INHIBITORY POTENTIAL OF POLYHYDROXYLATED FULLERENES AGAINST PROTEIN TYROSINE PHOSPHATASE 1B

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Inhibition of PTP1B by polyhydroxylated fullerenes was studied in silico and in vitro. The enzyme kinetics in the presence of polyhydroxy small gap fullerenes showed that reciprocal value of maximum velocity non-linearly increases with increasing the inhibitor concentration. Analysis of the dose-dependent curve of PTP1B inhibition suggests an apparent positive cooperativity with involvement of at least two binding sites for the hydroxylated fullerene cages. Molecular docking calculations indicated that highly hydroxylated fullerene C_{60} may occupy the active site and additional allosteric binding site with similar affinity. In silico analysis of a number of fullerenols with 6, 12, 18, 24, 30, and 36 hydroxyl groups showed that the inhibitory activity may depend on the degree of hydroxylation of the nanoparticles surface. These data provide some understanding of the mechanisms of inhibitory action of fullerenols on activity of protein tyrosine phosphatases.

K e y   w o r d s: protein tyrosine phosphatase, PTP1B, fullerenes, fullerenols.

Polyhydroxylated fullerene derivatives (fullerenols) are characterized by promising biological properties including immunomodulating, anticancer [1-3] and antimetastatic activities [4]. The ability of fullerenols to neutralize free radicals is associated with their possible use as antioxidants and radioprotective agents in living cells [5,6]. In addition, fullerenols are able to inhibit the activity of enzymes such as P450-dependent monooxygenase, Mg^{2+}-ATPase [7], M-MuLV reverse transcriptase [8], lysozyme [9], and monoamine oxidase B [10].

We have previously reported that fullerenol and some other fullerene derivatives can also be effective as inhibitors of human protein tyrosine phosphatases (EC 3.1.3.48) [11-13]. These regulatory enzymes catalyze dephosphorylation of tyrosine residues in signaling proteins, and abnormal changes in their activities may lead to development of serious diseases [14]. Some receptor and non-receptor protein tyrosine phosphatases are involved in the regulation of insulin signaling. Among them, protein tyrosine phosphatase 1B (PTP1B) provides dephosphorylation of the insulin receptor tyrosine residues contributing to type 2 diabetes risk [15, 16]. Negative role of PTP1B in obesity is associated with the blocking of transduction signal from the leptin hormone *via* dephosphorylation of Jak2 [17]. Furthermore, the enzyme may act as a promoter or suppressor of tumor development [18].

In this context, search for effective PTP1B inhibitors is essential for drug design [19], and the polyhydroxylated fullerenes can be considered as potential therapeutic agents exhibiting significantly lower toxicity in comparison with the unmodified ones [20-22]. The aim of the present report was to elucidate some characteristics of polyhydroxy small gap fullerenes as inhibitors of PTP1B *in vitro*. In addition, molecular docking was performed to predict the inhibitory potential of the nanoparticles with different number of hydroxyl groups against PTP1B as a favorable target.

Materials and Methods

PTP1B, *p*-nitrophenyl phosphate (*p*-NPP), 4-methylumbelliferyl phosphate disodium salt (4-MUP), ethylenediamine tetraacetate disodium salt (EDTA) and dithiotreitol (DTT) were purchased from Sigma-Aldrich. Preparation of polyhydroxy small gap fullerenes (Sigma-Aldrich) was used without additional purification as a representative fullerenol with about thirty oxy functionalities per one fullerene core of the common formula $C_{120}O_{30}(OH)_{30} \cdot 30H_2O \cdot 25Na^+$.

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For studying the inhibition of PTP1B *in vitro*, a typical reaction mixture consisted of 0.05 M bis-Tris buffer (pH 7.2), 100 mM NaCl, 3 mM EDTA, 1 mM DTT, 1% dimethyl sulphoxide, recombinant PTP1B$_{1-436}$ (1-436 amino acid residues) and *p*-NPP as an artificial substrate. In some of kinetic experiments, fluorogenic substrate 4-MUP was employed to analyze the inhibition of PTP1B$_{1-322}$. The mixture was incubated during 5 min at 37 °C. The reaction was started by adding the enzyme (in the case of *p*-NPP) or substrate (in the case of 4-MUP). The enzymatic hydrolysis of *p*-NPP was monitored spectrophotometrically at 410 nm by watching the accumulation of *p*-nitrophenol. The rate of 4-MUP hydrolysis was observed due to increasing concentrations of fluorescent product, 4-methylumbelliferone (excitation wavelength and emission wavelength were 360 and 450 nm, respectively). Hill slope was calculated using four-parameter equation from the dose-dependent inhibition curve and shown as the mean value ± standart error. Apparent IC$_{50}$ value was the inhibitor concentration that inhibits the enzyme activity by 50% and is given as the mean value ± standart deviation. Kinetic data of *p*-NPP and 4-MUP enzymatic transformations were analyzed using Lineweaver-Burk double reciprocal plots. The kinetic and statistical calculations were carried out using MS Excel software.

Computer-simulation studies of PTP1B (structures with PDB identification codes 1NL9 and 2CM8) were performed by Autodock Vina [23]. Additional calculations with Autodock 4.2 showed the similar positions of the hydroxylated fullerenes on the surface of PTP1B. Energetically favorable structures [24, 25] of the fullerene ligands were optimized by Avogadro software [26] using the MMFF94s force field.

Results and Discussion

Polyhydroxy small gap fullerenes with OH and ONa groups covalently attached to the nanoscale carbon core were used as a water-soluble polyhydroxy fullerene example [27] to inhibit PTP1B *in vitro*. The previous studies [12] showed that the polyhydroxylated fullerenes can display strong inhibitory effects towards this enzyme. In this paper, the two artificial substrates, *p*-NPP and 4-MUP, were applied to elucidate the kinetic characteristics of the inhibition.

Progress curves of *p*-NPP hydrolysis catalyzed by PTP1B in the presence of polyhydroxylated fullerenes (Fig. 1) showed a time-dependent decrease
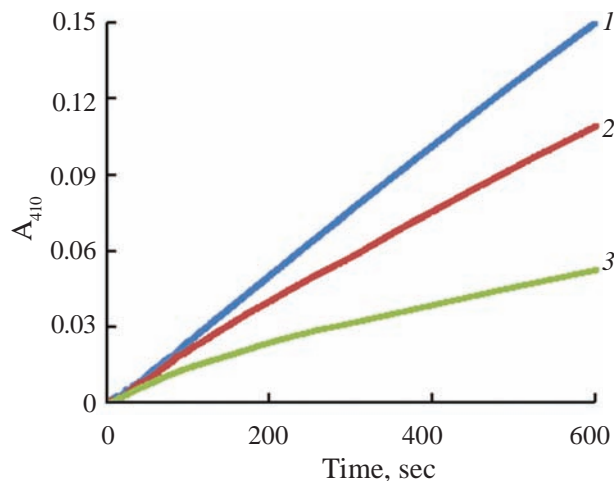

Fig. 1. The hydrolysis curves of p-NPP by the protein tyrosine phosphatase 1B in absence (1) and in the presence of polyhydroxylated fullerenes at concentrations of 0.0722 (2) and 0.271 µg/ml (3)

in the reaction rate. Reaching a steady-state velocity was observed after about the first five minutes of *p*-nitrophenol accumulation. Taking into account the slow-binding inhibition, the steady-state kinetics was used to analyze the enzyme inhibition.

The dose-dependent curve of PTP1B suppression by the polyhydroxylated fullerenes is presented in Fig. 2. Under assay conditions, the fullerenols have a high inhibitory potential with activity at concentration from 0.01 to 1 µg/ml (based on representative fullerene derivative C$_{60}$(OH)$_{30}$, molar concentrations of the inhibitor may be given in range from 5 to 500 nM). The apparent IC$_{50}$ value was 0.114 ± 0.033 µg/ml that is in agreement with
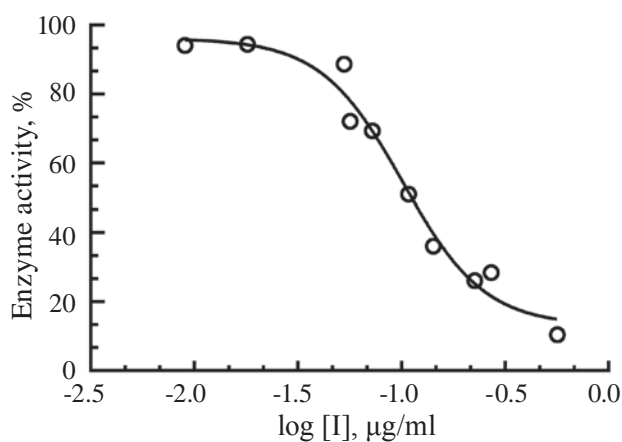

Fig. 2. Dose-dependent inhibition curve of PTP1B by polyhydroxylated fullerenes. p-NPP was used as a substrate

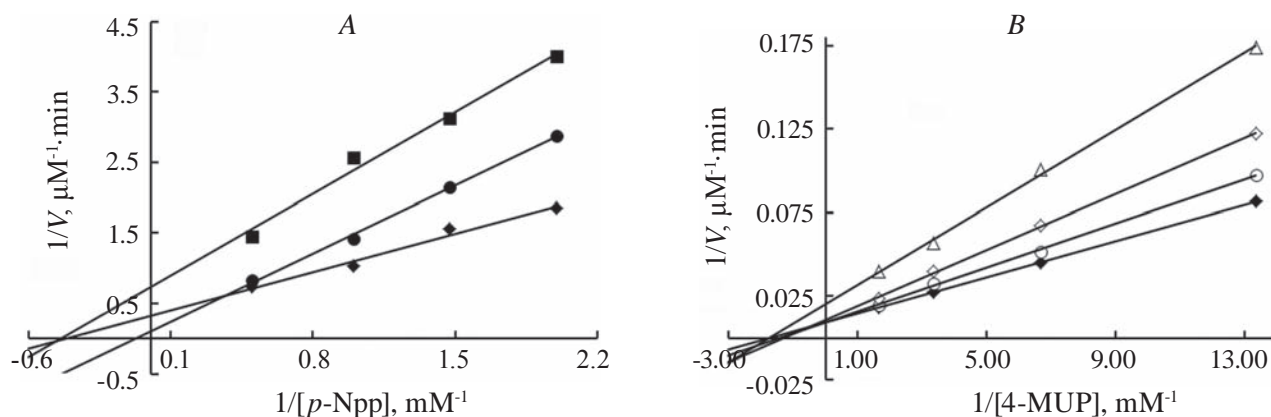*ISSN 2409-4943. Ukr. Biochem. J., 2015, Vol. 87, N 4*

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Fig. 3. Lineweaver-Burk plots of PTP1B inhibition by polyhydroxylated fullerenes. p-NPP (A) or 4-MUP (B) were used as a substrate. The inhibitor concentration were 0 (♦), 0.036 µg/ml (○), 0.054 µg/ml (●), 0.072 µg/ml (◊), 0.108 µg/ml (■), and 0.144 µg/ml (∆)

previously reported data [12]. The Hill coefficient value was found to be 2.20 ± 0.51. This suggests an apparent positive cooperative effect with involvement of at least two binding sites of PTP1B for the hydroxylated fullerene cages.

The plots of the steady-state velocity against the concentration of the substrate ($1/V$ versus $1/[S]$) were linear (Fig. 3, *A*, the values of r^2 are 0.98-0.99), however, the graphical analysis does not allow identifying the type of inhibition.

Similar results were obtained with 4-MUP as a substrate (Fig. 3, *B*, the values of r^2 are 0.99). The observed kinetic effects of the polyhydroxylated fullerenes at concentration 0.1 µg/ml may be correlated with self-assembly of the nanoparticles in the aqueous phase [28, 29]. At the same time, analysis of

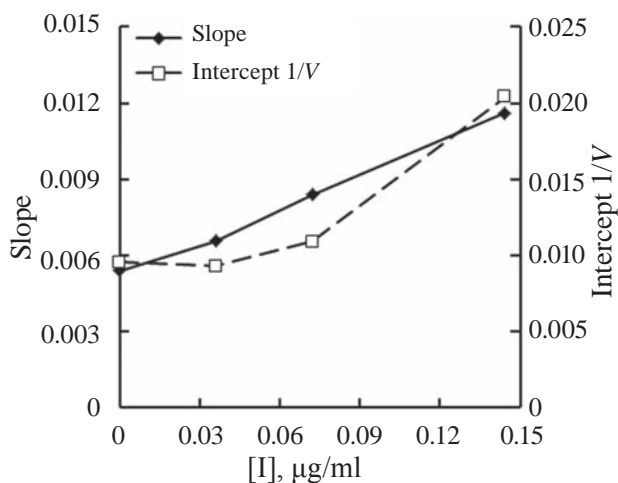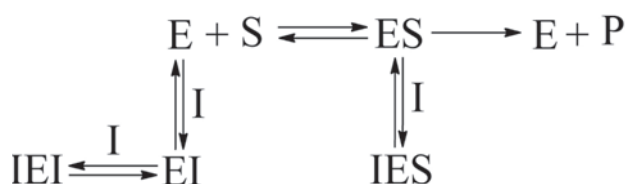*Scheme. Possible kinetic scheme of PTP1B inhibition by polyhydroxylated fullerenes*

the secondary replots of slope and intercept versus the inhibitor concentration (Fig. 4) shows that value of the intercept ($1/V_{max}$) increases non-linearly with increasing the inhibitor concentration but dependence of the slope on the inhibitor concentration is linear, which can be characteristic of S-linear I-parabolic mechanism of inhibition [30, 31]. The parabolic character of the dependence of $1/V_{max}$ on the inhibitor concentration is in accordance with interaction of fullerenol molecules with two binding sites of PTP1B. According to this suggestion, polyhydroxylated fullerenes interact with the enzyme to form EI, IEI, and IES inactive complexes (Scheme).

Optimization of ligand structures shows that attachment of hydroxyl groups to C_{60} leads to increase of molecular volume of the inhibitor, reduces aromaticity and consequently results in a distorted fullerene cage [24, 25]. Docking modeling of enzyme-inhibitor complexes (PDB code 1NL9) indicated that two wide cavities on PTP1B surface that are comparable to the size of the molecule of $C_{60}(OH)_{30}$ can be formed by the active site region and allosteric inhibitor binding site (Fig. 5). Docking binding energies were calculated to be -8.2 kcal/mol and -7.7 kcal/

Fig. 4. The dependence of the slope and intercept (data from Fig. 3, B) on the inhibitor concentration

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mol, respectively. Location of the inhibitor molecule in the active site is characterized by the presence of several hydrogen bonds and electrostatic interactions with Asp48, Glu115, Lys116, Lys120, Asp181, Ala217, Ser216, Gly220, Arg221, and Gln266 (Fig. 5, *A*). Additional forces that stabilize the enzyme-inhibitor complex are π-π and π-cationic interaction with Tyr46 and Lys120, respectively. Distance to catalytically important Cys215 is approximately of 3.72 Å. However, the molecule of the hydroxylated fullerene can not occupy the active site of PTP1B with closed WPD-loop (PDB code 2CM8). In this case (as well as in case of PTP1B with open WPD-loop), the fullerenol may be located at allosteric site at the distance of about 20 Å from the catalytically important Cys215 [32]. The molecule of $C_{60}(OH)_{30}$ occupies the region formed by helices α3, α4, α6, α7, and S-loop (Fig. 5, *B*) that are involved in conformational changes of PTP1B [32-34]. The inhibitor forms H-bonds and electrostatic interaction with Arg79, Arg199, Glu200, Gly202, Ser205, Glu207, Asp236, and Gln288.

The results of this docking revealed molecular fragments of representative hydroxylated fullerene C_{60} that are responsible for the interaction with the PTP1B. It should be noted that polyhydroxy small gap fullerene product is heterogeneous and rich in giant fullerenes larger than C_{60}. Hence their complexes with the enzyme also may be suggested including, for example, the interaction with dimeric fullerene derivatives.

Molecular docking simulations of polyhydroxylated fullerene derivatives $C_{60}(OH)_{6}$, $C_{60}(OH)_{12}$, $C_{60}(OH)_{18}$, $C_{60}(OH)_{24}$, $C_{60}(OH)_{30}$, $C_{60}(OH)_{36}$ as inhibitors of PTP1B (Fig. 6) showed that the inhibitory potential of these compounds depends on degree of hydroxylation of the fullerene. The efficiency of the inhibitors decreases with increasing the number of hydroxyl groups and remains almost stable in case of the fullerenols containing more than 18
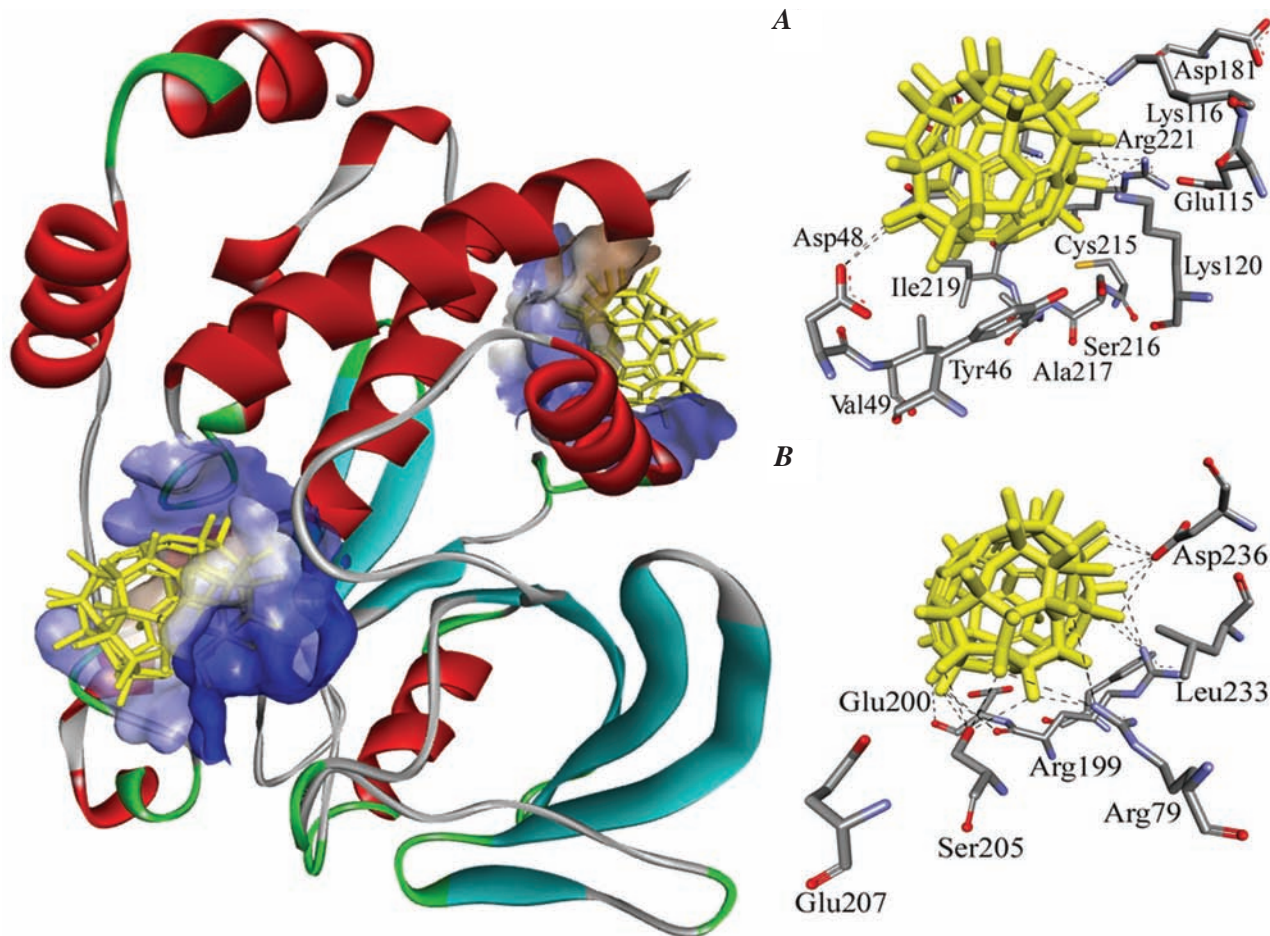

Fig. 5. Predicted binding modes of polyhydroxylated fullerene $C_{60}(OH)_{30}$ at the active site (A) and allosteric inhibitor binding site (B) of PTP1B with open WPD-loop (PDB identification code 1NL9)

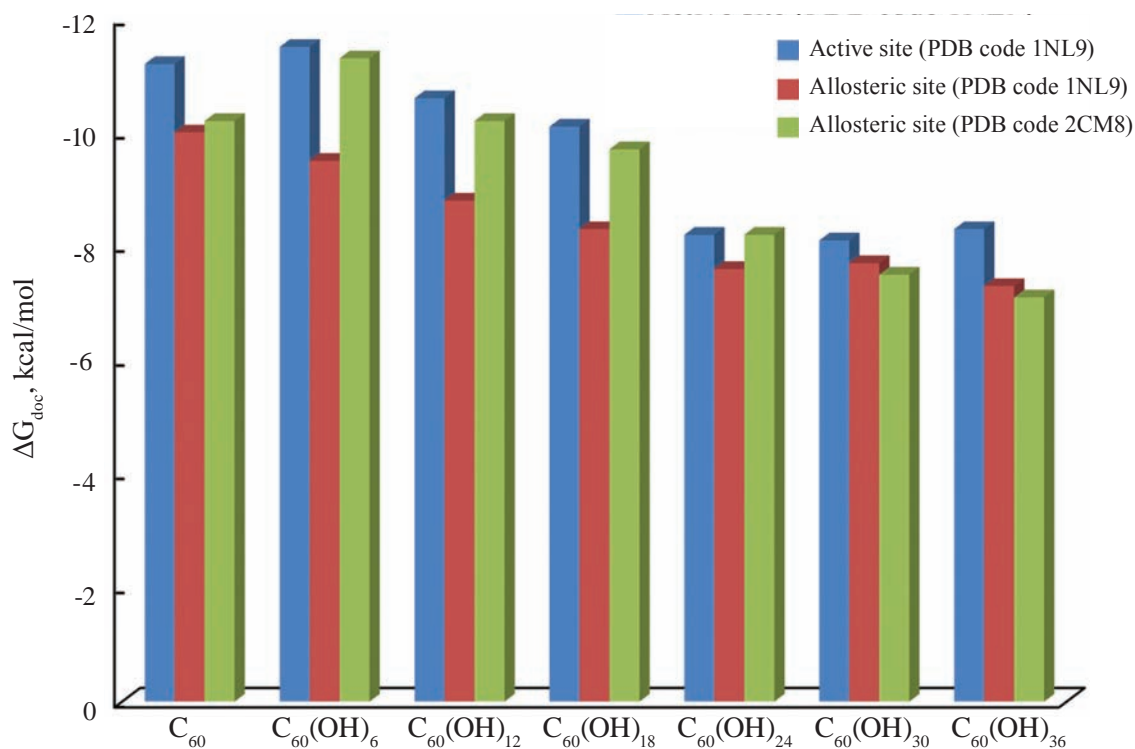ISSN 2409-4943. Ukr. Biochem. J., 2015, Vol. 87, N 4

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Fig. 6. Molecular docking energies of various polyhydroxylated fullerene derivatives bound to the PTP1B active site (PDB code 1NL9) and allosteric site (PDB codes 1NL9 and 2CM8)

OH groups. More hydrophilic nanoparticles bind to active and allosteric sites with approximately equal binding energies. In contrast, the fullerene derivatives with a lower degree of hydroxylation form more stable enzyme-inhibitor complexes interacting with hydrophobic residues at the active site of PTP1B. This is consistent with the fact that pyrrolidino[60]fullerene tris-carboxylic acid inhibits the activity of PTP1B in a competitive manner [12], whereas the polyhydroxylated fullerenes show the other inhibition types. It should also be noted that some of low functionalized fullerene derivatives exhibited selective inhibition of receptor-type protein tyrosine phosphatase CD45 (in contrary to PTP1B) which may be explained by significant hydrophobicity of CD45 surface [12, 13].

In conclusion, the results of kinetic studies showed that polyhydroxylated fullerenes may inhibit activity of PTP1B with positive cooperativity. Computer docking simulation indicated that $C_{60}(OH)_{30}$ particles occupy the substrate binding site and allosteric inhibitor binding site of the enzyme with similar docking energies. The inhibition of PTP1B may depend on the degree of fullerene surface hydroxylation. These data provide some basis for understanding the mechanisms of inhibitory action of fullerenols on activity of PTP1B and other protein tyrosine phosphatases.

ІНГІБУВАЛЬНА ЗДАТНІСТЬ ПОЛІГІДРОКСИЛЬОВАНИХ ФУЛЕРЕНІВ СТОСОВНО ПРОТЕЇНТИРОЗИНФОСФАТАЗИ 1В

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In silico та *in vitro* досліджено інгібування активності PTP1B полігідроксильованими фулеренами. Результати кінетичних досліджень ензиматичних реакцій за наявності полігідроксильованих фулеренів демонстрували нелінійне зростання оберненого значення уявної максимальної швидкості реакції з підвищенням концентрації інгібітора. Аналіз дозозалежної кривої припускає позитивну кооперативність із залученням щонайменше двох сайтів зв'язування для полігідроксильованих фулеренових фрагментів. Комп'ютерні розрахунки

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показали, що полігідроксильовані фулерени $C_{60}(OH)_{24-36}$ характеризуються приблизно однаковими значеннями енергії докінгу у разі взаємодії як з активним, так і з алостеричним сайтами зв'язування. Аналіз результатів молекулярного докінгу ряду фулеренолів з 6, 12, 18, 24, 30 і 36 OH-групами свідчить, що інгібуючий вплив залежить від ступеня гідроксилювання поверхні наночастинок. Ці дані є важливими для з'ясування механізмів інгібування активності протеїнтирозинфосфатаз фулеренолами.

Ключові слова: протеїнтирозинфосфатази, PTP1B, фулерени, фулереноли.

ИНГИБИРУЮЩАЯ СПОСОБНОСТЬ ПОЛИГИДРОКСИЛИРОВАННЫХ ФУЛЛЕРЕНОВ ПО ОТНОШЕНИЮ К ПРОТЕИНТИРОЗИНФОСФАТАЗЕ 1В

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In silico и *in vitro* исследовано ингибирование активности PTP1B полигидроксилированными фуллеренами. Результаты кинетических исследований энзиматических реакций в присутствии полигидроксилированных фуллеренов демонстрировали нелинейный рост обратного значения кажущейся максимальной скорости реакции при повышении концентрации ингибитора. Анализ дозозависимой кривой предполагает положительную кооперативность с вовлечением по меньшей мере двух сайтов для связывания полигидроксилированных фуллереновых фрагментов. Компьютерные расчеты показали, что полигидроксилированные фуллерены $C_{60}(OH)_{24-36}$ характеризуются примерно одинаковыми значениями энергии докинга при взаимодействии как с активным, так и с аллостерическим сайтами связывания. Анализ результатов молекулярного докинга ряда фуллеренолов с 6, 12, 18, 24, 30 и 36 OH-группами свидетельствует, что ингибирующее влияние зависит от степени гидроксилирования поверхности наночастиц. Эти данные являются важными для понимания механизмов ингибирования активности протеинтирозинфосфатаз фуллеренолами.

Ключевые слова: протеинтирозинфосфатазы, PTP1B, фуллерены, фуллеренолы.

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