Enzyme Tunnels and Gates As Relevant Targets in Drug Design

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Abstract: Many enzymes contain tunnels and gates that are essential to their function. Gates reversibly switch between open and closed conformations and thereby control the traffic of small molecules—substrates, products, ions, and solvent molecules—into and out of the enzyme's structure via molecular tunnels. Many transient tunnels and gates undoubtedly remain to be identified, and their functional roles and utility as potential drug targets have received comparatively little attention. Here, we describe a set of general concepts relating to the structural properties, function, and classification of these interesting structural features. In addition, we highlight the potential of enzyme tunnels and gates as targets for the binding of small molecules. The different types of binding that are possible and the potential pharmacological benefits of such targeting are discussed. Twelve examples of ligands bound to the tunnels and/or gates of clinically relevant enzymes are used to illustrate the different binding modes and to explain some new strategies for drug design. Such strategies could potentially help to overcome some of the problems facing medicinal chemists and lead to the discovery of more effective drugs.

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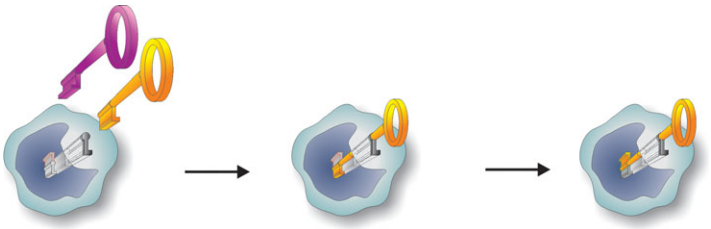


Figure 1. The lock–keyhole–key model. The *key* represents the substrate, which must pass through the *keyhole*, representing the tunnel, to reach the *lock*, that is, the active site, and react (adapted from Ref. 1; copyright: Wiley-VCH Verlag GmbH & Co. KGaA; reproduced with permission; see the online version for colored images).

1. INTRODUCTION

Natural systems and their mechanisms of operation are astonishingly complex. So too are the biomolecules that perform and regulate the physiological processes of life, which range from the hydration of simple carbon dioxide molecules to the replication of DNA by the synchronized stepwise action of several different biomolecules. Consequently, when seeking to investigate or manipulate these biochemical systems, it is necessary to employ a wide range of techniques and conceptual approaches. If we are to continue delivering new drugs and treatments, medicinal chemists cannot afford to remain wedded to existing paradigms and approaches; it is necessary to continuously search for new ones.

It is well known that most biomolecular systems contain voids, cavities, channels, tunnels, or grooves of some kind. These tunnels and channels often have functional roles, which typically involve transporting substances between different spatial regions. For example, they might connect inner cavities to the surface, one inner cavity to another, different parts of the protein's surface, or (in the case of membrane proteins, for example) different cellular environments. Many enzymes that have buried active sites must be able to transfer substrates and products to and from the bulk solvent in order to exercise their catalytic activity. The *lock–keyhole–key* model[1] has recently been proposed as a more realistic alternative to Fischer's *lock-and-key* model[2] or Koshland's *induced-fit* model[3] for describing the behavior of enzymes containing buried active sites (Fig. 1). In this model, the substrate, or *key*, must pass through a tunnel—the *keyhole*—to reach the active site—the *lock*—where it is converted into the product. This model implies that the access tunnels are important structural features that could contribute to the regulation of enzymatic functions and other biological processes.[1,4,5] As such, they can be regarded as potential hotspots for modulating the functioning of biomolecules inside living cells.

Enzyme tunnels often feature *molecular gates* that can specifically regulate the transport of substances through these biomolecular systems. Molecular gating is a dynamic process whereby individual small molecule substances are granted or denied access to particular sites on or within the macromolecule. Different gating processes can have very different timescales and roles. The gates of ion channels have been studied and targeted by drug designers for a long time. However, gates of other biomolecules such as those found in many enzymes have not been thoroughly investigated and are not so well understood. The functionality of gates depends on the inherently dynamic nature of all biomolecules, and they can be regarded as sophisticated structural tools that enable important functions to occur within complex biochemical systems.[4,6] It is increasingly acknowledged that dynamics are a fundamental property of biomolecules and contribute significantly to their function. It is therefore essential to take protein flexibility into account in any detailed study of structural biology or structure-based drug design.[7–11]

Our aim in this review is to highlight the importance of enzymes' tunnels and gates, and their relevance in drug discovery. While these structures are often overlooked, they may hold the key to solving longstanding problems and enable the discovery of very active and selective drugs. In the following sections we review the functional roles, structures, and localization of tunnels and gates. A system for classifying gates and tunnels is introduced and examples of therapeutic targets belonging to the different classes are presented. In addition, we discuss some representative case studies on pharmaceutically relevant targets complexed with synthetic inhibitors that bind to their tunnels and/or gates. These examples allow us to illustrate different types of binding to gates and tunnels, and the benefits of targeting these structural elements. We consider that collectively they constitute proofs of concept for several new strategies for designing new ligands targeting enzyme tunnels and gates.

2. ENZYME TUNNELS AND GATES

This section presents a detailed classification and description of enzyme tunnels and gates as functional structural features. The terminology used to describe tunnels and gates in the scientific literature is quite diverse, and the terms *tunnel* and *channel* are often used interchangeably. Here, we define *tunnel* as a transport pathway with a functional role that connects two points located in different regions of a protein's structure; those points may be on the protein's surface or inside a cavity. A molecular *gate* is a dynamic system consisting of individual or groups of residues, loops, secondary structure elements, or even domains that reversibly switch between *open* and *closed* conformations and thereby control the traffic of small molecules—substrates, products, ions, or solvent—into or out of the protein structure. For each structure type, we describe its structural basis, functional roles, localization, and potential as a target in a drug design.

A. Enzyme Tunnels

Many enzymes contain catalytic or binding sites that are not exposed to the solvent, but are buried within their cores. Buried active sites of this sort can enable very tight control over the catalytic process at different levels, but they require some means of communication with the bulk solvent, that is, a way of transferring substrates, products, cofactors, and solvent molecules between the external environment and the active site (or between one active site and another). This is the primary role of an *enzyme tunnel*. A secondary and related role of tunnels is in selecting the species that are permitted to undergo this transfer. For example, the presence of water molecules may hinder the enzyme-catalyzed reaction and so the enzyme would need to tightly control their passage. In other cases, it might be necessary to prevent the release of toxic intermediates from the enzyme's interior while transferring them between distinct active sites. In general, the existence of a tunnel makes it easier to select which substances are permitted to access specific parts of the protein from the complex mixture of molecules present in the cell. If the tunnel's geometry and physicochemical properties are well tuned, it may be able to exclude all but the desired substrates and thus ensure the enzyme's substrate specificity. This can be intuitively understood from the *lock–keyhole–key* model (Fig. 1), which implies complementarity between the *key* (substrate) and the *keyhole* (tunnel).

Tunnels are very common in enzymes, being found in all six major enzyme classes defined according to the NC-IUBMB classification system[12] (Table I). Three tunnel types can be delineated on the basis of their structural elements and molecular functions[1]:

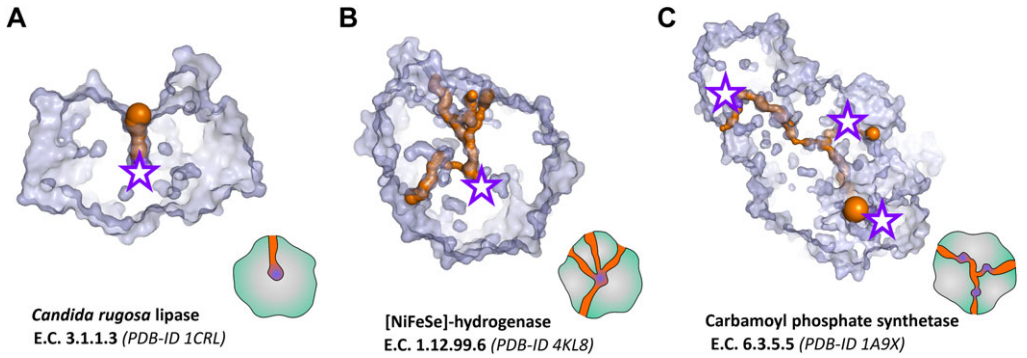


Figure 2. Different types of enzyme tunnels. Examples of enzymes containing (A) one tunnel or (B) multiple tunnels connecting the active site to the surface, or (C) tunnels connecting separate active sites in a single protein. Stars indicate the locations of catalytic sites. The tunnels are represented in orange (adapted with permission from Refs. 1 and 4; copyright Wiley-VCH Verlag GmbH & Co. KGaA; see the online version for colored images).

(1) Single tunnels connecting a buried cavity to the bulk solvent. The tunnel is the only pathway for the exchange of reagents, products, solvent, or ions between the buried cavity and protein's surroundings. One example of such enzymes is the lipase from *Candida rugosa* (Fig. 2A).
(2) Sets of two or more tunnels connecting the same buried cavity to the bulk solvent. The substrates, products, and solvent may have different preferences for specific tunnels. In most cases, there is one main tunnel and one or more secondary tunnels that function as alternative or auxiliary routes. [NiFeSe]-hydrogenase is an enzyme that contains multiple tunnels (Fig. 2B).
(3) Tunnels connecting different catalytic sites in multifunctional enzymes or enzyme complexes possessing multiple active centers. These tunnels steer the intermediate products in the right direction and prevent them from escaping into the medium, thus enhancing the enzyme's efficiency. They can also help to prevent side reactions by keeping labile intermediates away from the solvent, or even prevent toxic products from being released into the intracellular environment. Such phenomenon is commonly known as *substrate channeling* through molecular tunnels.[13] This should not be confused with *electrostatic channeling*, which refers to the steering of the intermediates between different catalytic sites driven by electrostatic fields and do not necessarily involve enzyme tunnels.[13–15] Carbamoyl phosphate synthase is a good example of a multifunctional enzyme with several tunnels connecting different active sites (Fig. 2C).

The *bottleneck* of a tunnel—its narrowest point—is often a hotspot for selectivity because it determines the maximum size of the substances that can pass through. Another important part of the tunnel is its *entrance* or *mouth*. This is the first point of interaction with the bulk solvent, and may play a vital role in substrate recognition. Similarly, the group of residues forming the bottleneck or the first shell of residues at the entrance of a tunnel can play a major role in determining its function. It is therefore necessary to consider several parameters when attempting to understand the function of a given tunnel, including the tunnel's *length* and *curvature*, *bottleneck radius*, *average radius*, *entrance residues*, and *bottleneck residues*.

The dynamic nature of the system must also be taken into account when investigating a tunnel's function. Because proteins are dynamic, the tunnel's geometry may vary significantly over time. While main tunnels are frequently permanent and readily identified by examining crystal structures, additional *transient tunnels* can only be identified by studying dynamic changes

Table I. Some Clinically Relevant Enzymes Featuring Molecular Tunnels and/or Gates

Class	Enzyme	Clinical relevance	Structural feature	References
EC1, oxidoreductases	Aldehyde dehydrogenase	Neurodegenerative disorders, cancer	Tunnel	54
	Catalase	Inflammation, tumor, anemia, diabetes mellitus, hypertension, vitiligo	Tunnel and gate	55–57
	Cholesterol oxidase	Bacterial pathogenesis	Tunnel and gate	19, 20, 58
	Choline oxidase	Bacterial pathogenesis	Gate	59
	Copper-containing amine oxidase	Wound healing, atherosclerosis, cell growth	Tunnel and gate	60, 61
	Cyclooxygenase	Pain, inflammation, cancer	Tunnel	62–64
	Cytochrome P450	Cancer, antibiotics, antiparasitic, drug metabolism	Tunnel and gate	18, 65–68
	Dihydrofolate reductase	Cancer, antibiotics, antiparasital	Gate	69–71
	Dihydroorotate dehydrogenase	Autoimmune and parasitic diseases, immunosuppression, cancer, inflammation	Tunnel	72, 73
	Enoyl-acyl carrier protein reductase	Antibacterial	Gate	74
	Lipoxygenase	Stroke therapy, inflammatory diseases	Tunnel and gate	75
	Monoamine oxidase B	Alzheimer, Parkinson and other neurodegenerative diseases	Tunnel and gate	76, 77
	Nitric oxide synthase	Neurological diseases, inflammation, rheumatoid arthritis, immune-type diabetes, stroke, cancer, thrombosis, infection susceptibilities	Tunnel and gate	78, 79
	Polyamine oxidase	Cell growth, proliferation, differentiation	Tunnel	80
	Proline utilization A	Bacterial pathogenesis	Tunnel and gate	81
	Xanthine oxidase	Cardiovascular and inflammatory diseases, chronic obstructive pulmonary disease, gout, ischemia	Tunnel and gate	82
EC2, transferases	β-Ketoacyl-acyl carrier protein synthase	Antibiotics	Tunnel	31, 83
	Anthranilate phosphoribosyltransferase	Tuberculosis	Gate	84

Continued

Table I. Continued

Class	Enzyme	Clinical relevance	Structural feature	References
	Aspartate transaminase	Antiparasital, antibiotics, cancer	Gate	85–87
	Catechol-*O*-methyltransferase	Schizophrenia, depression, Parkinson's disease	Gate	88
	DNA and RNA polymerases	Antivirals	Gate	32, 89–91
	Fatty acid synthase type I	Antivirotics, cancer	Tunnel and gate	92
	Glucosamine-6-phosphate synthase	Antifungal chemotherapy	Tunnel and gate	30, 93, 94
	Glutamine phosphoribosylpyrophosphate amidotranferase	Leukemia	Tunnel	93, 95
	Glutathione S-transferase	Antibiotics, cancer	Tunnel	96
	Glycogen phosphorylase	Diabetes	Tunnel	97
	Imidazole glycerol phosphate synthase	Antibiotics, herbicides	Tunnel and gate	93, 98, 99
	Octaprenyl pyrophosphate synthase	Antibiotics	Tunnel	48
	Peptidyl transferase center (ribozyme)	Antibiotics	Tunnel	100, 101
	Phospho-2-dehydro-3-deoxyheptonate aldolase	Antibiotics	Gate	102
	Polynucleotide kinase	Cancer	Tunnel and gate	103
	Protein kinases	Cellular metabolism, proliferation, survival, growth, angiogenesis	Gate	104
	Purine nucleoside phosphorylase	Gout, arthritis, cancer	Gate	105, 106
	Sulfotransferase	Xenobiotic metabolism, cancer	Gate	107
	Thymidylate synthase	Cancer	Gate	108, 109
	Transglutaminase 2	Celiac sprue	Tunnel and gate	110
EC3, hydrolases	β-Lactamase	Antibiotics	Gate	111, 112
	β-Secretase	Alzheimer's disease	Gate	113, 114
	γ-Secretase	Alzheimer's disease	Gate	115, 116
	Acetylcholinesterase	Obesity, Alzheimer's disease, dyslipidemia	Tunnel and gate	16, 117–119
	Aryl esterase	Coronary and heart disease	Tunnel	
	ATP-dependent protease HsIVU	Chronic stress disease, aging	Gate	120, 121

Continued

Table I. Continued

Class	Enzyme	Clinical relevance	Structural feature	References
	Autotaxin	Arthritis, cancer, neurological and cardiovascular diseases	Tunnel	122
	ClpP serine protease	Antibiotics	Tunnel and gate	123
	Cysteine protease	Chagas disease, other parasitic diseases	Gate	124–126
	Deacetylase LpxC	Antibiotics	Tunnel	127, 128
	Epoxide hydrolase	Vascular diseases	Tunnel and gate	129–131
	Histone deacetylase	Inflammation, cancer, neurodegenerative disorders, parasitic diseases	Tunnel	132, 133
	HIV protease	HIV infection	Gate	134, 135
	Leukotriene-A4 hydrolase	Inflammatory diseases	Tunnel	136
	Lipase	Atherosclerosis, chylomicronemia, obesity, Alzheimer's disease, and dyslipidemia associated with diabetes, insulin resistance	Tunnel and gate	137–139
	Neurolysin	Nervous and endocrine systems disorders	Tunnel	140
	Phospholipase A2	Nephropathy, vascular diseases, neurological disorders	Gate	141
	Prolyl endopeptidase	Neurological disorders, Chagas disease, cancer, celiac sprue (as therapeutics)	Tunnel and gate	142–144
	RNA triphosphatase	Anemia, Alzheimer's disease, leukemia, colitis, fungal infections	Tunnel and gate	145, 146
	Urease	Hepatic coma, infection stones, and peptic ulceration	Gate	147

Continued

Table I. Continued

Class	Enzyme	Clinical relevance	Structural feature	References
EC4, lyases	2-Amino-2-desoxyisochorismate synthase PhzE	Tryptophan deficiency	Tunnel and gate	148
	β-Hydroxyacyl-acyl carrier protein dehydratase FabZ	Gastric diseases	Tunnel and gate	42, 149, 150
	Carbonic anhydrase	Autoimmune disease, diuretics, anticancer, antiobesity, Alzheimer's disease	Tunnel and gate	151, 152
	Chondroitin AC lyase	Neurological disease	Tunnel and gate	153
	Aromatic L-amino acid decarboxylase	Parkinson's disease, Tourette's syndrome, schizophrenia, depression, cancer	Gate	154
	Tryptophan synthase	Tuberculosis, bacterial and protozoan infections	Tunnel and gate	93, 155, 156
EC5, isomerases	Glutamate racemase	Antibiotics	Tunnel	157, 158
	Methylmalonyl-CoA-mutase	Acidemia	Tunnel and gate	159, 160
	Oxidosqualene cyclase	Antibiotics	Tunnel and gate	161
	Squalene-hopene cyclase	Anticholesterol	Tunnel	162
	Triosephosphate isomerase	Tropical diseases, tuberculosis, Alzheimer's disease	Gate	163–165
EC6, ligases	Asparagine synthetase	Lymphoma	Tunnel and gate	44, 93, 166, 167
	Carbamoyl phosphate synthetase	Urea cycle defect	Tunnel and gate	45, 46, 93, 168, 169
	Cytidine triphosphate synthetase	Anticancer, antiparasitic	Tunnel and gate	170, 171
	Ubiquitin-conjugating enzyme E2	Cancer	Gate	172, 173

within the biomolecule. Transient tunnels switch between open and closed states depending on the protein's conformation. Despite their transience, they can be essential for the proper functioning of the enzyme, and many enzymes that are currently thought to possess only a single tunnel may in fact have other functional transient tunnels that can only be identified by examining the proteins' dynamics.[1,4,5,16,17] In some cases, the properties of the tunnels can only be properly characterized when studied in the presence of the ligands, which may induce their opening and influence their formation and persistence.[18]

Several studies have shown that the geometry, physicochemical properties, and dynamics of tunnels can profoundly influence enzymes' properties and catalytic behavior. For instance, mutagenesis of tunnel-lining residues or bottlenecks has been reported to modify enzymes' activity (e.g., cholesterol oxidase,[19,20] catalase,[21–23] cytochrome P450,[24–29] glucosamine-6-phosphate synthase,[30] β-ketoacyl-acyl carrier protein synthase,[31] RNA-dependent RNA polymerase,[32] lipase,[33,34] acetylcholinesterase,[35,36] epoxide hydrolase,[37] haloalkane dehalogenase,[38,39] tryptophan synthase,[40,41] 3-hydroxydecanoyl-acyl carrier protein dehydratase,[42] squalene-hopene cyclase,[43] asparagine synthetase,[44] and carbamoyl phosphate synthetase),[45,46] substrate specificity, and enantioselectivity (e.g., amine oxidase,[47] cytochrome P450,[24] octaprenyl pyrophosphate synthase,[48] lipase,[34,49,50] epoxide hydrolase,[37,51] haloalkane dehalogenases,[38,52] squalene-hopene cyclase),[43] and stability (e.g., haloalkane dehalogenases).[53] Some known pharmaceutical targets containing tunnels are listed in Table I. Inhibitors that interact with the tunnels of some of these targets have already been developed (see Section 3). However, there are many targets on the list for which no such inhibitors are yet available, and targets containing transient tunnels are heavily overrepresented among this group.

B. Molecular Gates in Enzymes

Tunnels are structural features that enable and control the access of small molecules and ions to the functional regions of an enzyme. This control can be exerted on the basis of size exclusion or chemical complementarity, and the movement of the small molecules through the tunnel may be driven by diffusion, an electrochemical gradient, or osmotic pressure, among other things. While in some cases it is possible for the properties of the tunnel alone to control which molecular species can access the functional sites, in other cases it may not be sufficient. More sophisticated regulation can be achieved by *molecular gates*, which are structural features that can reversibly switch between open and closed conformations, and which selectively permit the passage of certain molecules while barring others.[4–6,174] The definition of *molecular gate* can be extended beyond the tunnels to any kind of pathway in which *dynamic elements* regulate the access of individual molecules to restricted areas of the proteins. The gates of ion channels have been studied extensively by biochemists and structural biologists for some time, but the gates of enzymes and other proteins have received far less attention. Publications on this topic are dispersed throughout the literature, and there is no widely accepted terminology to describe such gates. Moreover, until recently, there was no systematic way of classifying different kinds of enzymatic gates.[6] This review focuses on the gates of enzymes, but many of the concepts that are presented can be generalized to describe and classify gates in other biomolecular systems.

Many gated enzymes have been described in the literature, and all six main classes of enzymes have members that contain some type of gate[6] (Table I). Molecular gates can contribute to the functioning of biochemical systems in a variety of ways. One of their main functions involves controlling the *access of substances* to the enzyme's tunnels and specific sites within the enzyme. A gate's properties, such as its hydrophobicity, electrostatic profile, opening amplitude, and opening/closing rates, can make it an efficient filter for ensuring selectivity and timeliness. Enzyme gates can also restrict the *access of the solvent*. Some enzyme-catalyzed reactions are very sensitive to water, making it necessary to exclude water molecules from the active site. In

particular, enzymes whose catalytic cycles involve the formation of reactive intermediates, such as cytochromes P450,[175] carbamoyl phosphate synthetase,[46] and imidazole glycerol phosphate synthase,[99] may use gates to prevent the destruction of these intermediates by adventitious water molecules. Gates can also influence the *synchronization* of molecular events taking place in different parts of the protein. This can be important in enzymes containing multiple active sites, in which the fluxes of intermediate products must be regulated. Gate-equipped tunnels can also prevent the escape of toxic intermediates into the cell. This occurs in enzymes such as carbamoyl phosphate synthetase,[46] asparagine synthetase,[44] glucosamine 6-phosphate synthase,[30] and glutamate synthase,[176] all of which have tunnels for ammonia transportation. In addition, the carbamoyl phosphate synthetases have tunnels for transporting carbamate, tryptophan synthase for indole,[41] and the carbon monoxide dehydrogenase/acetyl coenzyme A synthase for carbon monoxide.[177]

The key structural elements that define an enzymatic gate are (i) *door residues*, which are displaced during gating and whose displacement directly causes the gate to open or close; (ii) *anchoring* residues, which interact with the door residues and stabilize them in either the open or the closed state; and (iii) *hinge residues*, which make the structure flexible and allow it to move. Molecular gates can be classified based on their structures[6]; they may consist of single residues, groups of residues, secondary structure elements, or domains (Fig. 3). The *timescale* of a molecular gate is defined as the time required for the gate to transition from the open to the closed state, or vice versa. A gate's *amplitude* is related to the extent of conformational rearrangement that is required to achieve the transition between the open and closed states. Gates belonging to different structural classes have different opening amplitudes and timescales.

(1) The simplest gate type is the *wing gate*, which is opened or closed by the rotation of the side chain of a single residue. This is the most common gate type in enzymes. Their amplitudes are small (in the range of a few angstroms), but their timescales can range from picoseconds to microseconds. Each state can be stabilized by anchoring residues, which interact with the gating residue and hold it for some time in a certain conformation. The most residues most frequently involved in gates of this sort are W, F, and Y. One example of an enzyme containing a wing gate is α-amylase[178] (Fig. 3A); further examples can be found in Section 3 or in comprehensive review article by Gora et al.[6]
(2) A *swinging door gate* is opened and closed by the synchronized rotation of two side chains. The closed state may be stabilized by bonding interactions between the two side chains, such as π–π stacking (in F–F or F–Y pairs), hydrophobic interactions (in F–I, F–V, F–L, L–I, L–V, or R–L pairs), ionic interactions (in R–E or R–D pairs), or H–bonding (in, e.g., R–S pairs). This is the second most common type of gate, and the most common pair of residues comprising a swinging door gate is F–F. Their reported timescales range from picoseconds to microseconds. Methane monooxygenase hydroxylase is one example containing a swinging door gate[179,180] (Fig. 3B).
(3) An *aperture gate* is opened or closed by the simultaneous movement of the backbone atoms of several residues in a sort of a low-frequency "breathing" motion, without any need for side chain rotations. These backbone movements change the relative positioning of the residues in question, and the overall amplitude of the opening/closing process is substantially greater than in gates based solely on the movement of side chains. The timescales of such gates are highly dependent on the protein's rigidity, and range from nanoseconds to microseconds. Acetylcholinesterase is an enzyme that contains an aperture gate[16] (Fig. 3C).
(4) *Drawbridge* and *double drawbridge gates* open and close via the movement of one or two secondary structure elements, respectively; they frequently incorporate loops. The amplitudes of such gates are greater than those of the preceding classes, and they typically

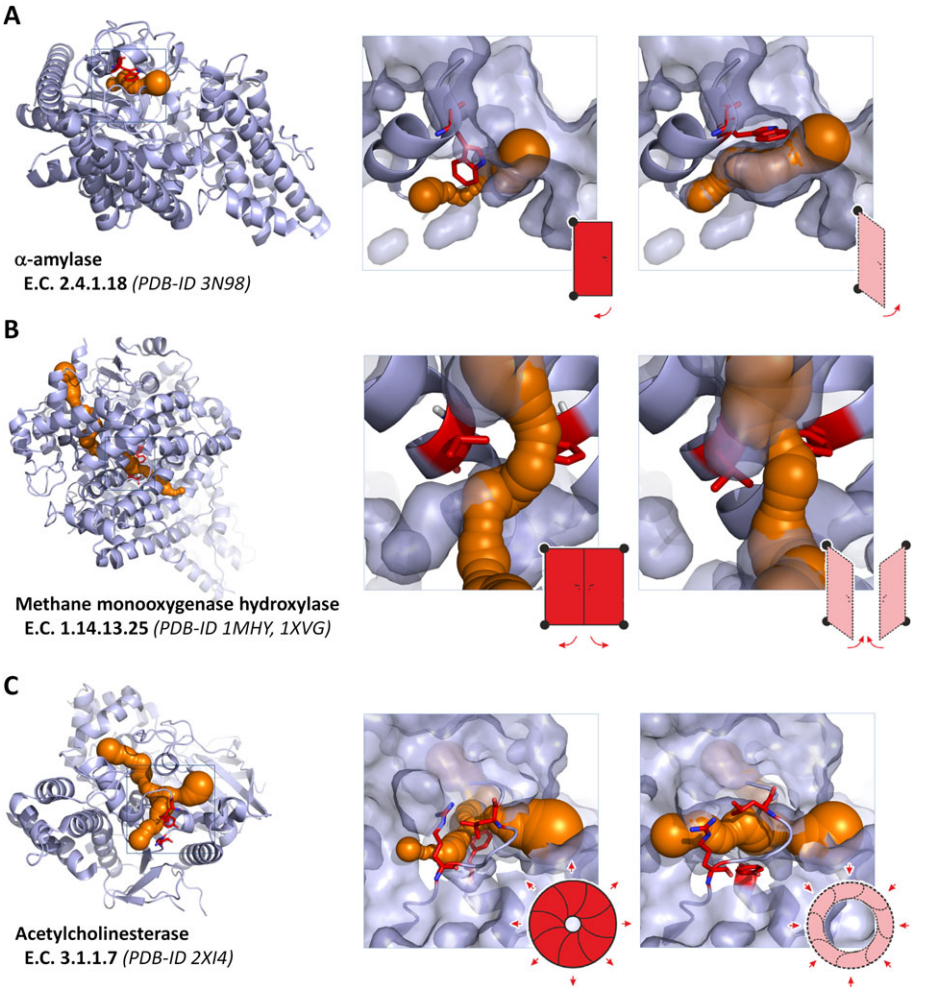


Figure 3. Classification of molecular gates. Representative enzymes containing (A) wing, (B) swinging door, (C) aperture, (D) drawbridge, (E) double drawbridge, and (F) shell gates. The closed and open conformations are shown in the middle and right-hand columns, respectively, and each conformation is presented alongside a stylized image depicting the mechanism of gate opening and closing. The gating elements are shown in red, and the gated access pathways are represented by the orange spheres (adapted with permissions from Refs. 4 and 6; see the online version for colored images).

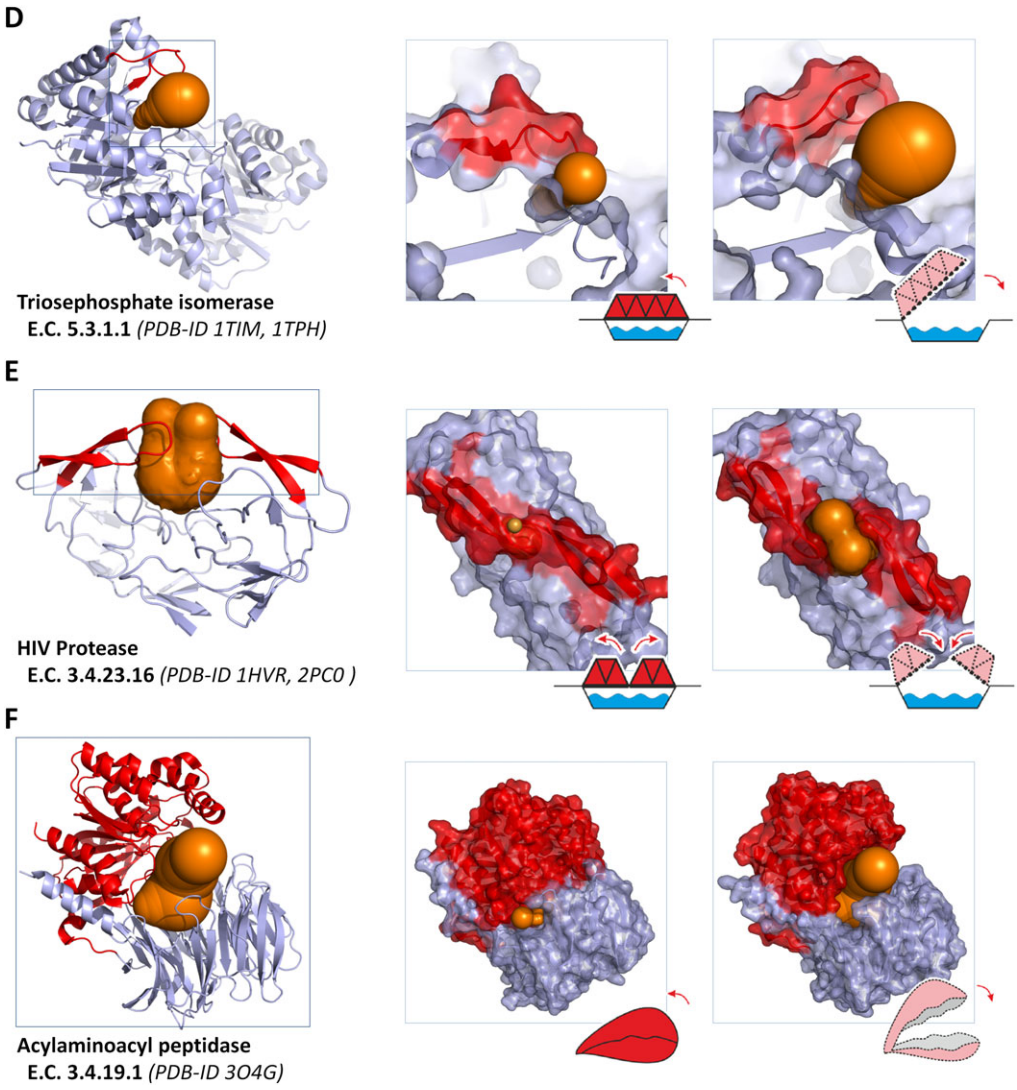


Figure 3. Continued.

control the access of large ligands or cofactors to the binding cavities. Such movements can be components of a complex system that opens, closes, or merges existing tunnels, and can even operate in cooperation with smaller gates that enable fine tuning of the ligand's accessibility. The timescales of such gates range from the nanoseconds to microseconds. Triosephosphate isomerase is an example of an enzyme with a drawbridge gate[165] (Fig. 3D), and HIV protease[181] an example containing a double drawbridge gate (Fig. 3E).

(5) A *shell gate* is characterized by the movement of entire protein domains. Gates of this kind are typically found in enzymes that catalyze reactions involving very large substrates, but they are also common in ion channels and ion pumps. Sometimes such large movements require an additional supply of energy, for example, in the form of adenosine triphosphate (ATP). Due to their amplitude and the size of the elements involved, the timescales of such gates can vary widely, from hundreds of nanoseconds to seconds. An example with a shell gate is the acylaminoacyl peptidase[182] (Fig. 3F).

Enzyme gates can operate *stochastically*, in which case their behavior can be understood using the formalism introduced by McCammon and co-workers to describe diffusion-controlled gates.[183,184] These authors approximated the gating process as a stochastic switch between the fully *closed* and the fully *open* states, and the overall binding rate as a function of the nongated binding rate and the rates of opening and closing. By comparing these two rates, two limit situations can be defined, corresponding to *fast* or *slow* gating. Molecular gates can also be *induced* to open or close by stimuli such as voltage changes or the binding of certain ligands. Such behavior is very common in ion channels, but they are beyond the scope of this review.

Gates may be found in a variety of *locations* within enzymes, including (i) at the *mouth* or the *bottleneck* of an access tunnel or channel, (ii) at the *entrance* to the active site, or (iii) at the *interface* between the cofactor binding site and the active site itself.[6] Tunnels play important roles in determining which substances can access specific regions of the enzymes and many of them incorporate gates that enable a finer level of molecular steering or regulation. In such cases, the gate is typically positioned at the tunnel's *mouth* or *bottleneck*. The entrance to the tunnel is the first point of contact with the bulk solvent and the substances dissolved in it, and, therefore, is a good site for a gate whose function is to select which molecules are permitted to enter. On the other hand, the tunnel bottleneck is the tunnel's narrowest point and often determines its permeability. The entrance to the *active site cavity* can also be a suitable location for a gate; such gates can easily assist in synchronizing the admission of reactants to the catalytic site, or ensuring the proper orientation of the catalytic residues when the substrate enters. Enzyme gates can also be positioned at the entrance to a *cofactor cavity*, and their opening and closing mechanisms may be linked to the movements of protein residues that interact with the cofactor. In some cases, the gate incorporates the cofactor itself, which can adopt different conformations to grant or deny the substrate access. While most known gates are located in one of the positions described above, it should be noted that some can be located in different positions.[6]

Many enzymes targeted in pharmaceutical research exhibit gating processes of some kind (Table I). In some of them, the gates have already been recognized as hotspots for binding of small molecules (Section 3). In other cases, they are unexplored but potentially useful targets for new drug design strategies.

3. THE BINDING OF SMALL MOLECULES TO TUNNELS AND GATES

In this section we discuss different aspects of the binding of small molecules to the tunnels or gates of clinically relevant enzymes, and the resulting changes in the enzymes' activity. We

critically review different types of binding, some reported strategies for designing effective binders, and the claimed and achieved benefits of these strategies. Twelve representative pharmaceutical targets complexed with small molecules, taken from the Protein Data Bank, are presented here to illustrate the concepts under discussion (Table II). Each example illustrates a particular binding mode and the potential benefits of such binding, and serves as a proof of concept for at least one of the drug design strategies discussed above. Cases were selected based on (i) the target's clinical relevance, (ii) the availability of experimental structures of the enzyme–ligand complex, (iii) the location of the ligand in the tunnel or gate of the enzyme, (iv) showcase of a variety of binding modes, (v) the inhibitory activity of the ligand, and (vi) the resolution of the structure. The most important factors were the clinical relevance of the enzyme, the existence of a crystal structure that clearly showed the ligands binding to the specified structural features, and showcasing diverse binding modes. Each one of these case studies is described in more detail in Table III. Unless stated otherwise, all numbered examples mentioned throughout this section refer to the corresponding entries in Tables II and III.

A. Types of Binding

A ligand that binds to a tunnel or gate in an enzyme and thereby inhibits or modifies the enzyme's activity can do so by interacting with (i) the *catalytic site*, (ii) a *tunnel*, (iii) a *gate*, or (iv) with two or more of these sites simultaneously, in so-called *mixed* binding (Fig. 4).

1. A ligand targeting an enzyme can bind directly to the *catalytic site* in order to prevent the enzymatic reaction. This type of binding is the most frequently observed type among known enzyme inhibitors.
2. A ligand can bind to *a tunnel* in a protein and block its main function as a transport pathway. Such ligands may extend across the tunnel's entire length, making many contacts with the tunnel-lining residues. This can lead to the formation of very stable complexes because of the large number of interactions between the ligand and tunnel, making the ligand a very potent inhibitor. Ligand–protein complexes of this sort are exemplified by the complexes of CYP51, DHODH, and LTA4H presented in Tables II and III (examples #2, 4, and 8). However, it is more common for a ligand to bind to a specific region of the tunnel where it forms strong stabilizing interactions with a few residues rather than extending across its whole length. These regions are typically the entrance to the catalytic site, tunnel bottleneck, or tunnel entrance. Interactions with the residues at the entrance to the catalytic site are perhaps most common in inhibitors that bind to the catalytic site, but such interactions by themselves are unlikely to confer much selectivity because residues around the active site are often highly conserved among related enzymes. Binding at the tunnel entrance has been observed for AChE (example #7), which has an inhibitor that interacts strongly with a group of aromatic residues, enabling it to block the transport through the tunnel and inactivate the enzyme with reasonable potency. As mentioned before, the tunnel mouth sometimes has features that contribute to substrate recognition, and specific interactions in this region can provide selectivity, as in the case of CYP51 (example #2). The tunnel bottleneck is another potential hotspot for the binding of ligands. Being the narrowest part of the tunnel, even a relatively small ligand may be able to form sufficient strong interactions to produce a stable adduct with the target enzyme and block transport through the tunnel. Moreover, the bottleneck residues may be less highly conserved than those in the active site, offering opportunities to develop highly specific and selective inhibitors. This is the case for CYP17A1 and PTC (examples #3 and 5).

Table II. List of Enzyme–Inhibitor Complexes in Which the Inhibitor Binds to a Tunnel or Gate

No.	Enzyme	Clinical relevance	Structural features	Binding modes	Benefits	Inhibitory activity	PDB ID	Resolution (Å)
1	Inducible nitric oxide synthase (*iNOS*) E.C. 1.14.13.39	Neurological diseases, inflammation, rheumatoid arthritis, immune-type diabetes, stroke, cancer, thrombosis, infection susceptibilities	Tunnel and *wing* gate	Catalytic site, tunnel and gate (*open state*)	Selectivity	$IC_{50} = 0.4\ \mu M$	3EBF	2.29
2	Sterol 14α-demethylase (*CYP51*) E.C. 1.14.13.70	Antiparasitic	Several tunnels	Catalytic site and substrate tunnel	Selectivity, potency, broad-spectrum activity	$K_d = 73$ nM	3K1O	2.89
3	Cytochrome P450 17A1 (*CYP17A1*) E.C. 1.14.14.19, E.C. 4.1.2.30	Breast and prostate cancer	Several tunnels	Catalytic site and substrate tunnel	Potency, selectivity	$IC_{50} = 3$ nM	3RUK	2.6
4	Dihydroorotate dehydrogenase (*DHODH*) E.C. 1.3.5.2	Autoimmune or parasitic diseases, cancer, immunosuppression	Tunnel	Tunnel	Selectivity, broad-spectrum activity	$K_i = 8$ nM	1D3H	1.8
5	Peptidyl transferase center (*PTC*) E.C. 2.3.2.12	Antibiotics	Tunnel	Tunnel	Affinity, broad-spectrum, enlarged space of binding modes	$K_i = 0.7\ \mu M$	1NJI	3.0
6	Serine/threonine protein kinase AKT1 (*AKT1*) E.C. 2.7.11.1	Cellular metabolism, proliferation, survival, growth, angiogenesis	*Shell* gate	Gate (*closed state*)	Selectivity	$IC_{50} = 58$ nM	3O96	2.7

Continued

Table II. Continued

No.	Enzyme	Clinical relevance	Structural features	Binding modes	Benefits	Inhibitory activity	PDB ID	Resolution (Å)
7	Acetylcholinesterase (*AChE*) E.C. 3.1.1.7	Neurological diseases	Two tunnels and gate	Main tunnel	Selectivity, wider space of binding modes	$K_i = 28\ \mu M$	2XI4	2.3
8	Leukotriene A_4 hydrolase/aminopeptidase (*LTA4H*) E.C. 3.3.2.6	Inflammatory diseases	2 Tunnels	Main tunnel	Affinity, selectivity, target one enzymatic function	$K_i = 2\ \mu M$	4L2L	1.65
9	Prolyl endopeptidase (*PREP*) E.C. 3.4.21.26	Neurological disorders, Chagas disease, cancer	*Shell* gate	Catalytic site and gate (*closed state*)	Binding affinity	$IC_{50} = 1.0\ \mu M$	2BKL	1.5
10	*Human immunodeficiency virus 1* protease (*HIV-1 protease*) E.C. 3.4.23.16	Antivirotics	*Double drawbridge* gate	Catalytic site and gate (*open state*)	Affinity, enlarged space of binding modes	$K_i = 20\ \mu M$	3BC4	1.82
11	UDP-3-*O*-[3-hydroxymyristoyl] *N*-acetylglucosamine deacetylase (*LpxC*) E.C. 3.5.1.33	Antibiotics	Tunnel	Catalytic site and tunnel	Potency, broad-spectrum activity, drug resistance	$K_i = 1$ nM	3NZK	1.8
12	β-Hydroxyacyl-acyl carrier protein dehydratase (*FabZ*) E.C. 4.2.1	Antibiotics	Tunnel and *wing* gate	Tunnel and gate (*open state*)	Broad-spectrum activity, drug resistance	$K_i = 1\ \mu M$	3D04	2.4

The table specifies the clinical relevance of the enzyme in question, the number of tunnels and gates it contains, inhibitor's binding mode, benefits achieved by binding through that mode, inhibitor's IC_{50} value, PDB ID of the complex, and resolution of the corresponding crystal structure.

3. When ligands bind to *a gate* of an enzyme, the most obvious and common binding position is at a point of contact between the moving elements. Depending on the nature of the gate, this may be the door residues, flexible secondary elements, or an entire domain. Ligands of this sort may target either the open or closed conformation of the gate, stabilizing and locking the conformation to which they bind and thereby disrupting the gating mechanism and the biological function of the target. For instance, AKT1 (example #6) is a serine/threonine protease with a gate that can be locked into its closed conformation by a potent inhibitor that binds between the two gate domains. Similarly, there is an HIV-1 protease inhibitor (example #10) that binds between the two flexible flaps of the enzyme's gate, locking it in the open conformation and thereby suppressing the enzyme's functionality.
4. The binding modes described above are clearly defined, with the ligand binding to a specific site somewhere along the tunnel's length. However, most ligands that interact with tunnels and gates bind via multiple modes. For instance, they may bind to both catalytic site and some of the residues comprising the tunnel. If the tunnel is gated, it may be that the ligand binds to the tunnel residues and gate interface, or to the catalytic residues and to some of the gating elements. Many different combinations of binding modes are possible, depending on the enzyme that is being targeted and the ligand design strategy that is adopted. Mixed-mode binding can be seen in the complexes of iNOS, CYP17A1, PREP, LpxC, and FabZ (examples #1, 3, 9, 11, and 12).

B. Benefits of Binding to Tunnels and Gates

The binding of ligands to tunnels or gates can confer important pharmacological benefits including (i) *high target selectivity*, (ii) *high binding affinity*, (iii) *a broad spectrum of activity*, (iv) *a broad spectrum of binding modes*, (v) *low drug resistance*, and (vi) *selective targeting of a single function*.

(1) *High target selectivity* is the first benefit, and probably the one most commonly achieved, by binding to tunnels or gates. Enzymes' catalytic sites and the surrounding residues are often highly conserved among members of the same enzyme family and sometimes across families. Conversely, tunnels are typically less highly conserved, and consist of many residues; as such, they are more likely to vary between members of a given family. The specific binding of ligands to nonconserved tunnel regions can thus enable selective and specific inhibition of the targeted enzyme. It is often the bottleneck that contributes most heavily to specific interactions within the tunnel, as in the cases of the CYP17A1 and PTC complexes listed in Tables II and III (examples #3 and 5, respectively), or the residues at the tunnel mouth, as exemplified by the complexes of CYP51, AChE, and FabZ (examples #2, 7, and 12, respectively). It is important to recall that protein dynamics can profoundly affect the binding of selective inhibitors. Because proteins are dynamic, the geometry of the tunnel fluctuates over time. This fluctuation may differ between related variants of a given enzyme due to differences in the tunnel-lining residues or the residues in the second and third shells with respect to the binding site. (anchoring residues). iNOS (example #1) is a very interesting case in which these relatively distant anchoring residues played an essential role in the discovery of isozyme-selective inhibitors because they give rise to differences in the dynamics of the tunnels in different nitric oxide synthases (NOSs). An inhibitor targeting the active site and tunnel of iNOS revealed 2.5 and 125 times lower IC_{50} than in the case of neuronal (nNOS) and endothelial (eNOS) isozymes. The conventional inhibitors targeting only the active site revealed similar IC_{50} for all three isozymes.[185] Highly selective inhibition can also be achieved by binding to enzyme gates.

Being more or less complex systems whose properties are affected by several factors (for instance, the door, hinge, and anchoring elements can all affect a gate's dynamics), gates can be quite specific for each member of an enzyme family. The case of AKT1 (example #6) shows how investigations into binding at the gate interface can result in the discovery of selective inhibitors. The majority of conventional AKT1 inhibitors compete with ATP for the active site resulting in nonselectivity against other AKT isozymes as well as against closely related kinases. However, targeting the gate interface of AKT1 revealed 4 and 40 times lower IC_{50} than in the case of AKT2 and AKT1 isozymes.[186]

(2) *High binding affinity* is the second major benefit of targeting gates and tunnels in ligand design. A tunnel can have a large accessible surface area, and thus offers many potential contact sites for a ligand. If many favorable contacts are made, a very stable enzyme–ligand complex will be formed. This is demonstrated by the complexes of iNOS, CYP51, CYP17A1, DHODH, PTC, LTA4H, and LpxC (examples #1–5, 8, and 11, respectively). A ligand that binds to an enzyme's gate may also form strongly stabilizing interactions with one of the gate's possible conformations, which can result in very potent inhibition. This can occur if the gate is flexible enough to accommodate the ligand and maximize their interactions. Such binding may stabilize either the closed (as in the cases of AKT1 and PREP, examples #6 and 9) or the open conformation of the gate (as in HIV protease, example #10).

(3) While selective inhibition of a specific enzyme is desirable in many cases, in others, *a broad spectrum of activity* is preferable. This is the case when developing antivirals or antibiotics, for instance, as such drugs are often required to inhibit enzymes of a given class produced by several different strains of viruses or bacteria. Some enzymes and other biochemical systems are common to many strains, with only minor differences. In such cases, they can be treated as a common target, with their tunnels or gates serving as the primary binding motifs. The enzymes CYP51, DHODH, PTC, LpxC, and FabZ all form complexes with such promiscuous inhibits (examples #2, 4, 11, and 12).

(4) *A broad spectrum of binding modes* here refers to the number and diversity of interactions available for ligand binding. For instance, a tunnel offers a wider spectrum of binding modes than a shallow cavity because it has more residues available for contact. This can be important when designing new ligands to target a certain protein. The same is true when binding to a gate; the existence of two distinct conformational states increases the number of binding possibilities, and the chance of finding a ligand with an ideal pharmacophore for binding to one conformation or the other. The inherent flexibility of tunnels and gates may also increase their ability to change their conformations to accommodate a particular ligand and form optimal binding interactions.

(5) Finally, binding to tunnels or gates can potentially *reduce drug resistance*. Resistance is a serious problem for many drugs due to the high mutability of some otherwise very attractive target proteins. In such cases, exploring new binding modes may increase the likelihood of identifying efficient inhibitors that can avoid or minimize the resistance problem. This is demonstrated by the cases of CYP51, DHODH, PTC, HIV protease, LpxC, FabZ (examples #2, 4, 5, and #10–12, respectively).

(6) Some enzymes have multiple functions; in such cases, the *selective targeting of a single function* may be preferable to inhibiting all of the enzyme's activities. LTA4H (example #8) illustrates this point nicely: The binding of a selective inhibitor to one of its two tunnels suppressed the hydrolysis of one substrate without reducing the general functionality of the catalytic site or the second tunnel. Consequently, the enzyme retained its activity toward other substrates. It is easy to imagine that a similar approach could be applied to other bifunctional enzymes containing different binding pockets or tunnels.

C. Prospective Drug Design Strategies

Most known enzyme inhibitors bind to catalytic sites or neighboring binding sites in order to exert their action. However, for a number of reasons, this strategy sometimes fails to deliver the desired results. As explained in the preceding section, inhibitors that target enzymes' gates and tunnels could have important advantages over more conventional active site-targeting inhibitors, and drug design strategies based on targeting tunnels and gates may succeed where conventional approaches fail. Here, we propose four different strategies for the design of new ligands targeting tunnels and gates: (i) modify a tunnel with *new contacts*, (ii) bind to a *specific region* of the tunnel, (iii) bind to an *auxiliary tunnel*, and (iv) bind to a *gating element*. These strategies are based on the different binding modes discussed in the preceding sections of this review. The approach of choice will depend on the system under investigation, the extent to which its structural features are known, and the desired pharmacological effect.

(1) If the main objective is to achieve potent inhibition, the best inhibitor would be that which forms the greatest number of *favorable contacts* with the tunnel. Therefore, the aim should be to design a ligand that complements the tunnel's geometry and physicochemical properties. Virtual screening and pharmacophore mapping inside the tunnel could be very useful in identifying an optimal lead. Some of the difficulties implementing this strategy can be (i) the dynamic properties of the target tunnel, which may significantly change its geometry in time; (ii) identifying the optimal tunnel to target when multiple tunnels are present; (iii) predicting the importance of the tunnel solvation, which may contribute unfavorably due to entropic effects; and (iv) limited applicability of bulky molecules due to their poor bioavailability and pharmacokinetic properties.

(2) Another way of achieving high activity or selectivity would be to adopt a drug design strategy that targets *a specific region* of a tunnel. Individual amino acids in the tunnel can be complemented by specific molecular fragments, leading to the design of ligands with high affinity. If the targeted region of the tunnel is lined with nonconserved residues, its complementation may provide high selectivity for a particular enzyme. Relevant regions are the tunnel bottleneck, tunnel mouth, and gating residues, and it is possible to target multiple regions simultaneously. The possible difficulty of this approach can be (i) identifying the ideal specific regions of the tunnel to target, (ii) ensuring that the ligand binds to the target regions and not elsewhere, and (iii) preventing unforeseen repercussions to the whole system due to targeting larger interaction networks.

(3) Certain enzymes contain multiple access tunnels, some of which are secondary or *auxiliary tunnels*. The biological function of these auxiliary tunnels is currently not well understood. Auxiliary tunnels may not be essential for the transport of the substrate or product, but can serve as alternative pathways for transportation of solvent molecules. Approaches for designing ligands targeting auxiliary tunnels would be similar to those discussed above. Transient tunnels are a particularly notable class of secondary tunnels that open only occasionally and are therefore always gated. Some inhibitors and other ligand types that bind to transient tunnels have been identified[67,68,187]; in general, a detailed description of the system's dynamics is needed to design drugs targeting these structures. Obtaining such descriptions can be challenging, but is possible with the existing technologies. One more drawback is that it may not be easy to fully understand the role of the auxiliary tunnel. But importantly, the targeting of secondary/transient tunnels provides a currently underexplored tool for discriminating between closely related enzyme variants in order to achieve selectivity. This approach would be challenging to implement but could be a powerful way of identifying new solutions to longstanding problems.

Table III. Extended Description of the Selected Enzyme Complexes Featuring Inhibitors That Bind to Tunnels and/or Gates

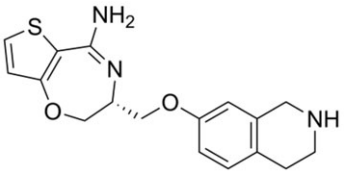
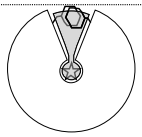
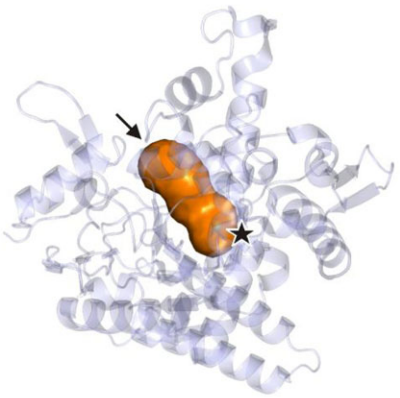
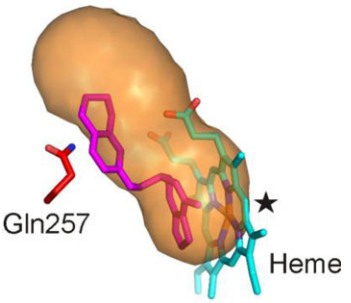
<table>
<tr><td>#1</td><td colspan="2">Enzyme: Inducible nitric oxide synthase (iNOS)

E.C. 1.14.13.39</td><td>PDB ID: 3EBF</td></tr>
<tr><td colspan="2">Function: Produces nitric oxide for signaling in response to cytokines or pathogen exposure, in order to kill bacteria, viruses or tumor cells. However, overproduction of nitric oxide by iNOS has been associated with several diseases

Clinical relevance: Neurological diseases, inflammation, rheumatoid arthritis, immune-type diabetes, stroke, cancer, thrombosis, infection susceptibility

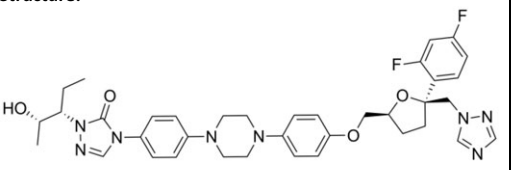
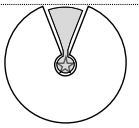
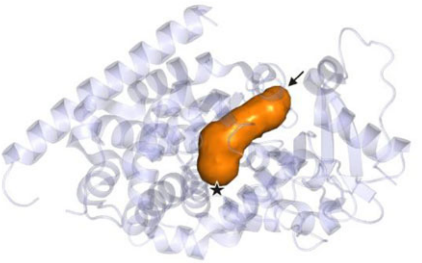
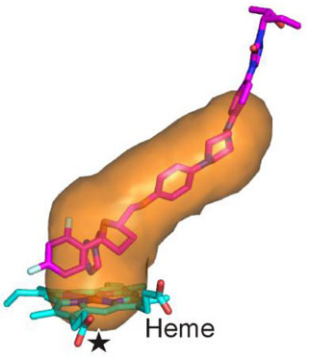
Structural features: One access tunnel with a wing gate</td><td colspan="2">Inhibitor: (3R)-3-(1,2,3,4-tetrahydroisoquinolin-7-yloxymethyl)-2,3-dihydrothieno[2,3-f][1,4]oxazepin-5-amine

IC₅₀ (Homo sapiens) = 0.4 μM (iNOS); 50 μM (eNOS); 1μM (nNOS)

Structure:
  </td></tr>
<tr><td colspan="2">Binding:
Catalytic site, tunnel and tunnel gate (open state)
  </td><td colspan="2">Benefit: Selectivity. The active sites of the three NOS isozymes are structurally conserved. Stabilization of the tunnel gate by the inhibitor induces distant isozyme-specific conformational changes in the non-conserved second- and third-shell residues (in terms of residue-wise distance from the bound inhibitor), making the inhibitor selective for the target over related enzyme variants</td></tr>
<tr><td colspan="2"> 
Cartoon: overall structure of iNOS from Mus musculus; black arrow: tunnel mouth; orange surface: tunnel; black star: catalytic site</td><td colspan="2"> 
Magenta sticks: the inhibitor blocking the tunnel and interacting with the tunnel gate (Gln257) and heme cofactor</td></tr>
<tr><td colspan="4">References: 185,195–197</td></tr>
</table>

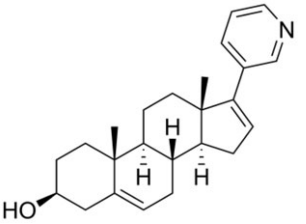
Continued

Table III. Continued

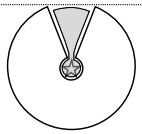
#2	**Enzyme:** Sterol 14α-demethylase (*CYP51*) **E.C.** 1.14.13.70	**PDB ID**: 3K1O
Function: Catalyzes an essential step in the biosynthesis of ergosterol, which is required for membrane construction, growth, development and division in parasites such as *Trypanosoma cruzi*, *Trypanosoma brucei* or *Leishmania* sp. **Clinical relevance:** Antiparasitic **Structural features:** Several access tunnels	**Inhibitor**: 4-[4-[4-[4-[[(2S,5S)-5-(2,4-difluorophenyl)-5-(1,2,4-triazol-1-ylmethyl)oxolan-2-yl]methoxy]phenyl]piperazin-1-yl]phenyl]-2-[(2S,3S)-2-hydroxypentan-3-yl]-1,2,4-triazol-3-one (*posaconazole*) K_d *(Neosartorya fumigata)* = 73 nM **Structure:** 	
Binding: Catalytic site and tunnel 	**Benefit**: Selectivity, potency, broad spectrum activity. Complete blockage of the tunnel results in strong inhibition of the target enzyme, while interactions at the tunnel mouth provide selectivity towards the parasitic enzyme but leave the human CYP51 homolog unaffected	
 Cartoon: overall structure of CYP51 from *Trypanosoma cruzi*; black arrow: tunnel mouth; orange surface: tunnel; black star: catalytic site	 Magenta sticks: posaconazole blocking the tunnel and coordinating the heme cofactor	
References: 68,198–200		
#3	**Enzyme:** Cytochrome P450 17A1 (*CYP17A1*) **E.C.** 1.14.14.19; **E.C.** 4.1.2.30	**PDB ID**: 3RUK
Function: Catalyzes the synthesis of numerous steroid hormones in humans. CYP17A1 has dual functions: its hydroxylase activity enables production of glucocorticoids, and, in combination with its lyase activity, catalyzes the biosynthesis of androgenic and estrogenic sex steroids. **Clinical relevance:** Breast and prostate cancer **Structural features:** One main access tunnel and many adjacent solvent tunnels	**Inhibitor**: (3S,8R,9S,10R,13S,14S)-10,13-dimethyl-17-pyridin-3-yl-2,3,4,7,8,9,11,12,14,15-decahydro-1H-cyclopenta[a]phenanthren-3-ol (*abiraterone*) IC_{50} = 3 nM **Structure:**	

Continued

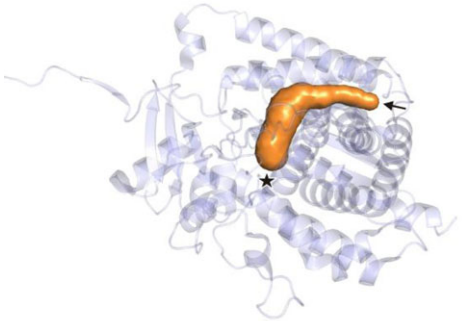
Table III. Continued



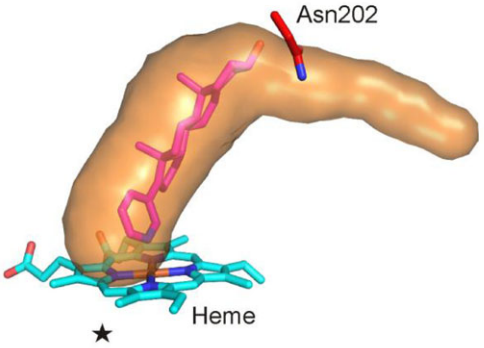
Binding: Catalytic site and tunnel



Benefit: Potency and selectivity. Interactions with tunnel residues conferred potency, and in some cases (notably, those with bottleneck residues) selectivity as well



Cartoon: overall structure of CYP17A1 from *Homo sapiens*; black arrow: tunnel mouth; orange surface: main tunnel; black star: catalytic site



Magenta sticks: abiraterone blocking the main tunnel and coordinating the heme cofactor; Asn202: the tunnel-bottleneck residue which is responsible for the inhibitor's selectivity within the P450 enzyme family

References: 65,66,175,201–203

#4	**Enzyme:** Dihydroorotate dehydrogenase (*DHODH*) **E.C.** 1.3.5.2	**PDB ID**: 1D3H

Function: Catalyzes the rate-limiting step in the *de novo* biosynthesis of pyrimidines. Rapidly proliferating cells, i.e., human T cells or parasitic cells, have an exceptional requirement for *de novo* pyrimidine biosynthesis.

Clinical relevance: Autoimmune or parasitic diseases, cancer, immunosuppression

Structural features: One access tunnel

Inhibitor: 6-fluoro-3-methyl-2-(4-phenylphenyl)quinoline-4-carboxylic acid (*brequinar analog*)

K_i = 8 nM

Structure:

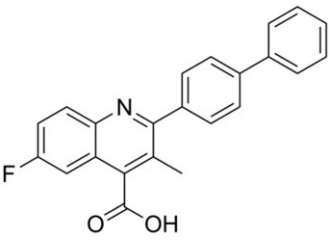


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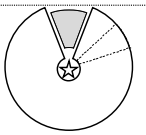
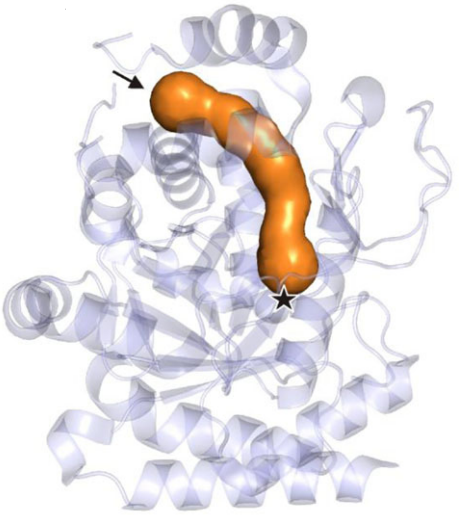
Table III. Continued

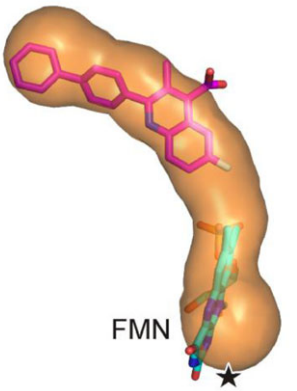
Binding: Tunnel



Benefit: Selectivity, broad spectrum activity. The active sites of the various DHODHs closely resemble one another. However, the access tunnels in human and pathogenic enzymes (e.g. from *Plasmodium falciparum* or *Helicobacter pylori*) differ markedly



Cartoon: overall structure of DHODH from *Homo sapiens*; black arrow: tunnel mouth; orange surface: tunnel; black star: catalytic site



Magenta sticks: the *brequinar* analog blocking the tunnel used by the second substrate (ubiquinone) to approach the cofactor for the redox reaction; FMN: flavin mononucleotide cofactor

References: 72,73,204

#5	**Enzyme:** Peptidyl transferase center (*PTC*) **E.C.** 2.3.2.12	**PDB ID:** 1NJI

Function: Part of the large subunit of the ribosomal RNA (rRNA), it is essential for the ribosome's main function, protein synthesis. It catalyzes the bond formation between the amino acids, supplied stepwise by aminoacyl-tRNAs, to build up the peptide chain bound at the peptidyl-tRNA molecule. It then catalyzes the termination of the reaction and the release of the fully assembled polypeptide by hydrolysis of the final peptidyl-tRNA

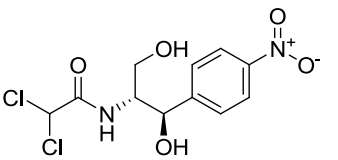
Clinical relevance: Antibiotics

Structural features: One tunnel, necessary for the product release

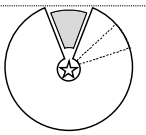
Inhibitor: 2,2-dichloro-*N*-((1*R*,2*R*)-1,3-dihydroxy-1-(4-nitrophenyl)propan-2-yl)acetamide (*chloramphenicol*)

K_i = 0.7 μM

Structure:



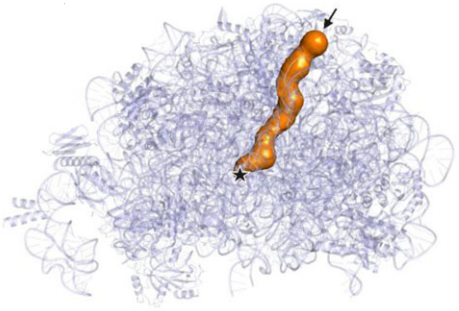
Binding: Tunnel (bottleneck)



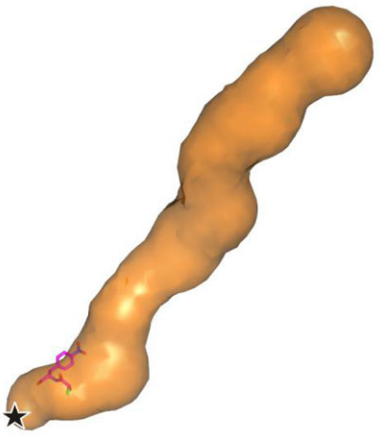
Benefit: Affinity, broad spectrum, enlarged range of binding modes. Most PTC inhibitors compete with substrates for binding and can suffer from drug resistance. Targeting the exit tunnel may *widen the space of binding* modes, which can help to tackle the problem of *drug resistance* to antibiotics

Continued

Table III. Continued



Cartoon: overall structure of PTC from *Haloarcula marismortui*; black arrow: tunnel mouth; orange surface: exit tunnel; black star: catalytic site



Magenta sticks: *chloramphenicol* bound at the bottleneck of the tunnel, blocking polypeptide release

References: 100,101,205,206

#6	**Enzyme:** Serine/threonine protein kinase AKT1 (*AKT1*) **E.C.** 2.7.11.1	**PDB ID**: 3O96

Function: Regulates many cellular processes, including metabolism, proliferation, survival, growth and angiogenesis. The phosphoinositide 3-kinase/AKT pathway is possibly the most frequently activated signal transduction system in human cancers.

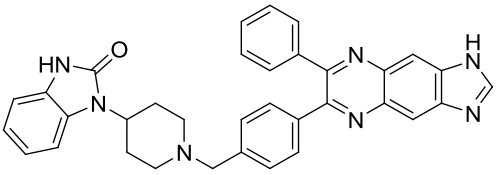
Clinical relevance: Cellular metabolism, proliferation, survival, growth, angiogenesis

Structural features: *Shell* gate

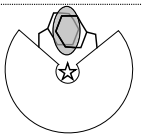
Inhibitor: 1-(1-(4-(7-phenyl-1H-imidazo[4,5-g]quinoxalin-6-yl)benzyl)piperidin-4-yl)-1H-benzo[d]imidazol-2(3H)-one

IC_{50} = 58 nM

Structure:



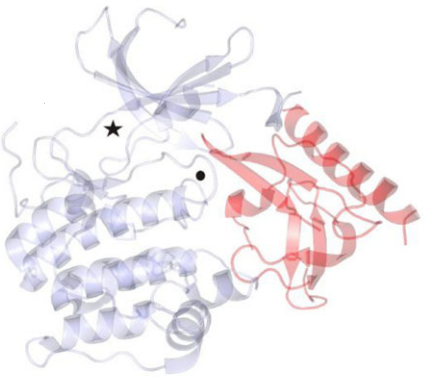
Binding: Gate (gate interface, closed state)



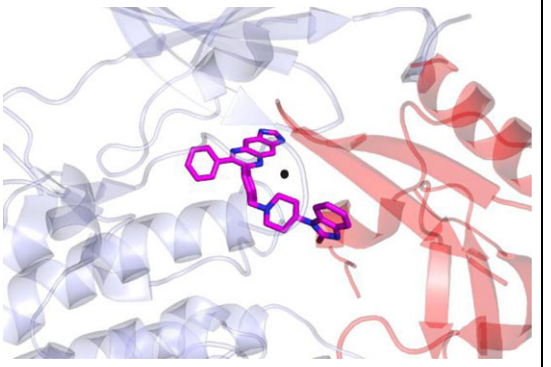
Benefit: Selectivity. Binding at the allosteric site (at the gate interface) blocks the gating mechanism needed for two regulatory steps and enabled the development of selective inhibitors. Most inhibitors that bind at orthosteric sites are not selective

Continued

Table III. Continued



Light blue cartoon: kinase domain; red cartoon: PH domain from *Homo sapiens*; black star: orthosteric site; black dot: allosteric site



Magenta sticks: inhibitor binding at the allosteric site (i.e. the gate interface)

References: 186,207,208

#7	**Enzyme:** Acetylcholinesterase (*AChE*) **E.C.** 3.1.1.7	**PDB ID**: 2XI4

Function: Plays a crucial role at cholinergic synapses in both the central and peripheral nervous systems. AChE terminates the impulse transmission by rapid hydrolysis of the neurotransmitter acetylcholine

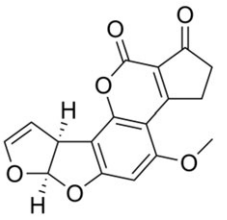
Clinical relevance: Neurological diseases

Structural features: One main access tunnel and one backdoor tunnel with an *aperture* gate

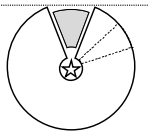
Inhibitor: (6aR,9aS)-4-methoxy-2,3,6a,9a-tetrahydrocyclopenta[c]furo[3',2':4,5]furo[2,3-h]chromene-1,11-dione (*aflatoxin* B_1)

K_i = 28 μM

Structure:



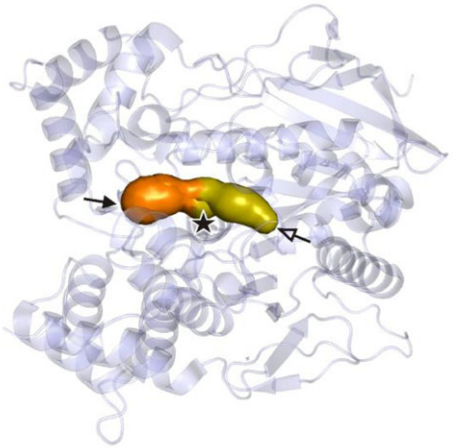
Binding: Mouth of main tunnel



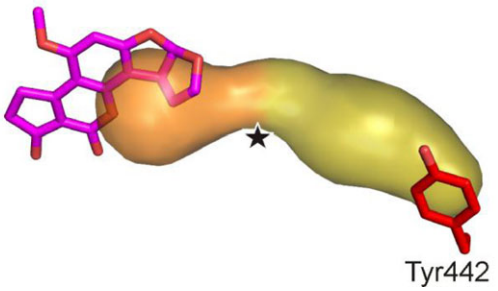
Benefit: Selectivity and an expanded range of binding modes. The inhibitor forms strong interactions at the tunnel mouth, achieving inhibition by blocking substance exchange. Binding at the peripheral anionic site (tunnel mouth) may also provide selectivity for AChE over butyrylcholinesterase

Continued

Table III. Continued



Cartoon: overall structure of AChE from *Torpedo californica*; full black arrow: main tunnel mouth; empty arrow: backdoor tunnel mouth; orange surface: main tunnel; olive surface: backdoor tunnel; black star: catalytic site



Magenta sticks: *aflatoxin B_1* blocking the main tunnel mouth; Tyr442: the *aperture* gate that controls the transit of substrate, products, and solvent. This gated backdoor tunnel explains the enzyme's high efficiency and residual activity when inhibited by tunnel blockers

References: 16,209–211

#8	**Enzyme:** Leukotriene A_4 hydrolase/aminopeptidase (*LTA4H*) **E.C.** 3.3.2.6	**PDB ID**: 4L2L

Function: Catalyzes hydrolysis of leukotriene A_4 (LTA4), an essential step in the formation of the proinflammatory lipid mediator, leukotriene B_4 (LTB4), a chemoattractant during the innate immune response. LTA4H also inactivates the neutrophil chemoattractant tripeptide Pro-Gly-Pro (PGP), which is a biomarker for chronic pulmonary disease.

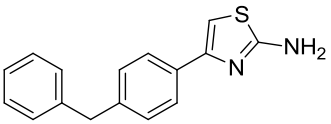
Clinical relevance: LTA4H is a target in acute and chronic inflammatory diseases such as nephritis, arthritis, dermatitis, chronic obstructive pulmonary disease and arteriosclerosis.

Structural features: Contains two access tunnels for the transport of LTA4 and PGP to the active site, respectively

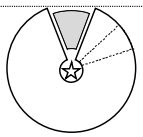
Inhibitor: 4-(4-benzylphenyl)thiazol-2-amine (*ARM1*)

K_i = 2 μM

Structure:



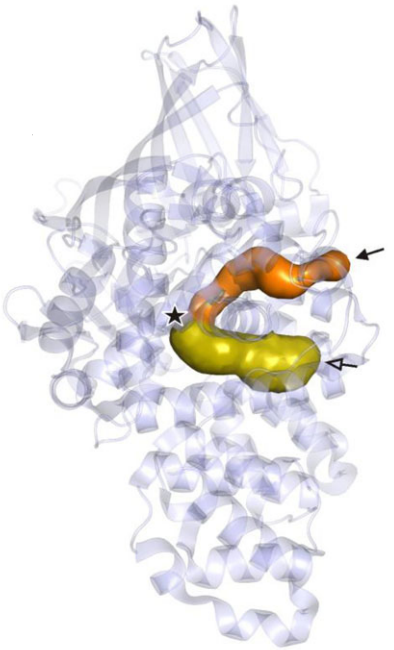
Binding: One main tunnel out of two



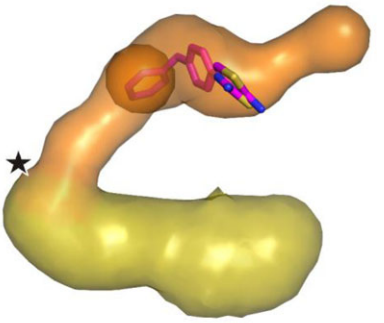
Benefit: Affinity, selectivity, and targeting of a single enzymatic function. Binding to only one tunnel blocks the formation of LTB4 without reducing the enzyme's ability to hydrolyze the undesirable PGP.

Continued

Table III. Continued



Cartoon: overall structure of LTA4H from *Homo sapiens*; black arrow: mouth of the tunnel used for transport of LTA4 (orange surface); empty arrow: mouth of the tunnel used for transport of PGP (olive surface); black star: catalytic site



Magenta sticks: the ARM1 inhibitor blocking the tunnel used for the access of LTA4 substrate

References: 212–214

#9	**Enzyme:** Prolyl endopeptidase (*PREP*) **E.C.** 3.4.21.26	**PDB ID**: 2BKL

Function: Cleaves peptides after proline residues. The human PREP is a cytosolic enzyme involved in the maturation and degradation of peptide hormones and neuropeptides.

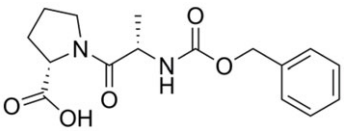
Clinical relevance: Neurological disorders, Chagas disease, cancer, celiac sprue (as therapeutics)

Structural features: *Shell* gate

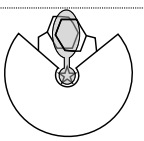
Inhibitor: (2S)-1-[(2S)-2-phenylmethoxycarbonylaminopropanoyl]pyrrolidine-2-carboxylic acid (*Z-Ala prolinal*)

IC_{50} (*Sus scrofa*) **=** 4 nM

Structure:



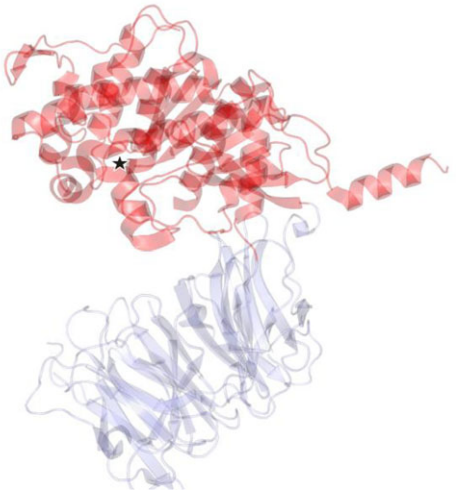
Binding: Catalytic site and gate (gate interface, closed conformation)



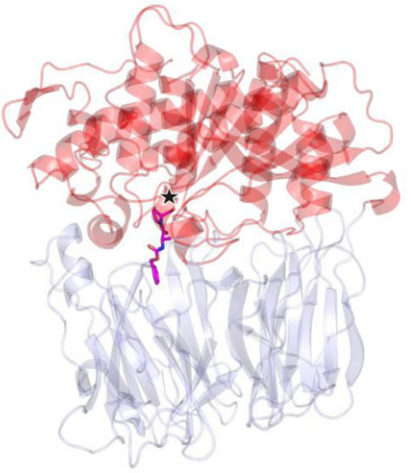
Benefit: Binding affinity. Inhibition of the gating mechanism prevents the substrate from binding to the active site

Continued

Table III. Continued



Unbound enzyme from *Novosphingobium capsulatum* in the open conformation (PDB ID: 1YR2); light blue cartoon: β-barrel domain; red cartoon: catalytic domain; black star: catalytic site



Magenta sticks: Z-Ala prolinal binding at the catalytic site and locking the gate in the closed state from *Myxococcus xanthus*

References: 143,215–218

#10	**Enzyme:** *Human immunodeficiency virus* 1 protease (*HIV-1 protease*) **E.C.** 3.4.23.16	**PDB ID**: 3BC4

Function: HIV protease is a retroviral aspartic endopeptidase that is essential for the life cycle of *human immunodeficiency virus* (HIV) because of its role in cleaving newly synthesized polyproteins into functional units. Without this protease's activity, the HIV virions remain non-infectious

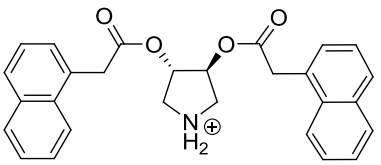
Clinical relevance: HIV protease is one of the prime targets in highly active antiretroviral therapies against the AIDS/HIV epidemic

Structural features: *Double drawbridge* gate

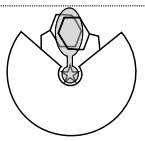
Inhibitor: (3*S*,4*S*)-pyrrolidine-3,4-diyl bis(2-(naphthalen-1-yl)acetate)

K_i = 20 μM

Structure:



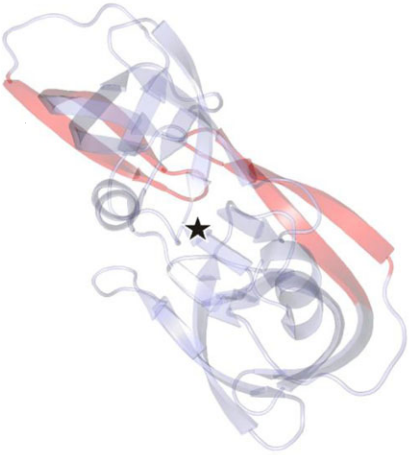
Binding: Catalytic site and gate (gate interface, open conformation)



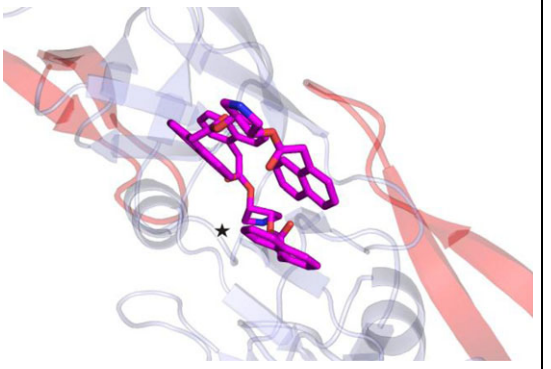
Benefit: Affinity, enlarged space of binding modes. Most HIV protease inhibitors target the active site, but they are also vulnerable to drug resistance. Targeting the open or closed conformations of the enzyme's gate expands the *space of binding modes*, which increases the chances of finding inhibitors that bind to less mutable residues and helps to avoid the problem of *drug resistance*

Continued

Table III. Continued



Unbound enzyme from virus HIV-1 in the closed conformation (PDB ID: 1HVR); blue cartoon: core domain; red cartoon: flexible gating flaps; black star: catalytic site



Magenta sticks: inhibitor bound at the gate interface in the open conformation

References: 219,181,220,221

#11	**Enzyme:** UDP-3-*O*-[3-hydroxymyristoyl] *N*-acetylglucosamine deacetylase (*LpxC*) **E.C.** 3.5.1.33	**PDB ID**: 3NZK

Function: Performs a critical step in the biosynthesis of lipid A, which is a membrane anchor of the outer leaflet of the outer membrane of Gram-negative bacteria. Lipid A is responsible for the bacterial cells' viability and induces toxicity in inflammatory responses

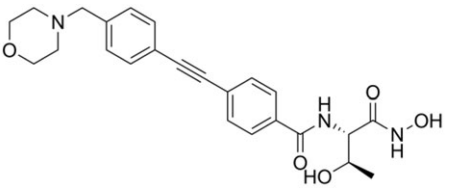
Clinical relevance: Antibiotics

Structural features: One access tunnel formed by a βαβ subdomain which is conserved among many Gram-negative bacteria. The βαβ subdomain contains tunnel-lining residues that are essential for time-dependent inhibition and antibiotic resistance

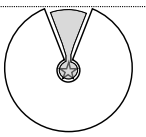
Inhibitor: N-[(2S,3R)-3-hydroxy-1-(hydroxyamino)-1-oxobutan-2-yl]-4-[2-[4-(morpholin-4-ylmethyl)phenyl]ethynyl]benzamide (*CHIR-090*)

K_i = 1 nM

Structure:



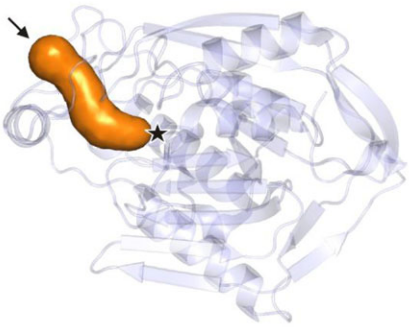
Binding: Catalytic site and tunnel



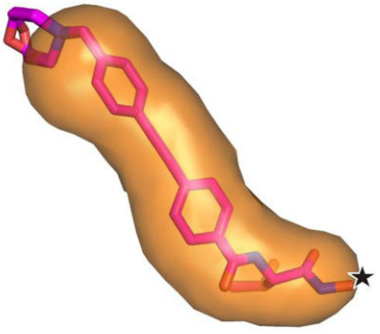
Benefit: Potency, broad spectrum activity, drug resistance. Blocking of the evolutionarily conserved tunnel might affect the viability of Gram-negative bacteria

Continued

Table III. Continued



Cartoon: overall structure of LpxC from *Yersinia enterocolitica*; black arrow: tunnel mouth; orange surface: tunnel; black star: catalytic site



Magenta sticks: CHIR-090 blocking the tunnel and chelating the catalytic zinc ion

References: 222–225

#12	**Enzyme:** *β*-hydroxyacyl-acyl carrier protein dehydratase (*FabZ*) **E.C.** 4.2.1	**PDB ID**: 3D04

Function: Catalyzes the essential step in biosynthesis of both saturated and unsaturated fatty acids in bacteria

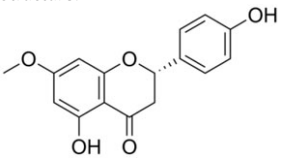
Clinical relevance: Antibiotics

Structural features: L-shaped access tunnel with two *wing* gates located at the entrance and the exit of the tunnel

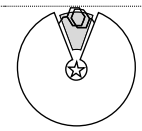
Inhibitor: (2S)-5-hydroxy-2-(4-hydroxyphenyl)-7-methoxy-2,3-dihydrochromen-4-one (*sakuranetin*)

IC_{50} = 2 μM

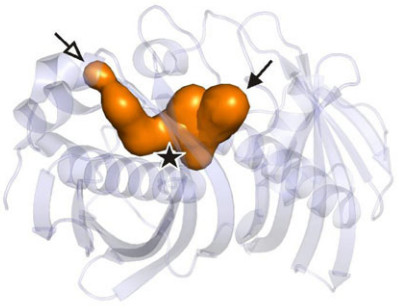
Structure:



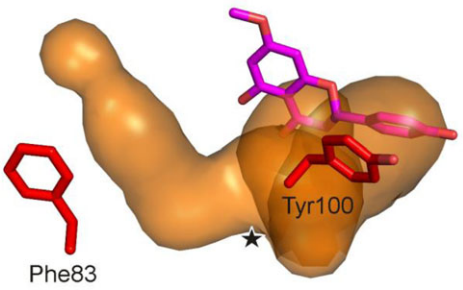
Binding: Tunnel mouth and tunnel gate (door residues, open conformation)



Benefit: Broad-spectrum activity, drug resistance. The structural differences between the fatty acid biosynthesis systems in bacteria and mammals might yield effective inhibitors with against pathogenic microbes



Cartoon: overall structure of FabZ from *Helicobacter pylori*; black arrow: mouth of the main tunnel; empty arrow: mouth of the backdoor tunnel; orange surface: tunnels; black star: catalytic site



Magenta sticks: sakuranetin blocking the tunnel mouth; Phe83 and Tyr100: the *wing* gating residues. The gates prevent the exposure of the active site to the bulk solvent, drive product release and determine the length of the accommodated acyl chains

References: 42,149,226

The table provides basic information about each enzyme and its clinical relevance and structural features, as well as the inhibitor, its activity and binding modes, and the main biological benefits of inhibition by the highlighted means (see the online version for colored images).

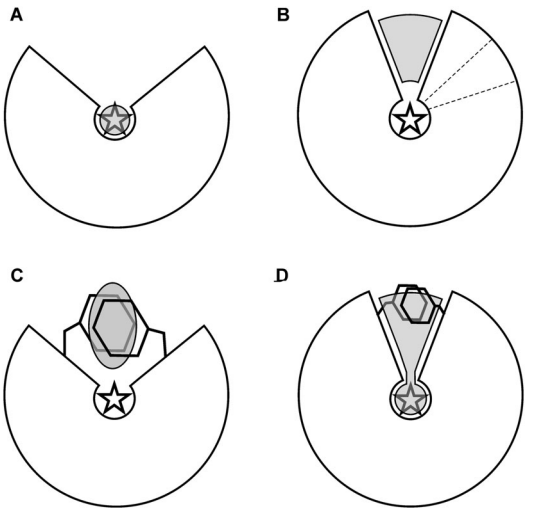


Figure 4. Schematic representation of the binding modes of inhibitors to enzymes containing tunnels or gates. Binding: (A) to the *catalytic site*, (B) to the main *tunnel*, (C) to a *gate*, and (D) *mixed* with all previous three sites. The star represents the catalytic site, the gray geometric objects the inhibitors, and the hexagonal structures the doors of a gate (C and D). A single protein can possess multiple tunnels (B).

(4) Targeting an *enzyme gate* will provide ligands that interact with the gating elements and thus preferentially stabilize (or destabilize) one of the gate's conformations. The most obvious binding sites for such ligands are the door residues. The rational design of such binders requires a thorough knowledge of both open and closed conformations. Ideally, the discovery of any putative gate-binding ligand should be validated by studying the dynamics of the corresponding ligand–protein complexes in order to confirm the relative stability of the gate's conformations. An alternative strategy, which might be more difficult to execute rationally, would be to target the gate's hinge and anchoring residues. This approach is exemplified by the successful identification of allosteric binders that inhibit a cytochrome P450 by rigidifying the whole system.[188] However, the function of hinge and anchoring residues is often complex and difficult to fully understand, and the consequences of the binding of individual ligands cannot be reliably predicted. Nonetheless, tools for identifying allosteric binding sites are under development.[189,190] Importantly, many ion channels and receptors of other kinds have been successfully targeted by developing ligands that bind to orthosteric or allosteric binding sites, and it has been shown that ligand binding to these elements, which define the flexibility of the protein's gates, can either block the channels or modulate their activity.[191–194] We therefore believe that similar approaches could be used to rationally design novel enzyme inhibitors or modulators.

One common downside to all the strategies outlined above relates to the flexibility of the targets and the difficulty of correctly describing their properties. The inherently dynamic nature of proteins implies that a tunnel's geometry can change substantially over time, and a tunnel structure determined by crystallography may be very different from the average ensemble that exists in solution, which may confound attempts at structure-based design. To deal with this problem, it is advisable to investigate the target's conformational space before engaging in extensive design work, using either computational methods such as molecular dynamics simulations, or experimental methods such as analyses of structural ensembles determined by nuclear magnetic resonance spectroscopy. Once the structure of a potential inhibitor has been designed, additional in silico studies on the dynamics of the protein–ligand complex may

provide theoretical validation of such design. However, it should be noted that transient tunnels and molecular gates can be difficult to detect, especially for the elements with slow dynamics.

4. CONCLUSIONS

Our aim in this review was to highlight the potential of enzyme tunnels and gates as targets for drug discovery. We first described the importance of molecular tunnels and gates for the regular functioning of enzymes and gave examples of clinical targets containing these features. A general system for classifying tunnels and gates was presented, and the key structural elements in each case were identified. The possible binding modes of individual ligands targeting enzyme tunnels and gates were then discussed together with the benefits that could be secured by binding in these ways. Finally, four different strategies for the design of new ligands targeting tunnels or gates were outlined. It was emphasized that the most appropriate strategy to use in any given case depends on the information available about the target, and the desired biological effect. In particular, we stressed the desirability of understanding the target's dynamic behavior before attempting to design drugs targeting gates and tunnels because the lack of information about the structures of the open and closed states could substantially reduce the probability of success. Many enzymes are likely to contain transient tunnels and gates with slow dynamics that have not yet been identified, and the analysis of these structures may enable the discovery of new drugs that can address important clinical needs. The different modes of binding to tunnels and ligands were illustrated with twelve complexes of inhibitors bound to tunnels or gates of clinically relevant enzymes. These examples serve as proofs of concept for the drug design strategies outlined in the review and demonstrate their potential to help overcome both new and longstanding challenges in medicinal chemistry.

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