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Synthesis of new phosphate derivative of benzothiazole and its inhibiting effect on two series of human neuroblastoma cell growth

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Abstract−The sodium salt of di ((1-hydroxy-2-benzothiazolium-1-yl) ethyliden-1,1-H-bisphosphonic acid) orthophosphate was synthesized and its toxicity and viability effects screened on two different human neuroblastoma cell lines. This novel derivative of benzothiazole provides a new compound in connection with research and therapeutic application for tumor cell growth inhibition. Benzothiazole was alkylated in reaction with bromoacetic acid and then converted to its H-bisphosphonic acid derivative in presence of H_3PO_3/$POCl_3$. The procedure led to formation of two molecules of corresponding H-bisphosphonic acid which attached together via a phosphate bridge. The investigated compound exhibits activities (IC_{50} value) ranging from 14-23 μM (corresponding to human neuroblastoma SK-BE (2) and SK-NM-C cells).

Key words: Benzothiazole, Bromoacetic Acid, Neuroblastoma, Viability, SK-BE (2), SK-NM-C

INTRODUCTION

Nitrogen-containing bisphosphonates (N-BPs) are known to play an important role in tumor cell growth inhibition, activation of gamma-delta T cells, treatment of osteoporosis through directly inhibiting farnesyl pyrophosphate (FPP) syntheses activity [1,2] or undecaprenyldiphosphate synthase enzymes, bone resorption diseases, cancer, immune disorders, immunotherapy, and infectious diseases. They have a high affinity for solid-phase calcium phosphate and bind strongly to the mineralized bone matrix. They are also potent activators of human and antiparasitic activity [3-10]. Numerous studies have described the ability of N-BPs to reduce the survival, profile ration, adhesion, migration, and invasion of tumor cells in vitro [11]. Most, if not all, of these antitumor effects of N-BPs in vitro are due to inhibition of (FPP) syntheses. A surprising advance has been the recognition that certain structural features can significantly enhance the activity of the compounds. It has become clear that changes to the structure of N-BPs might give rise to compounds capable of inhibiting other enzymes of mevalonate pathway that use isoprenoid lipids [12]. The phosphate-like structure substituted by a P-C-P bond is poorly metabolized by the biologic enzymes that customarily degrade foreign chemicals, causing N-BPs to circulate in and exit living organisms as the parent molecule. The hydroxyl group on the carbon atom is responsible for the affinity of N-BPs for bone surfaces [13]. Inhibition of (FPP) synthase also seems to account for the adverse effects of N-BPs in vivo and for the antitumor effects of N-BPs in vitro. The more potent N-containing bisphosphonates such as Pamidronate (Aredia®), Alendronate (Fosamax®), Zoledronate (Zometa®), Ibandronate (Boniva®) and Residronate (Actonel®) represent important drugs currently used to treat conditions such as osteoporosis, Paget's disease, and hypercalcemia due to malignancy [14,15].

Recently, two kinds of hydrolytically activated chemotherapeutic pro-drugs containing bisphosphonate, a bone-targeting moiety, were synthesized [16] due to increased mode of action and strength of the drugs. Also, the groups link to the carbon atom of the P-C-P chain influence the pharmacokinetics and the mode of action/strength of the drugs, respectively. In this study, benzothiazole (I) was alkylated using bromoacetic acid (II). The resulting compound 1-carboxymethyl benzothiazolium bromide (III) was reacted with H_3PO_3 (IV) and $POCl_3$ (V). Subsequent neutralization of reaction mixture led to formation of the sodium salt of di ((1-hydroxy-2-benzothiazolium-1-yl)ethyliden-1,1-H-bisphosphonic acid) orthophosphate (VI). We observed a shift to higher field of ^{31}P chemical shift, and large coupling constant ($^1J_{P\text{-}H}$=602 Hz) in ^{31}P-NMR and ^1H-NMR spectra related to compound (VI). Both of them were good evidences of the formation of H-BP, instead of BP product. H-BPs are not stable under hydrolytic conditions. So, the optimization of pH was the key step of the procedure. The structural similarity of H-BPs and pyrophosphates is very important. Valuable results were achieved via in vitro investigations in which the compound (VI) was treated with SK-BE (2) and SK-NM-C human cells. These re-

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sults are being reported here for the first time.

EXPERIMENTAL

All reagents used were purchased from Merck Company. All chemicals were reagent grade and were without further purification. The ^{1}H-NMR, ^{13}C NMR and ^{31}P-NMR were recorded with a Bruker 300 Avance Spectrometer in D_2O. IR spectra were obtained with an FT-IR (Jasco FT/IR-410) spectrophotometer. LC-Mass spectra were recorded with an Agilent-MSD ion trap-VL. The results of flame photometer were extracted from 410-Flame photometer (Sherwood Scientific).

1. Carboxymethyl Benzothiazolium Bromide (III)

Bromoacetic acid (2 mmol) was added to a solution of benzothiazole (2 mmol) in ethyl acetate (3 mL) and the reaction mixture stirred at room temperature for 48 hr., giving1-carboxymethyl benzothiazolium bromide as a pink precipitate. This was then filtered, washed with ethyl acetate (2×3) and dried in vacuum.

2. Di ((1-hydroxy-2-benzothiazolium-1-yl) Ethyliden-1,1-H-bisphosphonic Acid) Orthophosphate-sodium Salt (VI)

The resulting pink powder (III) was added to a mixture of H_3PO_3 (5 equivalents) and toluene (6 mL) and heated to 80 °C with stirring. After all solids had melted, $POCl_3$ (5 equivalents) was added dropwise and the reaction mixture vigorously stirred at 80 °C for 5 h. Upon cooling the supernatant was decanted and 6 N HCl (3 mL) added to the residue. The resulting solution was refluxed for 1 h, then most of solvent was removed in vacuum. After that, 2-Propanol (25 mL) was added to the reaction mixture and the yellowish oily product was neutralized with NaOH and then crystallized as the sodium salts from H_2O/2-PrOH.

3. Cell Culture

The human neuroblastoma SK-BE (2) and SK-N-MC cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) supplemented with FBS (10%, v v^{-1}). Cells were maintained in a humidified incubator supplied with 5% CO_2 for pH control and 95% air at 37 °C on tissue culture dishes. Cells were subcultured as needed by detaching the cells with 2.0 to 3.0 ml of Trypsin-EDTA 0.25% solution to flask and observe cells under an inverted microscope until cell layer was dispersed (usually within 5 to 15 minutes). For measuring the toxicity and viability the MTT assay was used. Cells were seeded in 96-well plates and each plate was seeded at 500,000 cells per well. After incubation for 24 and 48 hours, the cell culture media were aspirated; 150 ml of MTT (5 mg ml^{-1}) was added to each well and incubated for 4 hours. Afterwards, 100 ml of the MTT solubilization solution (isopropanol) was added to each well. The resulting purple formazan crystals were dissolved in acidic isopropanol and quantified by measuring absorbance at 630 nm and 490 nm using an ELISA micro plate reader. Data were calibrated to the appropriate calibration curve.

RESULTS AND DISCUSSION

Neuroblastoma is a tumor derived from the neural crest and is the third most common extra cranial solid neoplasm in children [17]. Metastasis at diagnosis is common in patients with neuroblastoma and sites most frequently involved include bone marrow, bone, lymph bodes, liver, and intracranial and orbital sites. Neuroblastoma rarely metastasizes to the lungs, brain, [18] or spinal cord; most develops from nerve cells inside the abdomen or chest. This type of cancer is most commonly diagnosed during early infancy. Osteoclasts are recruited by neuroblastoma cells that are present in the bone marrow and are involved in the formation of osteolytic lesions. Recent studies suggested direct antitumor effects of bisphosphonates. Investigations show that bisphosphonates could be clinically useful in patients with neuroblastoma who have developed bone metastases [19]. In the series of imidazolyl derivatives, the influence of three different ways to attach an imidazole ring to the carbon chain linker was previously investigated (A_1, A_2, A_3 in Fig. 1) [19]. Comparing the antiresorptive potency of these bisphosphonate compounds revealed the most active BP known to date has been obtained by linking the imidazole ring via a nitrogen atom to the bone hook (A_1). Based upon experimental results, bioactivity potency improves with increasing size and lipophilicity of the amino group, and the most attractive compounds are those that have a heteroaromatic substituent [19,20]. On the other hand, when the bioactive moiety is linked via a nitrogen atom to the bone hook, the hydroxyl group reduced the stability of the molecules. Those compounds that bear thiazole substituent are of specific interest. The 2-thiazolyl derivative (B) had potency comparable to that of Alendronate or at least 10 times that of pamidronate. Potency is strongly relying on substituent in posi-

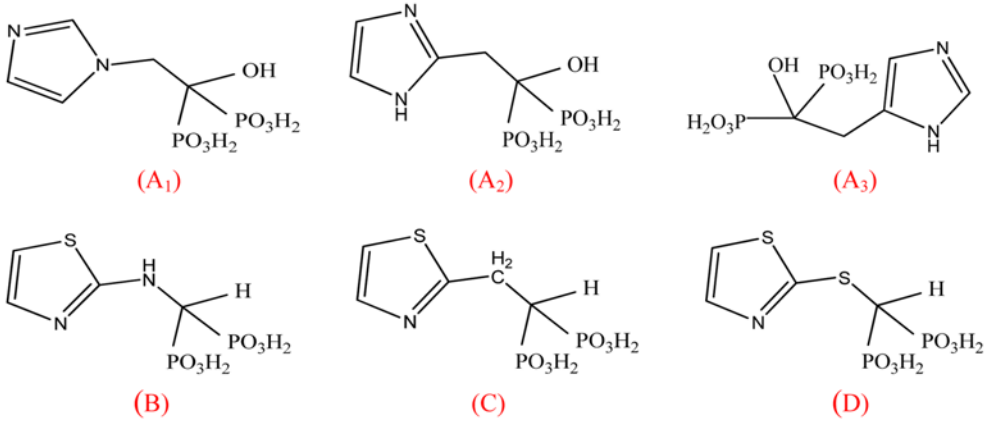
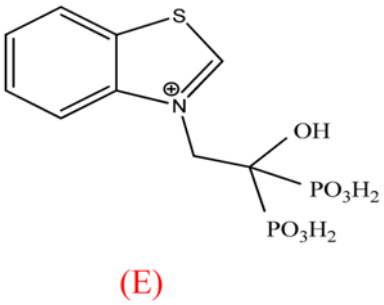


Fig. 1. (A_1, A_2 and A_3): Three different ways to attach an imidazole ring to the carbon chain linker; (B, C and D): Bisphosphonic acids containing a thiazole linked via different atoms.



(E)

Fig. 2. Molecular structure of compound (E).

tions 4 and 5, which may affect the serious interaction of basic amino functionality with the putative molecular target. Substitution of nitrogen linker atom of thiazole derivative (B) by a methylene or a sulfur bridge eventuated to compounds (C) and (D), which unexpectedly are much less potential (Fig. 1) [19].

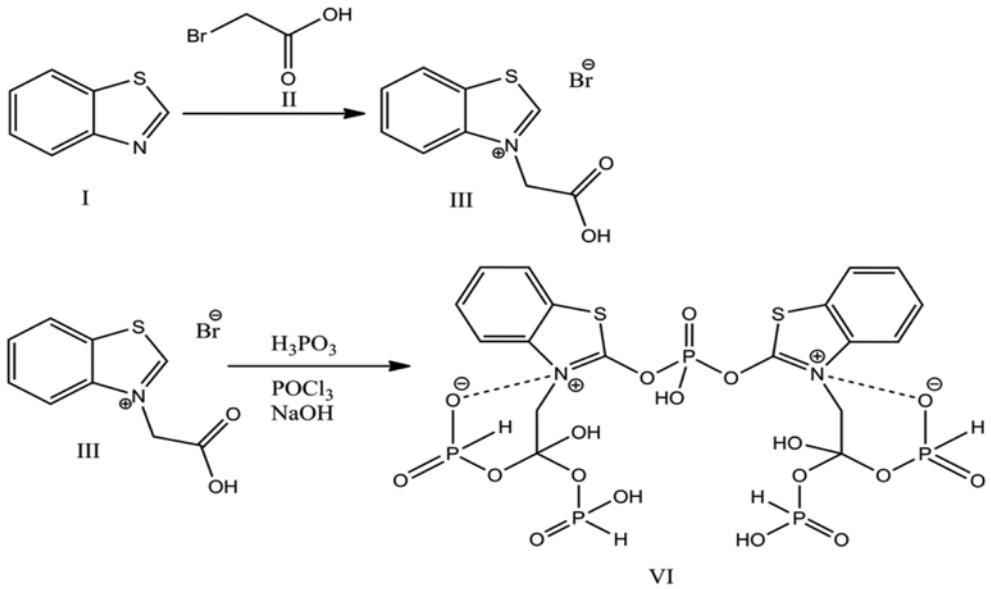
So, less bioactivity potency of BP compounds supposedly results from substitution of hydrogen and NH by OH and methylene groups, respectively. To compensate this negative effect, we decided to change the location of methylene linkage from 2-position to 1-status in those thiazolyl derivatives that contain both hydroxyl and methylene groups.

As said before, experimental data resulting from imidazole derivatives showed bioactivity was improved by the attachment of heteroaromatic ring to the carbon chain linker. So, we planned, and were interested, to study the synthesis and bioactivity potency of an individual molecule with molecular structure of (E) (Fig. 2).

In spite of what was expected, experimental results displayed a different reaction pathway and therefore unexpected bioactivity potency of resulted product. By choosing benzothiazole as initial material, reaction proceeded by phosphorylation of carboxyl group and was completed with formation of a phosphate bridge between two molecules of benzothiazolyl-1-yl methylene H-phosphonic acid (VI) (Scheme 1). To demonstrate our concept, we report here the design, synthesis and the vitro evaluation of the sodium salt of di ((1-hydroxy-2-benzothiazolium-1-yl)ethyliden-1,1-H-bisphosphonic acid) orthophosphate (VI) and screened the toxicity of this new type of H-bisphosphonates on two series of neuroblastoma cell lines. At the first step, the reaction of benzothiazole (I) and bromoacetic acid (II) yielded 3-carboxymethyl benzothiazolium bromide (III) as white needles. Subsequent reaction of (III) with H_3PO_3 (IV) and phosphorus trichloride (V) led to formation of an oily product which was neutralized with NaOH, then crystallized as the sodium salt from H_2O/2-PrOH. Spectroscopic data showed that dimerization took place through a phosphate bridge moiety. Compound (VI) was synthesized as shown

Table 1. Spectral data of newly prepared compounds

Compound	Spectral data
III	IR (KBr) (υ/cm^{-1}): 1574, 1625 (C=C, aromatic), 1733 (C=O), 2927 (CH_2, bending), 3026 (C-H, aromatic), 3447 (OH) ^{1}H-NMR (300 MHz, D_2O): δ 5.44-5.47 (2H, CH_2), 7.72-8.2 (4H, Aromatic), 10.21-10.24 (1H) ppm ^{13}C-NMR (300 MHz, D_2O): δ 54.25 (CH_2, 1C), 116.78-140.73 (aromatic, 6C), 164.13 (C=N), 169.69 (C=O) ppm
VI	IR (KBr) (υ/cm^{-1}): 1013 (P=O), 1455 (CH_2), 1570-1657 (C=C), (aromatic), 2925 (CH_2, bending), 3370 (OH) ^{1}H-NMR (500 MHz, D_2O): δ 5.61 (s, 2H, CH_2), 6.2-7.4 (d, J=602 Hz, P-H), 7.69-7.72 (t, J=9 Hz, H), 7.77-7.80 (t, J=9 Hz, H), 7.99-8.01 (d, J=6 Hz, H), 8.16-8.18 (d, J=6 Hz, H), 10.28 (s, P-OH) ppm ^{31}P-NMR (300 MHz, D_2O): δ 1.93-6 (P-H, d, J=602 Hz), 6.59 (P) ppm LC Mass: m/z=772 (acidic media) (M+1)) Flame photometer: Na (average=370 ppm, 0.05 gr. sample, 10 ml H_2O)



Scheme 1. Synthetic reaction pathway for preparation of compound (VI).

in Scheme 1. Temperature and pH controlling prevented the phosphonic-phosphate rearrangement [21-26].

The product in each step was characterized and confirmed by spectroscopic methods (IR, ^{1}H-NMR, ^{13}C-NMR, ^{31}P-NMR, LC-Mass and Flame photometer).

Larger amino group size as well as alkaline condition which removed total positive charge in molecular structure of (VI) led to an increasing of lipophilicity. The new pro-drug has been shown to reduce the growth of SK-BE (2) and SK-NM-C cell lines in vitro (Figs. 3 and 4).

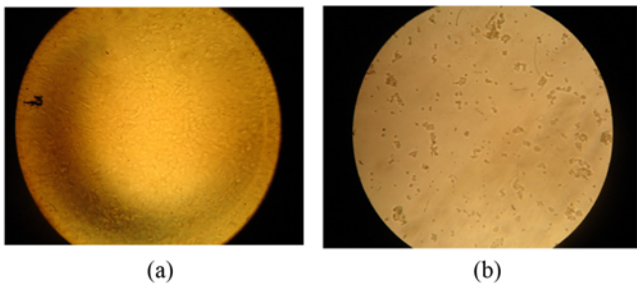


Fig. 3. Microscopic observation shows the new compound (VI) defected the SK-BE (2) cells after injection; (a) normal, (b) after injection.

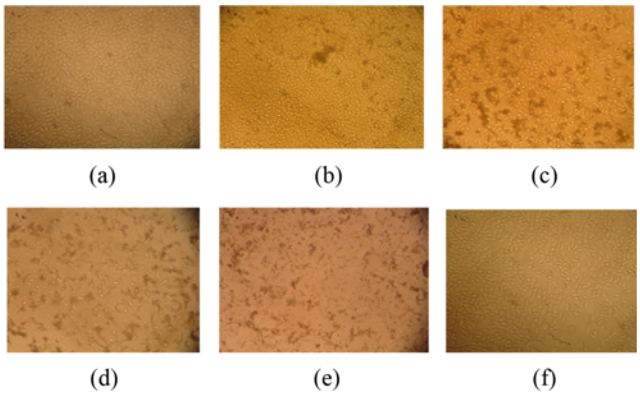


Fig. 4. Microscopic observation of the effects of new compound (VI) on human neuroblastoma SK-NM-C cells in different concentrations; (a) 5 mg, (b) 10 mg, (c) 15 mg, (d) 20 mg, (e) 25 mg, (f) 30 mg.

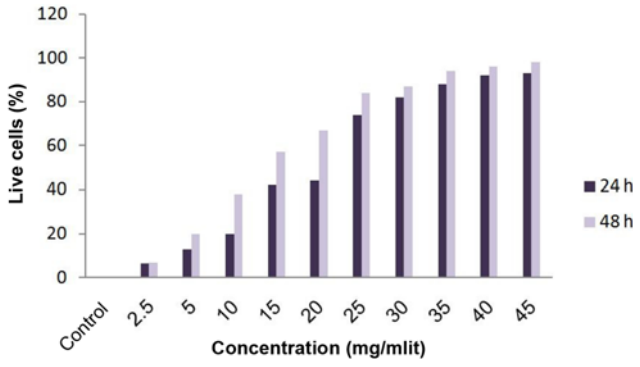


Fig. 5. Toxicity effect of the new compound (VI) on human neuroblastoma SK-BE (2) cells (in 24 h and 48 h). IC_{50} was measured as 12 mg ml^{-1}.

The SK-NM-C cell line was more sensitive than the other one in the same condition. After 72 h, GI concentrations (inhibiting cell growth by 50% compared to untreated controls) for (VI) ranged from 14-23 μM (Figs. 5-8).

At high concentrations of [OH^{-}], ring opening took place with gas (H_2S) evaluation. These results are of interest because they represent the first report of the activity of a novel H-bisphosphonic acid derivative with a phosphate bridge against two series of human neuroblastoma cell growth.

On the other hand, the effect of compound (VI) on the activity of acetyl cholinesterase was measured by using Ellman's method. The measuring of absorbance showed that (VI) acted as a good in-

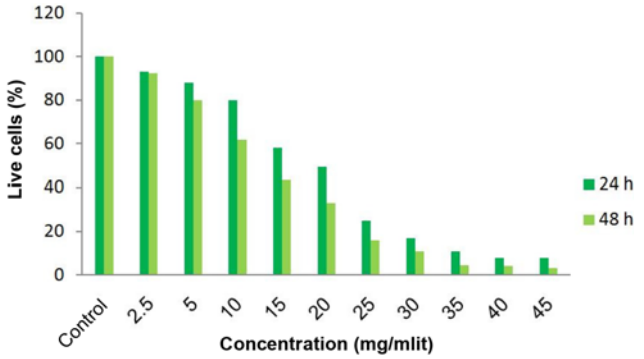


Fig. 6. The viability of SK-BE (2) cell growth at the risk of different concentration of compound (24 h and 48 h).

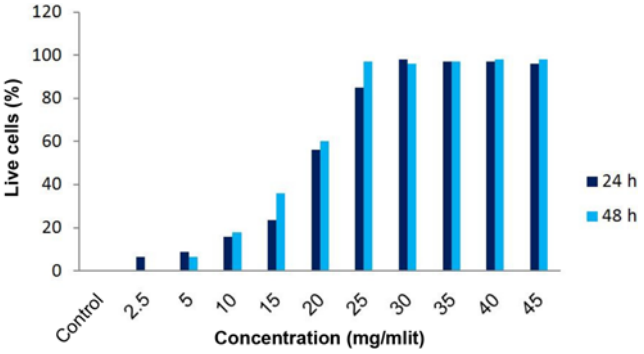


Fig. 7. Toxicity effect of the new compound (VI) on human neuroblastoma SK-NM-C cells (in 24 h and 48 h). IC_{50} was measured as 12 mg ml^{-1}.

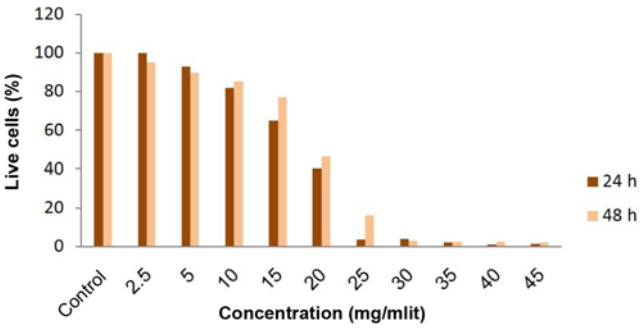


Fig. 8. The viability of SK-NM-C cells growth at the risk of different concentration of compound (24 h and 48 h).

hibitor for acetyl cholinesterase.

CONCLUSIONS

Bisphosphonates, and in particular N-BPs, which are among the leading agents with well-demonstrated antiosteoclastic activity, have shown promising results against several diseases associated with increased bone remodelling like osteoarthritis and metastatic cancer [2]. We synthesized the sodium salt of di((1-hydroxy-2-benzothiazolium-1-yl)ethyliden-1,1-H-bisphosphonic acid) orthophosphate (VI) as a new member of phosphonates and screened its toxicity and viability on two different human neuroblastoma cell lines. So benzothiazole was alkylated using bromoacetic acid, and 1-carboxymethyl benzothiazolium bromide (III) was obtained as white powder. This product was then converted to the corresponding phosphate derivative in the presence of H_3PO_3/$POCl_3$. The resulting oily product was neutralized by NaOH solution and yielded compound (VI) in 95%. For the first time, the procedure yields a dimer of corresponding H-bisphosphonate with five phosphor atoms. The data emphasize unique antitumor effect of (VI) in vitro and activities (IC_{50} value) ranging from 14-23 μM (corresponding to human neuroblastoma SK-BE (2) and human neuroblastoma SK-NM-C cells). Low viability and great efficacy have been shown by (VI). The structure of product in each step was characterized and confirmed by spectroscopic methods (IR, ^{1}H-NMR, ^{31}P-NMR, LC-Mass and Flame photometer).

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