Crystallization of aldose reductase with the inhibitors sorbinil (top) and tolrestat (bottom) reveals that their polar head groups both bind near the coenzyme, but that their hydrophobic moieties are at 90° to each other. Sorbinil binds with few changes in the enzyme structure, whereas the binding of tolrestat induces a displacement of Leu300 and Phe122. This figure was produced with permission from Podjarny et al (*Structure* **1997**, *5*, 601).
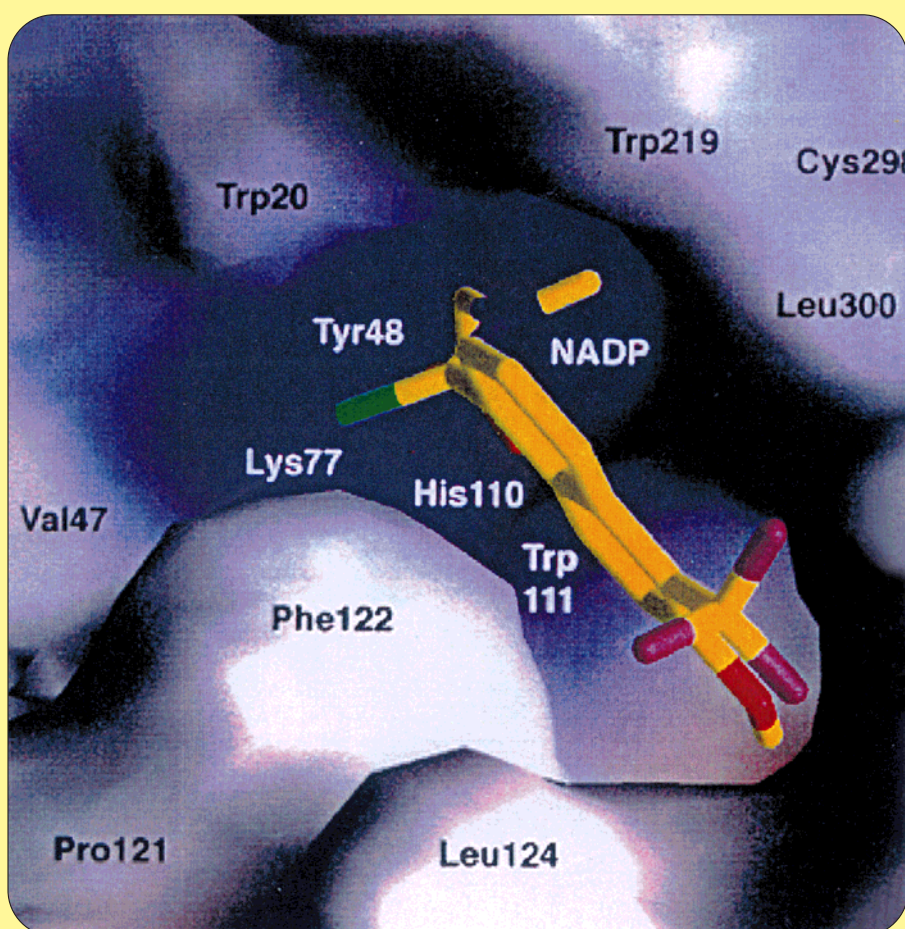
Hydrogen Bonding, Hydrophobic Interactions, and Failure of the Rigid Receptor Hypothesis

Andrew M. Davis and Simon J. Teague*

Why does experimental determination of the structure of drug–receptor complexes so often result in surprises? The literature is scattered with examples of drugs which bind to their receptors in orientations quite different from that expected from simple overlay or indeed from molecular modeling. Charge-reinforced hydrogen bonds are very strong, but neutral–neutral hydrogen bonds are much weaker and their contribution to the binding constant can range from 15-fold to zero. Hydrophobic interactions are less easily visualized, but appear to play a crucial role in the binding of many drugs to their receptors. In a number of cases, where the structure of a series of complexes was determined during structure–activity studies, tighter binding has been observed in analogues where hydrophobic interactions have been optimized, even at the expense of possible hydrogen bonds. The optimization of hydrophobic interactions also plays a major role in "induced fit" of receptors to ligands. One consequence of induced fit is that several molecules, with differing shapes, can bind and fit well to the same part of a certain receptor. Overemphasis of the importance of hydrogen bonds has often resulted in the incorrect prediction of binding orientation within a series of related ligands. The balance between hydrogen bonds and hydrophobic interactions is addressed to differing extents by existing molecular modeling packages. At present most assume the receptor is almost rigid. None are able to predict the large or small changes often observed in experimentally determined drug–receptor complexes. In validations some programs were more successful in docking polar ligands than hydrophobic ones. However, a balance between polar and hydrophobic properties is required for druglike molecules. Tight binding of drugs to receptors is achieved through polar interactions and most importantly through the optimization of *specific* hydrophobic interactions. The bulk properties of drugs, which control solubility, absorption, metabolism, and pharmacokinetic properties, is achieved mainly with polar groups, together with sufficient lipophilicity to achieve partition into lipid bilayers. The present status of rational design tools still necessitates the employment of a range of approaches in the discovery of new drugs.

Keywords: drug research · hydrogen bonds · hydrophobic effect · molecular modeling · protein structures

1. Introduction

The successful practice of medicinal chemistry is crucially dependent upon an understanding of the principles of molecular recognition. Surely the greatest success in the field of molecular recognition was one of the first, the discovery of the principles underlying the structure of DNA by Watson and Crick in the early 1950s.[1] The mutual recognition of adenine (A) and thymine (T) as well as guanine (G) and cytosine (C) bases for each other is determined by their hydrogen-bonding and shape complementarity. More recently a great deal of information about protein structure has become available from X-ray crystallography and NMR spectroscopy. This, together with the use of energy calculations and docking procedures from molecular modeling, has resulted in predictions of the bound geometry of new ligands and made the concept of rational design of drugs a reality. Although it is well accepted that the binding of a drug to its receptor is mediated by ion–ion interactions, hydrogen bonding, dipole–dipole interactions, lipophilicity, and shape complementarity, the relative contributions of each of these interactions is still surprisingly poorly understood. This is because, although many studies have tried to quantify these interactions through mathematical deconvolution of observed binding energies[2, 3] or experiment,[4, 5] few have attempted to experimentally dissect the relative contribution of these interactions under conditions that are relevant to the biological situation, that is, in water.

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The contribution of hydrogen bonding to drug – receptor interactions varies greatly. It is often overlooked that formation of a hydrogen bond in a drug – receptor complex is an exchange process, and that in order to form this bond similar bonds have to be broken between the drug and water and between the receptor and water. Likewise new hydrogen bonds are formed between the drug and receptor and between the previously hydrogen bonded water molecules and bulk water. The favorability of the drug – receptor hydrogen bond depends upon the overall energy change involved in breaking and forming all these hydrogen bonds. The work of Fersht et al.[6] and Williams et al.[7] represents two important and complementary approaches to quantifying the contribution of hydrogen bonding to drug – receptor binding interactions; structural alterations to the receptor and to the drug were investigated. Fersht and co-workers studied the coupling of tyrosine to ATP to yield tyrosyl-AMP, the first step in the transfer of tyrosine to t-RNA catalyzed by the enzyme tyrosyl t-RNA synthase.[6] Site-directed mutagenesis was used to probe the energetics of this coupling through the effects of mutations upon the kinetics of the reaction. Because the crystal structure of the complex was known, mutations could be interpreted directly as losses in specific hydrogen bonds, salt bridges, and hydrophobic contacts. Through a series of point mutations, they were able to determine that a neutral – neutral hydrogen bond contributes only about 0.5 – 1.5 kcal mol^{-1} in binding (equivalent to a 2- to 15-fold increase in affinity), but the presence of a charged hydrogen bond contributes up to 4.7 kcal mol^{-1} (equivalent to a 3000-fold increase in affinity).

Williams et al. studied the binding of model peptides of the D-ala-D-ala terminus of the growing peptidoglycan bacterial cell wall to the antibiotic vancomycin by NMR spectroscopy.[7] Structural modification of the peptide caused changes in the overall binding affinity to vancomycin. The NMR investigations provided information on both the macroscopic binding constants and individual interactions between the peptide and vancomycin. Williams et al. estimated the contributions of neutral – neutral hydrogen bonds to be only 0.5-1.5 kcal mol^{-1}, as observed by Fersht et al. Further confirmation of these estimates of the contribution of hydrogen bonds to binding can be found in a study of the affinities of fluorodeoxy sugar analogues to glycogen phosphorylase, where hydroxyl groups were serially replaced with hydrogen bond accepting fluorine atoms.[8] This study also found that a neutral – neutral hydrogen bond is worth up to about 1.5 kcal mol^{-1}, which is equivalent to a maximum 15-fold increase in binding. The degree of agreement between these studies is remarkable, and are further supported by many observations from drug design investigations.[9–11] Even though a hydrogen bond between a charged and a neutral component can contribute up to 3000-fold to the binding of a drug, a neutral – neutral hydrogen bond is worth less than 15-fold in binding. Very recently Williams and Westwall have revised even these very modest contributions to binding from neutral – neutral hydrogen bonds by suggesting they are a gross overestimate, and that their true local contribution to binding is near zero![12] Even more orthodox views concerning the importance of hydrogen bonds, for instance in the cross-linking of collagen 4-hydroxy-proline residues, has recently been called into question.[13]

The contribution of hydrophobicity to drug – receptor interactions is well described. Removal of the hydrophobic surface area from water, by binding into a hydrophobic region of a receptor, is generally worth a minimum of 28 cal-Å^{-2} mol^{-1}, which is the equivalent of 0.68 kcal mol^{-1} or a 3.2-fold increase in binding constant per methyl group.[14] This is similar to the energy gain in partitioning of a drug from water to a hydrophobic solvent such as n-octanol.[15] In some circumstances, where the complementarity between the drug's hydrophobic surface and the receptor is particularly high, the contribution can be significantly greater than this.[16] Large numbers of hydrophobic atoms are present in drug molecules, and it is apparent that hydrophobicity is a major source of binding in drug – receptor interactions. This conclusion is supported by a survey of the properties of marketed oral drugs as listed in the Physicians Desk Reference.[17] This shows that on average drugs contain only one to two donors and three to four acceptors, whereas the average number of hydrophobic atoms in a drug molecule is 16 (Figure 1).

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This view of the energetics of drug–receptor interactions is not new, though it does not appear to be universally appreciated. It is often assumed that, although hydrophobicity
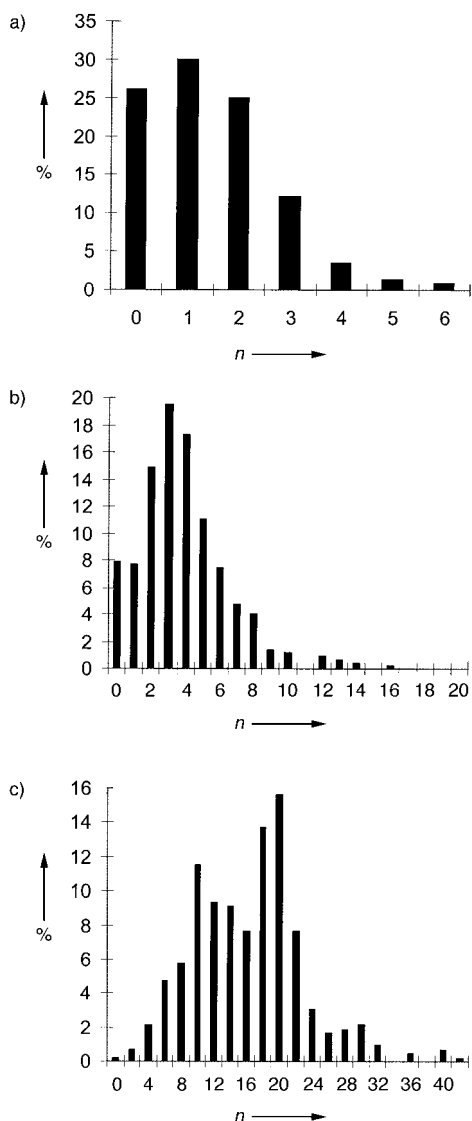

Figure 1. a) Distribution of hydrogen-bond donors in 415 oral drugs listed in the Physicians Desk Reference. b) Corresponding distribution of hydrogen-bond acceptors. c) Corresponding distribution of hydrophobic atoms. Ordinate: frequency in %; abscissa: number n of the donors, acceptors, or hydrophobic atoms.

makes an important contribution to binding, its contribution is nonspecific. Likewise drug–receptor hydrogen bonds are considered as important contributors to binding, and moreover, because of their directionality, important in determining the specificity of drug–receptor binding. These conclusions are reinforced by the large amount of X-ray structural data from protein–inhibitor complexes where hydrogen bonding and charge–charge interactions are clearly visible. But just seeing an interaction tells us nothing about its contribution to binding. In recent years the structures of protein–inhibitor complexes for a series of analogues in the course of investigation of structure–activity relationships have sometimes been used as a drug-design tool, often with surprising

results concerning the relative geometry or orientation of bound ligands. A few examples have been noted previously, but with the rapidly increasing numbers of protein–ligand complexes appearing in the literature[18] these surprising results are appearing with increasing frequency. In this review we highlight some of these examples and use them to support the hypothesis that the balance of contribution between polar and hydrophobic interactions in molecular recognition may need reevaluation. The importance of hydrophobicity and induced fit in drug–protein interactions is highlighted, together with the deficiencies of the predictive tools presently available to us.

## 2. Hydrophobic Interactions instead of Hydrogen Bonds

There are many occasions in the literature where affinity is enhanced through hydrophobic interactions, even at the expense of hydrogen bonds. The demonstration that DNA polymerase efficiently and faithfully pairs adenine with difluorotoluene deoxynucleoside when it has been incorporated in a DNA strand provides an elegant demonstration of the importance of shape fit to enzyme–substrate recognition.[19] The efficiency is only fourfold lower than that observed with thymidine, and the selectivity for adenine is 2.9–4.2 log units with respect to incorporation of C, T, or G.

Thymidine   Difluorotoluene deoxynucleoside
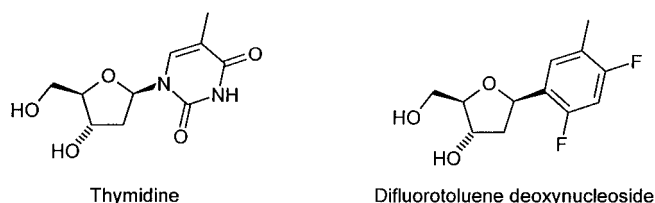
The current assumption that the number and strength of hydrogen bonds is the prime determinant of efficiency and fidelity in DNA synthesis may require reexamination. The participation of an unconventional hydrogen bond (C–F··· H–N) between the fluoro analogue and adenine could not be demonstrated even in favorable solvents such as chloroform. No inherent pairing selectivity was observed in the absence of DNA polymerase. It is interesting to note that the resulting double-stranded helix is actually destabilized by 4–5 kcal relative to thymidine in the same position; thus, hydrogen bonds are important in DNA duplex. However, this work provides evidence that they are much less important in the recognition of bases by DNA polymerase, which is a situation much more analogous to drug–receptor interactions.

The discovery of inhibitors of influenza neuraminidase (NA) has provided one example where hydrogen-bonding interactions can be replaced by additional hydrophobic binding.[20] Inhibitors of NA were discovered based upon transition state analogues of sialic acid cleavage from glycoconjugates. X-ray crystallographic studies of Neu5Ac and its analogues with NA revealed that the two terminal hydroxyl groups of the glycerol side chain form a bidentate

hydrogen-bonding interaction with Glu276. Removal of the glycerol side chain and replacement with a hydroxyl group gave **1** ($IC_{50} = 6300$ nM), which is in good agreement with data from Fersht et al. for the loss of a hydrogen bond between a

Sialic acid Nuc5Ac **1** R = H **2** R= $(CH_3CH_2)_2CH$
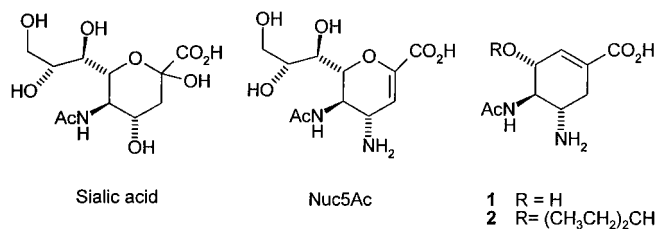
charged and a neutral component.[6] However, in a series of alkyl analogues, steady increase in inhibitory activity was observed that culminated for **2** ($IC_{50} = 1$ nM). X-ray crystallographic analysis of the complex of **2** with NA shows that the 3-pentyl group is bound against a large hydrophobic surface created by the hydrocarbon side chains of polar amino acids Glu276, Arg224, and hydrophobic residues Ala246 and Ile222! The carboxylate group of Glu276, to which the glycerol hydroxyl groups had been bound in Nuc5Ac analogues, was forced outward from the hydrophobic pocket. Interestingly it had previously been suggested[21] that hydrogen bonds were important in the molecular recognition of all carbohydrates, with one hydrogen bond often associated with each sugar hydroxyl group.

The structure–activity relationships of two series of HIV-2 protease inhibitors of the type **3** and **4** have been described.[22] They display very similar affinities despite the replacement of the P2 and P3 substituents in **3** by the lipophilic dimethylphenoxy substituent in **4**. X-ray crystallographic analysis of the protein–inhibitor complex of **3** reveals hydrogen bonds between the quinoline amide Asp29′ and Gly48′. The complex with **4** shows induced fit to the dimethylphenoxy group through a shift of 1 Å for Asp29′ and a shift of 4 Å for the side chain at Asp30′. The flap region (43′–48′) also undergoes a conformational change upon binding the more lipophilic inhibitor.

3

4
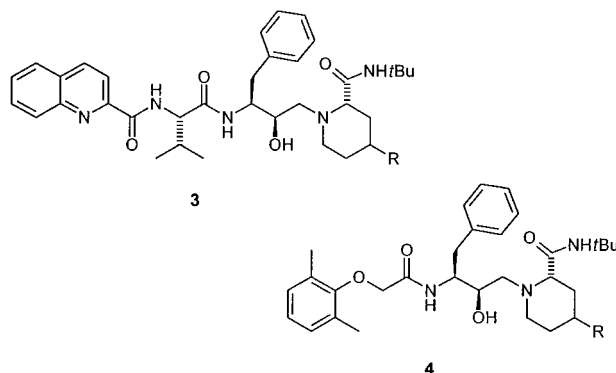
The complexes between thymidylate synthetase (TS) and the inhibitors CB3717 and 1843U89 ($K_d = 0.1$ nM) have been compared.[23] The two inhibitors lie in nearly identical
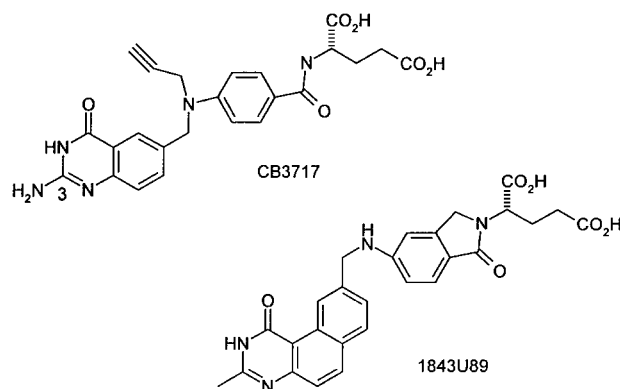
CB3717

1843U89

positions, despite the loss of the hydrogen-bonding guanidine group at position 3 in 1843U89; in CB3717 it was hydrogen bonded to Ala263 (Figure 2). The glutamate attachments occupy quite different positions in the two inhibitor complexes. Induced fit to the receptor is critical since the addition of an extra ring in 1843U89 results in the compound being 4 Å longer. 1843U89 takes up an L-shaped conformation with Ile79 forming a contact to both the isoindolinone group and the benzoquinazoline rings from its position on the inside of the "L". Residue Phe176 contacts the benzoquinazoline group
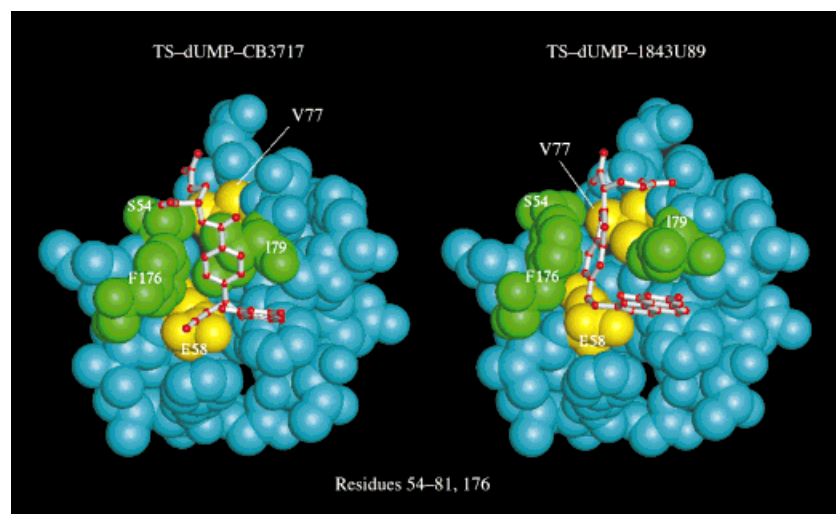

Figure 2. The binding of CB3717 and 1843U89 to thymidylate synthetase. The movement of Ile79 (I79) and Phe176 (F176) are apparent. This figure was produced with permission from Montfort and Weichsel.[23]

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on the outside face. The degree of induced fit caused by 1843U89 is dramatic, and involves roughly half the protein and includes residues on all sides of the binding pocket. The atoms of the main chain of Ile79 shift by approximately 1.5 Å and the side-chain atoms by 2.0–6.6 Å between the two complexes. The authors conclude that the discovery of 1843U89 "has implications for drug design, as 1843U89 could not have been obtained from current structure-based approaches".[23]

The complexes of matrix metalloproteinase inhibitors **5** and **6** (X = CH) with stromelysin (MMP-3) have been compared (Figure 3).[24] Replacement of the P3′ N-methyl amide group
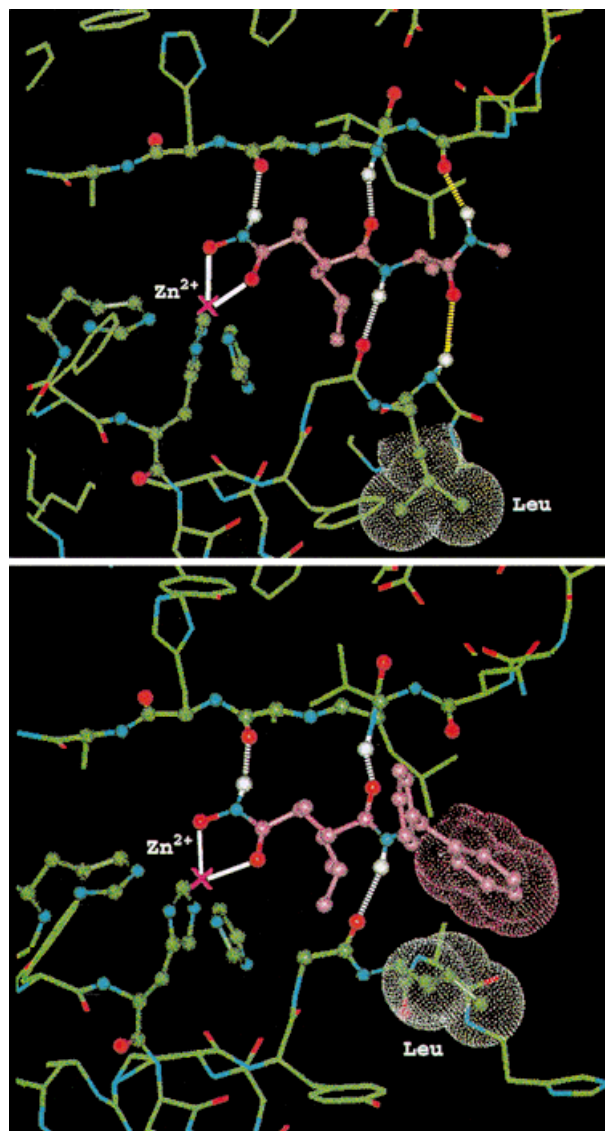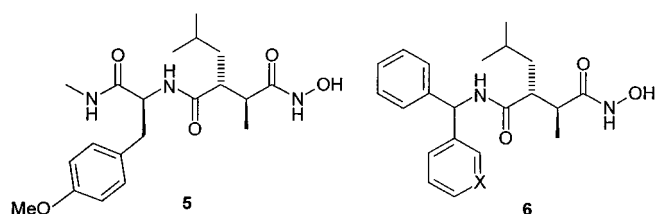

Figure 3. Complexes of **5** (top) and **6** (bottom) with stromelysin. Hydrophobic binding to a leucine residue replaces two hydrogen bonds. This figure was produced with permission from Decicco and DeGrado et al.[24]

in **5** by a phenyl ring (→**6**) proved to be possible despite the loss of two hydrogen bonds. The complex with **6** showed an unexpected shift of a loop (residues 222–231), which allowed a Leu residue to hydrophobically bind to the benzhydryl moiety. Having made this surprising discovery, the group was able to utilize the X-ray data and the newly created hydrophobic environment in this region of the complex to design further improvements to the inhibitors. Thus, by introducing a hydrogen bond acceptor (X = N) a further 16-fold improvement was observed, consistent with the formation of a neutral hydrogen bond.

Several different series of potent, active-site inhibitors of thrombin have been discovered to bind to the enzyme in surprising ways.[25–27] The dibasic benzo[b]thiophene inhibitors[25] unexpectedly place the hydrophobic benzo[b]thiophene nucleus in the S$_1$ pocket of the enzyme, the site which usually accommodates the basic side chain of arginine in the standard D-Phe-Pro-Arg sequence. A hydrogen bond from the hydroxyl group at position C6 of the benzo[b]thiophenes to Asp189 was observed in **7**, with the C6−H compound showing fourfold lower affinity. The relative importance and
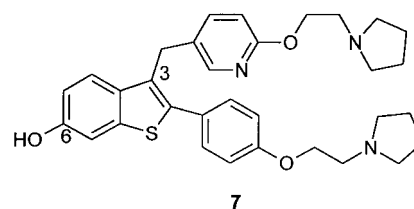
specificity of the hydrophobic interaction with the benzo[b]-thiophene was demonstrated, however, when replacement by thiophene, benzofuran, indole, or naphthyl rings all resulted in much more dramatic decreases in affinity. High-throughput screening thus provided an opportunity to break out into a new series of inhibitors. The group at Roche[26] has very ably and amusingly described their attempts to estimate relative affinities of inhibitor analogues based upon X-ray structure determination of the complex between thrombin and representative compounds in a series. Many experienced medicinal chemists will empathize with them for the difficulties they have encountered and agree with their conclusion that "It will continue to be important to critically examine the structure–activity relationships of any class of inhibitor for indications of unusual binding. The enzyme "sees", binds, and possibly adapts to the outside of the inhibitor folded in some low energy conformation, and is blissfully ignorant of the inhibitor's internal chemistry. The chemist on the other hand sees a compound as a core structure, decorated on the outside with interesting substituents. It is only fair to say that we must learn to look at inhibitors the way an enzyme does".

Some drugs possess several hydroxyl and polar groups, which in the many discussions of drug–receptor interactions would be termed "interactive". However, these assumptions are not always confirmed when the structure of a drug–receptor complex is determined experimentally. One example is the binding of digoxin to 26–10 Fab, a monoclonal antibody[28] which demonstrates almost exclusively hydrophobic interactions even though these interactive groups are present.

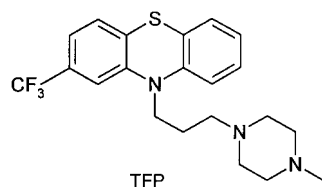*Angew. Chem. Int. Ed.* **1999**, *38*, 736–749

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Digoxin
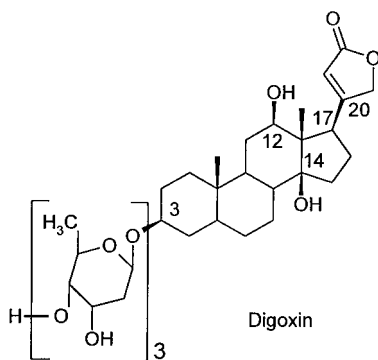
The effectiveness and specificity of shape complementarity and hydrophobic interactions is revealed in this complex. The antibody binds the drug with an affinity of 0.1 nM despite the lack of hydrogen bonds or charged group interactions. Digoxin binds with the lactone ring buried in a deep pocket at the bottom of the combining site and with the carbohydrate groups mostly exposed to external solvent. The lactone ring is flipped by 180° about the C17–C20 bond relative to the structure of unbound digoxin in the crystal. The drug is sandwiched between the aromatic rings of the V_H domain (Tyr33, Tyr47, Tyr50, and Trp100) which form the binding pocket. The surface complementarity is closest at the lactone and the steroid D-ring, decreases towards the periphery of the binding site, and has significant gaps between the surfaces around the hydroxyl groups at C12 and C14. The antibody also binds digitoxigenin, which lacks the 12-β-OH group, with equal affinity.

2.1. The Failure of the "Rigid Receptor" Hypothesis

Hydrophobic interactions are often associated with conformational changes of the receptor. Conformational change in a receptor upon binding a ligand is usually termed induced fit. In theory such changes could be the result of polar or hydrophobic interactions of the receptor with the ligand. However, examples in the literature are overwhelmingly the result of hydrophobic interactions. In these cases they could equally well be thought of as hydrophobic collapse of a receptor around a ligand. The relative preponderance of examples of hydrophobic binding may be a clear indication of the relative strengths of hydrophobic interactions compared to polar interactions.

Trifluoperazine (TFP) is an inhibitor of Ca^{2+}-calmodulin (Ca^{2+}-CaM).[29] Binding by TFP induces a major conformational change in the protein from an elongated dumbbell into a compact molecular form which can no longer interact with its target enzymes. Four TFP molecules per Ca^{2+}-CaM are observed in the X-ray crystal structure. The role of the net positive charge on the inhibitors is postulated to be stabilization of the resultant globular conformation, rather than providing essential binding capability. Several bind-

TFP

ing modes for TFP had previously been predicted, but none agreed with the observed structure for the complex. This was because of the large conformational change upon binding, unexpected hydrophobic interactions between two of the TFP molecules, the involvement of more than one region of the protein in forming a particular TFP binding site, and interaction of many of the involved regions of the protein with more than one TFP.

High-throughput screening has resulted in the discovery of Bay W1807, a potent inhibitor of glycogen phosphorylase (GP; Figure 4).[30] Bay W1807 binds to an allosteric binding
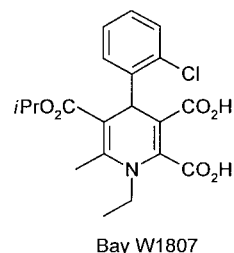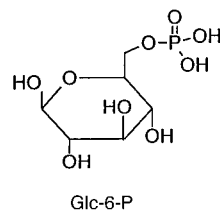
Glc-6-P   Bay W1807

site which binds a number of endogenous, phosphorylated molecules such as AMP and glucose 6-phosphate (Glc-6-P). Comparisons of the GP complexes of Bay W1807 and Glc-6-P reveal that they both bind their charged groups to the arginine residues of the pocket (Arg309 and Arg310), but that the increased affinity of Bay W1807 (in the nanomolar range) compared to that of Glc-6-P (in the micromolar range) is largely the result of additional hydrophobic interactions.

The chlorophenyl ring is sandwiched between Phe196 and Val45′, with the chlorine substituent making contacts with the aliphatic parts of Arg193 and Asp227. Favorable interactions exist between Tyr75 and the N-ethyl group and between the 2-propyl group, Trp67, Ile68, and the aliphatic part of Arg193. Major conformational change of the allosteric binding site is seen on binding of Bay W1807. Shifts in the Phe196 side-chain atoms of up to 2.9 Å occur as well as shifts of 1.2 Å in Val45′. Bay W1807 becomes almost completely buried, leaving only 7% of its surface area accessible to solvent. The authors comment that "Screening for effective ligand binding has identified high-affinity ligands that, by chance, are able to generate conformational changes that lead to high affinity, selective binding. Although such changes are easy to detect and rationalize in terms of the crystal structure they are much less easy to predict in a way that would facilitate structure-based drug design".

Many peptidic, rationally designed renin inhibitors have been discovered, based upon transition state analogues of the scissile Leu-Val moiety in human angiotensinogen. High-throughput screening[31] resulted in the discovery of 3,4-disubstituted piperidine **8** ($IC_{50} = 50\,\mu M$). Optimization resulted in a remarkable improvement in affinity culminating in **9** ($IC_{50} = 0.47\,nM$). X-ray crystallographic determination of a series of compounds obtained during this optimization process showed induced fit adaptation of the receptor pocket to accommodate the substituents at positions 3, 4, and 5 of the piperidine ring. The resulting compounds show excellent
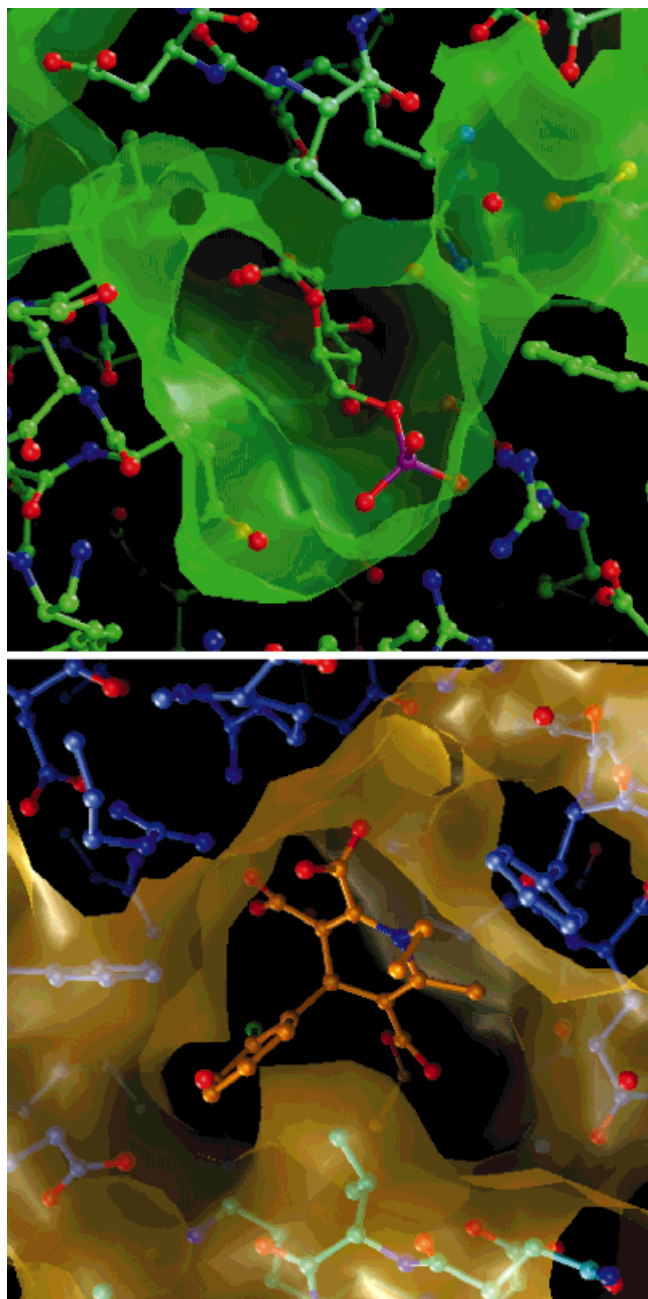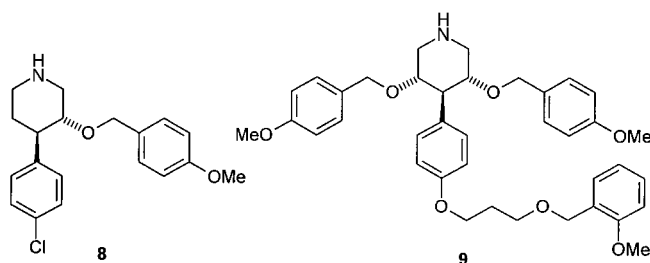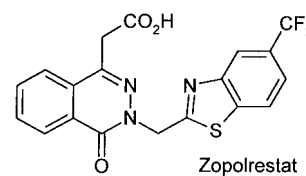
Figure 4. Top: The complex of glycogen phosphorylase b with glucose 6-phosphate. Bottom: The induced fit of the receptor to W1807. This figure was produced with permission from Johnson et al.[30]

metabolic stability, moderate bioavailability, and good duration of activity in various animal models.

Comparison of the structure of human aldose reductase with and without the 3 nM inhibitor zopolrestat bound reveals a

snugly fitting, hydrophobic pocket for the drug at the active site.[32] The formation of this pocket is ascribed to hinged-flap motions induced by the ligand. Once bound zopolrestat has an accessible surface area of 12.7 $Å^2$, a mere 6.4 % of that for the unbound inhibitor. The benzothiazole ring is sandwiched between Trp111 and Leu300, and the phthalazinone ring between Trp20 and Phe122. Ligand binding is accompanied by displacement of Leu300 towards the pocket and movement of loop 121 – 135, which enables Phe122 to participate in the binding pocket. The carboxylate group does play some part, being linked to His110 through a salt bridge; one oxygen atom mimics the carbonyl oxygen atom of the substrate. However, the authors comment that "The hydrophobic residues are the major determinants of molecular recognition. The marked preference of the enzyme for hydrophobic substrates (e.g. steroids) is consistent with this mode of binding the inhibitor". At least six ordered water molecules are also displaced, which may contribute up to 2 kcal mol^{-1} per water molecule to the entropic term in the overall binding energy.[33]

Crystallization of tolrestat and sorbinil with aldose reductase (AR) reveals that the polar head groups both bind near the coenzyme, but that the orientations of their hydrophobic moieties are at 90° to each other (Figure 5).[34] Sorbinil binds
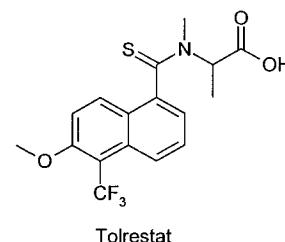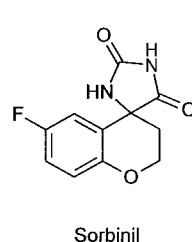
with few changes in the enzyme structure, but tolrestat binding induces a displacement of Leu300 and Phe122. The observed IC_{50} values for the two inhibitors are, however, very similar.

Conformational change as a consequence of hydrophobic interactions is observed with cofactors as well as drugs.[35] The cofactor NAD^+ induces loop closing in the enzyme isopropylmalate dehydrogenase (IMDH). Five loops from different stretches of primary sequence move up to 2.5 Å to form predominantly hydrophobic interactions with the cofactor. Despite the common prejudice that adenine contains "interactive" nitrogen atoms, this may not always be the case. Determination of the structure of glutamine 5-phosphoribosyl-1-pyrophosphate (PRPP), the regulatory enzyme of de novo synthesis of purine nucleotides, reveals the adenine base of AMP in the C site is sandwiched between the side chains of Tyr242 and Val349 but forms no hydrogen bonds with the protein.[36]

2.2. Receptor Plasticity and Multiple Ligands

Although the knowledge of several structurally diverse ligands for a given receptor is sometimes taken as suffient

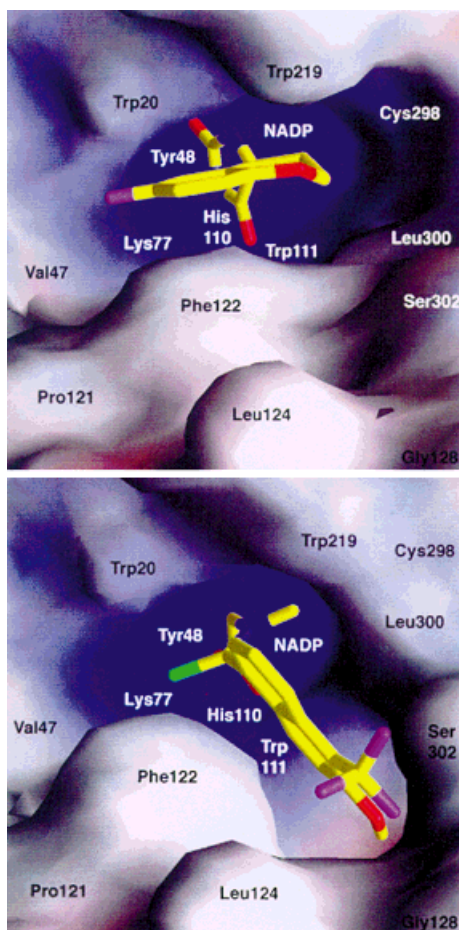*Angew. Chem. Int. Ed.* **1999**, *38*, 736 – 749

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Figure 5. Induced fit of aldose reductase to the inhibitors sorbinil (top) and tolrestat (bottom). This figure was produced with permission from Podjarny et al.[34]

evidence for several different binding sites, this may not necessarily be the case. A logical consequence of the importance of induced fit and hydrophobic interactions in receptor–ligand binding is that many structurally dissimilar ligands can interact with a biological target in similar orientations at the same binding site. Determination of the structures of HIV reverse transcriptase (RT) complexed with non-nucleoside inhibitors provides one such example Figure 6).[37]

Detailed structural comparisons are made between the complexes of HIV-RT and the four inhibitors nevirapine, a close analogue 1051U91, and the chemically more diverse structures α-APA and HEPT. These compounds share a common mode of binding to RT! The common features of

Nevirapine   α-APA   HEPT
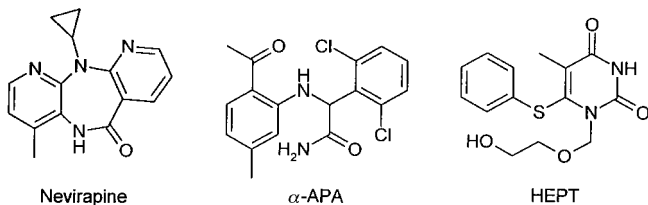
binding are largely hydrophobic interactions that arise from induced shape complementarity, achieved by reciprocal con-

formational rearrangement of both the enzyme and the compounds. Residues Leu100, Tyr181, and Tyr188 participate in extensive hydrophobic contacts with all four inhibitors. By contrast the binding pocket is "electrostatically bland" (i.e., electrostatic interactions play a minor role), with polar interactions between the receptor and the ligands varying widely. A comparison of the structure with a "consensus" structure shows that none of the Cα positions of the enzyme residues are displaced by more than 2.7 Å. However, the changes observed correspond precisely with variation in substituents of the inhibitors. Resistant virus strains have evolved which mostly possess binding site residues with smaller hydrophobic side chains such as Tyr181Cys and Tyr188Cys. These serve to underscore the importance of hydrophobic interactions at the binding site.

Several structurally diverse inhibitors of cyclooxygenase-2 (COX-2) are known to bind to the enzyme in very similar positions.[38] The distal ring of flurbiprofen overlaps with the bromophenyl ring of SC-558; the pyrazole of SC-558 superimposes on the fluorophenyl ring of flurbiprofen. The distal phenyl ring of flurbiprofen occupies an environment similar to that of the benzoyl ring of indomethacin. The carboxylate salts of flurbiprofen and indomethacin both bind to Arg120.

Flurbiprofen   Indomethacin   SC-558
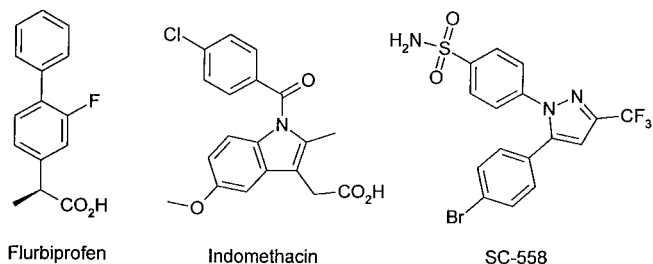
However, the carboxylate group of flurbiprofen and the hydrophobic trifluromethyl group of SC-558 bind in the same cavity! The phenyl sulfonamide substituent, which is so crucial to COX-2 selectivity, has its phenyl ring surrounded by hydrophobic residues and the sulfonamide group extending into a relatively polar region near the enzyme surface.

Comparison of the free and bound structures of the enzyme attests to the conformational changes which occur upon binding of the ligands. Many of these changes are explicable by reference to the structure of the enzyme. Kinetic evidence on the slow binding kinetics of inhibitors such as indomethacin had already indicated that large-scale conformational changes accompany binding of ligands. Many drug-discovery programs focus upon selectivity between related receptors as the key issue rather than potency per se. Thus, the challenge for rational drug design is acute, to produce ligands that bind at one site in preference to binding at a structurally closely related site.

3. Incorrect Prediction of Binding Orientation

There are many examples in the literature where emphasis upon the superposition of polar binding sites for putative ligands has resulted in the incorrect prediction of binding orientation. The relative importance of hydrophobic and
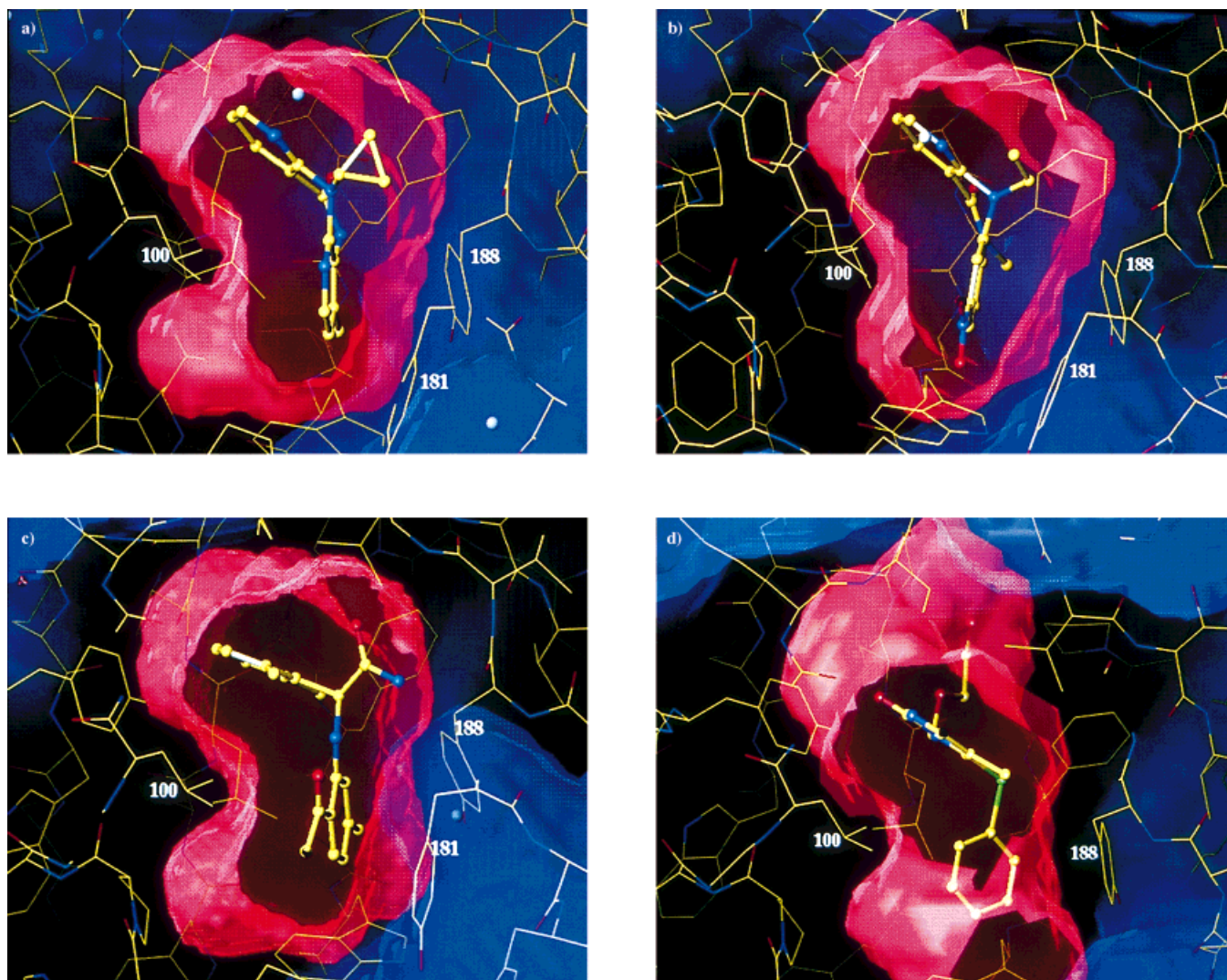
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Figure 6. The binding of a) nevirapine, b) 1051U91, c) α-AMP, and d) HEPT to HIV-1 RT. The pink surface delineates the pocket by showing the volume accessible to a small probe (1.4 Å). The adaptations of the pocket to the ligands are clearly visible. This figure was produced with permission from Ren, Stuart, and Stammers et al.[37]

polar interactions is especially important when attempting to predict the orientation of known ligands for a receptor in the absence of experimental observations. Weaknesses in the modeling programs for dealing with conformational change by the receptor and underestimation of hydrophobic interactions in the available force fields can result in a low level of confidence in the results. The literature is replete with examples of the difficulties encountered.
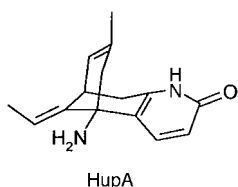
(−)-Huperazine (HupA) is a potent inhibitor ($K_i = 6$ nM) of acetylcholinesterase (AChE). Consideration of its pharmacophoric groups suggested a plausible orientation for HupA parallel to the acetylcholine molecule.[39] However, subsequent structure determination of the HupA–AChE complex revealed that its orientation in the active site gorge was orthogonal to that which was anticipated. Even though HupA has three potential hydrogen bonding sites only one hydrogen bond was observed. Its binding to AChE is through interaction of the

charged amine group with the aromatic rings of Trp84 and Phe330 together with several hydrophobic contacts to Trp84, His440, and with residues Gly118 through Ser122. Comparison with native AChE reveals conformational changes in the side chains of some of these aromatic residues, especially Trp84 and Phe330. Interestingly HupA lacks potentially complicating muscarinic effects, demonstrating how hydrophobic interactions may be exploited to achieve receptor selectivity.

The importance of conformational induction to signal transduction has been recently described.[40] 17β-Oestradiol and the oestrogen antagonist raloxifene bind to the same site but in different modes (Figure 7). Distinctly different conformations of the receptor are induced, providing evidence of the mechanism of antagonism. It is tempting to align the phenolic hydroxyl group of the A ring of oestradiol with that on position 6 of the benzothiophene moiety and the hydroxyl group on position 17 with that on the 2-phenyl substituent. However, a comparison of the two complexes shows that the later two are displaced by 5.1 Å. The group at C3 of raloxifene makes extensive hydrophobic contacts as well as a hydrogen

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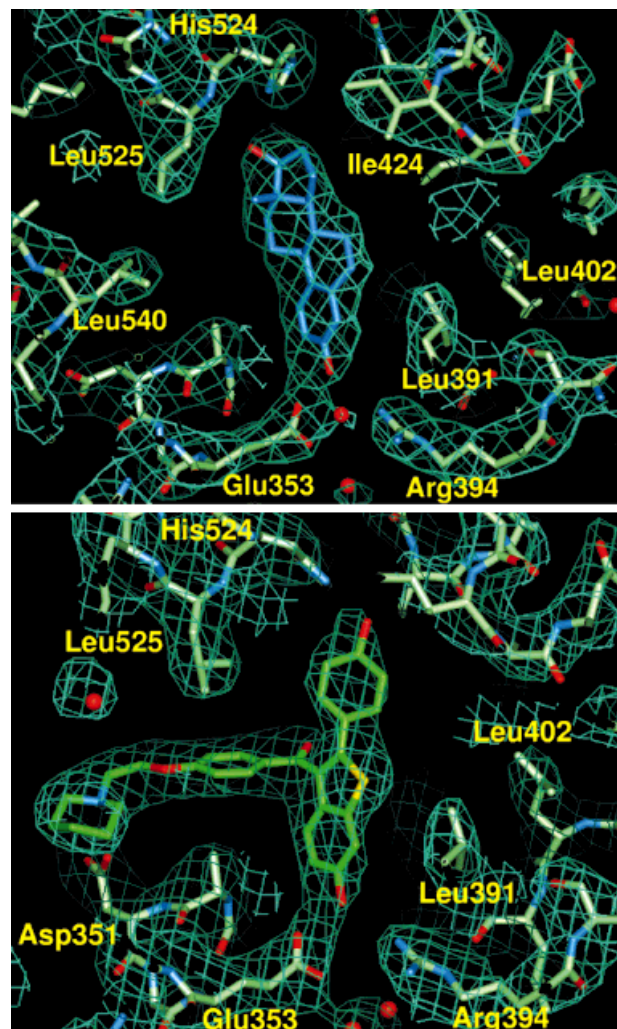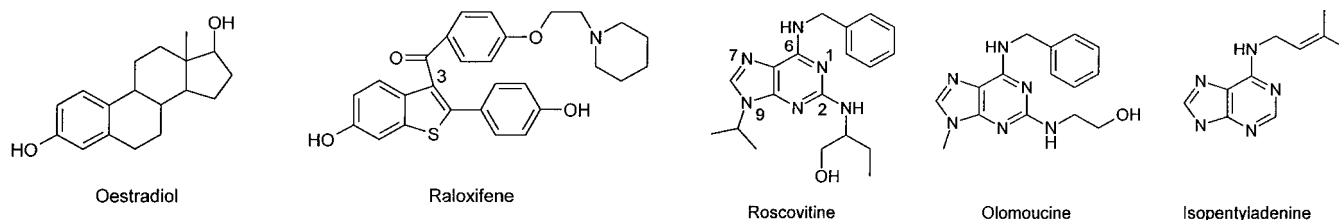Oestradiol   Raloxifene   Roscovitine   Olomoucine   Isopentyladenine

Figure 7. Oestrogen in the ligand binding domain of the oestrogen receptor (top) and the induced fit upon binding raloxifene (bottom). This figure was produced with permission from Pike and Hubbard et al.[40]

and His84. Ligand-specific differences in induced fit are seen when the two complexes are compared, such as a reorientation of the His84 residue. The reorientation of the purine ring systems results in observations that would be rather surprising in the absence of experimental structure determination. For example, substitution by hydrophobic residues at N9, which bears the ribophosphate in ATP, produces the most active compounds. Olomoucine binds in a very similar orientation to roscovitine.[42] The side chain of Ile10 is rotated by 120° relative to the conformation of the side chain in the ATP complex. The inhibitor isopentyladenine[41] shows yet another binding mode with the purine ring rotated by 180° about an axis through N3 and N7, with the substituent at N6 occupying the ribose pocket.

Kinases appear to be very flexible, and the adenine pocket is dominated by hydrophobic interactions. These factors may conspire to produce a permissive binding site which accepts a range of planar heterocyclic systems, many of which have been uncovered by high-throughput screening.

L868276 binds the benzopyran ring in the adenine pocket of CDK2.[43] The bicyclic ring system is in the same plane as the adenine in the ATP complex, but rotated by 60°. Significant motions of the side chains are seen upon binding L868276, with Asp145 and Lys89 moving away from the pocket and Ala144 moving closer. Many contacts to the pendant phenyl ring are observed, and the possible importance of these to selectivity can be outlined.

L868276   Stauroporine   10
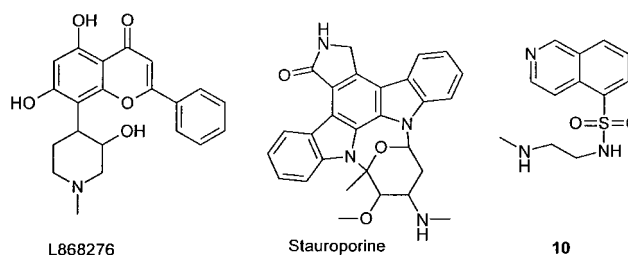
Stauroporine uses the indoylcarbazole group in its complexes with cAPK[44] and CDK2.[45] The positions of nearly all the residues that interact with stauroporine in its complex with cAPK are altered to accommodate this ligand, which is significantly larger than ATP. A hydrogen bond between Thr183 and N7 of ATP has no equivalent in the stauroporine complex, and the side chain of Phe327 is rotated to make a favorable $\pi - \pi$ interaction with one of the indole rings. In the stauroporine – CDK2 complex the side chain of Gln131 is displaced from the binding pocket. The complex of **10** with cAPK[46] reveals the isoquinoline moiety in the purine pocket with increased numbers of hydrophobic contacts to residues Ala70, Tyr122, Val123, Leu173, and Phe327 when compared to the ATP complex. Only one hydrogen bond acceptor

bond between the piperidine nitrogen atom and Asp351. Helix 12 is displaced and the transactivation of genes by the steroid complex disrupted.

The ATP binding sites of kinases provide a striking example of several superficially similar molecules that bind to the adenine site in completely different orientations. Roscovitine binds with the purine ring system flipped over compared to that in the ATP complex.[41] The atom N7 is close to the position of N1 in the ATP complex. The C2 substituent is placed in the "ribose pocket". The roscovitine – kinase interaction involves extensive hydrophobic interactions, dominated by contacts to Ile10, Leu83, and Leu134. The N6 benzyl group makes hydrophobic contacts with Ile10, Phe82,

group, equivalent to N1 of ATP, is conserved in all the inhibitor – kinase complexes determined so far. The presence of receptor flexibility and a permissive lipophilic binding pocket may combine to overcome initial reservations that the common ATP site in kinases would not provide a good target for the discovery of selective inhibitors.

4. Structure-Based Design and Drug – Receptor Interactions

Structure-based design is now an important part of many medicinal chemistry projects. The identification of pharmacophores for a database search or ligand design and the use of experimentally derived or homology-built receptors in ligand docking studies and database searches are two examples. Molecular modeling experts, medicinal chemists, and a number of the docking programs have traditionally put a heavy bias upon the alignment of functional groups for hydrogen bonding and directionality when perceiving pharmacophores.[47, 48] Although a number of successful docking validations and applications have appeared in the literature,[47–51] recent work suggests parameterization of some force fields may need to be more weighted towards hydrophobicity relative to hydrogen bonding.[47, 52, 53] To date the computational expense of an adequate treatment of solvation appears to have been the main drawback for an adequate treatment of hydrophobicity in these force fields. The largest validation so far undertaken for any docking program has been for the program GOLD.[47] In this study predictions of binding geometry for the redocking of 100 ligands removed from their respective proteins, taken from the Brookhaven crystallographic database, were examined in detail. The program GOLD was able to dock 71 % of the ligands studied with an acceptable agreement to that in the Brookhaven database. Analysis of the success and failure sets highlighted the difficulties faced by many docking programs. The program was successful in docking polar ligands, and tended to fail with more hydrophobic (druglike) ligands. It worked best when the ligand was extensively hydrogen bonded to the receptor site. Although full ligand flexibility is considered by GOLD, only very limited receptor flexibility is allowed (rotation around terminal bonds, for example, rotation of a hydroxyl group to maximize a hydrogen bond). This type of redocking study tends to disguise those examples where the structures of the apo-receptor is grossly different to that of the bound receptor. The assumption often made, that any significant induced fit occurring to the protein upon binding the ligand is likely to be constant across that ligand series, may be more difficult to accept based on some of the examples collected in this review. Recent developments in the program GRID,[54] which uses small molecular probes to identify binding locations in protein structures, have tried to address these problems by the inclusion of an empirical hydrophobic probe (DRY probe). This is to be used alongside the other molecular probes for hydrogen bonding, ionic interactions, and dipole interactions. Very importantly the latest version of GRID also allows for full flexibility of the receptor side chain.[55] This is intuitively a major step forward in addressing some of the problems we

have highlighted here. How successful it will be in reality still remains to be seen.

Evidence included in this review demonstrates the importance of induced fit in drug – protein interactions. Induced fit very often seems to be driven by hydrophobicity and can be viewed as hydrophobic collapse of receptors around a ligand. The degree of flexibility is not always that of domain rotations, but sometimes consists of subtle adjustment of specific residue positions to accommodate bulky ligands and achieve hydrophobic contacts. The changes are not always evident from gross measurements of protein shape (circular dichroism spectra). They are sometimes evident from kinetic measurements of the time-course of inhibition. It might be helpful if such time-course experiments were carried out in conjunction with modeling studies; indeed they might be used as a screen for cases which can be effectively modeled. Various methods are available to identify mobile segments of proteins such as determination of thermal B factors and NMR relaxation measurements.[56] It is possible that these could form the basis for incorporating a limited molecular dynamics strategy into a drug design scheme. Such a strategy might target hydrophobic interfaces between mobile portions of the protein. However a distinction should be drawn between the mobility of a protein loop in its native, unligated form and its absolute ability to assume more than one conformation.[57] The bound conformation may be energetically and kinetically accessible only in the presence of the ligand. Thus induced fit may be a means of selecting a productive mode without the entropic expense of immobilizing a flexible loop.

It should also be remembered that the observed binding affinity is a function of stability of the entire complex relative to ligand and apo-receptor. Thus, formation of new bonds between amino acids of the receptor upon ligand binding may have a substantial effect. This has been observed recently with dihydropyrancarboxyamides related to zanamivir.[58]

5. Drug Design and the Balance of Interactive Forces

The consequence of this emerging understanding of the relative importance of lipophilicity and induced fit compared to charge-reinforced and neutral – neutral hydrogen bonds may be a different way of thinking about drug design. One possibility is that medicinal chemists may seek increased potency and specificity, not necessarily by incorporating or maintaining hydrogen-bonding functionality and directionality, but by maximizing *specific* hydrophobic interactions. Where the initial ligands are rather polar molecules, the replacement of hydrogen-bonding groups by hydrophobic groups can have a number of beneficial effects. Sialic acid, NADPH, ATP, and most peptidic inhibitors would fall into this class. They tend to be afflicted with problems associated with poor absorption and rapid excretion. Introduction of lipophilic groups often increases potency, removes sites for conjugation, and increases plasma half-life (through increased distribution). Sometimes the journey from a polar starting point to useful drug is protracted, and several companies have revisited areas such as thrombin and renin using starting points generated by high-throughput screening as a means of

addressing these concerns. Compounds uncovered by high-throughput screens often present the medicinal chemist with the opposite problem, that of introducing polar functionality into rather lipophilic molecules. In this case placing polar functional groups where they are tolerated by the receptor is important whether or not an increase in affinity results. In this common medicinal chemical optimization the aim is often to find molecular modifications that reduce the overall bulk lipophilicity ($\lg P$) of the series whilst maintaining the important hydrophobic motifs. For instance, inclusion of an aliphatic ether into a potential drug in place of a methylene group may have several effects. It may alter potency by anything from zero to 15-fold, if it can act as an acceptor to a neutral donor in the receptor. However, the substitution would lower the value of $\lg P$ by two log units, increasing solubility by 100-fold (hence aiding absorption) and improving drug metabolism and pharmacodynamic properties. The observed tendency of present molecular modeling packages to predict the binding of polar molecules better than those with a more druglike balance of polar and lipophilic interactions is very interesting.[47] This balance of properties is crucial to the drug discovery process. Tight binding of drugs to receptors is achieved through polar interactions and most importantly through the optimization of specific hydrophobic interactions. The bulk properties of drugs, which control solubility, absorption, metabolism, and pharmacokinetic properties, is achieved mainly using polar groups together with sufficient lipophilicity to achieve partition into lipid bilayers. The Rules of 5 ($M_r < 500$, $\lg P < 5$, Σ(donors) < 5, Σ(N,O) < 10) from Pfizer[59] elegantly express the interplay between control of the number of hydrogen-bond acceptors and donors and the requirement to limit the overall value of $\lg P$ in order to achieve oral activity by the usual passive mechanisms.

6. Summary and Outlook

The contribution of hydrogen bonds to drug–receptor interactions is highly unpredictable. Charge-reinforced hydrogen bonds can contribute up to 3000-fold in binding. Neutral–neutral hydrogen bonds may contribute anything between zero and 15-fold. Hydrogen bonds can be "seen" and easily described in drug–receptor complexes, and this may lead to overemphasis of their importance relative to other binding interactions. Just "seeing" a hydrogen bond tells nothing about its contribution to binding. Hydrophobic interactions contribute a minimum of 3.2-fold increase in binding per methyl group. They play a major role in the affinity of most drugs for their receptors. This is indicated by counts of hydrophobic groups relative to acceptors and donors in oral drugs from the Physicians Desk Reference. Hydrophobic interactions appear to be the dominant driving force for induced fit of receptors around drugs. This failure of the rigid receptor hypothesis can also be described as hydrophobic collapse of a receptor around a drug. Ligands with increased affinity can sometimes be obtained by utilizing hydrophobic interactions and induced fit even at the expense of removing hydrogen bonds. Induced fit can allow beneficial hydrophobic

interactions to occur between drugs with different shapes at the same binding pocket in a given receptor. Existing molecular modeling packages have a poor balance between hydrogen bond and lipophilic interactions. At present they do not adequately take account of changes in receptor structure. The exploitation of specific hydrophobic interactions can be a very potent method of increasing affinity and selectivity for a receptor. The above observations also may be seen as potent arguments for retaining consideration of physicochemical properties and serendipity alongside rational design in the drug discovery process.

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