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CALIX[4]ARENE α-HYDROXYMETHYLPHOSPHONIC ACIDS AS POTENTIAL INHIBITORS OF PROTEIN TYROSINE PHOSPHATASES

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Key words: protein tyrosine phosphatases; inhibitors; calixarenes; docking

Calix[4]arene are known to be a promising scaffold for designing inhibitors of protein tyrosine phosphatases. In this work calix[4]arene mono- and bis-α-hydroxymethylphosphonic acids have been tested in vitro for the inhibitory activity against some therapeutically important protein tyrosine phosphatases. The results obtained have shown that these macrocyclic compounds can inhibit CD45, PTP1B, and SHP2 with IC_{50} values in the micromolar range. At the same time the inhibitors have demonstrated lower activity toward other protein tyrosine phosphatases such as TC-PTP and PTPβ. It has been found that mono-substituted calix[4]arene is more potent inhibitor of CD45 than the bis-substituted one and shows about 2-15 fold selectivity over TC-PTP, PTPβ, SHP2 and PTP1B. Model 4-hydroxyphenyl-α-hydroxymethylphosphonate displays at least one order lower activity than the phosphonate derivatives of calix[4]arene. Thus, the combination of a macrocyclic platform and α-hydroxymethylphosphonate group is essential for the inhibition activities of these compounds. Computer-simulated docking studies have been performed using AutoDock 4.2 programme by the example of PTP1B. The data obtained indicate that the inhibitors can bind in the active site of the enzyme. To clarify the inhibition mechanism the possible enzyme-inhibitor complexes have been considered using several crystal structures of PTP1B and all stereoisomeric forms of the inhibitors.

КАЛІКС[4]АРЕН α-ГІДРОКСИФОСФОНОВІ КИСЛОТИ ЯК ПОТЕНЦІЙНІ ІНГІБІТОРИ ПРОТЕЇНТИРОЗИНФОСФАТАЗ

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Ключові слова: *протеїнтирозинфосфатази; інгібітори; каліксарени; докінг*

Відомо, що калікс[4]арени є перспективною платформою для розробки інгібіторів протеїнтирозинфосфатаз. У цій роботі калікс[4]арен моно- та біс-α-гідроксиметилфосфонові кислоти були випробувані як інгібітори деяких терапевтично важливих протеїнтирозинфосфатаз. Отримані результати свідчили про те, що ці макроциклічні сполуки можуть інгібувати PTP1B, CD45 та SHP2 зі значеннями IC_{50} в мікромолярному діапазоні. Разом з тим, інгібітори демонстрували меншу активність відносно інших протеїнтирозинфосфатаз, таких як TC-PTP і PTPβ. Було встановлено, що моно-заміщений калікс[4]арен є кращим інгібітором CD45, ніж біс-заміщений макроцикл і виявляє приблизно 2-15-кратну селективність впливу відносно TC-PTP, PTPβ, SHP2 та PTP1B. Модельна сполука 4-гідроксифеніл-α-гідроксиметилфосфонат характеризується щонайменше на порядок гіршою активністю, ніж фосфонатні похідні калікс[4]арену. Таким чином, поєднання макроциклічної платформи та α-гідроксиметилфосфонатної групи відіграє важливу роль для інгібуючої здатності цих сполук. Молекулярний докінг було проведено з використання програми AutoDock 4.2 на прикладі PTP1B. Отримані результати показали, що інгібітори можуть зв'язуватися в активному центрі ферменту. Для з'ясування механізму інгібування були розглянуті можливі фермент-субстратні комплекси, сформовані з використанням різних кристалічних структур PTP1B та всіх стереоізомерних форм інгібіторів.

КАЛИКС[4]АРЕН α-ГИДРОКСИФОСФОНОВЫЕ КИСЛОТЫ КАК ПОТЕНЦИАЛЬНЫЕ ИНГИБИТОРЫ ПРОТЕИНТИРОЗИНФОСФАТАЗ

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Ключевые слова: *протеинтирозинфосфатазы; ингибиторы; каликсарены; докинг*

Известно, что каликс[4]арены могут быть перспективной платформой для конструирования ингибиторов протеинтирозинфосфатаз. В этой работе каликс[4]арен моно- и бис-α-гидроксиметилфосфоновые кислоты были испытаны как ингибиторы некоторых терапевтически важных протеинтирозинфосфатаз. Полученные результаты свидетельствовали о том, что эти макроциклические соединения могут ингибировать PTP1B, CD45 и SHP2 со значениями IC_{50} в микромолярном диапазоне. Вместе с тем, ингибиторы демонстрировали меньшую активность относительно других протеинтирозинфосфатаз, таких как TC-PTP и PTPβ. Было найдено, что моно-замещенный каликс[4]арен является лучшим ингибитором CD45 чем бис-замещенный макроцикл и проявляет приблизительно 2-15-кратную селективность по сравнению с ингибированием TC-PTP, PTPβ, SHP2 и PTP1B. Модельное соединение 4-гидроксифенил-α-гидроксиметилфосфонат характеризуется по меньшей мере на порядок меньшей активностью, чем фосфонатные производные каликс[4]арена. Таким образом, макроциклическая платформа и α-гидроксиметилфосфонатная группа играет важную роль для обеспечения ингибирующей способности соединений. Молекулярный докинг был проведен с использованием программы AutoDock 4.2 на примере PTP1B. Полученные результаты показали, что ингибиторы могут связываться в активном центре фермента. Для выяснения механизма ингибирования были рассмотрены возможные фермент-субстратные комплексы, сформированные с использованием различных кристаллических структур PTP1B и всех стереоизомерных форм ингибиторов.

A number of bioorganic studies have been focused on searching for inhibitors of protein tyrosine phosphatases (PTPs) [1]. The PTPs superfamily consists of 107 members that can be divided into different classes, which include the classical receptor and non-receptor PTPs [2]. Because of their involvement in various cellular processes, such as growth and proliferation, differentiation and survival or apoptosis [3], these enzymes are considered to be promising targets for new drug discovery today. Increase in activity or expression of PTPs may promote development of cancer, diabetes, obesity, Alzheimer's disease, Noonan syndrome, and other diseases [1]. For example, one of the non-receptor enzymes, protein tyrosine phosphatase 1B (PTP1B) was found to be a negative regulator of insulin, as well as leptin signaling pathways [4]. The experimental data with PTP1B knockout mice have shown that hyperactivity of this enzyme may lead to type 2 diabetes and obesity [5, 6]. The activity of protein tyrosine phosphatase SHP2 is associated with Noonan syndrome and the negative effect on several oncogene signaling pathways [7, 8]. It has been shown that SHP2 can be involved in pathogenicity of oncogenic bacterium *Helicobacter pylori* that is associated with stomach cancer [9]. One more protein tyrosine phosphatase CD45 belongs to the receptor-like PTPs and plays an essential role in signaling by T- and B-lymphocytes [10, 11]. This enzyme controls the immune response by dephosphorylating Src family kinases [12, 13]. Therefore, inhibitors of CD45 may be attractive as drugs for treatment of the tissue transplant rejection or autoimmune pathologies [14]. Thus, an opportunity to control the activity of at least several PTPs could lower risk of developing Type 2 diabetes and other diseases.

Calixarenes represent macrocyclic molecules that can be easily functionalized at either the upper or lower rim. Because of their unique three-dimensional structure they have used as important tools for investigations in bioorganic chemistry and medicine [15-18]. The calixarenes were found to exhibit antibacterial activity against gram-positive and gram-negative bacterial strains [19-21], antiviral [22, 23] and anticancer properties [24]. Finely, calixarenes can be used for biochemical recognition and separation of bioactive molecules such as amino acids, proteins, nucleotides, saccharides and steroids [25]. Experiments on mice have shown that some of calixarene derivatives have low or no toxicity [26].

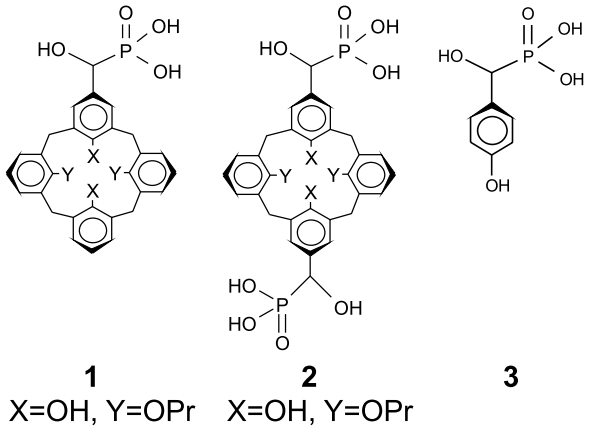

Fig. 1. Chemical structures of PTP inhibitors used in this work.

Based on understanding that the active site of PTP accommodates a phosphorylated tyrosine residue, it is likely that compounds with a non-cleavable phosphonate group, which mimic the phosphorylated tyrosine fragment, may be suitable for investigation as possible enzyme inhibitors. Recent studies have identified PTPs as targets for new bioactive phosphonate compounds [27, 28]. It has been shown also that preorganizing phosphonic or methylenebisphosphonic acids into calix[4]arene derivatives provides a promising approach to design effective inhibitors of alkaline phosphatase [29-31], Yersinia protein tyrosine phosphatase [32], and PTP1B [33].

Derivatives of 1-hydroxyphosphonic acid are shown to have a wide variety of the biological activity. They can be used as antihypertensive [34] and antiviral agents [35], antibiotics [36] and pesticides [37]. Series of the compounds featuring aryl or heteroaryl 1-hydroxyphosphonate derivatives were investigated as inhibitors of CD45 [38]. We have already determined that calix[4]arenes bearing hydroxymethylphosphonic acid fragments at the wide rim of the macrocycle are able to inhibit alkaline phosphatase and glutathione-S-transferase [39]. The present research was undertaken to evaluate the inhibitory activity of 1-hydroxymethylphosphonate derivatives of calix[4]arene toward some of protein tyrosine phosphatases.

Calix[4]arene α-hydroxyphosphonic acid **1** in the racemic form and compound **2** as a (RS)-stereoisomer (Fig. 1) were synthesized according to the protocols previously developed [39]. At physiological pH the phosphonic acid derivatives **1-3** can exist in phosphonate monoanionic and partially phosphonate dianionic forms. The compounds synthesized were evaluated in vitro for their inhibitory activity against commercially available PTP1B, TC-PTP, CD45, SHP2, and PTPβ. The activities of protein tyrosine phosphatases were determined by following the changes in the concentration of p-nitrophenol released during hydrolysis of p-nitrophenylphosphate used as a substrate. The values of IC_{50} were calculated from a dose-dependent curve as the concentration of the inhibitor, which decreased the enzyme activity by 50% in 5 min of incubation of the reaction mixture.

The results obtained are summarized in Table 1 macrocyclic compound **1** It has been found that macrocyclic compound is a potent inhibitor of CD45 (IC_{50} = 0.64 μM) and PTP1B (IC_{50} = 1.6 μM)**.** This macro-

Table 1

The values of IC_{50} (μM) of calix[4]arene α-hydroxymethylphosphonic acids **1** and **2** as inhibitors of protein tyrosine phosphatases[a]

Inhibitor	PTP1B	TC-PTP	SHP2	PTPβ	CD45
1	1.6	11	3.5	4.5	0.64
2	8.4	59	17	45	7.6
3	>10	>10	>10	N/D	N/D

[a] IC_{50} values determined with *p*-nitrophenylphosphate (in the concentrations equal to K_m values of enzymes) are the mean values of three assays with standard deviations within 25%.

cycle exhibits selectivity to CD45 over TC-PTP and shows a modest selectivity over PTP1B, PTPβ and SHP2. Compound **2** seems to be less potent inhibitor of PTPs than inhibitor **1**. Bis-substituted inhibitor **2** showed approximately the same values of IC_{50} in case of inhibiting CD45 and PTP1B and was less potent inhibitor against TC-PTP, PTPβ, and SHP2. The data obtained indicate that calix[4]arene inhibitors **1** and **2** are more effective in comparison with a model compound, 4-hydroxyphenyl-α-hydroxymethylphosphonate **3** (with IC_{50} values for PTP1B, TC-PTP, and SHP2 which were greater than 10 μM). The activity of calix[4]arene α-hydroxymethylphosphonates compared to the model compound can be explained by effects of both the phosphonate fragment and the molecular scaffold of the macrocyclic inhibitors.

To elucidate stereoselective effects of calix[4]arene-α-hydroxymethylphosphonates **1** and **2** on the activity of PTPs the inhibitors were docked into the active site of PTP1B. This enzyme selected for computer-simulation docking studies is represented by more than one hundred PDB files. It is known that PTPs catalyze the hydrolysis of phosphate monoesters by two-step mechanism [40]. In case of PTP1B, an important role in this action belongs to residues of Cys215, Arg221 (active pocket) and Asp181 (WPD-loop) [41]. All available X-ray crystal structures of PTP1B have the main difference, which can be attributed to open or closed conformation of WPD-loop [42]. As shown, the structures belonging to these two groups are also different and can be divided into subclasses [43]. To analyze the binding mode of the phosphonate inhibitors in the active site of PTP1B we applied the previously reported approach of dividing all PDB crystal structures into classes based on similarity of the binding profile. This was achieved by clustering all available conformations of PTP1B in the RSCB Protein Data Bank. Five centroids representing the enzyme structures with open WPD-loop (1NL9, 1PH0) and with closed WPD-loop (2CNF, 1Q6M, 2CM8) were used for docking of stereoisomeric forms of compounds **1** and **2**. According to the results obtained all stereoisomeric forms of α-hydroxyphosphonates are able to bind to the active site of PTP1B. The weak correlation between the experimental activity of the inhibitors and predicted free energies ΔG_{doc} is observed only for 2CNF. The values of binding free energies indicate that (*RS)*-isomer of **2** exhibits stronger binding affinity than its (*RR)*- or (*SS)*-analogues. Enantiomeric compounds (*S)*-**1** and (*R)*-**1** bind to the enzyme with the same affinity (Table 2).

Table 2

Docking results (AutoDock 4.2) of stereoisomers of calix[4]arene-α-hydroxymethyl-phosphonates **1** and **2** in the active site of PTP1B

Compounds	ΔG_{doc} (kcal/mol)				
	2CM8	2CNF	1Q6M	1PH0	1NL9
1(*R*)	-7.39	-8.84	-6.39	-4.92	-5.80
1(*S*)	-7.38	-8.86	-4.35	-5.10	-5.91
2(*RR*)	-7.19	-5.66	-7.21	-6.14	-5.92
2(*RS*)	-6.00	-8.24	-6.05	-4.49	-4.64
2(*SS*)	-6.80	-7.52	-3.72	-3.47	-5.35
3(*R*)	-7.54	-7.25	-8.13	-5.71	-5.90
3(*S*)	-8.36	-7.90	-8.22	-5.63	-5.95

Docking results have shown that the binding mode of calix[4]arene derivative **1** is similar to that of inhibitor **2**. The phosphonate residues of macrocycles **1** and **2** form a hydrogen bond with catalytic Cys215, and with other amino acid residues from the active pocket, such as Arg221, Ser216, and Ala217. Due to the closed conformation of WPD-loop, Phe182 is close to the upper rim of the macrocycle. In addition,

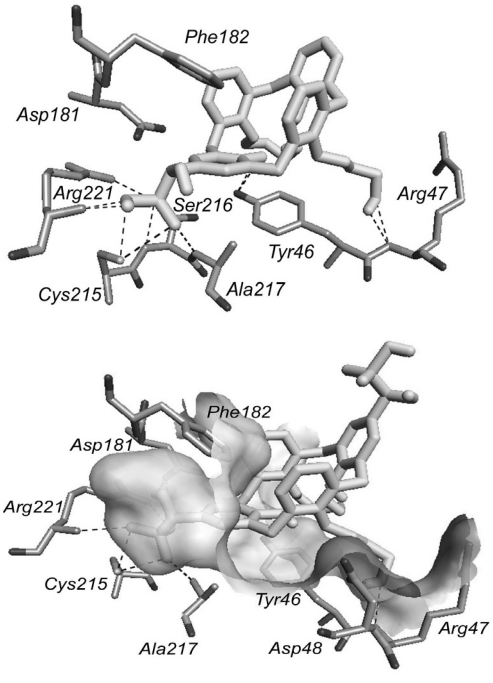

Fig. 2. The possible binding modes of *(S)*-calix[4]arene-mono-α-hydroxyphosphonate (top) and *(R)*-calix[4]arene-bis-α-hydroxyphosphonate (bottom) at the active site of PTP1B.

hydrophobic interactions are observed between the narrow rim of the macrocyclic platform and Tyr46, Arg47, and Asp48. According to the data (Tab. 1) experimentally obtained the second α-hydroxymethylphosphonate group of bis-substituted compound **2** does not interact with the enzyme surface (Fig. 2).

Experimental Part

The enzymatic reaction was carried out at 37ºC (PTP1B) and 30ºC (TC-PTP, SHP2, CD45, or PTPβ). The assay solution contained 50 mM Bis-Tris (pH 7.2), 1 vol. % of dimethyl sulfoxide, 100 mM NaCl, 1 mM DTT, 3 mM EDTA, 2 mM *p*-nitrophenyl phosphate (for PTP1B), and the inhibitor. The final volume was 0.5 ml. The mixture was preincubated for 5 min and the reaction was initiated by addition of the enzyme (6nM in the reaction mixture). The *p*-nitrophenol released was determined by measuring the absorbance at 410 nm (ε = 18.000 $M^{-1}cm^{-1}$).

Computer-simulated docking studies were performed using AutoDock 4.2 programme [44]. A ligand presented at the binding site was removed before calculations and then the enzyme was examined with inhibitors. The phosphoryl groups of the docked inhibitors were in the form of monoanions.

Conclusions

The data obtained in this study show that calix[4]arene-α-hydroxymetylphosphonic acids can inhibit CD45 and PTP1B with IC_{50} values in the micromolar range. The combination of the macrocyclic platform and α-hydroxymethylphosphonate group is essential for the inhibition activities of these compounds. The computer-simulated docking indicates that α-hydroxymethylphosphonate fragment of the macrocyclic inhibitor may compete with the phosphotyrosine-containing substrate for binding in the active site of PTP1B.

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