Enzyme Inhibitors

DOI: 10.1002/anie.201005673

From Conventional to Unusual Enyzme Inhibitor Scaffolds: The Quest for Target Specificity

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chemical biology · drug design · enzyme inhibitors · medicinal chemistry · selectivity

The tremendous challenge presented by the specific molecular recognition of single biomacromolecular targets within complex biological systems demands novel and creative design strategies. This Minireview discusses some conventional and unusual approaches for the design of target-selective enzyme inhibitors with a focus on the underlying chemical scaffolds. These include complicated natural-product-like organic molecules, stable octahedral metal complexes, fullerenes, carboranes, polymetallic clusters, and even polymers. Thus the whole repertoire of organic, inorganic, and macromolecular chemistry can be applied to tackle the problem of target-specific enzyme inhibition.

1. Introduction

Chemical compounds that are potent and selective modulators of biomacromolecular functions are highly valued reagents in biological and medicinal research, often called "chemical probes" or "molecular probes".[1–3] Such compounds are important in their own right even if they are never considered as drug candidates because of unsuitable pharmacokinetic or pharmacodynamic properties. A key criterion for the quality and usefulness of such synthetic compounds is the selectivity for a chosen target,[4] which can be a particular enzyme, cell surface receptor, nuclear hormone receptor, ion channel, transporter, or nucleic acid. Considering the large number of different proteins in a cell—the human genome contains around 20000–25000 protein-encoding genes[5]—in addition to the presence of nucleic acids, carbohydrates, membranes, cofactors, and other small molecules, it is a truly extraordinary challenge to design compounds that reach exclusive protein-target selectivity, often referred to as "target specificity".[6] This is even more of a dilemma for protein targets that are members of large and homologous protein families such as protein kinases, lipid kinases, and proteases.[7,8] One might question whether the typical small and structurally relatively simple organic molecules are theoretically even capable of reaching complete specificity owing to a limited number of possible interactions with the target in combination with a conformational flexibility that typically enables undesired binding promiscuity.

Thus, new and creative strategies for the design of highly target-specific bioactive compounds are sought in order to reach the envisioned precise control over the manipulation of biological processes. In this Minireview, some recent approaches towards the design of selective enzyme inhibitors will be discussed. Particular emphasis will be placed on the underlying chemical scaffolds, including natural-product-like, highly preorganized organic and metal-containing scaffolds, polymers, inert as well as reactive clusters, and structurally very simple molecules that exploit covalent or coordinative bond interactions with the active site for achieving high selectivity.

2. A Perfect Fit with Highly Preorganized Structures: From Natural-Product-like Molecules to Octahedral Metal Complexes

Complicated natural products often display exquisite target selectivities which make them an invaluable source for the development of new therapeutic agents[9] as well as ideal tools for the study of biological systems.[10] This high target selectivity can often be traced back to their preorganized three-dimensional scaffolds which perfectly complement the target-protein pockets in shape and functional-group presentation. Copying nature's approach, Diederich and co-

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workers reported an impressive example of using a complicated, natural-product-like scaffold for the design of selective thrombin inhibitors, a coagulation enzyme in the blood stream. Diederich et al. designed a class of rigid, tricyclic lactam inhibitors that bind in a highly preorganized fashion to the active site of thrombin, as shown in Figure 1 for the complex of thrombin with the tricyclic compound **1**.[11,12] The rigid tricyclic core structure serves to precisely orient substituents into three distinct subpockets at the active site. Accordingly, the hydrophobic selectivity (S1) pocket is filled with a phenylamidinium residue forming a bidentate salt bridge with the side chain of Asp189 at the bottom of the pocket, the large hydrophobic distal (D) pocket is occupied by a *para*-chlorobenzyl residue, and the narrow proximal (P) pocket is filled with an isopropyl residue. Tricycle **1** is a low-nanomolar-range inhibitor for thrombin ($K_i = 8$ nM) with a remarkable selectivity over the related serine protease trypsin by a factor of 1609. This selectivity can be explained by the position of the isopropyl group which occupies the P pocket in thrombin. This P pocket is unique to thrombin and formed by an insertion loop in the structure and is missing in other related serine proteases such as trypsin. However, it is important to emphasize the importance of the rigid, tricyclic core, which is a prerequisite to precisely target this P pocket with a proper substituent.

As illustrated by Diederich's protease inhibitor, a tricycle with five asymmetric carbon centers, globular and preorganized structures are typically based on sophisticated scaffolds including multiple stereocenters, which are often cumbersome to synthesize. This drawback can be traced back to the intrinsic limitation of carbon to linear, trigonal-planar, and tetrahedral bonding modes. It is therefore very attractive to devise alternative strategies towards globular compounds with defined shapes. One such approach uses metal complexes, in particular octahedrally coordinated metals, in which the central metal with its rich stereochemistry, in combination with tailored coordinated ligands, establishes the overall globular geometry of the compounds.[13–15]

In a recent example from our laboratory, the octahedral organoruthenium complex Λ-FL172 was designed as a selective inhibitor for the p21-activated kinase 1 (PAK-1) (Figure 2).[16,17] PAK-1 harbors a particularly open ATP-binding site, making it difficult to target with typical small organic scaffolds, but particularly suitable for filling with bulky and rigid octahedral complexes. The cocrystal structure of PAK1 with Λ-FL172 shown in Figure 2 reveals that a bidentate pyridocarbazole ligand of the ruthenium complex occupies the adenine binding site of ATP and interacts with the hinge region. The distance between the CO and the pyridine ligand in *trans* position stretches across the space between the flexible glycine-rich loop and the surface of the C-terminal domain and thus serves as a rigid yardstick of around 8 Å to discriminate between different sizes of protein kinase ATP-binding sites. As a cautionary note it is worth mentioning that although such a perfect fit with rigid structures is highly desirable for achieving potency and selectivity, it also requires especially careful design in which even minor deviations can have a dramatic effect on binding affinities. For example, adding to the scaffold of FL172 only a single methyl group or small fluorine atom at the *para* position of the phenyl substituent completely destroys its binding affinity because of an unavoidable steric clash with the glycine-rich loop (Figure 2).

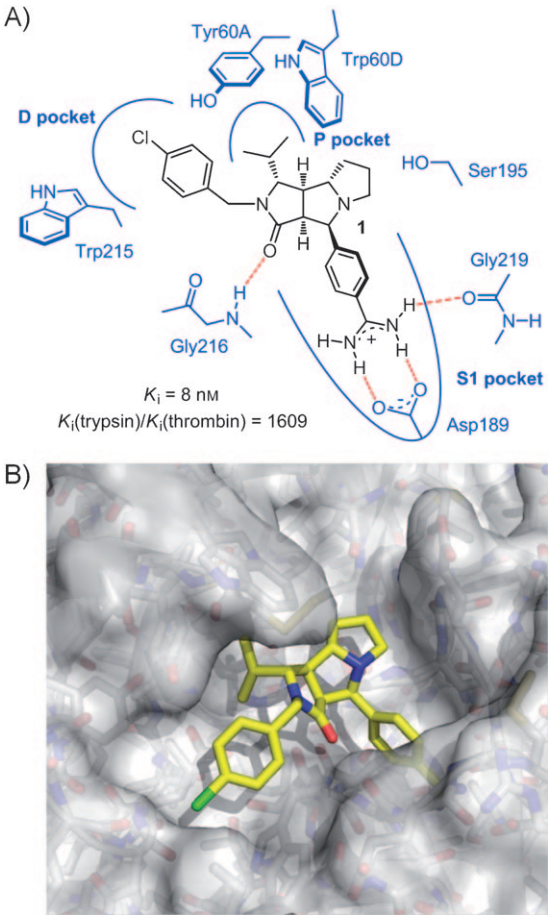
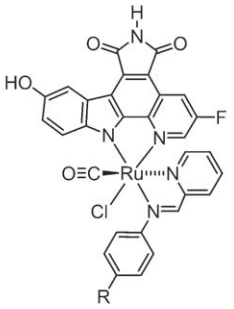


Figure 1. Thrombin inhibitor **1** with a tricyclic lactam framework. A) Schematic representation of the binding mode of inhibitor **1** in the active site of thrombin. The active site is defined by the catalytic center with the nucleophile Ser195, the selectivity (S1) pocket, a large hydrophobic distal (D) pocket, and a small proximal (P) pocket. Adapted from Ref. [12]. B) X-ray structure of the complex between thrombin and **1** (PDB code 2CF8), demonstrating the perfect shape complementarity.

Eric Meggers received his PhD from the University of Basel (Switzerland) in 1999 for research on long-range electron transport in DNA conducted under the guidance of Prof. Bernd Giese. After postdoctoral research on artificial metal-mediated base pairs in DNA with Prof. Peter G. Schultz at the Scripps Research Institute in La Jolla (USA), he joined the Chemistry Department at the University of Pennsylvania in 2002 as an Assistant Professor. Since 2007, Eric Meggers has been Professor of Chemical Biology at the Philipps-University Marburg. His current main research interests are the design of biologically active inert metal complexes and their stereoselective synthesis.



R = H: Λ-FL172: IC_{50} = 130 nM
R = CH_3: *rac*-FL224: 4% inhibition at 100 nM
R = F: *rac*-FL252: no inhibition at 100 nM

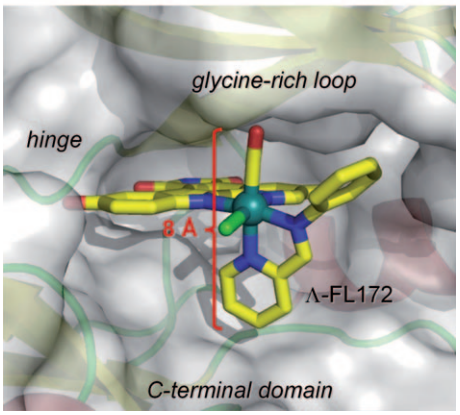


Figure 2. Cocrystal structure of the large and rigid octahedral ruthenium complex Λ-FL172 bound to the open ATP-binding site of the protein kinase PAK-1. IC_{50} values and activities at 100 nM were measured in the presence of 1 μM ATP.

3. Bigger and More Selective: Carboranes, Carbon Cages, and Polyoxometalates

Many elements of the periodic table display rich cluster chemistry which can be exploited for the design of enzyme inhibitors. Owing to their unusual geometry, such clusters most likely populate areas of chemical space[18] that cannot be accessed with purely organic structures. Clusters are novel and potentially useful scaffolds for the inhibition of certain enzymes since particularly large, flexible, or open enzyme active sites can be filled in unique ways. Clusters of carbon and boron (carboranes) and their metal derivatives (metallacarboranes) are an interesting class not only because of their unusual structures, but also because of thermal and metabolic stabilities, high lipophilicity, and the ability to form unusual proton–hydride (dihydrogen) bonds involving B–H groups.[19] For example, a series of metallacarboranes, specifically cobalt bis(dicarbollide) complexes in which two icosahedral cages share a common vertex, were recently reported as promising and novel frameworks for nonpeptidic inhibitors of HIV protease, a prime target for HIV therapy owing to its integral role in HIV replication.[20–22] It was discovered that the 3-cobalt bis(1,2-dicarbollide) anion (**2**) inhibits HIV-1 protease with a K_i value of 66 nM. The crystal structure of this metallacarbollide in complex with HIV-1 protease is shown in Figure 3.[20] HIV-1 protease is an aspartic protease in which the active site lies at the interface of two identical subunits. The crystal structure revealed that two carbollide anions bind to the symmetrical active site in the flap-proximal region of the hydrophobic S3 and S3′ subsites, holding the flap in a semi-open conformation. An open conformation of the flap is typical for uncomplexed structures, whereas inhibitor-bound structures typically show closed flap conformations. Inspired by this crystal structure with two metallacarbollides bound to the active site, derivatives were devised, in which two metallacarbollides are connected by flexible, hydrophilic linkers. This led to the discovery of the bis(metallocarbollide)

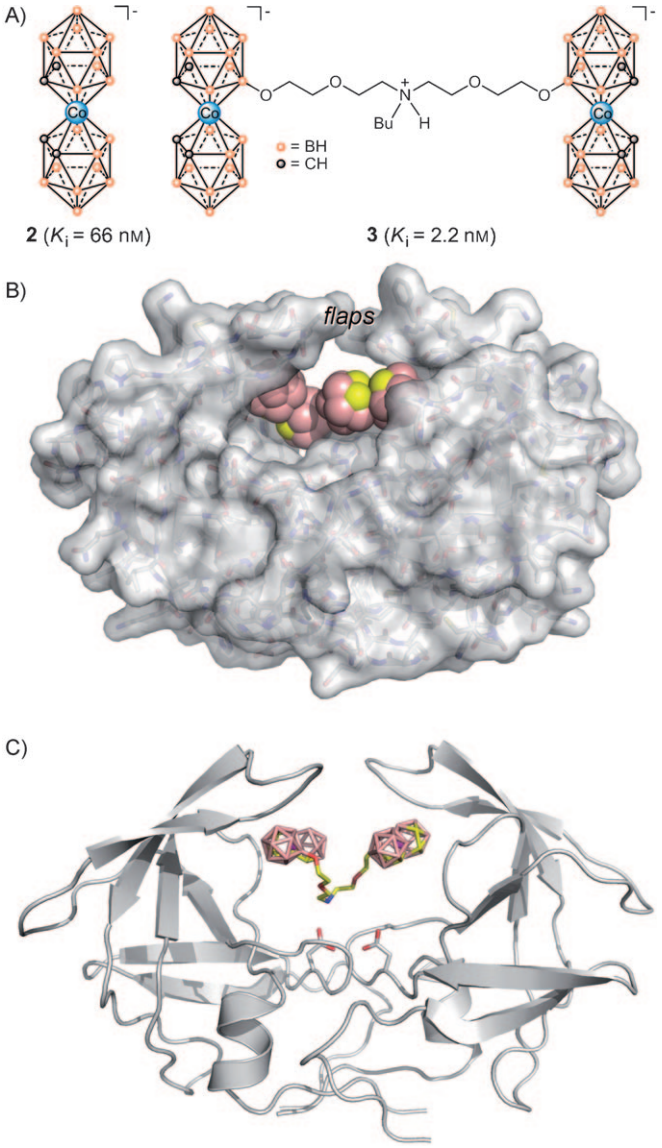


Figure 3. Cobalt bis(1,2-dicarbollide) clusters as HIV-1 protease inhibitors. A) Monomeric and dimeric metallacarborane inhibitors. B) Crystal structure of HIV-1 protease dimer with two bound cobalt bis(1,2-dicarbollide) anions **2** (PDB code 1ZTZ). Complexes **2** are shown as their van der Waals surfaces. C) A derivative of the bis-metallocarbollide compound **3** bound to HIV-1 protease with a modeled conformation of the disordered linker (PDB code 3I8W). The two catalytic aspartate residues are in close proximity to the protonated amine of the linker.

compound **3**, which was determined to be a low-nanomolar inhibitor for HIV protease with a K_i value of 2.2 nM. Compound **3** also exhibited a submicromolar EC_{50} value in antiviral tests and no toxicity in tissue culture, and was only weakly or not at all inhibited by other proteases such as cathepsin D, pepsin, trypsin, papain, and amylase.[21,22] Interestingly, this class of compounds also shows potent binding to some clinically relevant HIV protease mutants. This was explained by the novel binding mode of the metallacarboranes in the HIV protease binding pockets by means of unconventional B–H···H–X (X = N, O, C) proton–hydride hydrogen bonds and their propensity to adjust the position of the metallacarborane cage within the binding cleft.[22]

Beyond carboranes, large carbon-based cages such as fullerenes and diamondoids are unconventional but appealing scaffolds for large active sites because of their unusual sizes and shapes along with the prospect of placing substituents at well-defined positions on the periphery of these structures.[23–25] For example, the diphenyl C_{60} alcohol shown in Figure 4 is a nanomolar inhibitor of HIV-1 protease with a respectable K_i value of 103 nM.[26] Molecular modeling and molecular dynamics studies indicated that it nicely complements the large hydrophobic cavity region of the HIV-1 protease active site with the flexible flaps closing tightly around the C_{60} inhibitor, thereby expelling water from the cavity and leading to a favorable binding energy.[26,27] These results indicate that the combination of available methods for the defined derivatization of C_{60} along with computational and/or structure-based design should give access to even significantly more potent C_{60}-based HIV-1 protease inhibitors and potent inhibitors of other enzymes.[28]

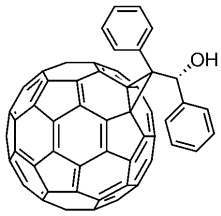


Figure 4. C_{60}-based HIV-1 protease inhibitor.

Completely inorganic clusters such as polyoxometalates (POMs) have been reported to exert antiviral, antitumor, and antibiotic activities.[29,30] Polyoxometalates are polyanionic clusters consisting of transition-metal oxyanions linked by edge-shared oxygen bridges to form closed three-dimensional frameworks, which in turn can enclose one or more heteroatoms. For example, the phosphomolybdate hexaanion $[P_2Mo_{18}O_{62}]^{6-}$ forms a Dawson structure and consists of a framework of 18 MoO_6 octahedrons connected through oxygen corners and surrounding two central PO_4 tetrahedrons. Such a Dawson type cluster, namely $K_6[P_2Mo_{18}O_{62}]$ (**4**), was identified in a recent study as a selective inhibitor for the protein kinase CK2 (Figure 5).[31,32] $K_6[P_2Mo_{18}O_{62}]$ was demonstrated to exhibit a remarkable potency for the protein kinase CK2 with an IC_{50} value of 1.4 nM at 100 μM ATP and at the same time a high selectivity in a panel of 29 kinases. Since POM **4** inhibits CK2 at less than equimolar concentrations and because POM structures are known for being prone to multiple equilibria depending on concentration, pH, and medium composition, the authors suggested that the powerful inhibition of CK2 by POM **4** is due to fragments of this compound. Kinetic studies, affinity chromatography, and trypsin proteolysis in addition to site-directed mutagenesis led to the conclusion that fragments of the POM cluster bind in an allosteric fashion outside the ATP and peptide substrate binding site at the activation segment. In CK2, this segment is stabilized by contacts to the N-terminal region which maintains CK2 in an active state. Coordination of POMs to the activation segment may disrupt these contacts, locking CK2 in an inactive conformation. Thus, the unique binding mode of this nonclassical kinase inhibitor may provide an exploitable mechanism for developing potent CK2 inhibitors by further functionalizing POMs with organic moieties.

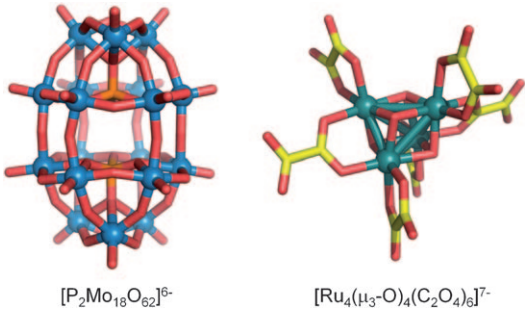


Figure 5. Polymetallic oxo clusters as enzyme inhibitors. $K_6[P_2Mo_{18}O_{62}]$ (**4**) and $Na_7[Ru_4(\mu_3\text{-}O)_4(C_2O_4)_6]$ (**5**) are low-nanomolar inhibitors for the protein kinase CK2 and HIV-1 reverse transcriptase, respectively.

It is worth mentioning that in contrast to labile POMs, anionic oxo clusters with incorporated organic ligands such as the ruthenium-oxo-oxalato cluster $Na_7[Ru_4(\mu_3\text{-}O)_4(C_2O_4)_6]$ (**5**) are actually stable under physiological conditions. Che et al. demonstrated complex **5** to be a highly potent inhibitor of HIV-1 reverse transcriptase with an IC_{50} value of 1.9 nM.[33] The further functionalization of this compound at one or several of the carboxylate moieties might give access to organic–inorganic hybrid structures with suitable pharmacological properties.

4. Plastic Inhibitors: Molecularly Imprinted Polymers

Molecularly imprinted polymers (MIPs) are frequently used for selective molecular recognition in analytical chemistry and for catalysis.[34,35] Haupt and co-workers recently demonstrated that MIPs are also promising materials for the generation of selective enzyme inhibitors.[36] In a clever strategy, a polymer microgel was polymerized around the active site of trypsin by using methacryloylaminobenzamidine **6** as a monomeric anchoring point in which the benzamidinium moiety is a well-established binder of the S1 pocket of trypsin (Figure 6). Monomer **6** was copolymerized with hydroxyethyl methacrylate and the cross-linker ethylene bisacrylamide to create highly solvated polymer particles (microgels) with controlled small sizes below 1 μm, which are molded around trypsin. Removal of trypsin afforded a polymer microgel **7** with the ability to inhibit trypsin with a nanomolar K_i value (79 nM). That is almost three orders of magnitude lower than the initial phenylbenzamidine monomer building block, thus demonstrating that the molded

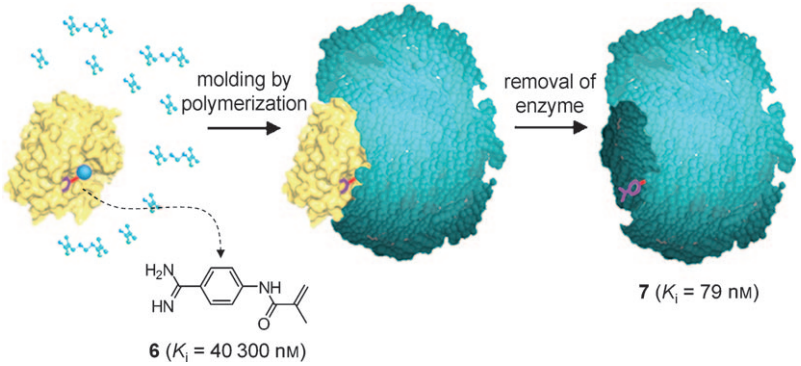


Figure 6. Microgel enzyme inhibitor by molecular imprinting. Methacryloylaminobenzamidine **6** is the monomeric building block containing a benzamidine moiety as a trypsin anchor point (colored purple, red, and blue, respectively). This is used to generate a polymer microgel (turquoise) molded around the enzyme trypsin (yellow), which is subsequently removed to afford a potent trypsin inhibitor **7**. Reproduced with modifications from Ref. [36] with permission of the American Chemical Society.

polymer significantly contributes to the binding affinity. Only weak and no inhibition was observed for the related proteases chymotrypsin and kallikrein, respectively.

This appears to be a promising technology for creating enzyme inhibitors for a variety of enzyme targets similar to the generation of antibodies for defined epitopes. In a future toolbox-like modular approach, additional substituents on the surface of the polymer microgel may be used to generate additional specific contacts or to target the polymer to certain receptors on the cell surface.

5. Reactive Enzyme Inhibitors: Simple, Reactive, and Selective

Particularly small and simple molecular scaffolds are highly attractive from an economical point of view but have the intrinsic disadvantage that they often cannot form enough weak interactions with the target protein to achieve high affinity and selectivity. However, the strategy of forming covalent inhibitor–enzyme complexes can overcome this limitation, because a significant amount of the binding energy arises from the bond formed between the small molecule and the enzyme, and one can expect that a limited number of additional noncovalent interactions are sufficient to achieve high potency and selectivity. In fact, a significant number of marketed drugs, for example, aspirin, the cyclooxygenase inhibitor that acts by acetylating serine in the active site, deactivate their enzyme targets by irreversible formation of covalent bonds within the active site.[37,38] A systematic review of known covalently modulated targets and their mechanism of action indicates that cofactor-mediated enzymes and enzymes bearing active-site cysteines or activated serines represent the most common targets for covalent modification.[38]

In a recent example, Taunton, Shokat, and co-workers reported a structured-based bioinformatics approach for designing a simple compound selective for the p90 ribosomal protein kinases RSK1 and RSK2.[39] The ATP-binding sites of the around 500 human protein kinases, which are the major target for the design of protein kinase inhibitors, are highly conserved, thus making the design of selective inhibitors highly challenging. A sequence alignment of 491 human protein kinases led to the identification of 11 protein kinases containing a reactive cysteine located in the flexible glycine-rich loop at a position that is typically occupied by a valine. Taunton and Shokat exploited this cysteine with the pyrrolo-[2,3-*d*]pyrimidine compound **8**, in which a fluoromethyl ketone moiety was designed to undergo a nucleophilic substitution with the cysteine side chain in the glycine-rich loop and to thus form a covalent bond (Figure 7). Exquisite selectivity for just the protein kinases RSK1 and RSK2 was then achieved with an additional toluyl substituent at the pyrrolopyrimidine scaffold that can reach into a deep hydrophobic pocket present in RSK1 and RSK2 but not in most others of the eleven protein kinases that bear a cysteine residue in the glycine-rich loop. This hydrophobic pocket is available in around 20% of all protein kinases which contain a small so-called "gatekeeper" amino acid at the entrance of this pocket. By designing a compound that exploits both the deep pocket (small gatekeeper amino acid) together with the reactivity of the cysteine in the glycine-rich loop, Taunton and Shokat reached exquisite selectivities for RSK1 and RSK2. This high selectivity was confirmed with a biotin-labeled inhibitor used in whole-cell lysates, in which out of thousands of reactive cysteine-containing proteins only RSK1 and RSK2 reacted with the reactive pyrrolopyrimidine. Thus, in this intriguing example, a simple organic molecule was designed to target selectively two protein kinases based on just two amino acids that distinguish RSKs from other protein kinases. These results are even more remarkable in the light of a recent study that revealed a correlation between binding selectivity and structural complexity of organic compounds as quantified by the relative content of sp^3-hybridized (shape complexity) and stereogenic carbon atoms (stereochemical complexity).[40]

Other cysteines within the ATP-binding site of protein kinases have been targeted with covalent bond formation, successfully resulting in the design of potent and selective inhibitors for the epidermal growth factor receptor (EGFR),

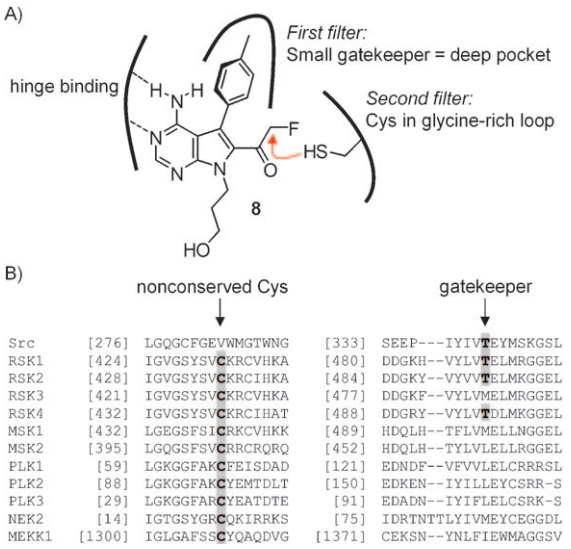


Figure 7. Design of protein kinase inhibitors guided by structural bioinformatics through the application of a double selectivity filter. A) The fluoromethylketone pyrrolopyrimidine inhibitor **8** is a classical ATP-competitive hinge binder but only binds to ATP-binding sites that fulfill two criteria simultaneously: a small gatekeeper amino acid and a cysteine in the glycine-rich loop. RSK2: $IC_{50} = 15$ nM (100 μM ATP) with greater than 600- and 200-fold selectivity over the C436V and T493M mutants, respectively. B) Sequence alignment of the 11 human kinases with a cysteine in the glycine-rich loop according to Ref. [39]. Of these kinases, only RSK1, RSK2, and RSK4 contain a small gatekeeper amino acid. The sequence of Src is shown as a reference.

the gatekeeper mutant EGFR T790M, Her2, and Bruton's tyrosine kinase (Btk), and to date five irreversible kinase inhibitors have been introduced into clinical study.[41] In these compounds acrylamide Michael acceptors serve as the electrophiles, which react with the solvent-exposed cysteines.

Reactivity based on the formation of coordinative bonds is also a highly suitable approach to provide strong contributions to the overall binding energy. For example, Fricker et al. recently reported a very simple oxorhenium(v) complex **9** (Figure 8) with an impressive low-nanomolar binding affinity for cathepsin B ($IC_{50} = 8.8$ nM).[42,43] Cathepsin B is a cysteine protease in which a cysteine is activated within a catalytic triad, resulting in an increased reactivity of the cysteine nucleophile. Kinetic studies and competition studies in combination with investigations by mass spectrometry reveal that the rhenium complex is an active-site-directed, time-dependent, slowly reversible inhibitor. This indicates coordination to the active-site cysteine by substituting the labile chloride ligand. This mode of action is analogous to the majority of organic cysteine protease inhibitors which form a reversible or irreversible covalent bond with the reactive cysteine in the active site. However, it is unexpected that this compound showed a 45-fold selectivity for cathepsin B over the related cathepsin K and some related parasite cysteine proteases. A structure–activity relationship around the tridentate 2,2′-thiodiethanethiolate ligand demonstrated the importance of a particular coordination sphere, which must be dictated by the size and shape of the active site of cathepsin B.

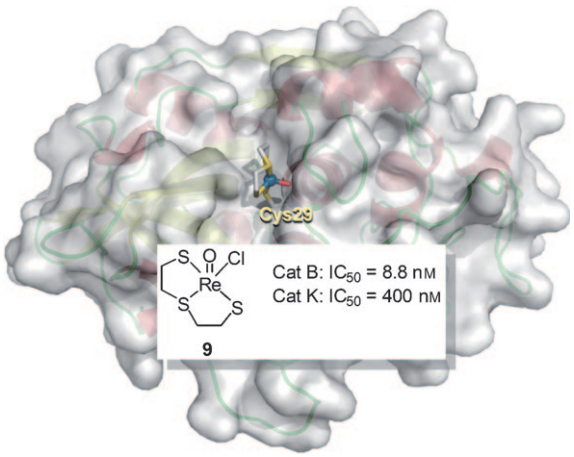


Figure 8. Oxorhenium(V) complex **9** as a selective and nanomolar inhibitor of cathepsin B. Shown is a putative docked binding mode of the complex in the active site of cathepsin B in which the active site Cys29 coordinates to rhenium by replacement of the chloride ligand.

6. Conclusions

Despite clearly powerful technologies such as combinatorial chemistry, high-throughput screening, sophisticated selection methods, computer-assisted drug design, and virtual compound screening, the design and discovery of completely target-specific compounds is still a more or less unsolved problem. In this Minireview we focused on the nature of the underlying chemical scaffolds, discussing interesting examples ranging from traditional to highly unconventional structures used for the design of potentially very selective enzyme inhibitors. Clearly, the scaffold of choice depends on the nature of the targeted enzyme, the shape and size of the active site, the type of enzyme-catalyzed reaction, and the availability of suitable reactive functional groups or cofactors. Although the strength of using more traditional organic scaffolds lies in the almost limitless possibilities for structural alterations, organic chemistry may not encompass the entire biologically relevant chemical space. Therefore, hopefully this short overview will inspire more biologists and chemists to leave traditional design pathways and to explore novel and unconventional chemical scaffolds for tackling the challenge of specific molecular recognition in complex biological systems.

I would like to thank Dr. Pavlína Řezáčová (Academy of Sciences of the Czech Republic, Prague) for sharing the modeled structure of a dimeric metallocarborane bound in the active site of HIV-1 protease which is the basis of Figure 3C, Prof. Bernold Hasenknopf (Université Pierre et Marie Curie, Paris, France) for providing the structure of the Dawson cluster shown in Figure 5, and Prof. Karsten Haupt (Compiègne University of Technology, France) for providing an image used for Figure 6.

Received: September 10, 2010
Published online: February 15, 2011

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