylic acids (*1c–5c*)

Paraformaldehyde (0.40 g, 0.0129 mol), methanol (20.20 ml), and triethylamine (0.14 ml) were put into a three-necked flask equipped with a condenser, magnetic stirrer, thermometer and dropping funnel and inert argon. The reaction mixture was heated to reflux temperature and held for 45 min, after which it became a clear solution. Aminocycloalkane-l-carboxylic acid (0.0083 mol) and triethylamine (1.70 ml) were added to this solution. The suspension was heated at 65–70 °C and after 3.5 h it became a clear solution. Dimethyl hydrogen phosphonate 0.883 ml (1.059 g, 0.0094 mol) was added to this solution for approximately 10 min. This reaction mixture was heated at 65–70 °C and held there for 3.5 h, after which it was cooled to room temperature and concentrated under reduced pressure. Compounds (**1c–5c**) were obtained from the methanol solution, after removing the non-reacted aminocycloalkane-l-carboxylic acid having lower solubility in methanol than the products (**1c–5c**).

*4.1.3.1. 1-[(Dimethoxyphosphono)methylamino]cyclopentanecarboxylic acid (**1c**).* White solid, 68.0% yield, mp = 218–220 °C, R_f = 0.4; IR (KBr, cm^{-1}): 3399 (NH), 2966–2877 (C–H), 1674 (C=O), 1303, 1255 (P=O), 1177, 1042 (P–O–C). ^{1}H NMR (250.13 MHz, D_2O), δ in ppm: 1.44–1.90 (m, 8H, CH_2); 2.51 (d, 2H, $^2J_{P-H}$ = 14.7 Hz, P–CH_2); 3.44 (d, 6H, $^3J_{P-H}$ = 11 Hz, O–CH_3). ^{13}C{^{1}H} NMR (62.90 MHz, D_2O), δ in ppm: 24.9 CH_2; 36.9 CH_2; 39.8 (d, $^1J_{P-C}$ = 146.0 Hz, P–CH_2); 57.9 (d, $^2J_{P-C}$ = 11.5 Hz, O–CH_3); 72.4 –C–; 183.7 C=O. ^{31}P{^{1}H} NMR (242.94 MHz, D_2O), δ in ppm: 24.48. Anal. Calcd for $C_9H_{18}NO_5P$ (251.22): C, 43.03%; H, 7.22%; N, 5.58%; P, 12.33%; found: C, 42.99%; H, 7.17%; N, 5.52%; P, 12.30%.

*4.1.3.2. 1-[(Dimethoxyphosphono)methylamino]cyclohexanecarboxylic acid (**2c**).* White solid, 58.3% yield, mp = 246–248 °C, R_f = 0.38; IR (KBr, cm^{-1}): 3369 (NH), 2942–2854 (C–H), 1610 (C=O), 1296, 1245 (P=O), 1091, 1054 (P–O–C). ^{1}H NMR (250.13 MHz, D_2O), δ in ppm: 1.28–1.96 (m, 10H, CH_2); 2.47 (d, 2H, $^2J_{P-H}$ = 14.69 Hz, P–CH_2); 2.89 (d, 6H, $^3J_{P-H}$ = 6.6 Hz, O–CH_3). ^{13}C{^{1}H} NMR (62.90 MHz, D_2O), δ in ppm: 22.7 $^{4}CH_2$; 25.3 $^{3,5}CH_2$; 33.3 $^{2,6}CH_2$; 42.0 (d, $^1J_{P-C}$ = 144.2 Hz, P–CH_2); 48.8 (d, $^2J_{P-C}$ = 7.5 Hz, O–CH_3); 64.2 –C–; 182.8 C=O. ^{31}P{^{1}H} NMR (242.94 MHz, D_2O), δ in ppm: 22.17. Anal. Calcd for $C_{10}H_{20}NO_5P$ (265.24): C, 45.28%; H, 7.60%; N, 5.28%; P, 11.68%; found: C, 45.21%; H, 7.58%; N, 5.22%; P, 11.61%.

*4.1.3.3. 1-[(Dimethoxyphosphono)methylamino]cycloheptanecarboxylic acid (**3c**).* White solid, 62.1% yield, mp = 209–211 °C, R_f = 0.45; IR (KBr, cm^{-1}): 3117 (NH), 2929–2854 (C–H), 1611 (C=O), 1280, 1235 (P=O), 1169, 1041

(P–O–C). ^{1}H NMR (250.13 MHz, D_2O), δ in ppm: 1.41–1.98 (m, 12H); 2.50 (d, 2H, $^{2}J_{P-H} = 15.1$ Hz, P–CH_2); 3.47 (d, 6H, $^{3}J_{P-H} = 6.6$ Hz, O–CH_3). ^{13}C{^{1}H} NMR (62.90 MHz, D_2O), δ in ppm: 22.6 $^{4,5}CH_2$; 29.2 $^{3,6}CH_2$; 37.2 $^{2,7}CH_2$; 38.8 (d, $^{1}J_{P-C} = 145.7$ Hz, P–CH_2); 51.4 (d, $^{2}J_{P-C} = 5.7$ Hz, O–CH_3); 67.9 –C–; 183.3 C=O. ^{31}P{^{1}H} NMR (242.94 MHz, D_2O), δ in ppm: 24.84. Anal. Calcd for $C_{11}H_{22}NO_5P$ (279.27): C, 47.31%; H, 7.94%; N, 5.02%; P, 11.09%; found: C, 47.21%; H, 7.90%; N, 5.01%; P, 11.03%.

*4.1.3.4. 1-[(Dimethoxyphosphono)methylamino]cyclooctanecarboxylic acid (**4c**).* White solid, 54.8% yield, mp = 206–208 °C, $R_f = 0.46$; IR (KBr, cm^{-1}): 3140 (NH), 2921–2849 (C–H), 1734 (C=O), 1305, 1212 (P=O), 1051 (P–O–C). ^{1}H NMR (250.13 MHz, D_2O), δ in ppm: 1.47–2.10 (m, 14H); 2.70 (d, 2H, $^{2}J_{P-H} = 14.8$ Hz, P–CH_2); 3.52 (d, 6H, $^{3}J_{P-H} = 7.3$ Hz, O–CH_3). ^{13}C{^{1}H} NMR (62.90 MHz, D_2O), δ in ppm: 22.1 $^{5}CH_2$; 24.7 $^{4,6}CH_2$; 27.8 $^{3,7}CH_2$; 32.2 $^{2,8}CH_2$; 39.3 (d, $^{1}J_{P-C} = 144.7$ Hz, P–CH_2); 52.0 (d, $^{2}J_{P-C} = 5.3$ Hz, O–CH_3); 68.1 –C–; 183.3 C=O. ^{31}P{^{1}H} NMR (242.94 MHz, D_2O), δ in ppm: 23.94. Anal. Calcd for $C_{12}H_{24}NO_5P$ (293.29): C, 49.14%; H, 8.25%; N, 4.78%; P, 10.56%; found: C, 49.03%; H, 8.19%; N, 4.75%; P, 10.46%.

*4.1.3.5. 1-[(Dimethoxyphosphono)methylamino]cyclododecanecarboxylic acid (**5c**).* White solid, 63.6% yield, mp = 221–223 °C, $R_f = 0.57$; IR (KBr, cm^{-1}): 3448 (NH), 2935–2862 (C–H), 1706 (C=O), 1353, 1235 (P=O), 1158, 1052 (P–O–C). ^{1}H NMR (250.13 MHz, D_2O), δ in ppm: 1.23–1.71 (m, 22H, CH_2); 3.16 (d, 2H, $^{2}J_{P-H} = 13.22$ Hz, P–CH_2); 3.84 (d, 6H, $^{3}J_{P-H} = 11$ Hz, O–CH_3). ^{13}C{^{1}H} NMR (62.90 MHz, D_2O), δ in ppm: 21.7 $^{3,11}CH_2$; 24.3 $^{7}CH_2$; 28.4 $^{4,5,6,8,9,10}CH_2$; 31.1 $^{2,12}CH_2$; 39.0 (d, $^{1}J_{P-C} =$ 153.39 Hz, P–CH_2); 56.8 (d, $^{2}J_{P-C} = 6$ Hz, O–CH_3); 72.9 –C–; 181.5 C=O. DEPT (62.90 MHz, D_2O), δ in ppm: 21.7 $^{3,11}CH_2$; 24.3 $^{7}CH_2$; 28.4 $^{4,5,6,8,9,10}CH_2$; 31.1 $^{2,12}CH_2$; 39.0 (d, P–CH_2); 56.7 O–CH_3. ^{31}P{^{1}H} NMR (242.94 MHz, D_2O), δ in ppm: 27.41. Anal. Calcd for $C_{16}H_{32}NO_5P$ (349.40): C, 55.00%; H, 9.23%; N, 4.01%; P, 8.86%; found: C, 54.87%; H, 9.11%; N, 4.00%; P, 8.72%.

4.2. Biological assays

4.2.1. In vitro assays. Chemicals, solutions and other materials

The cell culture flasks and the 96-well microplates were provided by NUNCLON (Denmark). MTT, FCS and cisplatin were purchased from Sigma Co. The stock solutions of tested compounds (20 mM) were freshly prepared in DMSO, and stored at 4 °C, protected from light for a maximum period of 1 week. The serial dilutions of tested compounds were prepared just before use. In the final dilutions obtained the concentrations of DMSO never exceeded 1%.

4.2.2. Cell lines and culture conditions

The cell lines HL-60 (acute promyelocyte leukemia), LAMA-84 and K-562 (chronic myeloid leukemia), DOHH-2 (non-Hodgkin lymphoma), HD-MY-Z (Hodgkin lymphoma) and 293T (human embryonal kidney) were supplied by DSMZ GmbH, Germany; the urinary bladder carcinoma EJ originated from the American Type Cell Culture, USA. Cells were cultured routinely in a controlled environment: 37 °C in 5% CO_2 humidified atmosphere. The human embryonal kidney 293T cells were cultured in Dulbecco's modified MEM medium, whereas all other cell lines were maintained in RPMI 1640; growth media were supplemented with 2 mM L-glutamine and 10% fetal calf serum. All cell lines were subcultured twice weekly to maintain continuous logarithmic growth.

4.2.3. Cytotoxicity assay

Cell survival was evaluated by using the MTT-dye reduction assay, which is based on the ability of viable cells to metabolize a yellow tetrazolium salt to violet formazan product that can be detected spectrophotometrically. The assay was carried out as previously described [34] with minor modifications [35]. Exponentially growing cells were plated in 96-well sterile plates at a density of 10^4 cells/well in 100 μl of medium and were incubated for 24 h. Thereafter the tested compounds were applied in concentrations ranging from 0.195 to 200 μM. After a 72 h continuous exposure 10 μl aliquots from a 5 mg/ml MTT solution were added to each well and the plates were further incubated for 4 h at 37 °C in a humidified 5% CO_2 atmosphere. The formazan crystals yielded were solubilized by addition of HCOOH (5%) acidified DMSO. The MTT-formazan absorbance was read on a Labexim LMR-1 multiplate reader.

4.2.4. Apoptosis assay

The characteristic for apoptosis mono- and oligonucleosomal fragmentation of genomic DNA was detected using 'Cell Death Detection' ELISA-kit (Roche Diagnostics, Germany). Cytosolic fractions of 1×10^4 cells per group (treated or untreated) served as antigen source in a sandwich ELISA, utilizing primary anti-histone antibody-coated microplate and a secondary peroxidase-conjugated anti DNA-antibody. The photometric immunoassay for histone-associated DNA-fragments was executed according to the manufacturer's instructions at 405 nm, using ELISA reader (Labexim LMR-1). The results were expressed as the oligonucleosome enrichment factor (representing a ratio of the absorption in the treated versus the untreated control samples).

4.2.5. Cytogenetical method

The cytogenetical investigation was conducted as described by Preston et al. [36]. Inbred male and female C57Bl mice, weighing 20.0 ± 1.5 g were kept at standard conditions at 20 °C and 12 h light/dark cycle, having free access to food and water. The compounds **1c–5c** were administered i.p. in doses of 10 and 100 mg/kg. Mitomycin C (Kyowa) 3.5 mg/kg was used as a positive control. The negative control animals were injected only with 0.9% NaCl.

Bone marrow chromosome aberration assay was performed on groups of animals each one consisting of 3 males and 3 females treated with the compound studied, and 5 pure control animals. The animals were injected i.p. with colchicine at a dose of

40 mg/kg, 24 and 48 h after the administration of applied chemicals or 0.9% NaCl solution and 1 h before isolation of the bone marrow cells. Bone marrow cells were flushed from femur and incubated for 20 min in a hypotonic (0.075 M) KCl solution at 37 °C. Thereafter the cells were fixed in methanol–acetic acid (3:1), dropped on cold slides and air dried. To examine the chromosome aberrations the slides were 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 in the bone marrow slides to score aberrations. The frequencies of abnormalities and the mitotic index were determined for each animal and then the mean ± standard error for each group was calculated.

4.3. Statistical analysis

Three-way analysis of variance (ANOVA) with fixed effects, followed by two-group Student's *t*-test and post hoc pairwise comparison test of Dunnett with a control was performed using BMDP4V, BMDP3D and BMDP7D programs [37]. Statistical significance was expressed as $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$; $p > 0.05$ – not significant. Unless otherwise stated, 8 animals were used per group.

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