Chiral Drugs

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The Active Site of an Enzyme Can Host Both Enantiomers of a Racemic Ligand Simultaneously**

Matthias Mentel, Wulf Blankenfeldt, and Rolf Breinbauer**

Dedicated to Professor Herbert Waldmann

Since Pasteur discovered the principle of chirality and its implications in interactions with biological systems,[1] the effect of chirality in drugs has been the subject of intense investigation. The most common case is that only one enantiomer of a racemic mixture binds to a biological receptor while the other can be regarded as "isomeric ballast" (Figure 1a). There are also cases in which the second enantiomer shows different behavior, ranging from agonistic or antagonistic binding to the same receptor to interactions with other biological targets, which can lead to cooperative, side, or even counterproductive effects.[2] Consequently, recent legal regulation requires that only single-enantiomer drugs may be marketed.[3,4] While the question of single-enantiomer drugs has been settled for the end of the drug-discovery process, racemic mixtures are still preferentially used in primary screens, mainly because of by the considerable efforts necessary to produce enantiomerically pure compounds and the pragmatic view that two compounds can be screened in one experiment.[5] If activity for a racemic mixture is found in an in vitro screen employing a defined target, isothermal titration calorimetry (ITC) experiments or cocrystallization with the racemate make it possible to identify the binding characteristics of the two enantiomers.[6] To the best of our knowledge such experiments have to date presented only data that can be rationalized by assuming that only one enantiomer is present in the protein receptor. In rare cases it has been demonstrated that both enantiomers bind individually in the binding pocket, but never at the same time (Figure 1b).[7] Here, we describe an unprecedented case of chiral recognition: we report the first crystal structure of a protein hosting both enantiomers of a racemic mixture simultaneously, thereby providing proof of a new type of enantiomer behavior which might have important implications for drug discovery (Figure 1c).

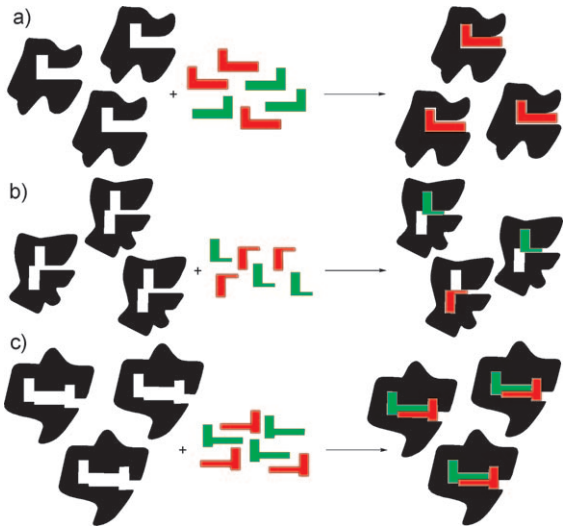

Figure 1. Three modes for the recognition of chiral drugs by a protein receptor: a) the target protein binds one enantiomer selectively from a racemic mixture, b) the ligand-binding pocket is able to host each enantiomer individually, and c) the ligand-binding pocket can host both enantiomers simultaneously.

During the course of our recent study investigating the role of the homodimeric PhzA/B enzyme of *Burkholderia cepacia* R18194 in phenazine biosynthesis,[8] we synthesized a series of achiral ligands that bound to the protein. Crystal structures of protein/ligand complexes revealed additional anchor points that should be targetable with suitable chiral molecules. Indeed, when PhzA/B crystals were soaked with racemic mixtures of these improved ligands, only one

[*] Dr. W. Blankenfeldt
Max Planck Institute of Molecular Physiology
Otto-Hahn-Strasse 11, 44227 Dortmund (Germany)
Fax: (+49) 231-133-2399
E-mail: wulf.blankenfeldt@mpi-dortmund.mpg.de
Prof. Dr. R. Breinbauer
Institute of Organic Chemistry
Graz University of Technology
Stremayrgasse 16, 8010 Graz (Austria)
Fax: (+43) 316-873-8740
E-mail: breinbauer@tugraz.at
Dr. M. Mentel, Prof. Dr. R. Breinbauer
Max Planck Institute of Molecular Physiology
Otto-Hahn-Strasse 11, 44227 Dortmund (Germany)
Dr. M. Mentel, Prof. Dr. R. Breinbauer
Institute of Organic Chemistry, University of Leipzig
Johannisallee 29, 04103 Leipzig (Germany)
Dr. M. Mentel
European Molecular Biology Laboratory (EMBL)
Meyerhofstrasse 1, 69117 Heidelberg (Germany)

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enantiomer was typically observed in the active center. These compounds emulate an intermediate of the twofold condensation reaction that the enzyme catalyzes, and their binding is characterized by polar interactions of their carboxylate groups with a motif formed by Y120/Q147/R160* (* indicates the second monomer) and with R41/S77, together with a hydrogen bond of the amine bridge with the catalytic residue E140 (Figure 2a).

To our surprise we discovered that one compound, *rac*-**1**, although of similar size and functional decoration, behaved completely differently: In the crystal structure the active site hosted two interacting ligand molecules of opposite chirality ((*R*)-**1** and (*S*)-**1**; Figure 2b). The two molecules bind in an orientation different from any of the other ligands previously investigated (exemplified in Figure 2a). Here, the carboxylate of the *R* enantiomer is sandwiched between the guanidino groups of R38 and R41, and the nitrogen atom of the piperidyl moiety, interacts with E140 and with the carboxylate group of the *S* enantiomer. The piperidyl ring is in a chair conformation, and the 6-amino-3-benzoate group occupies an equatorial position.

The 5-bromobenzoate moiety of the *S* enantiomer makes similar interactions as the ligand shown in Figure 2a, but the piperidyl ring is rotated to the other side of the bromobenzoate to also adopt a chair conformation, leading to interaction with C80. This displaces the side chain of W76, which in turn reorients the side chain of H73 and disorders the C terminus of the second monomer at residues beyond G162* (Figure 2b).

To test whether this unexpected simultaneous binding of the racemate arises from cooperativity between the two enantiomers, we performed soaking experiments with the pure enantiomers of **1**. Interestingly, while the *R* enantiomer displays the same binding mode as in the racemate complex (Figure 2c), the *S* form binds in a different orientation (Figure 2d), occupying approximately the same position as the analogue shown in Figure 2a. The difference is that the 6-amino-3-bromobenzoate group now occupies an axial position on the piperidine ring, which again adopts a chair conformation such that a hydrogen bond between the nitrogen and the side chain of E140 is formed. Accordingly, the C terminus is not disordered in this complex. This binding

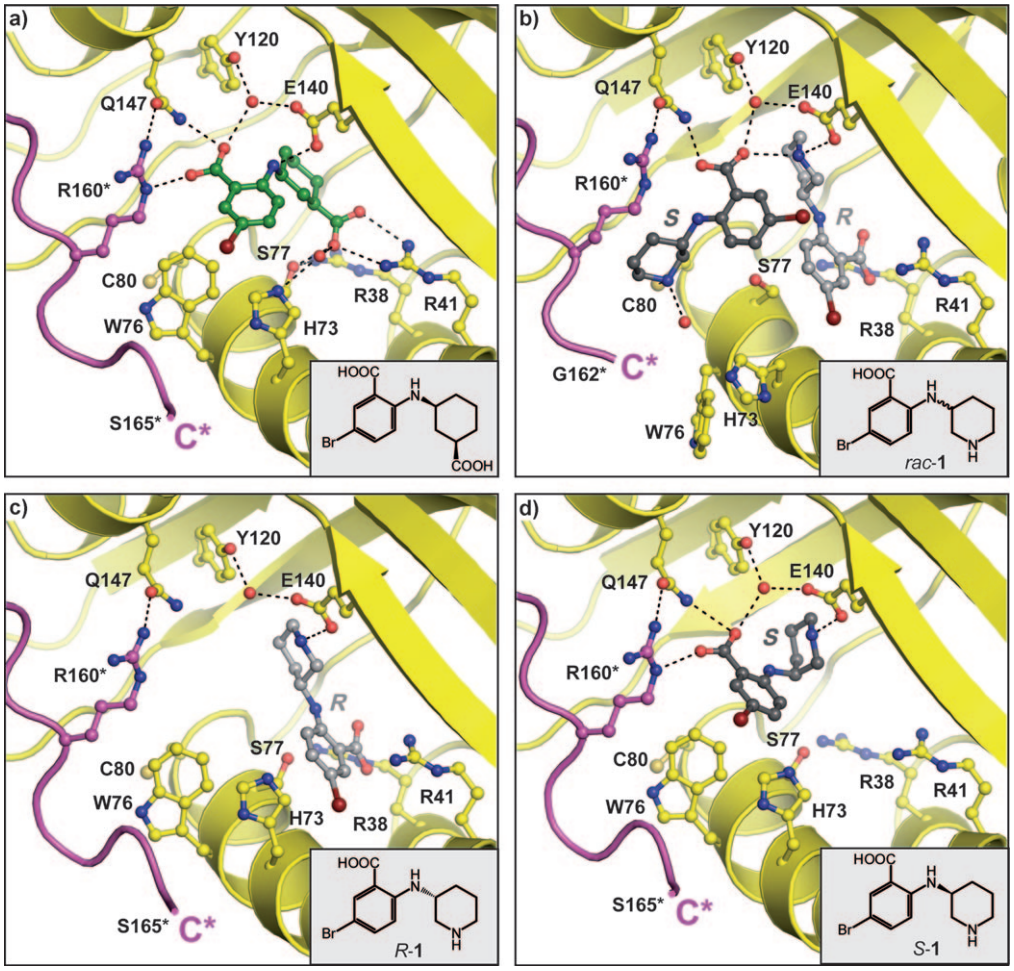

Figure 2. Binding of synthetic ligands to the active center of PhzA/B from *Burkholderia cepacia* R18194. Residues from the second monomer are shown in magenta and are marked by an asterisk (*). a) Binding mode observed with most ligands (dark green ligand, blue N, red O, dark red Br). b) Simultaneous binding of *rac*-**1** (dark and light gray). Note that parts of the C terminus of the second monomer are disordered. c) Binding of the pure *R* enantiomer (light gray). d) Binding of the pure *S* enantiomer (dark gray).

mode of (*S*)-**1** is mutually exclusive with its orientation seen in the racemate complex.

To rule out that the structures described above are an artifact of soaking crystals in high concentrations of the ligands (4 mM), we also performed a series of cocrystallization experiments. While this confirmed the complexes with the enantiopure compounds (as in Figure 2c,d), these experiments also led to the interesting observation that simultaneous binding of (*R*)-**1** and (*S*)-**1** is concentration dependent: At 1 mM only the *S* enantiomer is observed; at 2 mM the electron density indicates that 50% of the PhzA/B molecules bind the *S* enantiomer alone, whereas the other half binds the racemic mixture (Figure S2 in the Supporting Information); finally, at saturating concentrations (>10 mM at crystallization setup), only simultaneous binding as in Figure 2b is found. This indicates that different equilibria govern the binding of *rac*-**1**.

Binding was further analyzed by isothermal titration calorimetry (ITC). The single enantiomers displayed a 1:1 protein/ligand stoichiometry with (*S*)-**1** binding approximately three times tighter than the *R* enantiomer. In contrast, titration with the racemate gave a 1:1.5 stoichiometry with a lower affinity than for the enantiomers alone (Table 1, Figure 3). This deviation from the expected 1:2 complex and the apparent anticooperativity is interpreted in terms of partially competing binding equilibria: At the start of titration, all active sites are free and protein molecules begin to be populated with (*R*)-**1**, (*S*)-**1**, and *rac*-**1**. In the course of the experiment, the *S* enantiomer will partially displace (*R*)-**1** in some of the protein/(*R*)-**1** complexes and complete the protein/*rac*-**1** complex in others. At the end of titration, saturation of the binding sites will be reached and the protein/*rac*-**1** complex, which as the crystal structure suggests is the most stable assembly, will prevail. Ideally, this scenario should manifest itself in deviations from a sigmoidal titration curve as has been reported in studies of racemic protease inhibitors by Fokkens and Klebe.[6] However, to be observable by ITC, this would probably require significantly larger differences between the affinities of the enantiomers and potentially also the racemate (50- to 200-fold)[6] than in the case described here. As a consequence, the affinities of (*S*)-**1** towards the *R*-enantiomer complex to complete *rac*-**1** and that of *rac*-**1** to the protein alone could not be resolved in our ITC experiments.

Table 1: Binding thermodynamics as determined by ITC.

Ligand	K_D[a] [μM]	Number of sites[b]	ΔH[a] [kcal mol^{-1}]	$-T\Delta S$[a] [kcal mol^{-1}]
rac-**1**[c]	12.4 ± 0.82	1.53 ± 0.13	−5.01 ± 0.78	−1.7 ± 0.8
(*R*)-**1**	8.55 ± 2.6	1.02 ± 0.01	−2.09 ± 0.83	−4.8 ± 1
(*S*)-**1**	2.63 ± 1.1	1.00 ± 0.02	−6.13 ± 1.0	−1.5 ± 0.8

[a] Thermodynamic values are reported as the mean with a standard deviation obtained from three individual titrations. [b] The number of binding sites on the macromolecule refers to that of a single monomeric subunit. [c] The thermodynamic values refer to a single ligand molecule.

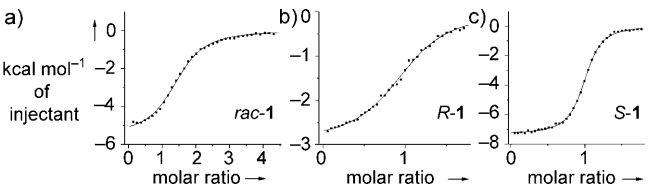

Figure 3. Titrations of PhzA/B with a) *rac*-**1**, b) (*R*)-**1**, and c) (*S*)-**1**. The different stoichiometry in the binding of the racemate compared to the binding of a single enantiomer is indicated by the molar ratio at the inflection point of the curve (see Table 1, number of sites).

We think that our findings add a relevant aspect to the discussion of chiral-drug action at primary drug targets such as enzymes, ion channels, and G-protein coupled receptors, which, by nature of their function, must have defined and specific binding pockets. Therefore, the observation made here for a specific enzyme synthesizing a secondary metabolite goes beyond recent findings of multiple ligand binding in promiscuous transporter proteins or P450 enzymes which, by their very nature, need to have large binding sites accommodating many different ligands.[9] While there is currently no alternative to continue the practice of using racemic mixtures for primary screening, the interpretation of binding data should therefore be performed with even more caution than before. On the other hand, the occurrence of a racemate bound to a binding site might offer new drug-discovery opportunities, especially following along the line of fragment-based drug discovery.[10]

Experimental Section

Compounds (*R*)-**1** and (*S*)-**1** were prepared according to standard Ullmann condensation reaction procedures[11] from 2-bromobenzoic acid (Aldrich) and *R*- and *S*-piperidin-3-amine, respectively (CNH Technologies). A detailed description of the synthesis will be reported elsewhere. Analytical spectra are given in the Supporting Information. ^{1}H NMR (400 MHz, D_2O): $\delta = 8.00$ (d, $^4J = 2.5$ Hz, 1H, Ar-H), 7.51 (dd, $^3J = 9.0$ Hz, $^4J = 2.5$ Hz, 1H, Ar-H), 6.74 (d, $^3J = 9.2$ Hz, 1H, Ar-H), 3.85 (dddd, $^3J = 8.7$, 8.9, 3.7, 4.0 Hz, 1H, CH), 3.46 (dd, $^2J =$ 13 Hz, $^3J = 3.2$ Hz, 1H, CHH_{eq}NH), 3.30 (ddd, $^2J = 13$ Hz, $^3J = 4.6$, 4.6 Hz, 1H, CHH_{eq}CHH), 3.05 (ddd, $^2J = 13$ Hz, $^3J = 9.9$, 3.3 Hz, 1H, CH_{ax}CHH), 2.98 (dd, $^2J = 13$ Hz, $^3J = 8.8$ Hz, 1H, CH_{ax}HNH), 2.19–2.09 (m, 2H, CHH), 2.08–1.98 (m, 2H, CHH), 1.91–1.79 (m, 1H, CHH), 1.74–1.63 ppm (m, 1H, CHH). ^{13}C NMR (75 MHz, [D_6]DMSO): $\delta = 168.8$ (COOH), 148.3 (C_{Ar}-NH), 136.8 (CH, C_{Ar}), 133.6 (CH, C_{Ar}), 113.9 (CH, C_{Ar}), 112.3 (C_{Ar}-Br), 105.4 (C_{Ar}-COOH), 46.0 (CH_2N), 45.7 (CHN), 42.8 (CH_2N), 28.5 (CH_2), 20.9 ppm (CH_2). HRMS (ESI$^+$): *m*/*z*: calcd for $C_{12}H_{16}BrN_2O_2^+$ $[M+H]^+$: 299.0390; found 299.0389. UV (MeCN/H_2O, 0.05% trifluoroacetic acid): $\lambda_{max} =$ 222, 262, 359 nm. M.p. 106 °C (subl.). $[\alpha]_D^{22}$((*R*)-**1**) = −11° (*c* = 0.48 g cm^{-3}, H_2O). $[\alpha]_D^{22}$((*S*)-**1**) = +12° (*c* = 0.32 g cm^{-3}, H_2O).

Expression and purification of *N*-terminal hexahistidine-tagged PhzA/B from *Burkholderia cepacia* R18194 was performed as described previously.[8] Pure protein was concentrated in 20 mM Tris·HCl pH 8.0, 150 mM NaCl, snap-frozen in liquid nitrogen, and stored at −80 °C until further usage. After determination of protein concentration by UV absorption at 280 nm using Lambert-Beer's law, ITC was carried out[8] employing 1 mM ((*R*)-**1**, (*S*)-**1**) or 2 mM (*rac*-**1**) solutions of ligands in 20 mM Tris·HCl pH 8.0, 150 mM NaCl and 0.1 mM PhzA/B in the same buffer. Crystallization of native PhzA/B was achieved with the vapor-diffusion hanging-drop method.[8] Crystals of the complexes were prepared by overnight soaking of native PhzA/B crystals in mother liquor supplemented with 4 mM of the respective ligand, or by cocrystallization with protein preincu-

bated with 1, 2, and 40 mM of the ligand as outlined in the Supporting Information. Crystals were washed in cryoprotectant supplemented with the same concentration of the ligand before data collection at 100 K on beamline X10SA of the Swiss Light Source (Villigen, Switzerland). Refinement followed the same strategy as described in reference [8]. $|F_O-F_C|$ difference electron densities of the ligands before incorporation into the model are shown in Figures S1 and S2 in the Supporting Information; full data collection and refinement statistics are shown in Table S1.[12]

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