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Analysis of Methylene-bisphosphonic Acids by *in silico* and *in vitro* Methods

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Abstract: On a global scale, Epstein-Barr virus (EBV) infects over 90% of the adult population and is responsible for ~1% of all human cancers. Fluorine is one of the most abundant elements on earth. However, it occurs extremely rarely in biological compounds. The introduction of the fluorine atom(s) into many biologically active molecules can bring about remarkable and profound changes in their properties. Development of potential drugs is closely related to *in silico* methods, which include PASS, QSAR, COMPARE-analysis and more.

The aim of this work was to analyze the potential biological activity and the target of action of derivatives of bisphosphonic acids by using *in silico* methods and examined received results by *in vitro* study. For this purpose, PASS software, web-server PharmMapper, PCR, MTT assay, trypan blue and neutral red assay were used.

According to PASS prediction two compounds (**10S20** and **10S21**) may possess antiviral activity, Pa/ Pi was 0,294/0,005 and 0,214/0,084, respectively. Also, all compounds may possess a cytochrome c as substrate. Several targets were identified by using molecular docking (PharmMapper). It was shown that a lot of possible targets are proteins, such as Gag-Pol protein (viral protein) and different kinds of protein kinases. A study *in vitro* shown anti-EBV activity for all compounds. On the other hand, derivatives of bisphosphonic acids had a high level of cytotoxicity on different lymphoblastoid cell lines.

Therefore, the *in silico* screening presents a good approach for the development of new anti-EBV agents. Our results showed, that derivatives of bisphosphonic acids may possess apoptosis modulating properties for treatment of lymphoproliferative diseases.

Keywords: Epstein-Barr virus, bisphosphonic acids, PASS, PharmMapper.

Epstein-Barr virus (EBV) is the most common and persistent virus infection in humans, with approximately 95% of the world‘s population sustaining an asymptomatic life-long infection. EBV was the first human tumour virus to be discovered. It is estimated that EBV accounts for more than 200,000 cases of cancer each year and that 1.8% of all cancer deaths is due to EBV-attributable malignancies [1, 2].

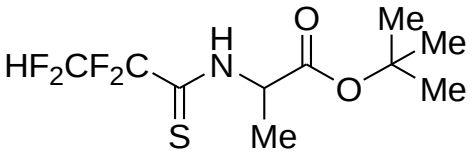
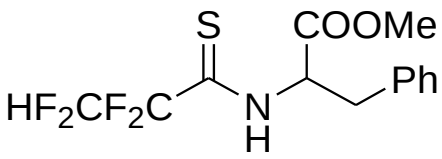
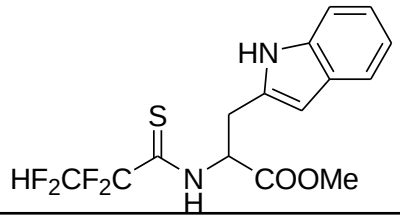
Computer-aided drug design approaches have emerged as attractive and complementary approaches to traditional high throughput screening [3]. Virtual screening has been applied to the successful identifications of biologically active molecules.

Of all commercialized pharmaceutical drugs, twenty percent contain fluorine, including important drugs in many different pharmaceutical classes. Fluorine is often added to drug molecules as even a single atom can greatly change the chemical properties of the molecule in desirable ways. Of all commercialized pharmaceutical drugs, twenty percent contain fluorine, including important drugs in many different pharmaceutical classes [4].

Phosphonates being hydrolytically stable, analogs of biogenic phosphates are widely used in antiviral drug design. Methylene-bisphosphonates (BPs) are mimics of inorganic pyrophosphate [5]. Today, these compounds have become a powerful family of pharmaceuticals for the treatment of skeletal complications of malignancy, Paget’s disease, osteoporosis, multiple myeloma, hypercalcemia and fibrous dysplasia.

With the view of finding the specific activity of these compounds, they were exploited for prediction of activity using PASS [6]. The predicted activity spectrum of a compound is estimated as Pa (probably activity) and Pi (probable inactivity).

In the present study, PASS predicted that the antiviral activity was expressed by the compound **10S20** and **10S21**. According to PASS, all studied compounds may be a substrate for cytochrome c, that might play an important role in the induction of apoptosis.

	Substances	Pa	Pi	Biological activity
10S20		0,485	0,134	CYP2H substrate
		0,308	0,005	Histone deacetylase SIRT1 inhibitor
		0,294	0,005	Antiviral (*Picornaviruses*)
10S21		0,715	0,033	CYP2H substrate
		0,341	0,057	Atherosclerosis treatment
		0,214	0,084	Antiviral (*Hepadnaviruses*)
10S22		0,397	0,219	CYP2H substrate
		0,234	0,012	Histone deacetylase SIRT1 inhibitor

Potential targets of fluorinated derivatives of methylene-bisphosphonic acids predicted by PharmMapper [7]

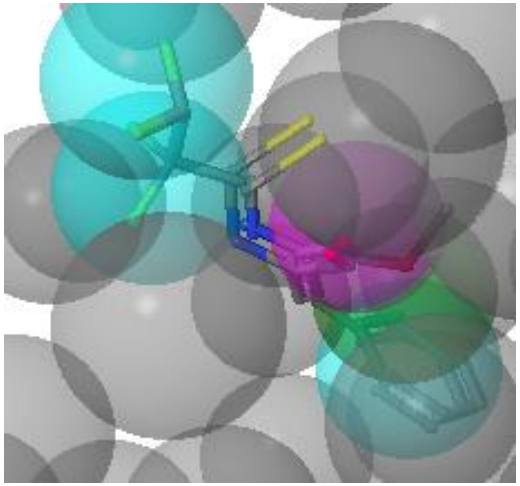
Substances 10S20		Substances 10S21		Substances 10S22	
Target name	Fit score	Target name	Fit score	Target name	Fit score
Acetylinesterase	4.771	Gag-Pol polyprotein	3.963	Leukotriene hydrolase	4.533
Dehydrogenase, mitochondrial	4.320	Cell division protein kinase 2	3.639	Gag-Pol polyprotein	4.468
Heat shock protein Hsp90-α	4.184	Serine/threonine-protein kinase	3.607	Purine nucleoside phosphorylase	4.288
Tyrosine-protein phosphatase	4.182	Mitogen-activated protein kinase 10	3.479	Protein-glutamine gamma-glutamyltransferase	4.078
Aspartate aminotranaferase	3.904	Mitogen-activated protein kinase 14	3.291	Mitogen-activated protein kinase 1	3.910

It was established, that majority of the targets are enzymes, such as protein kinases and apoptotic proteins. It was shown that compound **10S20** could interact with heat shock protein and other proteins. Both compounds, **10S21** and **10S22**, might play important role at induction of apoptosis by interacting with mitogen-activated protein kinase.

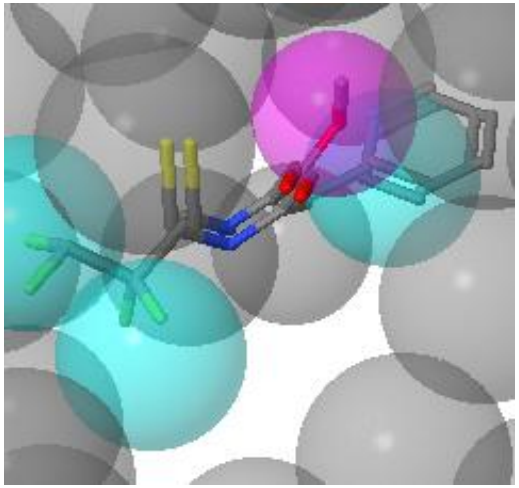
PharmMapper is a web server for potential drug target identification based on the use of a pharmacophore mapping approach. PharmMapper server works by ‘probing’ the ligand into a database of pharmacophore models of binding sites. It functions on the ligand-protein reverse docking strategy and reports potential target on the basis of normalized fit score.

Also, PharmMapper shown pharmacophore model for each target from list and helps to understand interaction between target and studied compound.

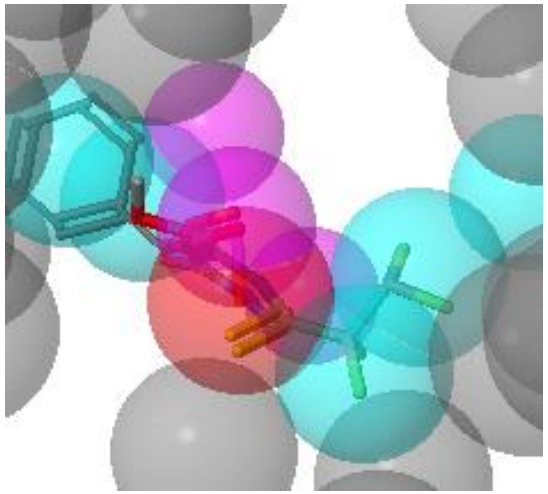
List of potential targets presents proteins, which are involved into different process. Identification of targets and prediction of possible biological activity allow to screen a large number of compounds.



Serine/threonine-protein kinase



Epidermal growth factor receptor

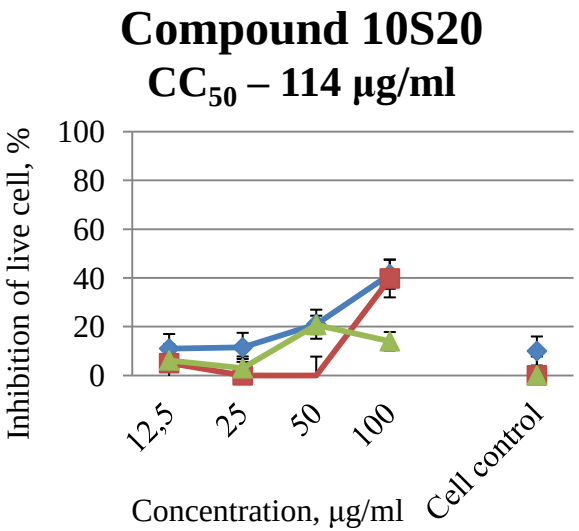


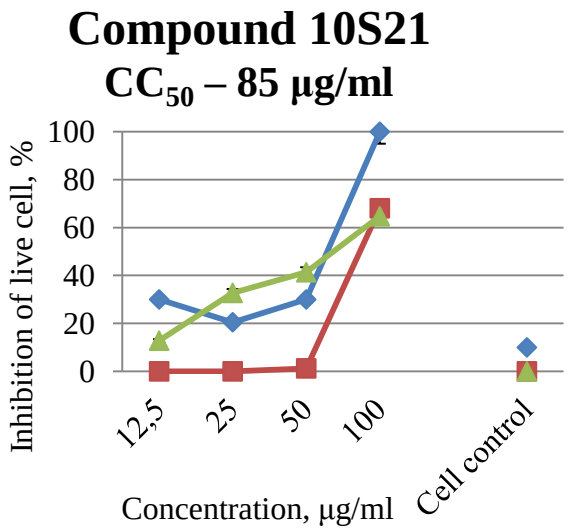
Mitogen-activated protein kinase

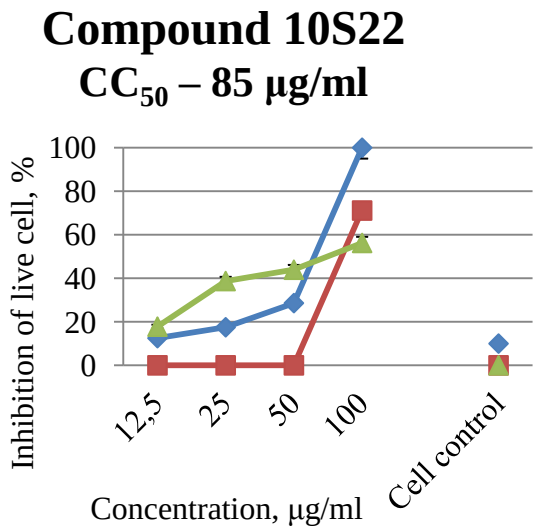
Any predicted property must be confirmed or disproved in the biological model. Accordingly, *in vitro* analysis of these compounds was carried out.

Determination of cytotoxicity of lead molecules is an integral component of any drug development process. Our results clearly show that all bisphosphonate acid derivatives are quite toxic on model Raji cell line.

Less toxic compound **10S20**, at high concentration of 100 μg/ml exhibited a percentage of inhibition cell of 40%. Compounds **10S21** and **10S22** shown a high level of cytotoxicity. Both compounds inhibited 100 % of living cells.

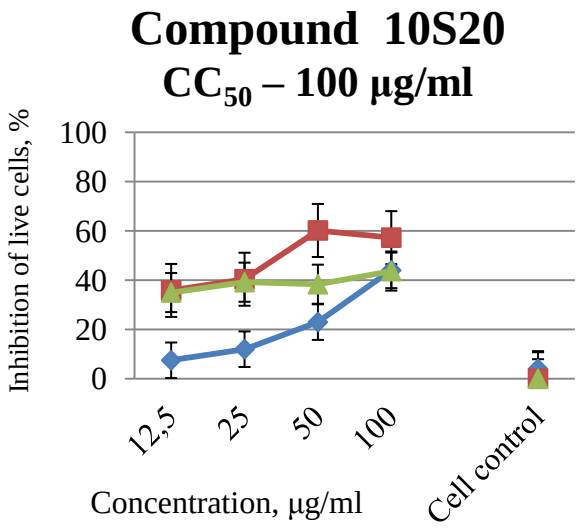


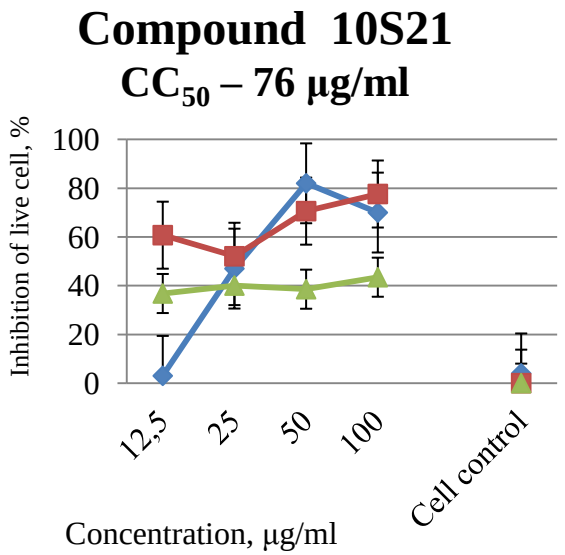


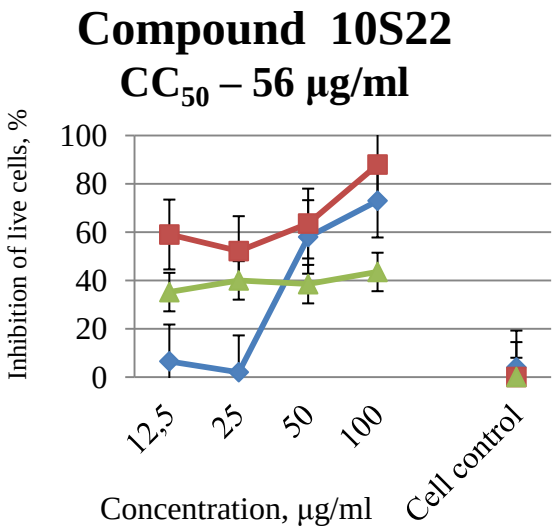


The cytotoxicity of compounds **10S20**, **10S21** and **10S22** on model Raji cell line using trypan blue (blue), MTT (red) and NRU (green) assays

The cytotoxicity of studied compounds on model B95-8 cell line used trypan blue (blue), MTT (red) and NRU (green) assay



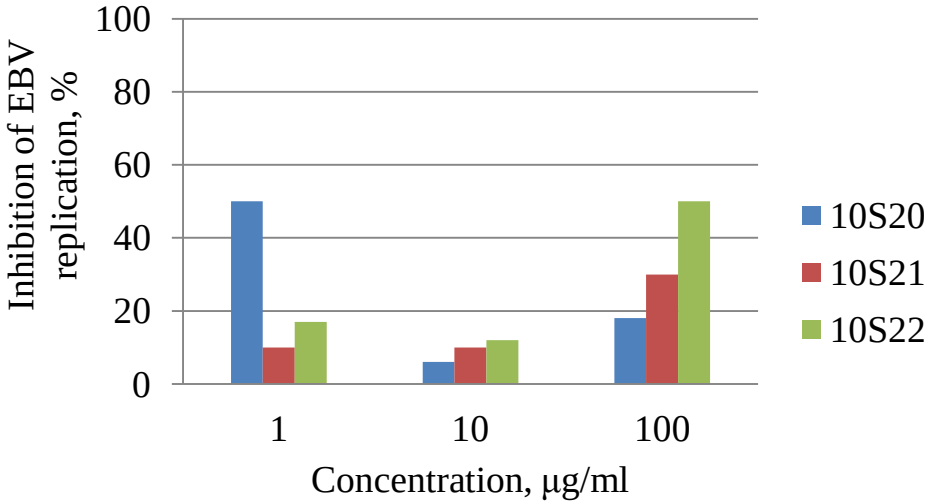




The present study showed a high level of cytotoxicity for bisphosphonate derivatives on model B95-cell line. All studied compounds inhibited living cells. The different assays showed activity on different compartments of the cell.

Thus, bisphosphonic acid derivatives may affect the mitochondrial system. Increasing inhibition of mitochondrial enzymes was detected by using MTT assay.

Antiviral activity of the test-agents was assessed by the degree of inhibition of EBV reproduction by quantitative RT- PCR method at concentrations 1-100 μg/ml for Raji cell cultures. Samples for analysis were taken after 48 h since this time point was an optimum both for the cell lines growth dynamics and for the EBV reproductive cycle.



Studied compounds	Raji CC_{50}[a]	Raji EC_{50}[b]	Raji SI[c]
10S20	114	1	114
10S21	85	20	4
10S22	85	100	1

a The 50% cytotoxic concentration of studied compound for Raji and B95-8 cell in μg/ml
b Concentration of compounds (μg/ml) producing 50% inhibition of EBV reproduction
c Selectivity index (SI) = CC_{50}/ EC_{50}

Study of an antiviral action of **10S22** against EBV in infected Raji cells showed that this compound at a maximum concentration of 100 μg/ml could effectively inhibit the viral DNA accumulation by 50%. Level of antiviral activity of compound **10S21** was lower. Increased concentrations of the compound may lead to an activation of the cell protection systems, such as DNA repair, for example. A slightly different response to increasing concentrations of the compound **10S20** was observed in the model Raji cell line. Inhibition of 50 % of EBV replication was determined at minimum concentration of 1 μg/ml.

SI is used to estimate the therapeutic effect of a drug and to identify drug candidates for further studies. Thus, compound **10S20** could be considered as promising new anti-EBV drug candidate for infection of EBV.

Conclusions

In the present study, the antiviral activity of derivatives of methylene-bisphosphonic acids was evaluated *in silico* and *in vitro* to understand their broad spectrum potential as anti-EBV agents.

According to PASS prediction and this study, it was shown that derivative of bisphosphonic acids **10S20** has a good selective index and might become a potential antiviral drug. The study demonstrates the ability of studied compounds to inhibit the virus replication machinery. Also, high level of cytotoxicity and antiviral activity of compounds **10S21** and **10S22** may contribute to the elimination of transformed cells from the population. All compounds contain amino acids in their composition, in particular irreplaceable. Thus, the effect of these compounds can be attributed to the change in the activity of the target protein.

Obtained and analyzed data let to relate the compound **10S20** to a perspective anti-EBV agent, and the **10S21** and **10S22** derivatives to apoptosis-inducing compounds that can be used in further research on antitumor action.

Reference

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