In vitro assay of the biological activity of bisphosphonates

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Abstract: *Nowadays, the use of bisphosphonates are the gold standard of treatment of bone diseases. Due to the high utility of these compounds, scientists still search for better and better structures, with a variety of substituents at carbon's atom and create new libraries or improve methods of their synthesis. To determine the potential effect of newly discovered bisphosphonates, for example antiosteoporotic activity, there are in vitro tests which allow to determine the half maximal inhibitory concentrations (IC_{50}) of cells. This paper describes in detail the methodology for test the biological activity of bisphosphonates. Shows the results of biological activity for synthesized bisphosphonates in relation to the commercial-incadronate and zoledronate.*

Keywords: *bisphosphonates, half maximal inhibitory concentration, cytotoxicity*

1. Introduction

Bisphosphonic acids are a group of valuable organic compounds. Bisphosphonates (BPs) are analogues of pyrophosphonate (PPi), which have stable, non-hydrolysable structure. Both are dimmers of two rests of phosphorous acid with this difference that PPi consists oxygen atom in the place of binding two monomers and bisphosphonate contains atom of carbon in that place. Present of carbon prevents bisphosphonates by hydrolysis. [1][2]

First bisphosphonic derivatives contained simple substituents as H, OH, Cl and CH_3 groups. Modern bisphosphonates consists long aliphatic chains or heterocyclic rings. Almost all of them have more or less influence on bone diseases' prevention.[3][4]

Ability to chelation of metal ions, which has charge +2, is caused by two ionized hydroxyl groups of rests of phosphorous acid. Because of that bisphosphonates are able to bind with hydroxyapatite (HAp) in bones (mineral salt with formula $Ca_5(PO_4)_3OH$).[4/5] Only ionized form of bisphosphonates are biologically active.[6] [7]

Bisphosphonates are an important class of anti-resorptive drugs used in the treatment of bone diseases, including osteoporosis. There is substantial evidence that BPs can have a direct effect on osteoclasts by mechanisms that may lead to osteoclast cell death by apoptosis. BPs can also inhibit proliferation and cause cell death in macrophages *in vitro*. From macrophages cells are formed osteoclast precursor cells. There is shown that the toxic effect of BPs on macrophages is also due to the induction of apoptotic, rather than necrotic, cell death. [8]

2. Materials and methods

2.1. Methodology

Testing antiproliferative activity of bisphosphonates involves the use of *in vitro* cell cultures.[9] [10] For this purpose, it is necessary to select particular cell line - mouse, macrophage-like, J774E, which is originated from the same precursors as osteoclasts, sensitive to bisphosphonates activity and allows to determine the impact of the test compounds on cell growth.[11] For this purpose mouse, macrophage-like J774E cells originating from the same precursors as osteoclasts were used. Such cells are well recognized as being sensitive to bisphosphonates, which most likely act by inducing apoptosis. [11] [12] [13]

Such cell culture, frozen in liquid nitrogen, has a bank of cell lines in Polish Academy of Sciences in Wrocław. To use the cell line, it is need to thawed them first, using a water bath at 37 °C with gentle stirring and put them into the culture medium. The prepared line is passaged 2 times a week in a RMPI (growth medium consist of: 10% FBS, 100 μg/ml streptomycin, 100U/ml penicillin and 2mM glutamine) in a Petri dishes. The cell growth is monitored under a microscope, allowing part of the material to the next stage of the experiment, and re-freezing the residue. Afterwards the cells are separated from the bottom (the surface proteins on the plasma membrane, which allow adhesion are destroyed) and can be collected from the culture vessel. Then in a Bürker chamber the cells density is counted, using a drop of cell suspension in a mixture

ratio of 1:1 to trypan blue.

In the next step, when the cell culture is ready for testing, cells need to be transferred into test plates. For this purpose, most often used are the flat-bottom 96-well plates, which are excellent coated with the mentioned adherent cells, in the log phase of their growth, with a portion of 100µl per well, in density of about $1x10^6$/ml. To verify the process of the test, the last line in the plate is omitted from the testing compounds impact and reserved to cells growth control and control of medium. Such prepared plate is incubated for 24h at 37 °C, 5% CO_2 in a humidified atmosphere to allow optimum conditions for their adhesion to the surface.

The testing compounds need *ex tempore* preparation. For this purpose they are dissolved in universal solvent – usually DMSO or NaOH. After that, in next step, the solutions are prepared in 4 different concentrations in culture medium (Fig. 1) – for example from 1 to 1000 µg/ml, using the curve to determine with high accuracy the concentration inhibiting cell proliferation cancer cells in 50% (IC_{50}). Samples of concentrations are imposed in 3 series of repetitions, 100µl per well. Incubation of the compounds with the cells takes 72h. The experiment should be repeated at least 3 times.

Parallel there are prepared controls of impact: solvents and references (commercial bisphospohonic drugs used in the pharmacological treatment of osteoporosis, for example: zoledronate).

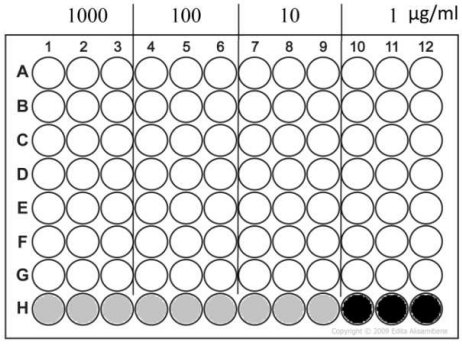

Fig. 1. Experiment on a 96-well.

A-G testing compounds, H - line control: 1-9 – the control cells, 10-12 - the control medium.

The results are read by the SRB test (intendend for adherent cells).[9] Cells were fixed by precipitation of proteins and DNA in 50% cold TCA (50µl per well), incubated at 4°C, for hour. After that, plates are rinsed 5x with tap water to delete the current acid and dried. Later, the wells are dyed by 0.4% sulfarodamine B in 1% acetic acid, 50µl per well and incubated in the dark for 30 min at room temperature. Rinse 5x with 1% acetic acid and dry. Add 150 µl TRIS buffer per well to dissolve bounded dye. Incubate 30 min in room temperature. The concentration of protein in the hole, corresponding to the number of proliferated cells. Density is automatically measured at 540 nm by spectrophotometer Synergy H4 (BioTek Instruments, USA) with zero at the absorbance of culture medium - a negative control. In next step, measured absorbance is converted to % inhibition of proliferation by ProLab-3 Programme (INFORM-TECH), and graphically performed into the curve (the dependence of inhibition of proliferation of the log concentration of test compound).

3. Calculations – Data handling

Using the repetitions experiences there are calculated the mean scores, IC_{50} and standard deviation.

$$\%\ \text{inhibition of proliferation} = \left[\left(\frac{Abs - Abs_M}{Abs_C - Abs_M}\right) x\ 100\right] - 100 \qquad (1)$$

Abs – Abs of specimen, Abs_M – Abs of medium, Abs_C – Abs of cells in medium

The obtained results are compared to the reference compounds. The accepted standard for assessment of activity of the test compounds during the screening carried out *in vitro* IC_{50} is the concentration ratio of not more than4µg/ml.[14]

The *in vitro* results were presented in terms of inhibitory concentration 50% (IC_{50}) values. The IC_{50} is the concentration of tested agent, which inhibits the proliferation of 50% of the cancer cell population. Average IC_{50} values for each preparation were calculated using data from three independent experiments.

4. Results

The bisphosphonates (1-10) obtained in Department of Bioorganic Chemistry were examined in biological activity test.

Table 1. Cytotoxic activity of the investigated bisphosphonates **1-10** against the mouse macrophage cell line J774.

	Compound		
IC_{50} (µg/ml) average ±SD	**1**	**2**	**3**
	180,86±16,62	114,12±18,81	136,82±23,46
	4	**5**	**6**
	209,17±24,78	94,78±12,87	92,86±14,48
	7	**8**	**9**
	175,11±16,21	44,9±7,43	24,88±5,17
	10	**Zoledronate**	**Incadronate**
	30,58±4,64	24,51±2,23	47,85±1,39

Results are presented as IC_{50} – the doze, which provides inhibition of proliferation in 50% of cell population (Table 1).

The figure 2 shows that synthesized bisphosphonic acids have antiosteoporosis activity. Comparing this results with control samples the antiosteoporosis potential is lower than potential of commercially used in medicine zoledronic and incadronic acids.

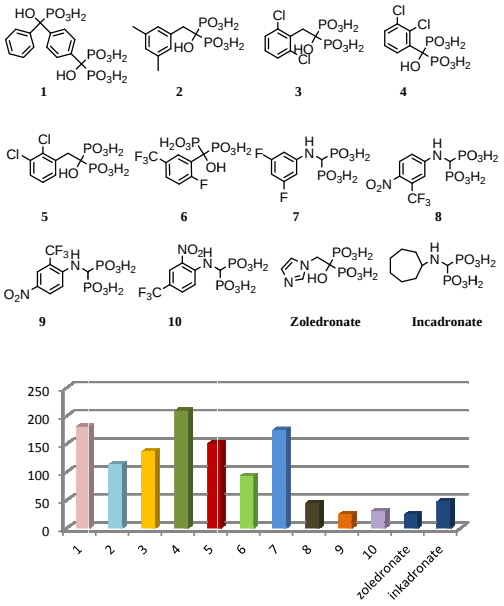

Fig. 2. Activity tests' results shown as IC_{50}.

5. Conclusions

Compounds were selected by rational selection. Selected structurally varied amino-containing bisphosphonates and hydroxyl – containing group. All tested bisphosphonates containing aromatic rings. Guided by structural similarity to commercial zoledronic and incadronic acids. The most active, in a test, proved to be compounds (on the charts): **8**, **9** and **10**.Their activity is higher than referent compound - incadronate. Compound **9** has the best profile of biological activity (inhibition of proliferation). Highly satisfactory results were obtained with compounds containing the six-membered ring in the structure. All of the ten tested compounds showed biological activity. Results shows that nitrogen atom plays the key role in antiresorption activity of bisphosphonates – all the nitrogen - containing bisphoshonates are more active than those non nitrogen. Compounds having an additional carbon between hydroxybisposphonate and aromatic ring have the better profile of activity than without carbon chain (compounds 4 and 5). Unable to determine the impact of changes in the position of substituents on the aromatic chain of the activity of the compound. The presence of fluoro atoms compared to the compound of chlorine increases the activity of bisphosphonates. The presence of the nitro group (8, 9 and 10) significantly increases the biological activity of compounds.

The data obtained suggest that the resulting compounds may be potential antiosteoporosis drugs.

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