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Preliminary communication

Synthesis, NMR characterization and in vitro antitumor evaluation of new aminophosphonic acid diesters

I. Kraicheva [a,*], A. Bogomilova [a], I. Tsacheva [a], G. Momekov [b], K. Troev [a]

[a] Institute of Polymers, Bulgarian Academy of Sciences, Acad. Georgi Bonchev St., Block 103-A, Sofia 1113, Bulgaria
[b] Laboratory of Experimental Chemotherapy, Department of Pharmacology, Pharmacotherapy and Toxicology, Faculty of Pharmacy, Medical University – Sofia, Sofia 1000, Bulgaria

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ABSTRACT

The synthesis of three novel α-aminophosphonic acid diesters *N*,*N*-dimethyl-[*N*′-methyl(diethoxyphosphonyl)-(2-furyl)]-1,3-diaminopropane, p-[*N*-methyl(diethoxyphosphonyl)-(2-furyl)]toluidine and p-[*N*-methyl(diethoxyphosphonyl)-(4-dimethylaminophenyl)]toluidine through an addition of diethyl phosphite to *N*,*N*-dimethyl-*N*′-furfurylidene-1,3-diaminopropane, *N*-furfurylidene-p-toluidine and *N*-(4-dimethylaminobenzylidene)-p-toluidine, respectively, is reported. The α-aminophosphonates have been characterized by elemental analysis, IR and NMR (^{1}H, ^{13}C and ^{31}P) spectra. The compounds were tested for antiproliferative effects against 4 human leukemic cell lines, namely LAMA-84, K-562 (chronic myeloid leukemias), HL-60 (acute promyelocyte leukemia) and HL-60/Dox (multi-drug-resistant sub-line, characterized by overexpression of MRP-1 (ABC-C1)) and were found to exert concentration-dependent cytotoxic effects. A representative aminophosphonate compound was shown to induce oligonucleosomal DNA fragmentation which implies that the induction of cell death through apoptosis plays an important role for its cytotoxicity mode of action.

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1. Introduction

Aminophosphonic acid derivatives constitute an important class of organophosphorus compounds on account of their versatile biological activity [1]. The generally low mammalian toxicity of these compounds makes them attractive for use in agriculture and medicine [2–5]. Numerous of them possess antifungal, pesticidal, herbicidal and plant growth regulatory activity and are of particular interest for agrochemistry [4–7]. Aminophosphonic acids are structural analogues of natural α-aminocarboxylic acids, and have been found to act as inhibitors of specific enzymes as HIV protease, thrombin and human collagenaze, and to suppress the growth of various tumors and viruses [3,8–13]. Moreover, some aminophosphonic acids inhibit bone resorption, delay the progression of bone metastases, exert direct cytostatic effects on a variety of human tumor cells and have found clinical application in the treatment of bone disorders and cancer [14,15]. Polymeric aminophosphonate analogues are used as bone seeking radiopharmaceuticals [16].

The pharmacological importance and utility of aminophosphonate derivatives have stimulated extensive studies on various aspects of their chemistry and biochemistry: synthetic routes, structural and spectral characterization and evaluation of their biological properties [14,17–20]. Among the numerous synthetic approaches to aminophosphonates, the addition of dialkyl phosphites to Schiff bases in the presence of sodium alkoxide and Lewis acids is the most convenient procedure [16,21].

In this work we report on the synthesis, spectroscopic characterization and preliminary antitumor evaluation of three novel aminophosphonic acid diesters. The cytotoxicity of the compounds was tested against a panel of human tumor cell lines, including the multi-drug-resistant model HL-60/Dox, characterized by the expression of MRP-1 drug efflux pump.

2. Results and discussion

2.1. Chemistry

The Schiff bases *N*,*N*-dimethyl-*N*′-furfurylidene-1,3-diaminopropane (**1**), *N*-furfurylidene-p-toluidine (**2**) and *N*-(4-dimethylaminobenzylidene)-p-toluidine (**3**) have been described earlier [22–24]. The spectroscopic characteristics of the azomethines **1** and **3** are given in the literature [24,25], but no NMR data about **2** are available. Therefore, we report here NMR spectroscopic data of this compound (see the Experimental section).

* Corresponding author. Tel.: +359 2 979 6633; fax: +359 2 870 0309.
E-mail address: kraicheva@yahoo.com (I. Kraicheva).

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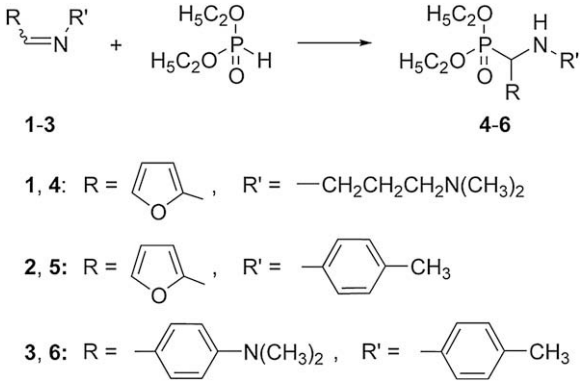
Three novel α-aminophosphonic acid diesters, *N*,*N*-dimethyl-[*N*′-methyl(diethoxyphosphonyl)-(2-furyl)]-1,3-diaminopropane (**4**), p-[*N*-methyl(dietoxyphosphonyl)-(2-furyl)]toluidine (**5**) and p-[*N*-methyl(diethoxyphosphonyl)-(4-dimethylaminophenyl)]toluidine (**6**), were synthesized through addition of diethyl phosphite to the azomethine bond of the Schiff bases **1**–**3**, according to Scheme 1.

The reaction was carried out using $NaOC_2H_5$ and CdI_2 as catalysts, as well as without catalyst. The addition of the phosphite to the Schiff bases was controlled by IR spectroscopy. In the presence of the catalysts the aminophosphonates **4**–**6** were obtained in good yields for 3 h, while in the absence of catalyst the reaction time was longer – up to 8 h. The products **4** and **5** are oils and **6** is crystalline solid. They are soluble in methanol, ethanol, benzene, chloroform.

The synthesized compounds **4**–**6** gave satisfactory elemental analyses, and their molecular structure was confirmed by IR and ^{1}H, ^{13}C and ^{31}P NMR spectroscopy. The IR spectra of **4**–**6** showed the expected [26] absorption bands at 3375–3306 and 1250–1239 cm^{-1}, which are attributed to NH and P═O stretching vibrations, respectively (see the Experimental section). The ^{1}H NMR spectra of compounds **5** and **6**, recorded in $CDCl_3$ solution, exhibit the signal of CHP proton as a doublet at 4.83 (**5**) and 4.65 (**6**) ppm. The same signal of **4** is masked by the $POCH_2$ multiplet signal and it was identified using H, C HMQC technique (Fig. 1). There is a correlation between the doublet signal at 4.06 ppm with $^2J_{PH} = 22.1$ Hz, situated in $POCH_2$ region of ^{1}H NMR spectrum of **4**, and the doublet at 54.68 ppm with $^1J_{PC} = 160.7$ Hz, observed in its ^{13}C NMR spectrum (Fig. 1). The values are typical for the proton and carbon atom from a CHP fragment [27,28].

The NH proton signal of **5** appears as a broad singlet. In the spectra of **4** and **6**, the NH proton signal is either not observed (**4**) or is partially overlapped with the doublet signal of CHP proton (**6**). The methyl protons from the ethoxy groups in **4**–**6** give two triplets, owing to the nonequivalence of these groups [27]. The $POCH_2$ proton signals appear as multiplets at about 4 ppm. The aliphatic methylene protons in **4** display three multiplets shifted upfield. The proton connectivities in these methylene groups, derived from HH COSY diagram, were used for the assignment of their proton signals. The furan proton signals in **4** and **5** are complicated due to the additional coupling with the ^{31}P nucleus [29,30]. They are observed as unresolved multiplets. Moreover, the signals for FurH-3 and FurH-4 of compound **4** are overlapped. The aromatic protons of **5** and **6** resonate in the expected region and the following HH connectivities are found from their HH COSY spectra: 6.54–6.92 ppm (ArH′) for **5** and **6** and 6.69–7.31 ppm (ArH) for **6**.



Scheme 1. Synthesis of α-aminophosphonic acid diesters **4**–**6**.

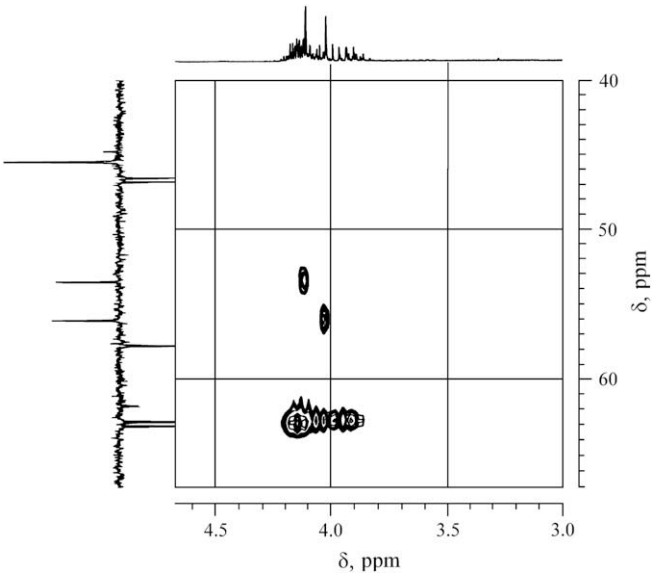


Fig. 1. CH COSY diagram of compound **4** in $CDCl_3$ showing the CHP and $POCH_2$ regions.

The assignment of the carbon atom signals in compounds **4**–**6** is based on the analysis of 1D ($^{13}C\{^1H\}$) and 2D (CH COSY and CH COLOC) NMR spectra and is in accordance with literature data for similar compounds [28,30]. Thus, $^{13}C\{^1H\}$ spectra of **4**–**6** reveal for the CHP carbon atom a doublet with large coupling constant (150–160 Hz), due to the one-bond interaction with phosphorus. Likewise, carbon–phosphorus coupling is observed for all carbon atoms in **4**–**6**, originating from aldehyde fragment: the furan carbons (**4** and **5**) and the aromatic carbons ArC (**6**). The doublet signal found from the spectra for the $NHCH_2$ carbon (**4**), as well as for the quaternary carbon ArC-1′ (**5** and **6**) shows three-bond coupling to phosphorus of 15.8 (**4**), 13.7 (**5**) and 15.1 (**6**) Hz. The large values of $^3J_{PC}$ observed at these atoms is due to the lone electron pair contribution of the adjacent nitrogen atom [28]. The other carbons from the amine (**4**) and toluidine (**5** and **6**) residue give singlets in the spectra. The nonequivalence of the ethoxy groups gives rise to the appearance of pairs of doublets for their methylene (**4**–**6**) and methyl (**4** and **6**) carbons. One doublet is found for the two ethoxy methyl carbons in **5** because of the identical chemical shifts of their signals.

The $^{31}P\{^1H\}$ NMR spectra of compounds **4**–**6** consist of one singlet in the region expected for the aminophosphonate derivatives – 21–25 ppm.

2.2. Pharmacology

The Schiff bases **2** and **3** and the aminophosphonates **4**–**6** were assessed for cytotoxicity against a panel of cell lines representative for some important types of human leukemia including the multidrug-resistant model HL-60/Dox. All compounds exerted concentration-dependent antiproliferative effects after 72 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 derivative **5** proved to be the most potent cytotoxic agents, which implies that the presence of both furyl and *N*-tolyl moieties is an important prerequisite for optimal activity for these compounds. The other Schiff base **3** and its aminophosphonate derivative **6** were far less active, whereas compound **4** exerted only marginal activity. All tested compounds

Table 1
Comparative cytotoxic activity of compounds **2**–**6** vs. cis-DDP in a panel of tumor cell lines after 72 h (MTT-dye reduction assay).

Cell line	IC_{50} value (μM)[a]					
	2	**3**	**4**	**5**	**6**	Cisplatin
LAMA-84[b]	39.9 ± 2.1	251.9 ± 7.3	>400.0	71.2 ± 2.4	119.4 ± 6.3	18.2 ± 1.7
K-562[b]	29.9 ± 1.9	212.9 ± 12.1	352.9 ± 11.7	22.9 ± 0.9	42.4 ± 3.0	25.7 ± 2.1
HL-60[c]	>400.0	>400.0	163.4 ± 5.3	74.8 ± 2.7	>400.0	7.8 ± 1.1
HL-60/Dox[c,d]	68.6 ± 4.0	226.1 ± 5.9	190.0 ± 4.7	115.2 ± 7.1	107.2 ± 4.1	14.5 ± 1.4

[a] Arithmetic mean ± standard deviation of at least 6 independent experiments.
[b] Chronic myeloid leukemia.
[c] Acute promyelocyte leukemia.
[d] Multi-drug-resistant (mdr) phenotype due to overexpression of MRP-1 (ABC-C1) transporter.

were generally less active as compared to the referent anticancer drug cisplatin, although compound **5** showed superior activity against K-562 cells. Nevertheless, an intriguing collateral sensitivity phenomenon was encountered with both Schiff bases **2** and **3** as well as with the aminophosphonate **6** i.e. while they failed to exert any significant antiproliferative effects against the sensitive cell line HL-60 they exhibited prominent cytotoxicity against the multi-drug-resistant variant HL-60/Dox. The mdr-phenotype in the latter cell line is due to the overexpression of MRP-1 protein (ABC-C1), a member of the ATP-binding cassette (ABC) family of transporters capable of unilateral energy-dependent efflux of different anti-cancer drugs out of tumor cells, thus rendering them resistant [31,32]. Hence, the occurrence of ABC-transporters overexpression is considered an important factor contributing to clinically relevant resistance of malignant tumors to chemotherapy [32–34]. Although the mechanistic aspects of the encountered collateral sensitivity necessitate further clarification the ability of compounds **2**, **3** and **6** to selectively inhibit MRP-1 expressing HL-60/Dox implies that these could be considered as promising leads for further development of agents active in chemotherapy refractory malignant disease.

The level of internucleosomal DNA fragmentation, a key feature of apoptotoic cell death after 24 h treatment with varying concentrations of the newly synthesized aminophosohonate compound **5** is presented in Fig. 2.

As evident from the results obtained, the exposure of LAMA-84 with the tested agent evoked concentration-dependent increase of the proportion of apoptotic cells as evidenced by the enrichment of cytosole with oligonucleosomal DNA fragments. These data unambiguosly indicate that the induction of apoptosis plays crucial role in the cytotoxic mode of action of the aminophosphonates under investigation.

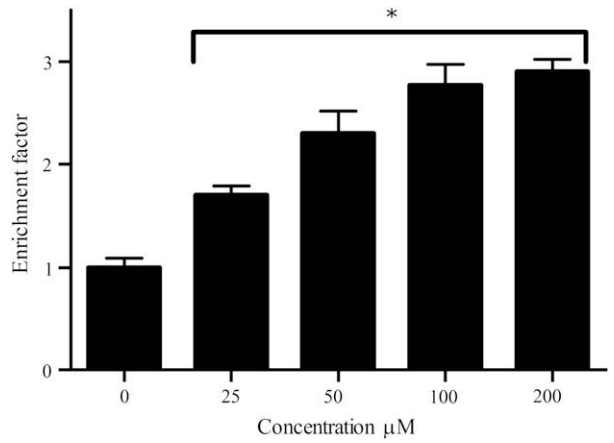


Fig. 2. Enrichment of the cytosole of LAMA-84 with mono- and oligonucleosomal DNA fragments after 24 h exposure with the aminophosphonate compound **5** (at 25, 50, 100 or 200 μM) was monitored using 'Cell-Death Detection™' ELISA. Each bar is representative for 4 separate experiments.

3. Conclusion

Novel α-aminophosphonic acid diesters, *N*,*N*-dimethyl-[*N*′-methyl(diethoxyphosphonyl)-(2-furyl)]-1,3-diaminopropane (**4**), p-[*N*-methyl(diethoxyphosphonyl)-(2-furyl)toluidine (**5**) and p-[*N*-methyl(dietoxyphosphonyl)-(4-dimethylaminophenyl)]toluidine (**6**) were obtained via addition of diethyl phosphite to the corresponding Schiff base (**1**–**3**). The reaction proceeded in the presence of sodium ethoxide and CdI_2, as well as without a catalyst. The molecular structure of the compounds **4**–**6** was studied by means of spectral methods.

The starting Schiff bases **2** and **3** and the α-aminophosphonates **4**–**6** were evaluated for cytotoxicity against 4 human leukemic cell lines, including the multi-drug-resistant model HL-60/Dox. All compounds exerted concentration-dependent antiproliferative effects after 72 h exposure. The cytotoxicity data obtained revealed that the Schiff base **2** and the corresponding aminophosphonate **5** provided to be the most potent cytotoxic agents among the tested compounds. It implies that the presence of both furyl and *N*-tolyl moieties is an important prerequisite for optimal activity in these substances. The studied compounds were far less active as compared to the referent anticancer drug cisplatin, except the aminophosphonate **5**, whose effect towards K-562 cell line was comparable with the referent. The Schiff bases **2** and **3** and aminophosphonate **6** failed to exert any significant antiproliferative effects against the sensitive cell line HL-60. At the same time they showed prominent cytotoxicity against the multi-drug-resistant variant HL-60/Dox. The ability of these compounds to selectively inhibit MRP-1 expressing HL-60/Dox indicates that they could be considered as promising leads for further development of agents active in chemotherapy refractory malignant disease. The level of internucleosomal DNA fragmentation after 24 h exposure of LAMA-84 with aminophosphonate **5** was monitored. The compound was shown to induce oligonucleosomal DNA fragmentation which implies that the induction of cell death through apoptosis plays an important role for its cytotoxicity mode of action.

Taken together the biological data give us reason to consider the presented Schiff bases and α-aminophosphonates as a novel class of antiproliferative agents. The observed collateral sensitivity of multi-drug-resistant cancer cell lines and the established activity of a representative compound to trigger apoptosis at sub-cytotoxic levels suggest that these compounds necessitate further more detailed pharmacological evaluation.

4. Experimental

Diethyl phosphite (Fluka Chemie AG, Buchs, Switzerland) was purified by vacuum distillation. 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 Bruker Vector 21 FT-IR spectrophotometer. ^{1}H, $^{13}C\{^{1}H\}$, DEPT, HH COSY, CH COSY and CH COLOC NMR spectra in $CDCl_3$ were recorded on a Bruker DRX-250 250 MHz spectrometer at r.t. and tetramethylsilane (TMS) as an internal standard. $^{31}P\{^{1}H\}$ NMR spectra in $CDCl_3$ were taken on a Bruker 500 MHz spectrometer (compound **4**) and a Bruker DRX-250 250 MHz spectrometer (compounds **5** and **6**) using 85% H_3PO_4 as an external standard.

4.1. Synthesis

4.1.1. Schiff bases (**1**–**3**)

N,*N*-Dimethyl-*N*′-furfurylidene-1,3-diaminopropane (**1**) was prepared from furfural and N,N-dimethyl-1,3-diaminopropane following a previously described procedure in Zondler et al. [22]; b.p. 65–67 °C/0.5 mmHg; *N*-furfurylidene-p-toluidine (**2**) was synthesized according to Head and Jones [23]; m.p. 41–42 °C. ^{1}H NMR ($CDCl_3$), δ (ppm), J_{HH} (Hz): 8.22 (s, 1H, CH═N); 7.52 (dd, $^3J = 1.3$, $^4J = 0.5$, 1H, FurH-5); 7.13 (pseudo-s, 4H, ArH); 6.86 (dd, $^3J = 3.5$, $^4J = 0.5$, 1H, FurH-3); 6.46 (dd, $^3J = 3.5$ and 1.7, FurH-4); 2.30 (s, 3H, $ArCH_3$). ^{13}C{^{1}H} NMR (62.90 MHz, $CDCl_3$), δ (ppm): 152.2 (FurC-2); 148.7 (ArC-1′); 146.8 (CH═N); 145.4 (FurC-5); 136,0 (ArC-4′); 129.7 (ArC-3′,5′); 120.8 (ArC-2′,6′); 115.8 (FurC-3); 112.0 (FurC-4); 20.9 ($ArCH_3$). *N*-(4-Dimethylaminobenzilidene)-p-toluidine (**3**) was synthesized following a well known procedure [35]; m.p. 117–118 °C.

4.1.2. Aminophosphonates (**4**–**6**)

Compounds **4**–**6** were synthesized from diethyl phosphite and the corresponding Schiff base **1**–**3** following the procedures given for **4**.

4.1.2.1. N,N-*Dimethyl-[N′-methyl(diethoxyphosphonyl)-(2-furyl)]-1,3-diaminopropane (**4**)*

4.1.2.1.1. Method A: in the presence of C_2H_5ONa. N,N-Dimethyl-*N*′-furfurylidene-1,3-diaminopropane (**1**) (3.87 g, 21.5 mmol) and diethyl phosphite (2.97 g, 21.5 mmol) were mixed and stirred, and a saturated solution of C_2H_5ONa was added dropwise. The mixture was stirred for 3 h at 65 °C. Then it was evaporated under reduced pressure at 65 °C. The reaction product was precipitated twice in diethyl ether from a benzene solution and dried to constant weight in vacuum. Yield: 5.60 g (82%); oil.

4.1.2.1.2. Method B: in the presence of CdI_2. Diethyl phosphite (1.67 g, 12.10 mmol) dissolved in dry benzene (5 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 a reflux condenser. After stirring for a quarter an hour at r.t. to the reaction mixture was added *N*,*N*-dimethyl-*N*′-furfurylidene-1,3-diaminopropane (2.18 g, 12.11 mmol). The mixture was heated for 3 h at 50 °C with stirring. Then benzene was removed in vacuo and the reaction product was purified and dried following the procedure given in method A. Yield: 3.08 g (80%).

4.1.2.1.3. Method C: in the absence of catalyst. N,N-Dimethyl-*N*′-furfurylidene-1,3-diaminopropane (2.82 g, 15.66 mmol) and diethyl phosphite (2.16 g, 15.65 mmol) were dissolved in benzene (5 ml) and placed in a flask. The mixture was stirred for 8 h at 65 °C. After removal of benzene in vacuo, the oily residue was purified according to the procedure given in method A. Yield: 3.64 g (73%). Anal. calcd. (%) for $C_{14}H_{27}N_2O_4P$: C, 52.83; H, 8.49; N, 8.81. Found: C, 52.61; H, 8.33; N, 8.72. IR (neat), ν (cm^{-1}): 3375 (ν_{NH}); 1499, 1465 ($\nu_{C=C(Fur)}$); 1243 ($\nu_{P=O}$); 1163, 1027 ($\nu_{P-OEt,\ C-O-C}$). ^{1}H NMR ($CDCl_3$), δ (ppm), J_{HH} (Hz), J_{PH} (Hz): 7.37 (m, 1H, FurH-5); 6.33 (m, 2H, FurH-3,4); 4.20–3.83 (m, 6H, OCH_2, CHP, NH); 4.06 (d, $^2J = 22.1$, 1H, CHP); 2.53 (m, 2H, $C\underline{H}_2NH$); 2.25 (m, 2H, $C\underline{H}_2N(CH_3)_2$); 2.16 (s, 6H, $N(CH_3)_2$); 1.58 (m, 2H, $CH_2C\underline{H}_2CH_2$); 1.29 and 1.18 (2 t, $^3J = 7.1$ and 7.1, 6H, CH_3). ^{13}C{^{1}H} NMR ($CD\underline{Cl}_3$), δ (ppm), J_{PC} (Hz): 150.02 (d, $^2J = 2.7$, FurC-2); 142.29 (d, $^4J = 2.9$, FurC-5); 110.48 (d, $^4J = 2.3$, FurC-4); 108.83 (d, $^3J = 7.5$, FurC-3); 63.05 and 62.77 (2d, $^2J = 6.9$ and 6.9, OCH_2); 57.66 ($\underline{C}H_2N(CH_3)_2$); 54.68 (d, $^1J = 160.7$, CPH); 46.59 (d, $^3J = 15.8$, CH_2NH); 45.38 ($N(CH_3)_2$); 27.60 ($CH_2\underline{C}H_2CH_2$); 16.39 and 16.26 (2d, $^3J = 5.9$ and 5.8, CH_3). ^{31}P{^{1}H} NMR (202 MHz, $CDCl_3$), δ (ppm): 22.16.

*4.1.2.2. p-[N-Methyl(dietoxyphosphonyl)-(2-furyl)]toluidine (**5**).* Compound **5** was prepared from *N*-furfurylidene-p-toluidine (**2**) and diethyl phosphite using them in molecular ratio 1:1 and following the procedures given for **4**. Oil; yield: 73% (method A), 77% (method B), 70% (method C). Anal.calcd. (%) for $C_{16}H_{22}NO_4P$: C, 59.44; H, 6.81; N, 4.33. Found: C, 59.20; H, 6.67; N, 4.21. IR (neat), ν (cm^{-1}): 3307 (ν_{NH}); 1618, 1519, 1443 ($\nu_{C=C(Ar,\ Fur)}$); 1250 ($\nu_{P=O}$); 1150, 1014 ($\nu_{P-OEt,\ C-O-C}$). ^{1}H NMR ($CDCl_3$), δ (ppm), J_{HH} (Hz), J_{PH} (Hz): 7.32 (m, 1H, FurH-5); 6.92 (m, 2H, ArH-3′,5′); 6.54 (m, 2H, ArH-2′,6′); 6.33 (m, 1H, FurH-3); 6.26 (m, 1H, FurH-4); 4.83 (d, $^2J = 23.9$, 1H, CHP); 4.46 (br.s., 1H, NH); 4.08, 3.85 and 3.63 (3 m, 4H, OCH_2); 2.15 (s, 3H, $ArC\underline{H}_3$); 1.25 and 1.15 (2 t, $^3J = 7.3$ and 7.1, 6H, CH_3). ^{13}C{^{1}H} NMR ($CD\underline{Cl}_3$), δ (ppm), J_{PC} (Hz): 149.27 (d, $^2J = 1.7$, FurC-2); 143.52 (d, $^3J = 13.7$, ArC-1′); 142.14 (d, $^4J = 3.2$, FurC-5); 129.40 (ArC-3′,5′); 127.82 (ArC-4′); 113.86 (ArC-2′,6′); 110.50 (d, $^4J = 2.6$, FurC-4); 108.46 (d, $^3J = 7.1$, FurC-3); 63.26 and 62.99 (2d, $^2J = 6.9$ and 6.9, OCH_2); 50.27 (d, $^1J = 159.8$, CHP); 20.10 ($Ar\underline{C}H_3$); 16.02 (d, $^3J = 6.3$, CH_3). ^{31}P{^{1}H} NMR (101 MHz, $CDCl_3$), δ (ppm): 21.03.

*4.1.2.3. p-[N-Methyl(diethoxyphosphonyl)-(4-dimethylaminophenyl)]-toluidine (**6**).* Compound **6** was obtained using *N*-(4-dimethylaminobenzylidene)-p-toluidine (**3**) and diethyl phosphite in molecular ratio 1:1 of the reagents and following the procedures described for **4**. The product was recrystallized from ethyl alcohol to produce **6** as colorless crystalline powder. Yield: 73% (method A), 76% (method B), 68% (method C); m.p. 101–102 °C. Anal. calcd. (%) for $C_{20}H_{29}N_2O_3P$: C, 63.83; H, 7.71; N, 7.45. Found: C, 63.69; H, 7.57; N, 7.29. IR (KBr pellet), ν (cm^{-1}): 3306 (ν_{NH}); 1614, 1520, 1480, 1445 ($\nu_{C=CAr}$); 1234 ($\nu_{P=O}$); 1164, 1024 (ν_{POEt}). ^{1}H NMR ($CDCl_3$), δ (ppm), J_{HH} (Hz), J_{PH} (Hz): 7.31 (m, 2H, ArH-2,6); 6.91 (m, 2H, ArH-3′,5′); 6.69 (m, 2H, ArH-3,5); 6.54 (m, 2H, ArH-2′,6′); 4.65 (d, $^2J = 23.4$, 1H, CHP); 4.61 (br s, 1H, NH); 4.12, 3.94 and 3.70 (3m, 4H, OCH_2); 2.91 (s, 6H, $N(CH_3)_2$); 2.18 (s, 3H, $ArC\underline{H}_3$); 1.29 and 1.15 (2t, $^3J = 7.1$ and 7.1, 6H, CH_3). ^{13}C{^{1}H} NMR ($CD\underline{Cl}_3$), δ (ppm), J_{PC} (Hz): 150.09 (d, $^5J = 1.9$, ArC-4); 144.22 (d, $^3J = 15.1$, ArC-1′); 129.50 (ArC-3′,5′); 128.52 (d, $^3J = 5.7$, ArC-2,6); 123.09 (d, $^2J = 2.5$, ArC-1); 113.99 (ArC-2′,6′); 112.48 (d, $^4J = 2.5$, ArC-3,5); 62.99 and 62.97 (2d, $^2J = 6.9$ and 6.9, OCH_2); 55.60 (d, $^1J = 152.9$, CHP); 40.40 ($N(CH_3)_2$); 20.26 ($Ar\underline{C}H_3$); 16.39 and 16.22 (2d, $^3J = 5.7$ and 5.7, CH_3). ^{31}P{^{1}H} NMR (101 MHz, $CDCl_3$), δ (ppm): 24.79.

4.2. Biological assays

4.2.1. Drugs, chemicals and solutions

Formic acid, 2-propanol, and L-glutamine were purchased from AppliChem GmbH, Darmstadt, Germany. Doxorubicin, fetal calf serum (FCS) and RPMI-1640 medium were purchased from Sigma–Aldrich GmbH, Steinheim, Germany. The tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was supplied from Merck, Darmstadt, Germany. The referent antineoplastic drug cisplatin was used as a commercially available sterile dosage form for clinical application (Cisplatin injection, Pfizer, USA). Stock solutions of the tested compounds were freshly prepared in DMSO and promptly subset to serial dilution in RPMI-1640 to yield the desired working solutions. At the final concentrations obtained cells were never exposed to DMSO concentrations exceeding 1%.

4.2.2. Cell lines and culture conditions

The cell lines used in this study namely HL-60 (acute myelocyte leukemia), its multi-drug-resistant sub-line HL-60/Dox, K-562, and LAMA-84 (chronic myeloid leukemias), were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ GmbH, Braunschweig, Germany). They were cultured in controlled environment – RPMI-1640 liquid medium supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine, in cell culture flasks, housed at 37 °C in an incubator 'BB 16-Function Line'

Heraeus (Kendro, Hanau, Germany) with humidified atmosphere and 5% CO_2. Cell cultures were maintained in logarithmic growth phase by supplementation with fresh medium two or three times weekly. The mdr-phenotype of HL-60/Dox was maintained by culturing cells in the presence of 0.2 μM doxorubicin. In order to avoid synergistic interactions HL-60/Dox were maintained in anthracycline-free medium (90% RPMI-1640, 10% FCS) for at least 72 h prior to the cell viability experiments.

4.2.3. Cytotoxicity assessment (MTT-dye reduction assay)

The cell viability after exposure to the tested compounds was assessed using the standard MTT-dye reduction assay as described by Mosmann [36] with minor modifications [37]. The method is based on the reduction of the yellow tetrazolium dye MTT to a violet formazan product via the mitochondrial succinate dehydrogenase in viable cells. In brief, exponentially growing cells were seeded in 96-well flat-bottomed microplates (100 μl/well) at a density of 1×10^5 cells per ml and after 24 h incubation at 37 °C they were exposed to various concentrations of the tested compounds for 72 h. For each concentration at least 6 wells were used. After the incubation with the tested compounds 10 μl MTT solution (10 mg/ml in PBS) aliquots were added to each well. The microplates were further incubated for 4 h at 37 °C and the MTT-formazan crystals formed were dissolved through addition of 100 μl/well 5% HCHO-acidified 2-propanol. The MTT-formazan absorption was registered using a microprocessor controlled microplate reader (Labexim LMR-1) at 580 nm. Cell survival fractions were calculated as percentage of the untreated control. In addition IC_{50} values were derived from the concentration–response curves.

4.2.4. Apoptosis assay

The hallmark for apoptosis oligonucleosomal DNA fragmentation was examined using a commercially available 'Cell-death detection' ELISA™ kit (Roche Applied Science). This method allows semi-quantitative determination of the characteristic for the apoptotic process histone-associated mono- and oligonucleosomal DNA fragments using 'sandwitch' ELISA. Briefly, exponentially growing LAMA-84 cells were exposed to varying concentrations of compound **5** and thereafter cytosolic fractions of 1×10^4 cells per group (treated or untreated, in quadruplicate) served as an 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 manufacturers' instructions at 405 nm, using a microplate reader (Labexim LMR-1). The results are expressed as the oligonucleosome enrichment factor (representing a ratio between the absorption in the treated vs. the untreated control samples).

4.3. Data processing and statistics

The cell survival data were normalized as percentage of the untreated control (set as 100% viability), were fitted to sigmoidal dose response curves and the corresponding IC_{50} values (concentrations causing 50% suppression of cellular viability) were calculated using non-linear regression analysis (GraphPad Prizm Software for PC). The statistical processing of biological data included the Student's *t*-test whereby values of $p \leq 0.05$ were considered as statistically significant.

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