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.

Key words: drug design; protein tunnels; protein gates; drug binding; selectivity; specificity

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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 has recently been proposed as a more realistic alternative to Fischer’s lock-and-key model or Koshland’s induced-fit model 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. 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. 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.
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$^{3}$:

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(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. 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. 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 given tunnel may 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.
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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. 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.

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, catalase, cytochrome P450, glucoseamine-6-phosphate synthase, β-ketoacyl-acyl carrier protein synthase, RNA-dependent RNA polymerase, lipase, acetylcholinesterase, epoxide hydrolase, haloalkane dehalogenase, trypophan synthase, 3-hydroxydecanoyl-acyl carrier protein dehydratase, squalene-hopene cyclase, asparagine synthetase, octaprenyl pyrophosphate synthase, lipase, epoxide hydrolase, haloalkane dehalogenases, squalene-hopene cyclase, and stability (e.g., haloalkane dehalogenases). 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.

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. 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 (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.

D

Triosephosphate isomerase
E.C. 5.3.1.1 (PDB-ID 1TIM, 1TPH)

E

HIV Protease
E.C. 3.4.23.16 (PDB-ID 1HVR, 2PC0)

F

Acylaminoacyl peptidase
E.C. 3.4.19.1 (PDB-ID 3O4G)
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\textsuperscript{165} (Fig. 3D), and HIV protease\textsuperscript{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\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{6}

Many enzymes targeted in pharmaceutical research exhibit gating processes of some kind (Table 1). 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>
<thead>
<tr>
<th>No.</th>
<th>Enzyme</th>
<th>Clinical relevance</th>
<th>Structural features</th>
<th>Binding modes</th>
<th>Benefits</th>
<th>Inhibitory activity</th>
<th>PDB ID</th>
<th>Resolution (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>iNOS</td>
<td>Neurological diseases, inflammation, rheumatoid arthritis, immune-type diabetes, stroke, cancer, thrombosis, infection susceptibilities</td>
<td>Tunnel and wing gate</td>
<td>Catalytic site, tunnel and gate (open state)</td>
<td>Selectivity</td>
<td>IC₅₀ = 0.4 μM</td>
<td>3EBF</td>
<td>2.29</td>
</tr>
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<td>2</td>
<td>CYP51</td>
<td>Antiparasitic</td>
<td>Several tunnels</td>
<td>Catalytic site and substrate tunnel</td>
<td>Selectivity, potency, broad-spectrum activity</td>
<td>Kₐ = 73 nM</td>
<td>3K1O</td>
<td>2.89</td>
</tr>
<tr>
<td>3</td>
<td>CYP17A1</td>
<td>Breast and prostate cancer</td>
<td>Several tunnels</td>
<td>Catalytic site and substrate tunnel</td>
<td>Potency, selectivity</td>
<td>IC₅₀ = 3 nM</td>
<td>3RUK</td>
<td>2.6</td>
</tr>
<tr>
<td>4</td>
<td>DHODH</td>
<td>Autoimmune or parasitic diseases, cancer, immuno-suppression</td>
<td>Tunnel</td>
<td>Tunnel</td>
<td>Selectivity, broad-spectrum activity</td>
<td>Kᵢ = 8 nM</td>
<td>1D3H</td>
<td>1.8</td>
</tr>
<tr>
<td>5</td>
<td>PTC</td>
<td>Antibiotics</td>
<td>Tunnel</td>
<td>Tunnel</td>
<td>Affinity, broad-spectrum, enlarged space of binding modes</td>
<td>Kᵢ = 0.7 μM</td>
<td>1NJ1</td>
<td>3.0</td>
</tr>
<tr>
<td>6</td>
<td>AKT1</td>
<td>Cellular metabolism, proliferation, survival, growth, angiogenesis</td>
<td>Shell gate</td>
<td>Gate (closed state)</td>
<td>Selectivity</td>
<td>IC₅₀ = 58 nM</td>
<td>3O96</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Continued
Table II. Continued

<table>
<thead>
<tr>
<th>No.</th>
<th>Enzyme</th>
<th>Clinical relevance</th>
<th>Structural features</th>
<th>Binding modes</th>
<th>Benefits</th>
<th>Inhibitory activity</th>
<th>PDB ID</th>
<th>Resolution (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>Acetylcholinesterase (<em>AChE</em>)</td>
<td>Neurological diseases</td>
<td>Two tunnels and gate</td>
<td>Main tunnel</td>
<td>Selectivity, wider space of binding modes</td>
<td>$K_i = 28 \mu M$</td>
<td>2XI4</td>
<td>2.3</td>
</tr>
<tr>
<td>8</td>
<td>Leukotriene A$_4$-hydrolase/aminopeptidase</td>
<td>Inflammatory diseases</td>
<td>2 Tunnels</td>
<td>Main tunnel</td>
<td>Affinity, selectivity, target one enzymatic function</td>
<td>$K_i = 2 \mu M$</td>
<td>4L2L</td>
<td>1.65</td>
</tr>
<tr>
<td>9</td>
<td>Prolyl endopeptidase (PREP)</td>
<td>Neurological disorders, Chagas disease, cancer</td>
<td><em>Shell</em> gate</td>
<td>Catalytic site and gate (closed state)</td>
<td>Binding affinity</td>
<td>IC$_{50} = 1.0 \mu M$</td>
<td>2BKL</td>
<td>1.5</td>
</tr>
<tr>
<td>10</td>
<td><em>Human immunodeficiency virus</em> I protease</td>
<td>Antivirals</td>
<td><em>Double drawbridge</em> gate</td>
<td>Catalytic site and gate (open state)</td>
<td>Affinity, enlarged space of binding modes</td>
<td>$K_i = 20 \mu M$</td>
<td>3BC4</td>
<td>1.82</td>
</tr>
<tr>
<td>11</td>
<td>UDP-3-O-[3-hydroxymyristoyl]N-acetylglucosamine deacetylase (LpxC)</td>
<td>Antibiotics</td>
<td>Tunnel</td>
<td>Catalytic site and tunnel</td>
<td>Potency, broad-spectrum activity, drug resistance</td>
<td>$K_i = 1 \text{nM}$</td>
<td>3NZK</td>
<td>1.8</td>
</tr>
<tr>
<td>12</td>
<td>β-Hydroxyacyl-acyl carrier protein dehydratase (FabZ)</td>
<td>Antibiotics</td>
<td>Tunnel and <em>wing</em> gate</td>
<td>Tunnel and gate (open state)</td>
<td>Broad-spectrum activity, drug resistance</td>
<td>$K_i = 1 \mu M$</td>
<td>3D04</td>
<td>2.4</td>
</tr>
</tbody>
</table>

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. 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\textsubscript{50} than in the case of AKT2 and AKT1 isozymes.

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\textsuperscript{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>
<thead>
<tr>
<th>#1</th>
<th>Enzyme: Inducible nitric oxide synthase (iNOS)</th>
<th>PDB ID: 3EBF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Function:</strong> 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.</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Clinical relevance:</strong> Neurological diseases, inflammation, rheumatoid arthritis, immune-type diabetes, stroke, cancer, thrombosis, infection susceptibility</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Structural features:</strong> One access tunnel with a wing gate</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Inhibitor:</strong> (3R)-3-(1,2,3,4-tetrahydroisoquinolin-7-yloxymethyl)-2,3-dihydrothieno[2,3-f][1,4]oxazepin-5-amine</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>IC$_{50}$ (Homo sapiens) = 0.4 μM (iNOS); 50 μM (eNOS); 1 μM (nNOS)</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Structure:</strong> Binding: Catalytic site, tunnel and tunnel gate (open state)</td>
<td>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></td>
<td>Cartoon: overall structure of iNOS from <em>Mus musculus</em>; black arrow: tunnel mouth; orange surface: tunnel; black star: catalytic site</td>
<td>Magenta sticks: the inhibitor blocking the tunnel and interacting with the tunnel gate (Gln257) and heme cofactor</td>
</tr>
<tr>
<td></td>
<td>References: 185, 195–197</td>
<td></td>
</tr>
</tbody>
</table>
### Table III. Continued

<table>
<thead>
<tr>
<th>#2</th>
<th>Enzyme: Sterol 14α-demethylase (CYP51)</th>
<th>PDB ID: 3K1O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Function: Catalyzes an essential step in the biosynthesis of ergosterol, which is required for membrane construction, growth, development and division in parasites such as <em>Trypanosoma cruzi</em>, <em>Trypanosoma brucei</em> or <em>Leishmania sp.</em></td>
<td>Inhibitor: 4-[4-[4-[4-[[[25,55]-5-(2,4-difluorophenyl)-5-(1,2,4-triazol-1-ylmethyl)oxolan-2-yl]methoxy]phenyl]piperezin-1-yl]phenyl]-2-[[25,35]-2-hydroxypentan-3-yl]-1,2,4-triazol-3-one (posaconazole)</td>
<td></td>
</tr>
<tr>
<td>Clinical relevance: Antiparasitic</td>
<td>$K_I$ (<em>Neosartorya fumigata</em>) = 73 nM</td>
<td></td>
</tr>
<tr>
<td>Structural features: Several access tunnels</td>
<td>Structure:</td>
<td></td>
</tr>
<tr>
<td><strong>Binding:</strong> Catalytic site and tunnel</td>
<td><strong>Benefit:</strong> 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</td>
<td></td>
</tr>
</tbody>
</table>

---

<table>
<thead>
<tr>
<th>#3</th>
<th>Enzyme: Cytochrome P450 17A1 (CYP17A1)</th>
<th>PDB ID: 3RUK</th>
</tr>
</thead>
<tbody>
<tr>
<td>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.</td>
<td>Inhibitor: (3S,8R,9S,10R,13S,14S)-10,13-dimethyl-17-pyridin-3-yl-2,3,4,7,8,9,11,12,14,15-decaydro-1H-cyclopenta[a]phenanthren-3-ol (abiraterone)</td>
<td></td>
</tr>
<tr>
<td>Clinical relevance: Breast and prostate cancer</td>
<td>$IC_{50}$ = 3 nM</td>
<td></td>
</tr>
<tr>
<td>Structural features: One main access tunnel and many adjacent solvent tunnels</td>
<td>Structure:</td>
<td></td>
</tr>
</tbody>
</table>

---

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
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: Dihydrorotate dehydrogenase (DHODH) | PDB ID: 1D3H |
--- | --- | --- |
E.C. 1.3.5.2 |  | |

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:
**Table III.** Continued

<table>
<thead>
<tr>
<th>Binding: Tunnel</th>
<th>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 <em>Plasmodium falciparum</em> or <em>Helicobacter pylori</em>) differ markedly</th>
</tr>
</thead>
</table>

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

<table>
<thead>
<tr>
<th>#5</th>
<th>Enzyme: Peptidyl transferase center (PTC)</th>
<th>PDB ID: 1NJI</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.C. 2.3.2.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Function:</strong> 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</td>
<td><strong>Inhibitor:</strong> 2,2-dichloro-N-(1R,2R)-1,3-dihydroxy-1-(4-nitrophenyl)propan-2-yl)acetamide (<em>chloramphenicol</em>)</td>
<td></td>
</tr>
<tr>
<td><strong>Clinical relevance:</strong> Antibiotics</td>
<td><strong>$K_i = 0.7 , \mu M$</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Structural features:</strong> One tunnel, necessary for the product release</td>
<td><strong>Structure:</strong></td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>#6</th>
<th>Enzyme: Serine/threonine protein kinase AKT1 (AKT1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E.C. 2.7.11.1</td>
</tr>
<tr>
<td></td>
<td>PDB ID: 3096</td>
</tr>
</tbody>
</table>

**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:**

![Inhibitor structure](image)

**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

References: 100,101,205,206

---

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.
### Table III. Continued

<table>
<thead>
<tr>
<th>#7</th>
<th>Enzyme: Acetylcholinesterase (AChE) E.C. 3.1.1.7</th>
<th>PDB ID: 2X14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Function:</td>
<td>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</td>
<td></td>
</tr>
<tr>
<td>Clinical relevance:</td>
<td>Neurological diseases</td>
<td></td>
</tr>
<tr>
<td>Structural features:</td>
<td>One main access tunnel and one backdoor tunnel with an aperture gate</td>
<td></td>
</tr>
<tr>
<td>Inhibitor:</td>
<td>(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&lt;sub&gt;1&lt;/sub&gt;)</td>
<td></td>
</tr>
<tr>
<td>$K_i = 28 \mu M$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Structure:</td>
<td><img src="image" alt="Structure of AChE inhibitor" /></td>
<td></td>
</tr>
</tbody>
</table>

**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.

References: 186, 207, 208
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 B₁* 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

<table>
<thead>
<tr>
<th>References</th>
<th>16,209–211</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme:</td>
<td>Leukotriene A₄ hydrolase/aminopeptidase (<em>LTA4H</em>)</td>
</tr>
<tr>
<td>E.C.</td>
<td>3.3.2.6</td>
</tr>
<tr>
<td>PDB ID:</td>
<td>4L2L</td>
</tr>
<tr>
<td>Function:</td>
<td>Catalyzes hydrolysis of leukotriene A₄ (<em>LTA4</em>), an essential step in the formation of the proinflammatory lipid mediator, leukotriene B₄ (<em>LTB4</em>), a chemoattractant during the innate immune response. LTA4H also inactivates the neutrophil chemoattractant tripeptide <em>Pro-Gly-Pro</em> (<em>PGP</em>), which is a biomarker for chronic pulmonary disease.</td>
</tr>
<tr>
<td>Clinical relevance:</td>
<td>LTA4H is a target in acute and chronic inflammatory diseases such as nephritis, arthritis, dermatitis, chronic obstructive pulmonary disease and arteriosclerosis.</td>
</tr>
<tr>
<td>Structural features:</td>
<td>Contains two access tunnels for the transport of LTA4 and PGP to the active site, respectively</td>
</tr>
<tr>
<td>Binding:</td>
<td>One main tunnel out of two</td>
</tr>
<tr>
<td>Benefit:</td>
<td>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.</td>
</tr>
</tbody>
</table>
Table III. Continued

<table>
<thead>
<tr>
<th>#9</th>
<th>Enzyme: Prolyl endopeptidase (PREP)</th>
<th>PDB ID: 2BKL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Function:</strong></td>
<td>Cleaves peptides after proline residues. The human PREP is a cytosolic enzyme involved in the maturation and degradation of peptide hormones and neuropeptides.</td>
<td><strong>Inhibitor:</strong> (2S)-1-[(2S)-2-phenylmethoxy carbonylamino propanoyl]pyrrolidine-2-carboxylic acid (Z-Ala prolinal)</td>
</tr>
<tr>
<td><strong>Clinical relevance:</strong></td>
<td>Neurological disorders, Chagas disease, cancer, celiac sprue (as therapeutics)</td>
<td><strong>IC$_{50}$ (Sus scrofa) = 4 nM</strong></td>
</tr>
<tr>
<td><strong>Structural features:</strong></td>
<td>Shell gate</td>
<td><strong>Structure:</strong></td>
</tr>
<tr>
<td><strong>Binding:</strong></td>
<td>Catalytic site and gate (gate interface, closed conformation)</td>
<td><strong>Benefit:</strong> Binding affinity. Inhibition of the gating mechanism prevents the substrate from binding to the active site</td>
</tr>
</tbody>
</table>

References: 212–214

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
### Table III. Continued

<table>
<thead>
<tr>
<th>#10</th>
<th>Enzyme: Human immunodeficiency virus 1 protease (HIV-1 protease)</th>
<th>PDB ID: 3BC4</th>
</tr>
</thead>
</table>

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

\[ K_i = 20 \mu M \]

**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.

---

**Unbound enzyme from Novosphingobium capsulatum** in the open conformation (PDB ID: 1YR2); light blue cartoon: \( \beta \)-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

---

**Table III.**

Unbound enzyme from *Novosphingobium capsulatum* in the open conformation (PDB ID: 1YR2); light blue cartoon: \( \beta \)-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*
### Table III. Continued

<table>
<thead>
<tr>
<th>#11</th>
<th>Enzyme: UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase (LpxC)</th>
<th>PDB ID: 3NZK</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>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</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Clinical relevance: Antibiotics</td>
<td></td>
</tr>
<tr>
<td></td>
<td>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</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inhibitor: N-[[25,3R]-3-hydroxy-1-(hydroxym amino)-1-oxobutan-2-yl]-4-[2-[4-(morpholin-4-ylmethyl)phenyl]ethynyl]benzamide (CHIR-090)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$K_i = 1, \text{nM}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Structure:</td>
<td></td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="Inhibitor Structure" /></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Benefit: Potency, broad spectrum activity, drug resistance. Blocking of the evolutionarily conserved tunnel might affect the viability of Gram-negative bacteria</td>
<td></td>
</tr>
</tbody>
</table>

References: 219,181,220,221

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
Table III. Continued

<table>
<thead>
<tr>
<th>#12</th>
<th>Enzyme: 6-hydroxyacyl-acyl carrier protein dehydratase (FabZ)</th>
<th>PDB ID: 3D04</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Function:</strong></td>
<td>Catalyzes the essential step in biosynthesis of both saturated and unsaturated fatty acids in bacteria</td>
<td></td>
</tr>
<tr>
<td><strong>Clinical relevance:</strong></td>
<td>Antibiotics</td>
<td></td>
</tr>
<tr>
<td><strong>Structural features:</strong></td>
<td>L-shaped access tunnel with two wing gates located at the entrance and the exit of the tunnel</td>
<td></td>
</tr>
<tr>
<td><strong>Inhibitor:</strong></td>
<td><em>(2S)-5-hydroxy-2-(4-hydroxyphenyl)-7-methoxy-2,3-dihydrochromen-4-one (sakuranetin)</em></td>
<td></td>
</tr>
<tr>
<td><strong>IC&lt;sub&gt;50&lt;/sub&gt;</strong></td>
<td>2 μM</td>
<td></td>
</tr>
<tr>
<td><strong>Structure:</strong></td>
<td><img src="image" alt="Structure" /></td>
<td></td>
</tr>
<tr>
<td><strong>Benefit:</strong></td>
<td>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</td>
<td></td>
</tr>
</tbody>
</table>

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).
(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. 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. 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. 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

**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).
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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TUNNELS AND GATES IN DRUG DESIGN


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**Lukas Daniel** received his Ph.D. in Biology from the Masaryk University, Czech Republic, in 2016. He has been a member of the Loschmidt laboratories since 2009, where he has been responsible for independent design of computational experiments, analysis of results, and transfer of outputs to other researchers. Lukas has been involved in collaborative projects with academic and industrial partners aiming at virtual screening of enzymes involved in pathogenesis, protein engineering, and molecular modeling of enzyme's substrate specificity. The outputs of his research yielded into novel patentable pharmaceuticals, molecular probes, fluorescent substrates, and the bioinformatic tool CAVER Analyst 1.0.

**Tomas Buryska** received his M.Sc. degree in Biochemistry in 2013 and since then he has been a Ph.D. student of Microbiology at Masaryk University. He gained substantial part of his knowledge on a 6-month stay at the University of Cambridge in United Kingdom and during shorter stays at the University of Southampton. Tomas has been working in the Loschmidt laboratories at Masaryk University since 2009. His specialization lies in the high-throughput methods, microfluidics, protein engineering, and enzyme kinetics. He gained experience in projects focused on identification of novel enzyme substrates and inhibitors identified by in silico methods. Since 2011, he focuses on the application of microfluidics in life sciences.

**Zbynek Prokop** works as an Associate Professor in the Loschmidt Laboratories at Masaryk University, Czech Republic, were he leads research team engaged in study of fundamental principles of enzyme mechanism and kinetics. He obtained Ph.D. degree in environmental chemistry from Masaryk University and extended his expertise during his research stays at University of Cambridge, ETH Zurich, University of Groningen, and University of Ghent in Belgium. Among the awards and distinctions, he received Werner von Siemens Award for Excellence in Innovation and Alfred Bader Prize for Bioorganic Chemistry. He is the co-author of 52 publications and 3 international patents. He is a co-founder of the first biotechnology spin-off from Masaryk University Enantis Ltd.

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**Jiri Damborsky** is Josef Loschmidt Chair Professor of Chemistry and Professor of Biochemistry at the Faculty of Science at Masaryk University, Czech Republic. Research in his group is focused on protein and metabolic engineering. The group develops new concepts and software tools for protein engineering (CAVER, HOTSPOT WIZARD, PREDICTSNP) and uses them for the rational design of enzymes. He has published >170 original articles, 14 book chapters, and 6 international patents. He is the holder of the EMBO/HHMI Scientist award of the European Molecular Biology Organization and the Howard Hughes Medical Institute. He is a co-founder of the first biotechnology spin-off from Masaryk University, Enantis Ltd.