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ORIGINAL ARTICLE

Cytotoxic activity of new racemic and optically active *N*-phosphonoalkyl bicyclic *β*-amino acids against human malignant cell lines

Petar T. Todorov · Diana W. Wesselinova · Nikola D. Pavlov · Jean Martinez · Monique Calmes · Emilia D. Naydenova

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Abstract The cytotoxic effects of novel racemic and optically active constrained *N*-phosphonoalkyl bicyclic *β*-amino acids were tested against a panel of human tumor cell lines. All of the compounds investigated exhibited different concentration-dependent antiproliferative effects against the HT-29, MDA-MB-231, HepG2 and HeLa cell lines after 24 h treatment. The most sensitive cells were the HeLa cells at various concentrations of the four compounds tested. The aminophosphonate **3** exerted the most pronounced antiproliferative effect against the HeLa cells (inhibition of the cell vitality up to 70% at 0.5 mg/ml) and was not toxic to the normal Lep3 cells at lower concentration. Furthermore, the *N*-phosphonophenyl derivatives **1** and **2** displayed antiproliferative effect against mainly the MDA-MB-231 tumour cells at higher concentration.

Keywords Cytotoxic activity · Human tumor cell lines · Aminophosphonates · *N*-phosphonoalkyl *β*-amino acids

P. T. Todorov (✉) · N. D. Pavlov · E. D. Naydenova
Department of Organic Chemistry, University of Chemical Technology and Metallurgy, 1756 Sofia, Bulgaria
e-mail: pepi_37@abv.bg

D. W. Wesselinova
Institute of Experimental Morphology, Pathology and Anthropology with Muzeum, Bulgarian Academy of Sciences, Acad. G. Bonchev Street, Bl. 25, 1113 Sofia, Bulgaria

J. Martinez · M. Calmes (✉)
Institut des Biomolécules Max Mousseron (IBMM), UMR 5247, CNRS-Université Montpellier 1 et 2, Université Montpellier 2, place E. Bataillon, 34095 Montpellier Cedex 5, France
e-mail: Monique.Calmes@univ-montp2.fr

Introduction

The α-aminophosphonic acid and aminophosphonate derivatives are considered as an important class of amino-acid mimetics. They have reached a position in the research works aimed at discovery, understanding, and modification of physiological processes in living organisms (Rozenfeld et al. 2003; Hirschmann et al. 1994; Naydenova et al. 2010; Troev 2006). The α-aminophosphonic acids are found to effectively compete with their amino acid counterparts for binding to enzyme active centers or other cellular targets (Kafarski and Lejczak 2001). Thanks to the tetrahedral configuration at the phosphorus atom, α-aminophosphonic acids act as stable analogues of the unstable tetrahedral-carbon transition state in peptide hydrolysis and therefore act as enzyme inhibitors (Kafarski and Lejczak 2000, 2001; Orsini et al. 2010). Compounds of this class are currently attracting interest in organic and medicinal chemistry. 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 (Naydenova et al. 2010; Orsini et al. 2010). The variety of biological activities expressed by aminophosphonic acid derivatives is high. In quite different fields such as agriculture and human health, several applications such as enzyme inhibitors, antifungal agents, herbicides, plant growth regulators and pesticides, immune system activators, neuroactive, antitumour, antiviral, and antibacterial compounds have been reported (Kafarski and Lejczak 2001; Naydenova et al. 2010; Troev 2006).

Concerning the cyclic aminophosphonic acid derivatives, their interest in both synthetic and medicinal chemistry has been investigated. Thus, the synthesis of a new class of α-aminophosphonates containing an adamantly group has been reported with the aim of obtaining a new class of biological compounds (Kabachnik et al. 2009).

Furthermore, the effectiveness of novel organocatalyst based upon cyclic β-aminophosphonate derivatives has been demonstrated in asymmetric Michael additions of ketones to nitrostyrene (Widianti et al. 2010). In medicinal chemistry, a series of cyclic aminoalkyl phosphonates exhibiting antitumour activity on human cell lines—A549, HeLa, Hep2, and LoVo—has been recently reported (Wang et al. 2008). At the same time, we have reported structure–activity relationship of 1-[(dimethoxyphosphono)methylamino]cycloalkanecarboxylic acids with 5-, 6-, 7-, 8- and 12-membered rings as potential antineoplastic agents (Naydenova et al. 2008). Our group determined 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 cytotoxic activity. The results obtained revealed that the increased number of methylene groups in the ring resulted in a marked augmentation of the cytotoxic activity.

In this context, we have focused our attention on the synthesis and biological evaluation of new α-aminophosphonates **1**–**4** (Fig. 1b) possessing a bicyclo[2.2.2]octane-moiety.

These compounds were prepared from an original rigid bicyclic β-amino acid: the 1-aminobicyclo[2.2.2]octane-2-carboxylic acid **I** (Fig. 1a) bearing an amino group at the bridgehead (Songis et al. 2007). Compound **I**, previously described by our group, combines the particular structural properties of constrained cyclic amino acids (Fülöp 2001; Park and Kurth 2002) and those of β-amino acids that are more resistant than α-amino acids to enzymatic degradations (Gademann et al. 1999). The interest in the bicyclic amino acids is highlighted by the publication of several investigations (Roberts et al. 1953; Wynn and Vaughan 1968; Reynolds et al. 2001; Arad et al. 2002). Some of these synthetic derivatives exhibit noticeable biological activities; for example, 2-aminobicyclo[2.2.2]octane-2-carboxylic acids selectively disturb levels of neutral amino acids in the cerebral cortex, while dihydroxylated 1-aminobicyclo[2.2.2]octane-4-carboxylic acids have been used as scaffolds for antiviral agents (Zand et al. 1974; Smith et al. 1999).

Herein, we describe the antitumor activity of new racemic and optically active constrained *N*-phosphonoalkyl bicyclic β-amino acids: i.e. (2*RS*,1′*RS*)/(2*RS*,1′*SR*)-, (2*R*,1′*R*)/(2*S*,1′*S*)-, and (2*R*,1′*R*)/(2*R*,1′*S*)-1-(*N*-dimethoxyphosphono(1′-phenyl)methyl) aminobicyclo[2.2.2]octane-2-carboxylic acid (**1** and **2**), and (*RS*)-, (*R*)-1-(*N*-dimethoxy phosphonomethyl) aminobicyclo [2.2.2]octane-2-carboxylic acid (**3** and **4**).

Materials and methods

All reagents were used as purchased from commercial suppliers without further purification. Solvents were dried and purified by conventional methods prior to use.

The racemic and enantiopure chiral (*R*)-1-aminobicyclo[2.2.2]octane-2-carboxylic acid **I** was prepared as previously described using a racemic or asymmetric Diels–Alder reaction between the 1-(benzyloxycarbonylamino)cyclohexadiene and the (*R*,*S*) or (*R*)-benzyl-4-(3-acryloyloxy-4,4-dimethyl-2-oxopyrrolidin-1-yl)benzoate (Calmès et al. 2005; Songis et al. 2007). The asymmetric Diels–Alder reaction, which is among the most important carbon–carbon bond forming reaction, is widely used to prepare six-membered rings with several stereogenic centers in a regio and stereo-controlled way (Carruthers 1990; Fringuelli and Tatichi 2002).

The *N*-phosphonoalkyl bicyclic β-amino acids **1**–**4** were prepared as previously described by a Kabachnik–Fields reaction by reacting the constrained bicyclic amino acid (*RS*) or (*R*)-**I** with paraformaldehyde or benzaldehyde, and dimethyl-H-phosphonate [$HP(O)(OMe)_2$] (Todorov et al. 2011).

General procedure for the preparation of the *N*-phosphonoalkyl bicyclic β-amino acids **1**–**4**

Paraformaldehyde or benzaldehyde (1.827 mmol), methanol (5 ml), and triethylamine (190 µl) were put into a three-necked flask equipped with a condenser, magnetic stirrer, thermometer and dropping funnel and argon inert. The reaction mixture was heated to reflux temperature and held there for 45 min, after which it became a clear solution. 1-Aminobicyclo[2.2.2]octane-2-carboxylic acid (*RS*) or (*R*)-**I** (1.175 mmol) and triethylamine (0.24 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 122 µl (146.5 mg, 1.331 mmol) was added to this solution for approximately 10 min. This reaction mixture was heated at 65–70°C and after maintaining this temperature for 5.5 h, it was cooled

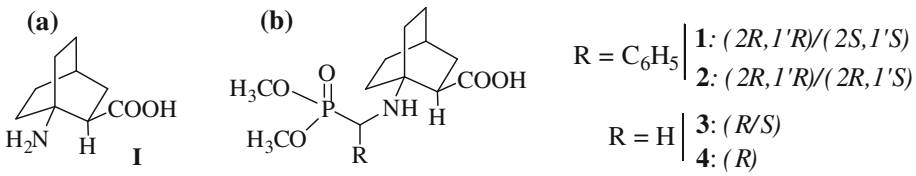


Fig. 1 **a** 1-Aminobicyclo [2.2.2]octane-2-carboxylic acid (**I**) and **b** *N*-phosphonoalkyl β-amino acids (**1–4**)

to room temperature and concentrated under reduced pressure. The crude compound was dissolved in methanol and the non-reacting (*R*)-1-aminobicyclo[2.2.2]octane-2-carboxylic acid was removed by precipitation with diethyl ether and collected by filtration. The filtrate was evaporated to give a residue, which was purified, by flash column chromatography on silica gel using dichloromethane/cyclohexane/methanol (7/2.5/0.5) as eluents to yield the expected compounds.

HPLC, MS and $^{1}H/^{13}C$ NMR data of all the compounds are identical to those previously described (Todorov et al. 2011).

Determination of the cytotoxic activity

Cytotoxicity of the substances was measured in vitro, using the following cultivated human tumour cell lines. Compounds **1**, **2**, **3** and **4** were evaluated for their cytotoxicity to human colorectal carcinoma cell line HT-29, breast cancer cells MDA-MB-231, cervical cancer cell line HeLa, human liver carcinoma cell line HepG2, and normal human diploid cell Lep3 (as controls) by using the MTS—3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt—test.

MTS test

Cells were seeded in 96-well flat-bottomed microplates (Orange Scientific) at a concentration of 2×10^4 cells/well. After 24th hour, cells were covered with DMEM medium containing different concentrations of the tested compound. Each concentration was applied in 3 wells. Samples of cells grown in non-modified medium served as a control. After 24 h incubation, the MTS colorimetric assay of cell survival was performed as described in the protocol of "Promega". This consisted of 24 h incubation with MTS solution at 37°C under 5% carbon dioxide and 95% air. The absorbance of each well at 490 nm was read by an automatic microplate reader (Absorbance Reader "Tecan"/Austria). Relative cell viability, expressed as a percentage of the untreated control (100% viability), was calculated for each concentration. Concentration-response curves were constructed manually for each experiment. All data points represent an average of three independent assays.

All cells were incubated with tenfold dilutions of 5 mg/ml (mother solution).

CellTiter 96 Non-Radioactive Cell proliferation assay, Technical Bulletin #TB112, Promega Corporation USA. Revised 12/99.

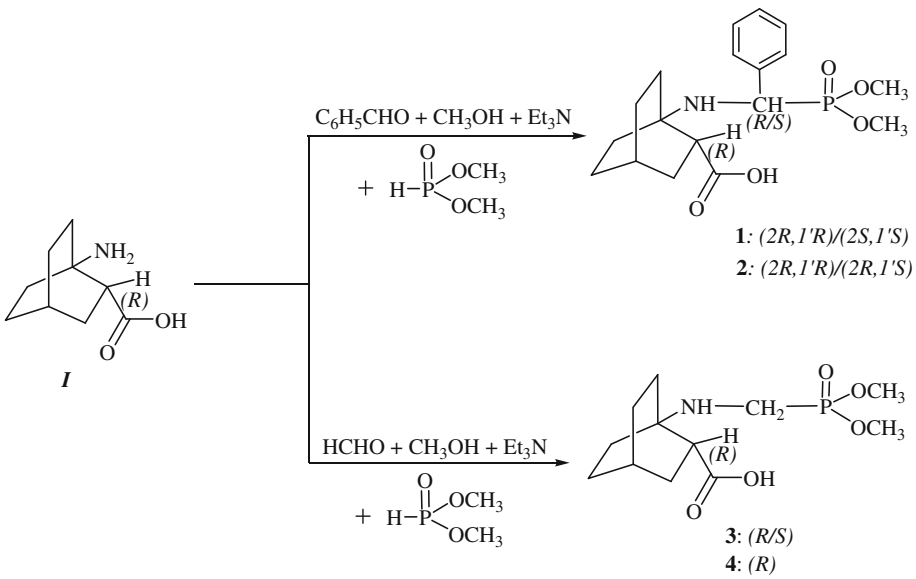
Statistical analysis

Statistical deviations were calculated automatically by Excel 2007 software program and the IG_{50}—by the "Origin" PC-program.

Results and discussion

Chemistry and biology

This work is a continuation of our previous study on the synthesis, genotoxic, antiproliferative, and cytotoxic effects of new α-aminophosphonic acids (Naydenova et al. 2008, 2010). The newly synthesized compounds **1**–**4**, presented in Scheme 1, have been prepared from compounds **I** by a Kabachnik–Fields reaction according to the procedure described by us (Todorov et al. 2011).



Scheme 1 Synthetic pathway for synthesis of the novel *N*-phosphonoalkylbicyclic *β*-amino acids **1**–**4**

The 1-aminobicyclo[2.2.2]octane-2-carboxylic acid (*RS*)- or (*R*)-(**I**) was prepared following the previously described procedure (Akkari et al. 2004a, b; Calmès et al. 2005; Songis et al. 2007).

The investigated compounds **1–4** were evaluated for their cytotoxicity against a panel of four tumor cell lines of human origin (HT-29, MDA-MB-231, HepG2 and HeLa cell lines) and normal human diploid cells Lep3 (as controls) by using the MTS—dye reduction assay. The results are shown in Figs. 2, 3, 4, 5, 6 and Table 1. As expected, the different cell lines reacted to the incubation with the separate compounds in different ways.

In all events where the vitality was around 100% the *N*-phosphonoalkyl derivatives **1–4** were not toxic at their experimental concentrations and did not stimulate the cells to proliferate (for example Fig. 2, substance **1** in concentrations below 0.5 mg/ml; Fig. 3, substance **3** in concentrations below 0.5×10^{-1} mg/ml; Fig. 5, substances **2** and **3** in lower concentrations). Vitality above 100% means an induction of the proliferative response (for example, Fig. 2, substances **2** and **4** in all concentrations above 0.5 mg/ml; Fig. 3, sub-

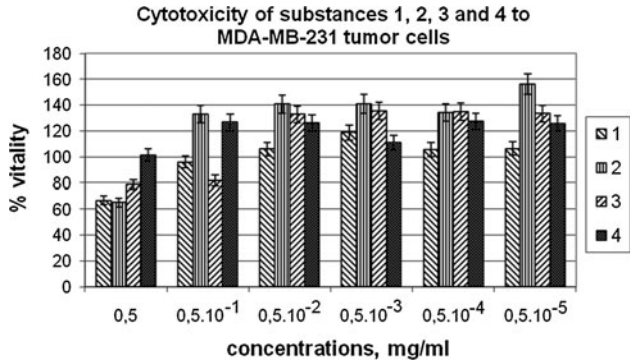


Fig. 2 Cytotoxic activity of tested compounds **1–4** in a breast cancer cells MDA-MB-231 after 24 h treatment

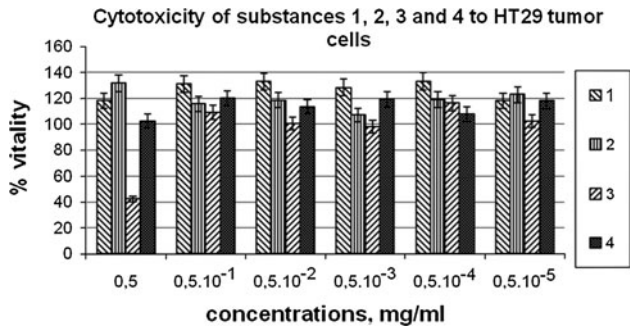


Fig. 3 Cytotoxic activity of tested compounds **1–4** in a human colorectal carcinoma cell line HT-29 after 24 h treatment

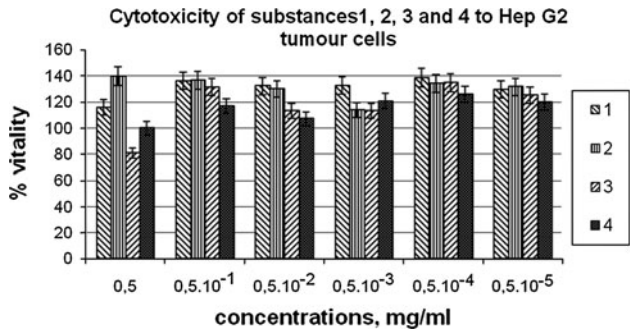


Fig. 4 Cytotoxic activity of tested compounds **1–4** in a human liver carcinoma cell line HepG2 after 24 h treatment

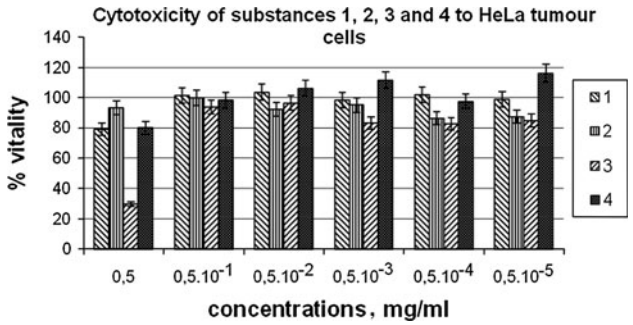


Fig. 5 Cytotoxic activity of tested compounds **1–4** in a cervical cancer cell line HeLa after 24 h treatment

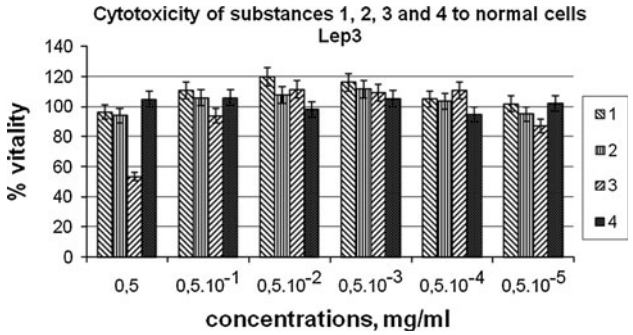


Fig. 6 Cytotoxic activity of tested compounds **1–4** in a normal human diploid cell Lep3 after 24 h treatment

Table 1 In vitro cytotoxicity after 24 h treatment

Cell lines	$IC_{50} \pm SE$ (μM) Compounds	
	2	**4**
HT29	102.10 ± 3.29	ND
HepG2	198.00 ± 3.75	ND
HeLa	9.12 ± 2.06	ND
Lep3	ND	64.90 ± 1.84

ND cytotoxicity not detected

stance **1** and **2** in all concentrations; substance **3** in concentrations above 0.5×10^{-2} mg/ml; Fig. 4, all the substances in concentrations above 0.5×10^{-2} mg/ml; Fig. 5, substance **4** in concentrations above 0.5×10^{-2} mg/ml).

The racemic 1-(*N*-dimethoxyphosphonomethyl)aminobicyclo[2.2.2]octane-2-carboxylic acid **3** proved to be the most active cytotoxic compound among the four compounds tested with inhibition of the cell vitality up to 70% for HeLa cell lines at 0.5 mg/ml (Fig. 5). In fact, the HeLa cells were the most sensitive to the tested compounds and their vitality after exposure to compounds **2** and **3** hardly reached 80% even at low concentration (0.5×10^{-3} to 0.5×10^{-5}) (Fig. 5). Substance **1** and **4** showed milder influence to them with the vitality around or above 100%. However, we could notice that even when compound **3** was not toxic at lower concentration for normal cells Lep3, it was toxic enough at higher concentration (0.5 mg/ml) (Fig. 6). In comparison it can be noticed the absence of toxicity for normal cells Lep3 of the corresponding optically pure (*R*)-1-(*N*-dimethoxy phosphonomethyl)aminobicyclo[2.2.2]octane-2-carboxylic acid **4** at 0.5 mg/ml. This compound displayed at the same concentration a 20% antiproliferative effect on HeLa cells (Figs. 5, 6). On the other hand, *N*-phosphonophenyl derivatives **1** and **2** that are also not toxic to the normal cells at higher concentration, displayed a sufficient antiproliferative effect against both the HeLa tumour cells and mainly the MDA-MB-231 tumour cells (Figs. 2, 6).

The HT29 and the HepG2 cell lines were not influenced (cytotoxic effect) by most of the tested compounds and their vitality was above 100% in all used dilutions with exception of the highest concentration where compound **3** partially suppressed these cells. As seen in Figs. 3 and 4 using different concentrations of the *N*-phosphonoalkyl derivatives (0.5×10^{-1} to 0.5×10^{-5} mg/ml) the cell vitality was always above 100% which means that the cells undergo proliferation.

It is important to mention that IC_{50} calculations of all substances revealed best values for three of the cell lines. Nice results were obtained with compound **2** against HT29, HepG2 and HeLa cells were their calculated IC_{50} were comparatively high, not seen from the vitality calculations (see Figs. 2, 3, 4). Again HeLa cells showed best sensitivity ($IC_{50} = 9.12$ μM). Separate experiments were performed (not shown in the text) with higher concentrations 0.5 and 0.5×10^{-1} mg/ml) of the tested compounds but their cytotoxicity did not increase, respectively. Close to our results are the examinations of Wang et al. (2008). These authors tested the antitumour activity of cyclic aminoalkyl phosphonates on HeLa and Hep2 cell lines (similar to our HepG2). Some of their prepared compounds have proved to be potent cytotoxic agents against tumor cell lines by inducing 50% inhibition of the cell proliferation at micro-molar concentrations, with cytotoxicity ($IC_{50} = 0.019$–5.15 μM) comparable to that observed in our experiment for the compound **2** (Wang et al. 2008).

In summary, we have demonstrated that the newly synthesized *N*-phosphonoalkylbicyclic *β*-amino acids possessed cytotoxic activity against human malignant cell lines. Antitumour investigations of the aminophosphonates **1**–**4** were performed. As expected, the substances showed different activity depending on cell line and amount of the compound used. Overall, the HeLa cells were the most sensitive to all of tested compounds. Thus, pronounced cytotoxic effects against HeLa cells were observed for compounds **1**, **3** and **4** after 24-h incubation. The same remark can be made against MDA-MB-231 cells for compounds **1**, **2**, and **3** but mainly at 0.5 mg/ml. In the case of both HepG2 and HT 29 cells only compound **3** displayed an antiproliferative effect at 0.5 mg/ml. The normal cell line was little affected, almost identically, in all compound concentrations excepted for compounds **3** that cause a noticeable toxic effect at 0.5 mg/ml. All our results did not allow to link a specific activity (cytotoxicity or induction of the proliferative response) in the presence or the absence of a substituent near the phosphonate group.

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