LETTER

In Silico Drug Screening Approach for the Design of Magic Bullets: A Successful Example with Anti-HIV Fullerene Derivatized Amino Acids

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Abstract: A database has been derived from recently reported [60]fullerene derivatives, and their binding scores with HIV-1 PR have been computed using docking techniques. Computational methods have been used to predict which derivatives may have high binding affinities, and for these compounds biological tests have been performed with purified PR. Experimental results confirm the high binding scores of fullerene derivatives predicted from the docking calculations. Our measurements showed that the fullerene derivative (Fmoc-Baa) has about three times better inhibitory binding (K_i = 36 nM) than the most active fullerene-based inhibitor (K_i = 103 nM) currently available.

To the Editor: Currently drug design follows several paths to the development of new leads. The "*classical*" method in which the synthesis of novel analogues is based on the chemical intuition of medicinal chemists has had significant success in the past, but it is time-consuming and no longer considered efficient with the plethora of potential targets. Instead, the design and synthesis of new analogues based on knowledge of the molecular basis of the disease has gained prominence.[1,2] The application of computational tools and physicochemical methodologies leads a rational and more efficient approach.[1−11] *In silico* drug design has supported pharmaceutical research for over three decades. Two major design principles have evolved; the receptor-based approach and the ligand-based method.[9] These strategies, particularly, in combination with fragment based lead discovery, are considered to be complementary to high-throughput screening, which still represents the major source of innovation for many pharmaceutical companies.[9]

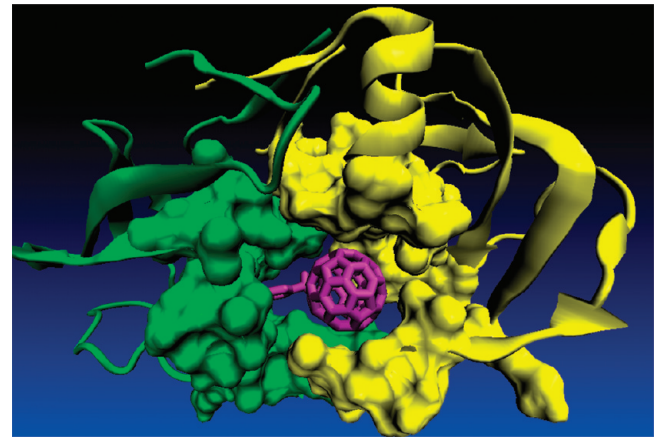

Figure 1. Perfect fit of a fullerene derivative at the active site of the HIV-1 PR (active site residues have been shown as molecular surface for clarity).

In silico drug design has many advantages compared to the classical approach: (a) it is more time and cost efficient; (b) it utilizes the conformational properties of the active site; (c) it reduces the number of wet experiments; and (d) it offers the possibility of replacing some animal tests with suitable *in silico* models.[9−11] Certain receptor active sites may have similarities in hosting potential drug leads. The medicinal chemist must be well acquainted with the environment of the receptor site under study. The shape and extent of the cavity, its lipophilicity profile, and polar group distribution are among the stereoelectronic features that must be studied thoroughly. A comparison of the stereoelectronic characteristics of binding sites may open new avenues to test and select potent drug leads targeted for other receptor sites. Herein we report that this approach can be successfully used by employing fullerene derivatives, which have already been synthesized for various biomedical applications.

The inhibition of human immunodeficiency virus type I aspartic protease (HIV-1 PR) by fullerene analogues has been demonstrated by Friedman et al.,[12] and the complexation of HIV-1 PR with fullerene compounds has been supported by molecular modeling studies.[12,13] These studies showed that the fullerene derivatives can be perfectly accommodated inside the binding pocket of HIV-1 PR (Figure 1). However, the binding affinity (K_i) values of "first generation" fullerene inhibitors were not significant ($K_i \sim 10^{-6}$ M).[8] Thus, further structural investigation is required in order to propose new HIV-1 PR/fullerene complexes with better binding affinities.

Considering the urgent need for new anti-HIV drugs, we used the *in silico* drug screening approach which is the least time-consuming, lowest cost procedure for the efficient rational drug design. Thus, a database that includes more than 100 fullerenes has been derived by searching the [60]fullerene derivatives, synthesized in the past decade, in order to predict which of those

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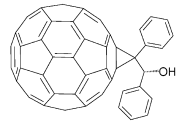
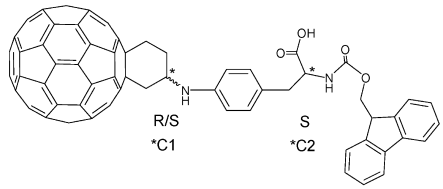
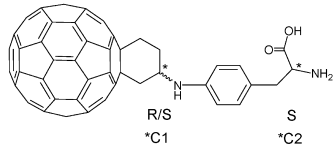
Table 1. Computed Binding Scores and Experimental Binding Energies for the Fullerene Derivatives (Compounds **2** and **3**) Presented in This Work[a]

Compound No.	Structure	Computational Binding Score with HIV-1 PR (kJ/mol)	Experimental Binding Energies (kJ/mol)
1		-36.66	-40.13
2		-43.53 (*C1, S) -39.40 (*C1, R)	-42.75
3		-37.60 (*C1, S) -36.56 (*C1, R)	-39.75

[a] For comparison, the derivative with the best currently available binding affinity (compound **1**) is also presented. Observed binding affinities (103 nM, 36 nM, and 120 nM for compounds **1**, **2**, and **3**, respectively) have been converted to the same units with computational binding scores for easy comparison.

have high affinity with HIV-1 PR. Most of these fullerenes were synthesized for other biomedical applications. Since the X-ray structure of HIV-1 PR enzyme is available, molecular docking simulations were performed using these derivatives. One group of compounds that shows particular promise are fullerene-based amino acid derivatives and their structural analogues we have recently reported.[14−16] Their higher affinities are connected with (i) the ability of pendant groups to form H-bonds with the catalytic site of the enzyme as well as (ii) the van der Waals interactions between the fullerene cage and the nonpolar surface of active site amino acid residues. Of these derivatives, the structures that showed better binding scores than the currently reported most potent fullerene-based HIV-1 PR inhibitor[13] (compound **1**, Table 1) were collected and subjected to biological testing.

As reported herein, our computational approach has proven successful. Biological tests for the candidates showing the most promising binding affinity in the applied molecular dynamics (MD) assisted molecular docking simulations have confirmed the high activity of these derivatives. The bucky amino acid (Baa, compound **3**, Table 1) showed similar binding affinity (K_i = 120 nM) with compound **1** (K_i = 103 nM). Furthermore, the 9-fluorenylmethoxycarbonyl protected Baa (Fmoc-Baa, compound **2**, Table 1) has about three times better activity (K_i = 36 nM) than the most active currently available fullerene derivative. This is considered to be an important finding because (i) a new anti-HIV fullerene derivative is proposed which is more active than those which have been reported[13] and (ii) it is expected to stimulate further research for even more effective anti-HIV drugs which are of immediate need at this time.

Computational Details. Since the X-ray structure of the HIV-1 PR complexed with fullerene-based inhibitors has not yet been reported, the initial structure has been taken from the HIV-1 PR complexed with a haloperidol derivative at 2.2 Å resolution (PDB code; 1AID).[17] The water molecules and the inhibitor were then removed, and all hydrogen atoms were added to the system. Ionization states for ionizable amino acid residues were assigned according to the standard pK_a values of amino acids. The geometry of the enzyme has been optimized by using the Tripos molecular mechanic force field of the Sybyl molecular modeling package.[18] Before docking, conformational analysis was performed on the ligands using the MultiSearch module of Sybyl (version 6.8). This option locates the various energy minima for a set of molecules, stored in a database, by randomly perturbing torsions, minimizing, and eliminating duplicates. The rotatable bonds were selected for perturbed torsions using the MMP2 minimizer in Sybyl with energy convergence of 0.01 kcal/mol (0.0418 kJ/mol). The maximum number of conformers for each molecule was set to 30, and the top 10 lowest energy conformers were used in docking simulations; only the best docked complex was considered for further analysis. The FlexX docking program (version 1.11.0 L) of Sybyl has been used for docking simulations. The FlexX docking method allows flexibility in the ligands, while it keeps the receptor rigid. The scoring function of FlexX, that was developed by Böhm,[19] was used. Since the docking scores are affected by the conformations of active site residues of the receptor, MD simulations have been performed for the HIV-1 PR/fullerene complex. As an initial test, the most potent reported fullerene derivative **1** was docked at the binding site of the enzyme, and the docked complex with the best binding score has been used as input in the MD simulations which were performed with the Gromacs 3.3.1 software package,[20] employing the gmx force field.[21] Canonical NVT ensemble at 300 K was used with periodic boundary conditions, and the temperature was kept constant by the Berendsen thermostat.[22] Electrostatic interac-

tions were calculated using the particle mesh Ewald method.[23] Cut-off distances for the calculation of Coulomb and van der Waals interactions were 1.0 and 1.4 nm, respectively. Prior to the MD simulations, the energy of the full system has been optimized without constraints using the steepest descent integrator for 5000 steps. The system was then equilibrated via a 100 ps MD simulations at 300 K. Finally, a 4 ns simulation was performed with a time step of 2 fs. The convergence criteria have been tested by the potential energy versus time plot and showed sufficient convergence (left in Figure S1, in the Supporting Information). Each receptor backbone structure from a trajectory is compared with the reference (initial) structure in order to obtain the receptor backbone rmsd versus time (right in Figure S1, in the Supporting Information). The average coordinate file of HIV-1 PR from the last 1 ns trajectory file of MD simulations has been used in the redocking calculations. A more detailed explanation about the computational methods we used can be found in our recent publication.[8] The active site in the docking runs included all atoms within a radius of 6.5 Å around the critical amino acids: Asp25, Asp25′, Ile50, and Ile50′ (residues 25 and 50 on chains α and β; each of the chains include 99 amino acids).[12,13]

Inhibition Assay. The K_i values were determined by a spectrophotometric assay using the chromogenic peptide substrate LysAlaArgValNle*NphGluAlaNle-NH2 as described by Weber et al.[24] Typically, 8−10 pmol of HIV-1 PR was added to 1 mL of 0.1 M sodium acetate buffer (pH 4.7), 0.3 M NaCl, and 4 mM EDTA, containing the substrate at a concentration near the K_m of the enzyme and various concentrations of the inhibitor dissolved in DMSO (from 0.1 mM to 0.01 nM). The final concentrations of DMSO were kept below 2.5% (v/v). Substrate hydrolysis was followed as a decrease in absorbance at 305 nm using a Cary 3−500 UV-vis spectrophotometer (Cambridge, UK). The HIV-1 PR remained stable over the whole reaction time. Inhibition data were analyzed using the equation for competitive inhibition according to Williams and Morrison.[25] The solubility of the compounds was sufficient for all concentrations used in biological and solubility experiments. More specifically, in *in vitro* tests, the DMSO/water mixture has been used as a 1:9 ratio, and no precipitation problems have been observed. For the solubility studies, the compounds were tested from 0.004 to 0.4 mg/mL, and in all cases the derivatives were soluble. They have been shown to be essentially nontoxic except at very high concentrations. Our previous studies[15,26] have shown that, for concentrations of less than 0.04 mg/mL, cell viability is maintained.

The binding interactions of potent novel fullerene derivatives **2** and **3** are compared with those of previously reported fullerene derivative **1**. Figure 2 shows the best binding pose of fullerene **2** at the catalytic site of the HIV-1 PR, which was obtained from docking simulations.

There are two chiral centers *C1 and *C2 at compounds **2** and **3** (Table 1); *C2 has an (S) configuration, but *C1 can have both configurations. Thus, both (S) and (R) configurations have been taken into consideration in the docking simulations. Compounds that have an (S) configuration at *C1 (**2S** and **3S**) have ∼4.0 kJ/mol and ∼1.0 kJ/mol better binding energies than the corresponding (R) configuration (**2R** and **3R**, correspondingly). The location of the best binding complex poses from docking for

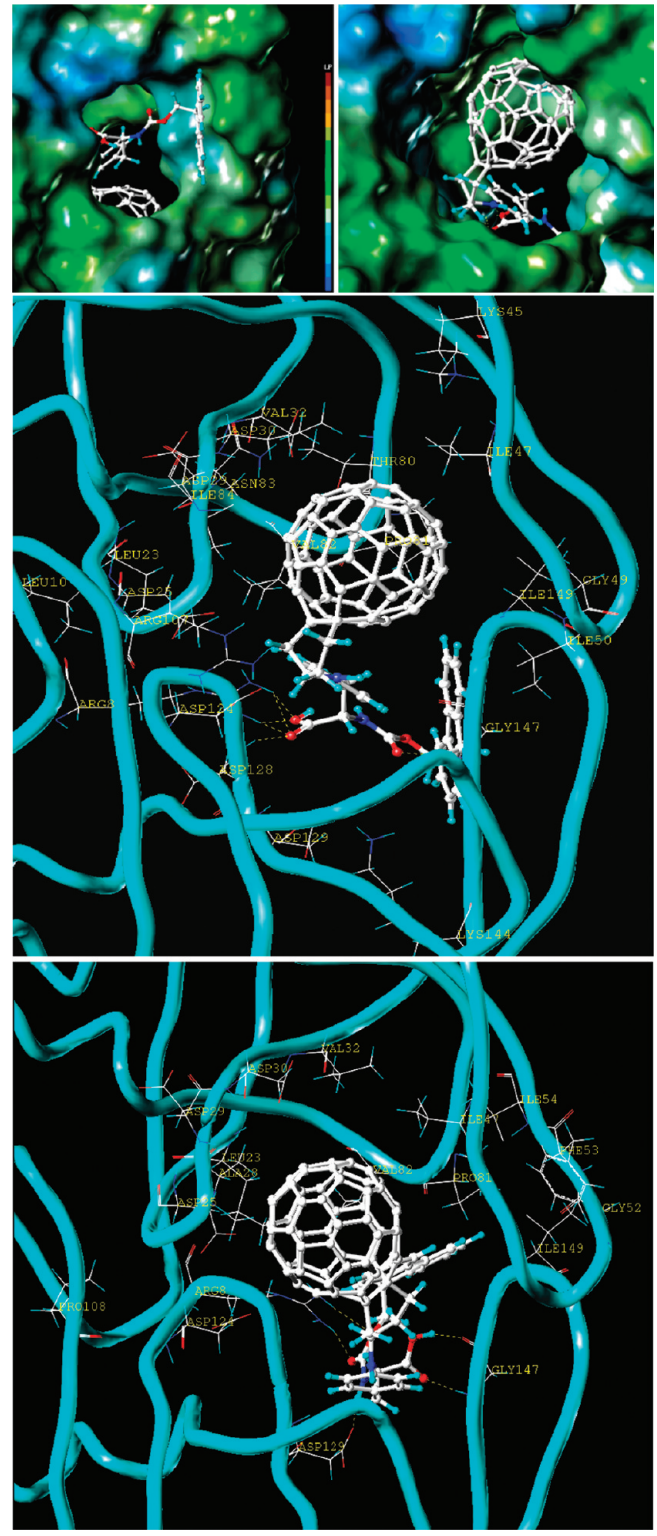


Figure 2. (top) The location of **2** (from both side views) at the binding pocket of HIV-1 PR from docking calculations. MOLCAD lipophilic potential surface was calculated for the receptor with the Connolly method. Brown color denotes the most lipophilic areas and blue color denotes the most polar areas. (middle) The binding interactions of **2S** with the active site residues of HIV-1 PR. (bottom) The binding interactions of **2R** with the active site residues of HIV-1 PR.

compounds **2R** and **2S** is similar at the binding pocket of HIV-1 PR. Compound **2S** forms six H-bonds with the binding site amino acid residues (Arg8, Asp29′, Asp30′, and Gly48′) of HIV-1 PR (Figure 2, middle), and compound **2R**

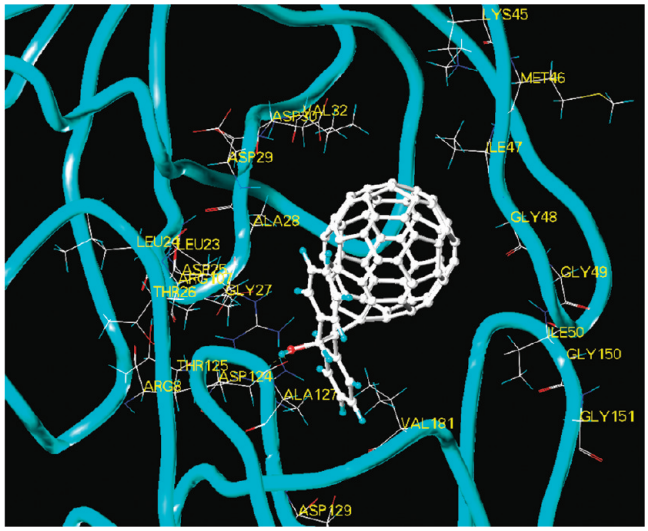


Figure 3. The binding interactions of **1** with the active site residues of HIV-1 PR.

forms five H-bonds with the active site residues (Arg8, Asp30′, and Gly48′) of HIV-1 PR (Figure 2, bottom). In addition, van der Waals interactions are observed between the nonpolar unsubstituted fullerene cages of **2S** and **2R** with the nonpolar moieties of residues such as Leu23, Ala28, Val32, Ile47, Ile50, Gly52, Phe53, and Pro81 of HIV-1 PR (Figure 2, middle and bottom). On the substituted part of **2S** and **2R**, the nonpolar surfaces also form further van der Waals interactions with nonpolar segments of the binding cavity residues (Figure 2, middle and bottom).

However, compound **1** forms only two H-bonds with Asp25′ and Ala28′ (Figure 3). The van der Waals interactions of its nonpolar surface are observed with nonpolar segments of HIV-1 PR residues (e.g., Leu23, Ala28, Val32, Ile47, Ile54, Pro81, Val82, and Ile84) (Figure 3). In addition, compound **1** shows van der Waals interactions between its phenyl substituent with Val82′ (Figure 3). The position of the best binding energy poses from molecular docking simulations of compounds **3R** and **3S** is similar at the binding pocket of HIV-1 PR. Compound **3S** formed four H-bonds with Arg8 and Asp30′; and compound **3R** formed three H-bonds with Arg8, Asp30′, and Gly48′ (Supporting Information, Figure S2). In addition, similar van der Waals interactions for compounds **2S** and **2R** are observed between the nonpolar unsubstituted fullerene cages of **3S** and **3R** with nonpolar surfaces of binding site residues. The presence of additional H-bonding and van der Waals interactions of compound **2** as compared to compounds **1** and **3** is consistent with the higher binding efficiency of the former. The experimental binding affinity values have shown that the calculations predicted satisfactorily the above property of compound **3** but overpredicted the degree of enhancement for derivative **2.** This may be attributed to its higher flexibility.

In conclusion, *in silico* screening approach has been used in order to propose potent fullerene analogues as anti-HIV drugs. Two of the most promising derivatives showing significant binding scores were subjected to biological studies that confirmed the efficacy of the new compounds. The results showed that new leads can be discovered possessing higher bioactivity. Our *in silico* screening approach, accompanied with biological tests, opens a new avenue for the discovery of more efficient anti-HIV drugs.

Abbreviations: Baa, bucky amino acid; Fmoc-Baa, fluorenylmethoxycarbonyl protected Baa; HIV-1 PR, human immunodeficiency virus type I aspartic protease.

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Supporting Information Available: Theoretical background for docking studies, screening of potential energy and enzyme backbone rmsd throughout the MD simulations, and binding interactions of **3S** and **3R** with active site residues of HIV-1 PR. This material is available free of charge via the Internet at http://pubs.acs.org.

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