Inorganic Polyhedral Metallacarborane Inhibitors of HIV Protease: A New Approach to Overcoming Antiviral Resistance

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HIV protease (PR) is a prime target for rational anti-HIV drug design. We have previously identified icosahedral metallacarboranes as a novel class of nonpeptidic protease inhibitors. Now we show that substituted metallacarboranes are potent and specific competitive inhibitors of drug-resistant HIV PRs prepared either by site-directed mutagenesis or cloned from HIV-positive patients. Molecular modeling explains the inhibition profile of metallacarboranes by their unconventional binding mode.

Introduction

HIV protease (PR)[a] cleaves the Gag and Gag-Pol polyproteins of the virus into structural proteins and replication enzymes to yield infectious viral particles. Prevention of this processing by mutation or chemical inhibition of PR leads to the production of immature progeny virus particles;[1] inhibitors of HIV PR are therefore effective virostatics. There are nine FDA-approved PR inhibitors currently on the market, and several others are making their way through the pharmaceutical pipeline.[2] All of the clinically active compounds identified to date are competitive inhibitors that target the PR active site. Most of them contain nonhydrolyzable peptide isosteres that mimic the transition state of substrate hydrolysis.

The evolution of drug resistant PR variants due to the rapid selection of resistant viral strains is a major complication of anti-HIV therapy.[3,4] The development of new, effective PR inhibitors with a novel mechanism of action capable of inhibiting multidrug resistant species and/or showing a high genetic barrier to resistance development[4,5] is still very relevant.

In our search for new structural types of versatile compounds that inhibit HIV PR, we identified a group of inorganic carbon/boron cluster complexes as promising frameworks for a novel class of nonpeptide PR inhibitors (PIs).[6] Our main attention has been focused on cobaltacarboranes, specifically the [3-cobalt bis(1,2-dicarbollide)]$^-$ ion **GB-18** (see Figure 1). There are several reports describing boron cluster-containing compounds as potential pharmaceuticals (antineoplastic and cytotoxic agents, estrogen agonists and antagonists, protein kinase C modulators), building blocks for BNCT (boron neutron capture therapy) pharmaceuticals, and agents for radioimaging.[7] On the other hand, not much is known about the toxicity of metallacarboranes in animals. Some early studies suggest low toxicity of metallacarboranes of the structural type of [3-cobalt bis(1,2-dicarbollide)]-ion in rats.[8]

In this paper, we show that cobaltacarboranes are able to inhibit a panel of recombinant HIV-1 PR species bearing signature resistance-conferring mutations for various FDA-approved PR inhibitors as well as highly resistant PR species amplified from HIV-positive patients failing antiretroviral therapy using HIV PR inhibitors. We provide a molecular model based on the recently published X-ray structure of a PR−metallacarborane complex that illuminates the structural reason for the activity of this novel family of HIV PR inhibitors.

Chemistry

The synthesis and characterization of all metallacarboranes, discussed in this paper, has been described previously.[6] Briefly, the cesium salt of **GB-18** was converted to a sodium salt by simple extraction metathesis.[9] The ring opening of 8-dioxane-3-cobalt bis(1,2-dicarbollide)[10] under mild conditions provided a series of *exo*-skeletally modified metallacarborane cluster anions **GB-21, GB-48,** and **GB-80**.[6] Compounds **GB-48** and **GB-80** involve the same symmetric structural pattern, in which two cobalt bis(1,2-dicarbollide) subunits are connected via a flexible hydrophilic spacer. The presence of an amino group in the center of the chain originates their unique zwitterionic-anionic character.

Results and Discussion

Selected metallacarboranes (Figure 1) were tested in vitro as potential inhibitors of the recombinant PR variants listed in Table 1. PRs 1−4 are variants prepared by in vitro

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[a] Abbreviations: BNCT, boron neutron capture therapy; FDA, Food and Drug Administration; HAART, highly active antiretroviral therapy; HIV-1, human immunodeficiency virus type 1; k_{cat}, catalytic rate constant; K_i, inhibition constant; K_m, Michaelis constant; Nle, norleucine; Nph, *p*-nitrophenylalanine; PR, protease; QM, quantum mechanics; wt, wild-type.

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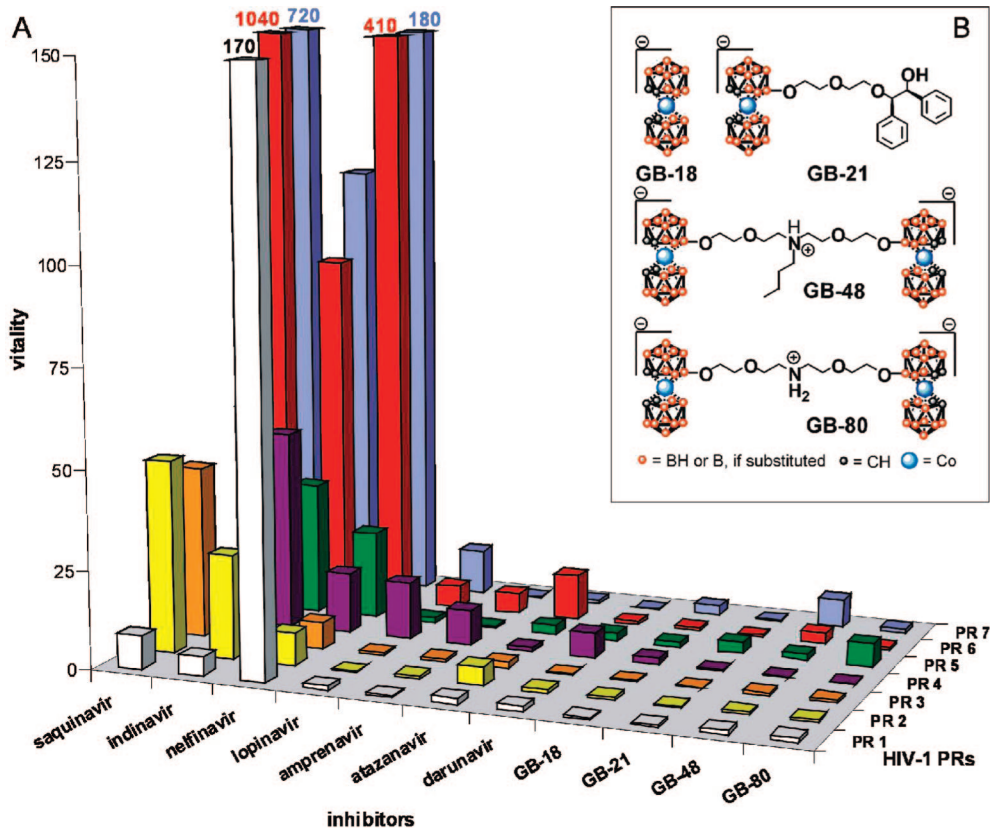

Figure 1. (A) Vitalities of seven clinical inhibitors and metallacarborane compounds with the panel of PR mutants. (B) Structural formulas of metallacarborane inhibitors used in this work. All compounds were prepared as Na^+ salts.

mutagenesis that bear signature primary mutations responsible for decreased susceptibility to nelfinavir, ritonavir, indinavir, and lopinavir, whereas PRs 5−7 are enzymes amplified from HIV-1 positive patients heavily pretreated with various PR inhibitors. Note that the latter group of PRs have up to 17 amino acid exchanges compared to the wild-type enzyme (PR7). All of the proteases were expressed in *Escherichia coli,* refolded from inclusion bodies, purified to homogeneity, and fully enzymologically characterized as previously reported.[11,12] Their enzyme kinetic parameters (K_m and k_{cat}) are summarized in Table S1 (see Supporting Information).

In Vitro Resistance Profile. Four metallacarborane compounds were selected and tested for their ability to inhibit a panel of selected proteases. These compounds exhibit classical competitive binding (data not shown), and no significant toxicity in tissue cultures was observed in the concentration range up to 50 μM. For comparison, seven clinically available inhibitors (saquinavir, indinavir, nelfinavir, lopinavir, amprenavir, atazanavir, and darunavir) were included in our inhibition assay. Table 1 summarizes the inhibition constants (K_i values) and relative increases in K_i values compared to the wild-type PR. Metallacarborane compounds proved to be potent inhibitors of wild-type PR and preserved their inhibitory capacity toward all tested PRs. The relative inhibition values (K_i mutant PR/K_i wt PR) for compounds **GB-18**, **GB-21**, **GB-48**, and **GB-80** range from 0.6 to 20, whereas relative inhibition values for the clinically available inhibitors range from 0.8 to 4500.

To correlate the relative K_i values with the changes in catalytic efficiency caused by the mutations in PR, the catalytic efficiency of the mutated PRs must be taken into account. The term "vitality" was introduced[13] as a measure of the relative capability of a mutated enzyme to cleave its substrate in the presence of an inhibitor. It is is defined as $\upsilon = (K_i\, k_{cat}/K_m)_{MUT}/(K_i\, k_{cat}/K_m)_{WT}$ and predicts the therapeutic effect of a given PI. Figure 1 depicts the relative vitalities of the panel of resistant PR species PR1-PR7 with compounds **GB-18**, **GB-21**, **GB-48**, and **GB-80** and comparison with clinically available inhibitors. Metallacarboranes show a low relative loss of activity, as demonstrated by the values of vitalities for all PR variants tested (Figure 1). While the specific mutations in the PR lead to dramatic (50-fold to 1000-fold) increases in vitality for saquinavir, indinavir, and nelfinavir, the increase in vitality is less dramatic for lopinavir, amprenavir, and darunavir (PR4, which harbors mutations V32I and I47A, shows the highest vitality). Metallacarborane compounds **GB-18**, **GB-21**, **GB-48**, and **GB-80** show a rather low relative loss of inhibition potency, as seen from the relative values of vitalities for all the PR variants in Figure 1.

Inhibitor Binding to HIV PR. The crystal structure of the wild-type enzyme in complex with **GB-18** (PDB accession 1ZTZ) revealed a unique binding mode of metallacarboranes in the HIV PR active site.[6] Two molecules of **GB-18** bind to the flap-proximal part of the enzyme active site cleft and hold the flaps in a semi-open conformation, occupying two hydrophobic pockets that are formed by the side chains of residues Pro81, Ile84, and Val82 and covered by the flap residues Ile47, Gly48, and Ile54. Interestingly, the two molecules (designated Cb1 and Cb2) of **GB-18** bind asymmetrically into the symmetrical active site (Figure 2A).

Mutations in the residues that form the inhibitor binding site are present in several of the protease variants tested. The primary mutations I47A, I54V, V82A, and I84V cause spatial

Table 1. K_i Values [nM] for the Inhibition of PR Mutants by Seven Clinically Available Inhibitors and by Metallacarboranes **GB-18**, **GB-21**, **GB-48**, and **GB-80**[a]

inhibitor	wild-type	PR 1	PR 2	PR 3	PR 4	PR 5	PR 6	PR 7
saquinavir	0.04 ± 0.01 (1)	0.51 ± 0.07 (13)	13 ± 2 (25)	12 ± 1 (290)	0.22 ± 0.01 (5.5)	2.9 ± 0.3 (73)	180 ± 15 (4500)	71 ± 6(1800)
indinavir	0.12 ± 0.02 (1)	0.88 ± 0.07 (7)	21 ± 4 (180)	13 ± 1.2 (110)	56 ± 6 (470)	5.5 ± 0.3 (46)	47 ± 3 (390)	33 ± 1 (280)
nelfinavir	0.07 ± 0.01 (1)	18 ± 1 (260)	3.8 ± 1.1 (54)	3.2 ± 0.2 (46)	10 ± 1 (140)	2.1 ± 0.3 (30)	130 ± 9 (1800)	32 ± 2 (450)
lopinavir	0.018 ± 0.009 (1)	0.026 ± 0.006 (1.5)	0.029 ± 0.007 (1.6)	0.060 ± 0.004 (3.3)	2.4 ± 0.5 (130)	0.029 ± 0.006 (1.6)	0.44 ± 0.09 (24)	0.50 ± 0.03 (28)
amprenavir	0.18 ± 0.02 (1)	0.13 ± 0.04 (0.7)	0.90 ± 0.10 (5)	0.82 ± 0.13 (4.5)	15 ± 3 (82)	0.15 ± 0.05 (0.8)	4.1 ± 0.3 (22)	0.13 ± 0.05 (0.7)
atazanavir	0.024 ± 0.005 (1)	0.055 ± 0.006 (2.3)	0.76 ± 0.04 (32)	0.23 ± 0.01 (9.4)	0.17 ± 0.02 (7.1)	0.076 ± 0.004 (3.2)	1.2 ± 0.4 (50)	0.054 ± 0.003 (2.3)
darunavir	0.0053 ± 0.0036 (1)	0.011 ± 0.001 (2)	0.032 ± 0.011 (6)	0.0043 ± 0.0007 (0.8)	0.31 ± 0.04 (58)	0.015 ± 0.004 (2.8)	0.02 ± 0.06 (3.7)	<0.001 (<0.2)
GB-18	66 ± 30 (1)	39 ± 14 (0.6)	330 ± 5 (5)	150 ± 35 (2.2)	870 ± 44 (13)	120 ± 20 (1.8)	210 ± 20 (3.1)	410 ± 30 (6.2)
GB-21	20 ± 5 (1)	13 ± 0.5 (0.7)	34 ± 9 (1.7)	39 ± 4 (2)	41 ± 1 (2.1)	82 ± 6 (4.1)	41 ± 5 (2.1)	9.1 ± 3.7 (0.5)
GB-48	2.2 ± 1.2 (1)	3.7 ± 2.2 (1.7)	9.0 ± 3.4 (4.1)	11 ± 4 (5)	2.7 ± 1.3 (1.2)	4.6 ± 2.7 (2.1)	24 ± 5 (11)	39 ± 6(18)
GB-80	4.9 ± 2.1 (1)	8.1 ± 1.5 (1.7)	22 ± 5 (4.5)	21 ± 8 (4.3)	5.0 ± 1.1 (1)	40 ± 17 (8.1)	13 ± 3 (2.6)	14 ± 1 (2.9)

[a] These inhibition constants were determined by spectrophotometric assay at the pH optimum of the protease (pH 4.7). Numbers in parentheses show relative increases in K_i values for the corresponding recombinant mutant proteases as compared with wild-type. The recombinant proteases bear the following mutations in comparison to the consensus wild-type HIV PR strain BH10: PR 1-D30N/N88D, PR 2-M46I/A71V/V82T/I84V, PR 3-A71V/V82T/I84V, PR 4-V32I/I47A, PR 5-L10I/I15V/E35D/N37S/R41K/I62V/L63P/A71V/G73S/L90M, PR 6-L10I/L24I/L33F/M46L/I54V/L63P/A71V/V82A/I84V, PR 7-L10F/L19I/K20R/L33F/E35D/M36I/R41K/F53L/I54V/L63P/H69K/A71V/T74P/I84V/L89M/L90M/I93L.

Figure 2. (A) Overall view of wild-type PR−**GB-18** complex crystal structure (PDB code 1ZTZ[6]). The PR dimer is shown in ribbon representation with two molecules of **GB-18** (represented as spheres) bound to the flap-proximal part of the enzyme active site. The two mutations at positions 82 and 84, which were studied by molecular modeling, are indicated (B). (C) Detail of two molecules of **GB-18** (Cb1 and Cb2) bound to the active site of PR wild-type (panel B) and to the V82T/I84V variant (panel C). Metallacarboranes (pink, boron; black, carbon; white, hydrogen; cyan, cobalt) as well as residues 82/82′ and 84/84′ of PR (yellow, carbon; white, hydrogen; red, oxygen; blue, nitrogen) are shown as sticks. Also, the PR solvent accessible surface is shown colored by charge from positive (blue) to negative (red). Dihydrogen bonds are shown as dashed lines with d_{HH} distances in Å. The figure was prepared with PyMol 0.99 (DeLano Scientific LLC, 2002).

enlargement of the S3 and S3′ substrate binding pockets. We employed molecular modeling to study the impact of mutations at residues 82/82′ and 84/84′ on interaction with **GB-18**. Metallacarboranes interact with biomolecules via unconventional B−H···H−X dihydrogen bonds, where X stands for N, O, or C atoms. The characteristic distance between interacting hydrogens d_{HH} is 1.8−2.3 Å.[14] In our optimized active-site structural models, we searched for differences in dihydrogen bonding between **GB-18** and either wild-type PR or a V82T/I84V PR mutant. In the mutant, the dihydrogen bond between the first molecule of **GB-18** (Cb1) and Thr82 is weaker than the interaction with Val82 (d_{HH} is longer by 0.1 Å), while the interaction with residue 84 is lost due to the I84V mutation (Figure 2B,C). Residues 82′ and 84′ are unable to interact with the second molecule of **GB-18** (Cb2) in the V82T/I84V PR variant due to distance constraints.

The strength of C−H···H−B dihydrogen bonds in carborane−amino acid complexes has been calculated as 2.1−5.8 kcal/mol.[14] Our experimental data show that the presence of primary mutations in the S3/S3′ subsites does not have a dramatic effect on metallacarborane inhibition compared to other inhibitors that occupy these subsites (e.g. saquinavir and indinavir). We observed 2- and 6-fold decreases in the binding affinity of **GB-18** toward PRs 2 and 3 (which contain V82T and I84V substitutions), respectively, as compared to the wild-type PR (Table 1). The changes in Gibbs free energy are thus 0.4 and 1 kcal/mol, respectively. These values are smaller than our estimate of 2.1−5.8 kcal/

mol, corresponding to the loss of the dihydrogen bond found in our models. We propose that repositioning of **GB-18** in the mutated PR binding site might explain the preserved inhibitory capacity of metallacarboranes. Indeed, two different modes of binding into identical binding sites were observed in the crystal structure,[6] implying that the metallacarborane cage has a certain degree of freedom in its binding position and that this position can be adjusted in response to binding pocket alterations.

Conclusions

Substituted metallacarboranes are potent, specific, and selective competitive inhibitors of wild-type and mutated HIV PRs. We explain their potential to inhibit a variety of PI-resistant PR species due to their novel binding mode in the PR binding pockets via unconventional proton−hydride hydrogen bonds (dihydrogen bonds) and their ability to adjust the position of the metallacarborane cage within the HIV PR substrate binding cleft. We conclude that boron clusters are promising pharmacophores for potent and specific inhibition of drug-resistant HIV protease mutants.

Experimental Methods

Resistant PRs were prepared by site-directed mutagenesis (HIV-1 PR 1−4) as previously described[11,12] or selected under the pressure of clinically available PIs (HIV-1 PR 5−7).[15] HIV-positive patients receiving highly active antiretroviral therapy (HAART) at the Faculty Clinic Bulovka in Prague within a long-term epidemiological study have been followed for the presence of resistant HIV species. Selection of the patients for this study was based on genotyping and clinical markers suggesting development of resistance to protease inhibitors.[15,16]

Inhibition Assay. The inhibition constants (K_i) were determined by spectrophotometric assay using the chromogenic peptide substrate LysAlaArgValNle*NphGluAlaNle-NH_2 as previously described.[11] Typically, 8−10 pmol of 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 substrate at a concentration near the K_m of the enzyme and various concentrations of inhibitor dissolved in DMSO. 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 UNICAM UV500 UV−vis spectrophotometer (Thermo, Cambridge, MA). The HIV PR remained stable over the whole reaction time. Inhibition data were analyzed using the equation for competitive inhibition according to Williams and Morrison.[17]

Inhibitor Synthesis. The synthesis and characterization of all metallacarboranes, discussed in this paper, has been described previously.[6]

Molecular Modeling. The effects of the Val82Thr and Ile84Val mutations in HIV-1 PRs 2 and 3 on the binding of compound **GB-18** were studied using molecular modeling. We added hydrogen atoms to the X-ray structure of the complex of wild-type HIV-1 PR with **GB-18** (PDB code: 1ZTZ)[6] using the program InsightII 2000 (Accelrys Software Inc., 2000). For quantum calculations, we selected the two molecules of **GB-18**, designated Cb1 and Cb2, and parts of active-site PR residues (Val32, Ile47, Gly48, Gly49, Ile54, Thr80, Pro81, Val82, Ile84, Ile47′, Gly48′, Thr80′, Pro81′, Ile84′) together with the peptide found in the PR active site and three structural water molecules. The model for the resistant variant was created by replacing the Cγ2 atoms of Val82/Val82′ by Oγ1, followed by deleting Cδ methyl groups from residues Ile84/Ile84′ and subsequent protonation.

Quantum chemical optimizations of the models were performed: (i) for hydrogen atoms only of the wild-type PR-compound **GB-18** complex and (ii) for the whole model except the anchoring atoms of the resistant PR variant−**GB-18** complex. The QM calculations were performed with the Turbomole 5.7[18] program package augmented with London dispersion energy.[19]

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Supporting Information Available: List of PR variants analyzed in this study, including the specific mutations and enzyme characteristics (K_m, k_{cat}, and catalytic efficiencies [k_{cat}/K_m]). This material is available free of charge via the Internet at http://pubs.acs.org.

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