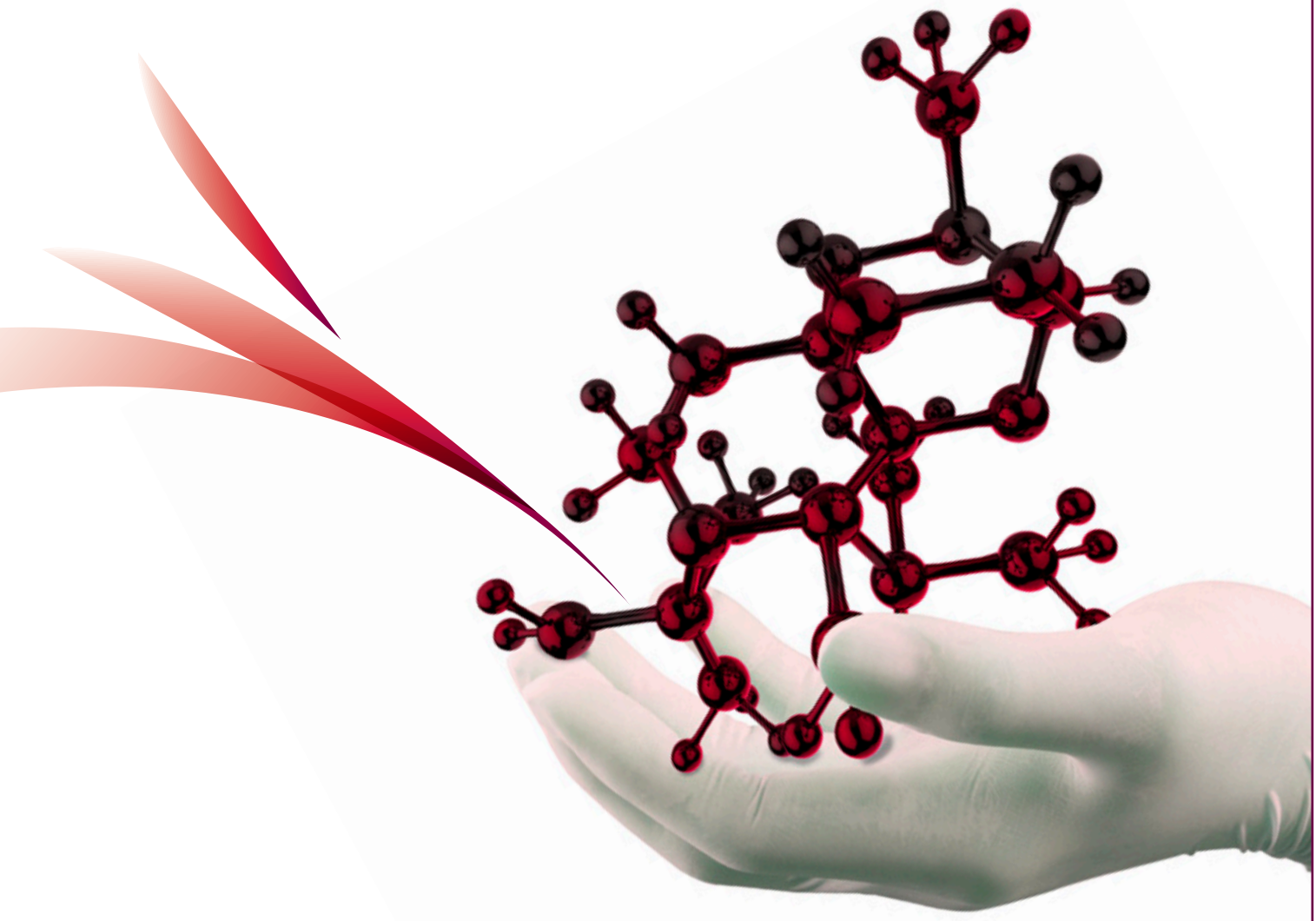
1st Edition

An Introduction to Inhibitors and Their Biological Applications



Merck Millipore is a division of MERCK

This is an interactive PDF document with clickable links.

Jump to the sections of your choice from either the main or chapter section listings.

Click on catalogue numbers and other resources to go directly to the web for additional information.

Click on at the bottom of any page to return to the main Table of Contents.

By:
Chandra Mohan, Ph.D.
Kevin D. Long, Ph.D.
Manpreet Mutneja, Ph.D.

Acknowledgements:
We are grateful to Dr. Chandreyee Das and Mary Ann Ford, Marketing Communications for critically reading and reviewing the manuscript and for their valuable suggestions to enhance its value to the readers. Our special thanks to Liza Benson, Design for her painstaking work in organizing and perfecting its presentation.

EMD Millipore

An Introduction to Inhibitors and Their Biological Applications

A word to our customers...

Intracellular and extracellular physiological cascades are regulated by activation and inhibition of various enzymes involved in these pathways. Investigating and understanding the mechanism of enzyme inhibition has become the basis of development of pharmaceutical agents. Biologically active natural and synthetic inhibitors have been developed and special emphasis has been placed on investigations that define their structure-function relationships in an effort to understand the origin of their biological properties. A powerful complement to the assessment of these agents is the preparation and subsequent examination of key partial structures, deep-seated structural modifications and the corresponding unnatural enantiomers of natural products.

Although activation of enzymes has been exploited therapeutically, most effects are produced by enzyme inhibition that normalizes an overactive pathway. Years of research have shown that inhibitors are useful for mechanistic studies—they reveal how enzymes interact with their substrates, what role inhibitors play in enzyme regulation, and, based on structure-activity relationships, how to develop drugs that inhibit aberrant biochemical reactions.

We are pleased to present you our first edition of "An Introduction to Inhibitors and Their Biological Applications," a basic guide for use of inhibitors in biological research that will serve as a reference guide in your laboratory. This practical resource is a part of our continuing commitment to provide useful product information and exceptional service to you, our customers. If you have used Merck Millipore (Calbiochem®, Novagen®, Chemicon®, Upstate®) products in the past, we thank you for your support and confidence in our products, and if you are just beginning your research career, please call us and give us an opportunity to demonstrate our exceptional service to you.

Table of Contents

Enzyme Inhibitors

1

1.1 Why are enzyme inhibitors important?

Although enzymes are absolutely essential for life, abnormally high enzyme activity can lead to disease conditions. Hence, overactive enzymes are attractive targets for development of inhibitor molecules to alleviate disease conditions. Manipulation of enzyme catalysis with inhibitors is critical for prevention of infectious diseases, intervention in cell cycle and cell growth, treatment of hypertension, control of inflammatory response and more. Besides acting as therapeutic agents, inhibitors also play important roles in biological and clinical research.

- Inhibitors serve as major control mechanisms in biological systems
- They can regulate metabolic activities
- They either block or slow down the rate of biochemical reactions
- A number of therapeutically important drugs act by inhibiting specific enzymes
- Reversible inhibitors can be used during enzyme purification
- Covalent inhibitors are usually used to identify active site amino acids
- Immobilized and fluorochrome-tagged inhibitors can be used to localize and identify intracellular sites of enzymes
- Many inhibitors are potent poisons
- Some inhibitors are used in pesticides and herbicides

1.2 Why use small molecules to study biological processes?

Chemical biology has been used successfully in both gain-of-function and loss-of-function approaches to study a variety of biological processes. For example, in chemical genetics, either small organic molecules or peptides are used to activate or inhibit specific proteins/enzymes involved in specific signaling pathways. This allows researchers to analyze the phenotype when a specific cellular protein is induced or suppressed. Small molecules offer a powerful approach to temporally and spatially modulate individual proteins and processes that can control biological phenotypes. These molecules have also helped to advance our understanding of the biological pathways that influence stem cell fate. They are used to modulate self-renewal, survival, direct reprogramming, and differentiation of pluripotent stem cells. Further, these molecules have helped to identify signaling molecules that define and maintain the extensive intracellular communication networks that control cell growth, differentiation, and metabolism.

Compared to other approaches, such as overexpressing genes from plasmid DNA or genetic knockdowns, chemical genetics is technically simple to perform in cell culture and requires fewer resources and less time. Compared to the RNAi approach, small molecules provide the advantage of acting quickly and directly on the protein target, and the effects can often be reversed rapidly by simply washing (Figure 1). The fast action of most small molecules also makes them ideal for live imaging. In addition, the use of small molecules can provide dose response information.

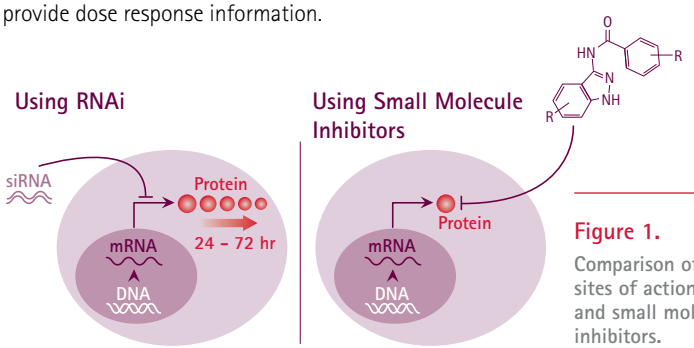


Figure 1.
Comparison of the sites of action of RNAi and small molecule inhibitors.

Some advantages of small molecule inhibitors over RNAi

RNAi

- Challenge with primary cells and difficult-to-transfect cells
- Challenge with mRNAs encoding proteins with long half-life
- Challenge with mRNAs with strong secondary structure and difficult to knock down
- Gradual protein depletion; slowly reversible
- Difficult to determine dose response during gradual depletion

Small Molecule Inhibitors

- Frequently cell-permeable
- No need for transfection agents
- Small molecules act by inhibiting or inactivating specific proteins
- Fast, often inhibitor-based drugs are reversible inhibitors
- Dose response is informative
- Dose can be easily manipulated

1.3 The active site

Enzymes are specific in the reactions that they catalyze: each enzyme usually acts on a single substrate or pair of substrates (in the case of bimolecular reactions). However, some enzymes may act on closely related substrate molecules, often with different efficiencies. Enzymes possess a small region (typically only about 20 amino acids), known as the active site, where the reaction occurs. Enzymes catalyze biological reactions by binding the substrate(s) to the active site. This binding changes the distribution of electrons in the chemical bonds of the substrate(s), lowering the activation energy of the reaction and enabling generation of the final product. This product is released from the active site, allowing the enzyme to regenerate itself for another reaction cycle. The substrate can interact with the active site through ionic interactions, hydrogen bonding, hydrophobic nonpolar interactions, and coordinate covalent bonding to a metal ion activator.

The active site organization is complementary to that of the substrate molecule, which allows only one substrate to bind to the site. However, in a few cases, similar molecules can also act as substrates, but with less than optimal reaction kinetics. This is popularly known as the lock-and-key model. However, not all reactions can be explained based on lock and key model. Hence, an induced fit theory was proposed, which takes into account that substrates also play a role in determining the final shape of the enzyme, which means enzyme shape can be flexible (Figure 2). In the induced fit model of enzyme-substrate binding, the shape of the active site of the unbound enzyme is not the exact complement of the shape of the substrate.

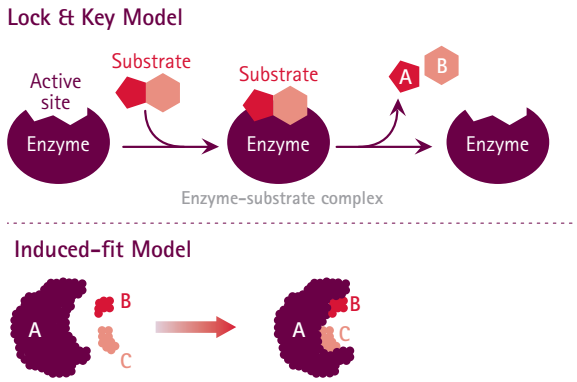


Figure 2.
Lock–and-key and induced fit models of substrate binding

Based on the induced fit theory, one can explain the phenomenon where certain compounds can bind to the enzyme, but fail to react rapidly because the enzyme structure has been distorted or the molecule is too small to induce a proper alignment. However, the enzyme does bind to the substrate, and, after binding, a conformational change occurs in the active site that results in a new shape, which is complementary to the shape of the substrate. Since almost all enzymes are proteins, the nature of amino acid side chains in the vicinity of the active site is also very important.

Enzymes may have varying degrees of specificity for multiple substrates. Some enzymes have absolute specificity for only one substrate; others may react with substrates with similar functional groups, side chains, or positions on a chain. The least specific enzymes catalyze a reaction at a particular chemical bond regardless of any other structural features.

1.4 Some useful terms

K_m: It is the substrate concentration, [S] at which half maximum velocity of reaction is observed under given set of conditions (Figure 3). It is an inverse measure of the binding strength between substrate and the enzyme where lower K_m means higher affinity and lower the concentration of substrate required to achieve maximum reaction rate. K_m values are dependent upon pH, temperature, and other reaction conditions.

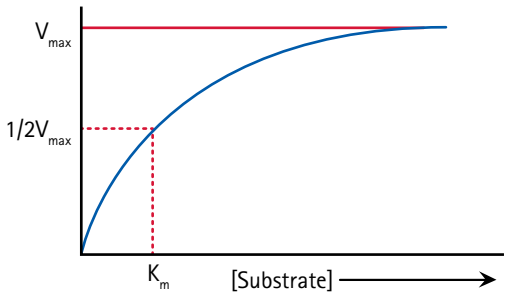


Figure 3.
Relationship between substrate concentration and maximum velocity of reaction.

V_{max}: It is the maximum velocity of reaction under given conditions (Figure 3). V_{max} is reached when all enzyme sites are saturated with the substrate. This will happen when substrate concentration [S] is greater than K_m so that $[S]/([S]+K_m)$ approaches 1.

EC_{50}: Clinical efficacy of a drug, reported as the drug concentration required to produce 50% of the maximum effect (may be inhibitory or stimulatory effect). The inhibitory response is halfway between the baseline and maximum after exposure to inhibitory molecule for a selected period of time. This term is used usually with pharmaceuticals.

IC_{50}: Concentration required to produce 50% inhibition (Figure 4). The amount of inhibitor required depends on various factors, such as substrate concentration, target accessibility, cell permeability, duration of incubation, type of cells used, etc. It is best to survey the literature to determine the initial concentration. If published K_i or IC_{50} values are known, one should use 5 to 10 times higher than these values to maximally inhibit enzyme activity. If K_i or IC_{50} values are unknown, then one should try a wide range of inhibitor concentrations and use Michaelis-Menten kinetics (see below) to determine the K_i value. It is not unusual to see either no inhibition or even a reverse effect when high concentrations of inhibitors are used (please see Prozone effect, section 1.9).

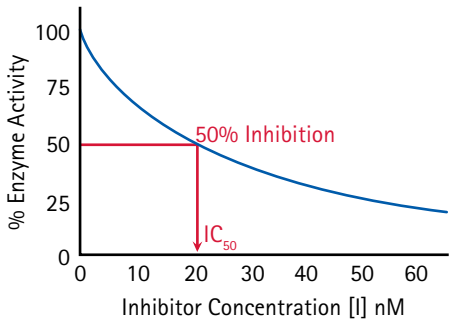


Figure 4.
Relationship between inhibitor concentration and IC_{50}.

K_i (Inhibition constant): Inhibitor concentration at which 50% inhibition is observed. Cheng and Prusoff (1973)* developed an equation that simplifies the calculation of K_i when IC_{50} is known:

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_m}}$$

*Cheng Y. and Prusoff W. H. (1973). Biochem. Pharmacol. 22: 3099-3108.

where [S] is the concentration of substrate, and K_m is the substrate concentration (in the absence of inhibitor) at which the velocity of the reaction is half-maximal. The K_i of an inhibitor for inhibition of a particular substrate (fixed K_m) is constant. For a different substrate, K_m is different, and so is the K_i.

ED_{50} refers to the median effective dose (as opposed to concentration) at which 50% of individuals exhibit the specified quantal effect. It is a measure of reasonable expectance of a drug effect, but not necessarily equal to the prescribed dose.

TAKE NOTE:

- IC_{50} is the functional strength of the inhibitor and is not an indicator of its affinity.
- IC_{50} value for a compound may vary between experiments, depending on experimental conditions.
- K_i reflects the binding affinity of the inhibitor. It is an absolute value.

Watch Out

A lower IC_{50} generally means a more potent inhibitor, which also could mean higher toxicity at lower doses.

1.5 The significance of K_m and V_{max}

The Michaelis-Menten constant, K_m, defined as the substrate concentration at which half maximum velocity is observed, varies considerably from enzyme to enzyme and also varies with different substrates for the same enzyme. When the substrate concentration is equal to the K_m value, half of the enzyme's active sites are occupied by substrate molecules.

- K_m is a constant with units M
- K_m is a constant derived from rate constants
- K_m is, under true Michaelis-Menten conditions, an estimate of the dissociation constant of enzyme and substrate.
- Small K_m means tight binding; high K_m means weak binding

In the Michaelis-Menten model for enzyme kinetics, it is assumed that enzyme first reacts with substrate to form an enzyme-substrate complex that breaks down to form product and free enzyme. Hence, K_m can be expressed in terms of three rate constants (k_1, k_{-1}, and k_2)

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

$$K_m = \frac{k_{-1} + k_2}{k_1}$$

K_m also depends on temperature, nature of substrate, pH of the reaction medium, ionic strength, and any other reaction condition. Hence, it is important to characterize enzyme-substrate reactions under specifically defined conditions. Any variation in K_m value indicates the presence of either an activator or an inhibitor in the reaction medium.

At very low substrate concentrations, the initial velocity of the reaction is proportional to the substrate concentration [S] and the reaction is of first order with respect to substrate. As [S] is increased, the initial rate of reaction declines and is not proportional to [S]. Under these conditions, the reaction is of mixed order. And, with further increase in [S], reaction rate becomes independent of [S] and asymptotically approaches a constant rate. At this point, the reaction is of zero order and enzyme is considered to be saturated with substrate. The time course of the formation of enzyme-substrate complex and product can be represented as shown in Figure 5.

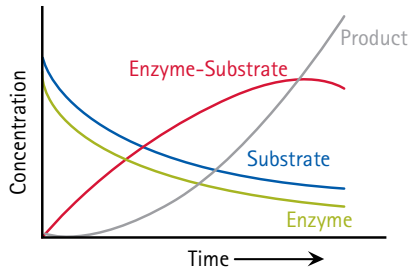


Figure 5.
Temporal dependence of relative concentrations of enzyme reaction components.

V_{max}, the maximum rate of reaction, is the rate at which the total enzyme concentration is present as the enzyme–substrate complex. V_{max} represents the maximum achievable rate of reaction under given conditions.

- V_{max} is a constant with units s^{-1}
- V_{max} is the theoretical maximal rate of the reaction; in reality, it is never achieved.
- Reaching V_{max} requires that all enzyme molecules are bound to substrate
- V_{max} is asymptotically approached as [S] is increased

If the initial enzyme concentration is known, then the value of k_2 can be determined from V_{max}. Since k_2 is first order rate constant, it is expressed per unit time (per minute or per second). It is also known as the turnover number or the catalytic constant, k_{cat}. The turnover number is the number of substrate molecules that can be converted to product in a given period of time under conditions where enzyme is completely saturated with substrate.

It is easy to estimate the turnover number by measuring the reaction rate under saturating substrate conditions (where [S] is greater than K_m). Generally, under physiological conditions, $[S]/K_m$ is less than 1. If [S] is greater than K_m, then the initial velocity (V_0) of the reaction can be written as follows:

$$V_0 = \frac{k_2}{K_m}[E]_0[S]$$

$$= \frac{k_{cat}}{K_m}[E]_0[S]$$

In this equation, k_{cat}/K_m is a measure of catalytic efficiency, with a larger value corresponding to the formation of more product.

Michaelis–Menten kinetics cannot be applied for irreversible inhibition, because the inhibitor forms a strong covalent bond with the enzyme and cannot be removed. Hence, the effectiveness of irreversible inhibitor is determined by the rate at which this binding takes place. A very common example of irreversible inhibition is diisopropylfluorophosphate (DFP) reaction with acetylcholinesterase (AChE). DFP forms a covalent bond with the hydroxyl group of the serine residue at the active site of AChE. The complex formed is so stable that normal nerve function is restored only after new enzyme is synthesized.

Allosterically regulated enzymes also do not fit into Michaelis-Menten equations. Here, instead of a hyperbolic reaction curve, a sigmoidal curve is obtained. These enzymes possess multiple binding sites and their activity is regulated by the binding of inhibitors or activators.

How to determine K_m and V_{max}: Lineweaver-Burk Plot

K_m and V_{max} can be determined experimentally by incubating the enzyme with different concentrations of substrate. The results can be plotted as a graph of velocity or rate of reaction (V) against concentration of substrate [S]. This will produce a hyperbolic curve. The reaction velocity and K_m have the following relationship.

$$V = \frac{V_{max}}{1 + \frac{K_m}{[S]}}$$

Even under expert hands, it is difficult to fit the best hyperbola through all the experimental points to determine V_{max} accurately. Scientists have developed methods to rearrange the Michaelis-Menten equation to allow more precise fitting to the experimental points and estimate V_{max} and K_m. However, with each method, there are some advantages and disadvantages.

The Lineweaver-Burk double reciprocal plot (Figure 6) is one of the most common methods that rearranges the Michaelis-Menten equation as follows:

$$\frac{1}{V} = \frac{K_m}{V_{max}} \left(\frac{1}{S}\right) + \frac{1}{V_{max}}$$

When we plot 1/V against 1/[S], a straight line is obtained and Y intercept = $1/V_{max}$; gradient = K_m/V_{max}; and X intercept = $-1/K_m$. Lineweaver-Burk plots are the most widely used for linearizing the data and they give the most precise estimates of K_m and V_{max}. However, this method places undue weight on the points obtained at lower substrate concentrations, i.e., the highest values of 1/[S] and 1/V).

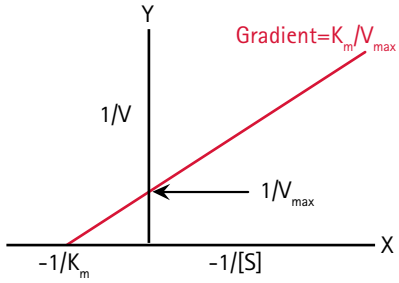


Figure 6.
A typical Lineweaver-Burk plot.

Eadie-Hofstee Plot

Another method for graphically representing enzyme kinetics is the Eadie-Hofstee plot (Figure 7), where reaction rate is plotted as a function of the ratio between reaction rate and substrate concentration.

This plot rearranges the Michaelis-Menten equation as follows:

$$V = \frac{V_{max} - K_m \times V}{[S]}$$

When we plot reaction rate V against V/[S] it gives a straight line where Y intercept is V_{max}; X intercept is V_{max}/K_m; and slope gradient is $-K_m$. Unlike the Lineweaver-Burk plot, this method gives equal weight to all data points in any range of substrate concentration. A disadvantage of this method is that neither X nor Y axes represent independent variables, and both are dependent on reaction rate. Any experimental or instrument error will affect both axes to a larger extent.

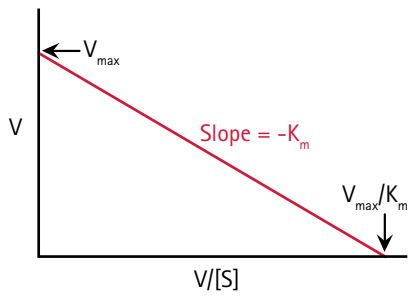


Figure 7.
A typical Eadie-Hofstee plot.

1.6 Catalytic efficiency

When we compare the rates of reactions of different enzymes acting on a substrate or same enzyme acting on different substrates, calculating relative catalytic efficiencies can inform us which enzyme is best suited to which substrate(s) under a given set of conditions.

If an enzyme is acting under steady-state conditions, then the kinetic parameters of that enzyme to consider are: k_{cat} (the catalytic constant for the conversion of substrate to product) and K_m (the Michaelis-Menten constant). Here k_{cat} is the turnover number, which indicates how much substrate is converted to product in a specific period of time. The ratio of k_{cat}/K_m is equal to the catalytic efficiency, which is then used to compare enzymes. This measure of efficiency is helpful in determining whether the rate is limited by the creation of product or the amount of substrate in the reaction mixture. Generally, the upper limit of k_{cat}/K_m is determined by the rate of diffusion of substrate to the active site of enzyme.

Under certain conditions, an enzyme may act on two somewhat related substrates. Here, one has to consider the relative rates of reaction with each substrate, because each substrate has its own K_m value. However, if K_m is the sole determinant of enzyme specificity, then, as the ratio $[S]/K_m$ increases above 1, k_{cat} becomes the best parameter to determine which substrate is better. With two substrates (x and y) being acted upon by the same enzyme, simultaneously, the following equation can be created for the relative velocities of reactions.

$$V_x / V_y = \frac{(k_{catx} - K_{mx})[S_x]}{(k_{caty} - K_{my})[S_y]}$$

Here V_x and V_y are velocities of enzyme reaction with substrates x and y. Substrate concentrations of two substrates are $[S_x]$ and $[S_y]$.

1.7 Classification of enzyme inhibitors

By virtue of their catalytic nature, enzymes accelerate the rate of a reaction without getting altered by their participation in the reaction. The ability of an enzyme to catalyze a reaction can be reduced by binding various small molecules (inhibitors) to the active site or, sometimes, at a site away from the active site.

Enzymatic activity depends on a number of factors. The most important factors that affect enzyme activity are: enzyme concentration, the amount of specific enzyme substrate, pH of the reaction medium, temperature, and the presence of activators and inhibitors.

Enzyme inhibitors are usually low molecular weight compounds that combine with the enzyme to form an enzyme-inhibitor complex, either reducing or completely inhibiting the catalytic activity of the enzyme and therefore reducing the rate of reaction. Binding of an inhibitor to the active site of enzyme can block the entry of substrate to the site. Alternatively, some inhibitors can bind to a site other than the active site and induce a conformational change that prevents the entry of substrate to the active site. Based on the type of interaction with the enzyme, inhibitor binding can be classified as either reversible or irreversible (Figure 8).

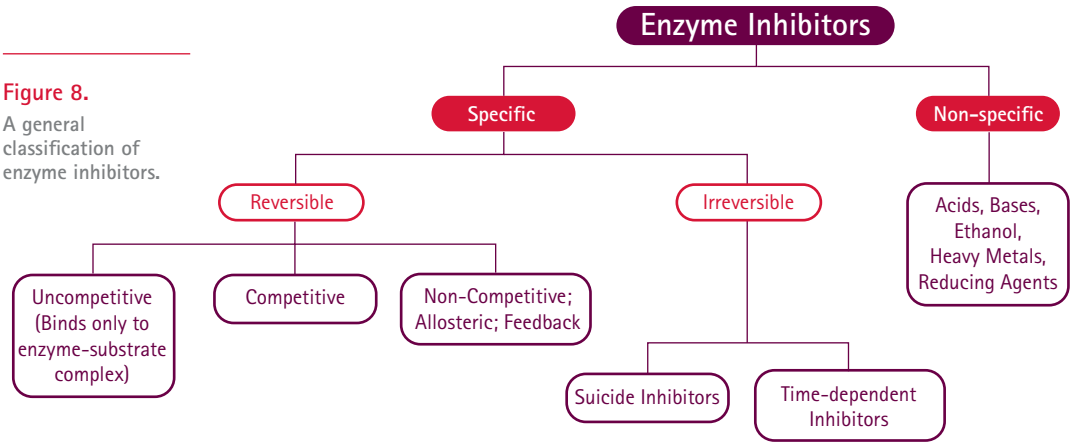


Figure 8.
A general classification of enzyme inhibitors.

1.7.1 Reversible inhibitors

Reversible inhibitors can be classified as competitive, non-competitive or uncompetitive. They bind to enzyme with noncovalent interactions, such as hydrogen bonds, ionic bonds, and hydrophobic interactions (Figure 9).

Irreversible inhibitors can react with the enzyme covalently and induce chemical changes to modify key amino acids that are required for enzymatic activity.

Also it is important to note that when enzymes can act on different substrates, inhibitors can display different types of inhibition depending on which substrate is being considered. This can occur because the active site may have different binding sites, one for each substrate. Hence, an inhibitor can compete with substrate A at the first binding site and act on a second binding site in a non-competitive manner with respect to substrate B.

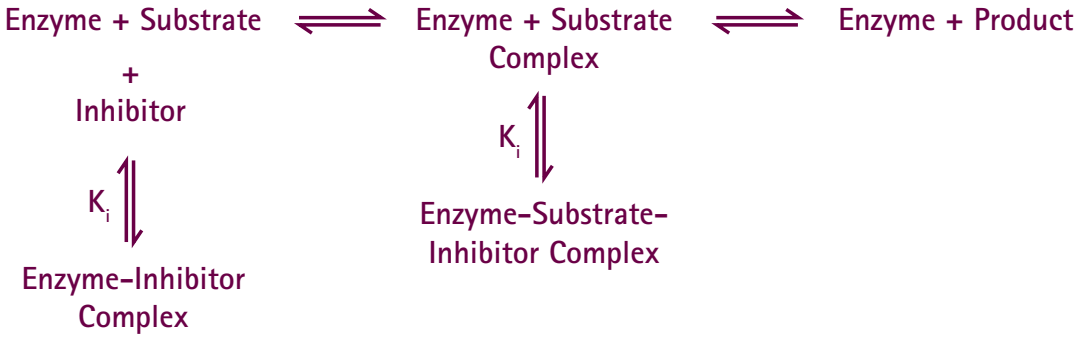


Figure 9.
A general scheme of reversible enzyme inhibition.

1.7.1.1 Competitive inhibitors

In competitive inhibition, the inhibitor usually has structural similarity with the natural substrate and competes with the substrate for access to the active site (Figure 10). The inhibitor has affinity for the active site, and if it binds more tightly than the substrate, then it is an effective competitive inhibitor. Conversely, if it binds less strongly, it is considered as a poor inhibitor. In competitive inhibition, the inhibitor can bind only to the free enzyme and not with the enzyme-substrate complex. Hence, inhibition can be overcome by increasing the concentration of substrate in the reaction mixture. By outcompeting with substrate, V_{max} can still be achieved. However, in the presence of inhibitor, substrate concentration has to be increased to achieve V_{max}. This will increase the K_m value.

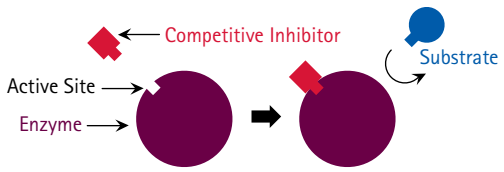


Figure 10.
A simplified model of competitive inhibition.

For competitive inhibition, one can determine K_i, the inhibition constant, which is the dissociation constant for the enzyme-inhibitor complex. The lower the K_i value, the lower is the amount of inhibitor required to reduce the rate of reaction. This relationship can be simplified as:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

$$+$$

$$I$$

$$K_i \uparrow\downarrow$$

$$EI$$

$$K_i = \frac{[E][I]}{[EI]}$$

Watch Out

When studying the effect of competitive inhibitors, add an appropriate amount of substrate to the incubation medium. Excessive amount of substrate will outcompete inhibitor. Always run a control reaction without inhibitor.

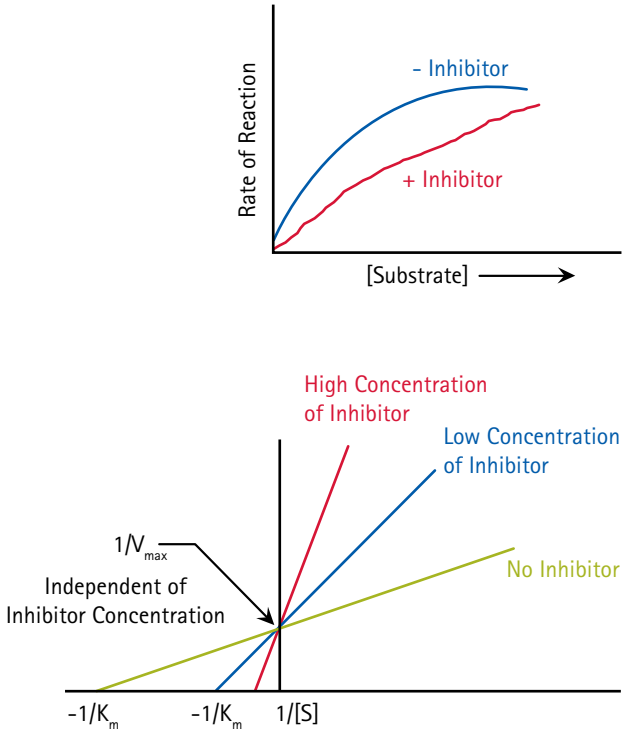


Figure 11.
Kinetics of competitive inhibition.

The result of competitive inhibition can be presented in a Lineweaver-Burk plot (Figure 11). Since K_m increases as a result of competitive inhibition, the X-intercept moves closer to the origin in the presence of an inhibitor. As we increase inhibitor concentration, K_m will increase further and X-intercept will move even closer to the origin. Note that all lines go through the same Y-intercept, because a competitive inhibitor does not affect V_{max}.

An example of competitive inhibitor is methotrexate, an anticancer agent. It inhibits the activity of dihydrofolate reductase, the enzyme that participates in tetrahydrofolate synthesis. Another example is sildenafil citrate, which is structurally similar to cyclic-GMP (cGMP) and competitively inhibits the activity of phosphodiesterase V, and the resulting accumulation of cGMP causes smooth muscle relaxation.

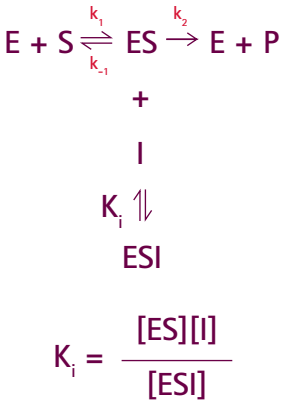
Sometimes an enzyme may follow the kinetics of partial competitive inhibition. This process is similar to competitive inhibition, but the enzyme-substrate-inhibitor complex (ESI) may exhibit partial activity. This type of inhibition displays lower V_{max}, but K_m is not affected. With complete competitive inhibition the velocity of reaction tends to be zero when inhibitor concentration is increased; however, with partial inhibition the enzyme is converted into a modified, but still somewhat functional enzyme-substrate-inhibitor (ESI) complex.

1.7.1.2 Uncompetitive inhibitors

Uncompetitive inhibitors bind only the enzyme-substrate complex. Uncompetitive inhibition should not be confused with noncompetitive inhibition. The inhibitor does not bind to the active site of the enzyme and it does not have to resemble the substrate. In uncompetitve inhibition, V_{max} is reduced because of the removal of activated enzyme-substrate complex. The amount of ESI complex depends on the concentration of the inhibitor. The elimination of ES complex also results in reduced K_m.

In uncompetitive inhibition, both K_m and V_{max} decrease at the same time and at the same rate. In other words, V_{max}/K_m is unaltered. Figure 12 shows that with uncompetitive inhibitor, $1/V_{max}$ is increased. Hence, the Y-intercept moves up. Inhibition also increases $1/K_m$ to a degree that maintains the ratio of K_m/V_{max}, which is the slope of the curve. For this reason, Lineweaver-Burk plots for uncompetitive inhibition are parallel, with and without inhibitor.

Uncompetitive inhibition is relatively rare, but may occur in multimeric enzyme systems. Evolutionarily, this is important because uncompetitive inhibition in a metabolic pathway can have larger effects on the concentrations of metabolic intermediates than competitive inhibition and may even increase toxicity. An example of a uncompetitive reversible inhibitor is oxalate, which inhibits lactate dehydrogenase.



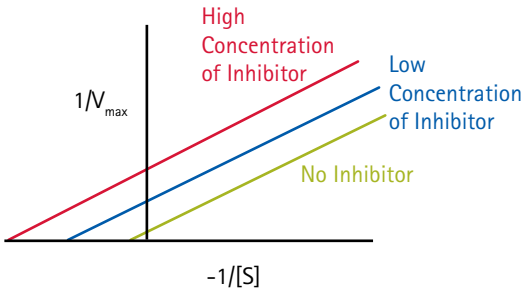


Figure 12.
Kinetics of uncompetitive inhibition.

1.7.1.3 Mixed type inhibitors

In certain cases, an inhibitor can bind to both the free enzyme (with dissociation constant K_i) as well as the enzyme- substrate [ES] complex (with dissociation constant K'i). However, their affinities are different, hence, $K_i \neq K'i$. Here, the inhibitor binding can be reduced by adding more substrate, but inhibition cannot be totally overcome as in competitive inhibition. Mixed type inhibitors interfere with binding and reduce the effectiveness of turnover. This type of inhibition is mostly allosteric in nature, where the inhibitor binds to a site other than the active site to cause a conformational change in the enzyme structure, reducing the affinity of substrate for the active site. Hence, K_m is increased while V_{max} is reduced (Figure 13). An example of mixed-type inhibition is that of xanthine oxidase by palladium ions.

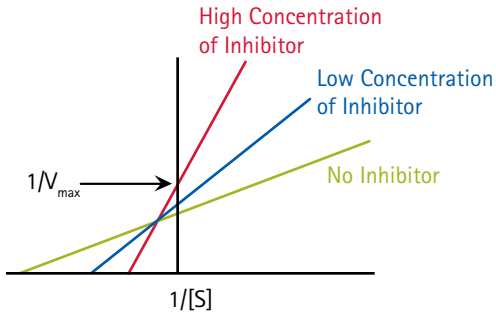
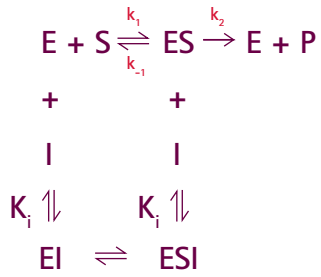


Figure 13.
Kinetics of mixed type inhibition.

1.7.1.4 Noncompetitive inhibitors

In noncompetitive inhibition, the binding of the inhibitor reduces enzyme activity, but does not affect the binding of substrate (Figure 14). Hence, the degree of inhibition is dependent only the concentration of the inhibitor. These inhibitors bind noncovalently to sites other than the substrate binding site. Inhibitor binding does not influence the availability of the binding site for substrate. Hence, the binding of the substrate and the inhibitor are independent of each other and inhibition cannot be overcome by increasing substrate concentration. Noncompetitive inhibitors have identical affinities for enzyme and enzyme-substrate complex; therefore, $K_i = K'_i$. Hence, V_{max} is reduced, but K_m is unaffected. V_{max} cannot be attained in the presence of a noncompetitive inhibitor. Equilibria for noncompetitive inhibition can be simplified as follows:



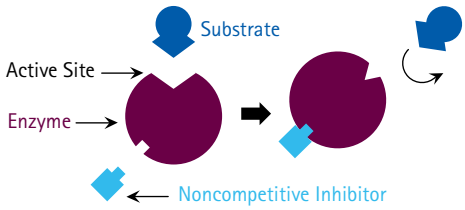


Figure 14.
A simplified model of noncompetitive inhibition.

The effect of a noncompetitive inhibitor is graphically presented in Figure 15. Since the Y intercept is $1/V_{max}$, as V_{max} decreases, $1/V_{max}$ increases. However, K_m remains the same for any concentration of the noncompetitive inhibitor. Hence, all lines go through the same X-intercept. An example of noncompetitive reversible inhibitor is digitalis, which blocks the activity of Na^+-K^+ ATPase and is used for treatment of cardiac arrhythmia.

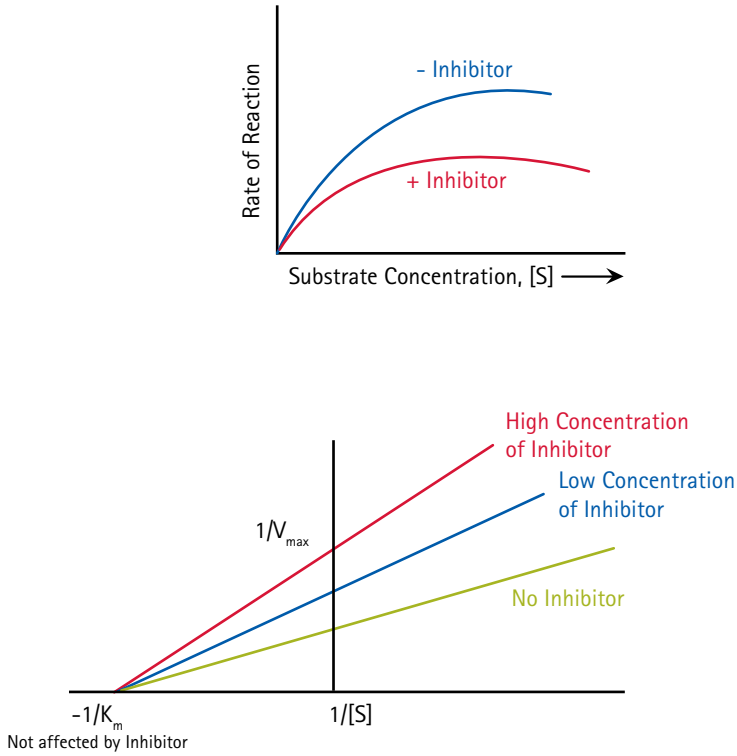


Figure 15.
Kinetics of noncompetitive inhibition.

1.7.1.5 Allosteric inhibitors

Allosteric enzymes belong to a group of enzymes that do not obey Michaelis-Menten kinetics. They generally have a regulatory role in the cell. They function through reversible, non-covalent binding of effector molecules (activators and inhibitors) to their regulatory site. Binding of activator promotes active shape and enzyme activity, whereas binding of inhibitor to the regulatory site causes the allosteric enzyme to adopt the inactive shape and cause a reduction in activity (Figure 16). Their kinetics generates a sigmoidal curve instead of a hyperbolic curve. This is because these enzymes possess multiple binding sites and can bind to more than one substrate molecule. They exhibit saturation with the substrate when [S] is sufficiently high. In this sigmoidal reaction curve, the substrate concentration at which reaction velocity is half- maximal cannot be designated as K_m, because the Michaelis-Menten model does not apply Instead the symbol $[S]_{0.5}$ or $K_{0.5}$ is used to represent the substrate concentration at which half maximum velocity is observed.

Allosteric inhibition occurs when the binding of one ligand decreases the affinity for substrate at other active sites. A classical example of allosteric inhibition is that of phosphofructokinase (PFK). PFK catalyzes the phosphorylation of fructose-6-phosphate to form fructose-1-6-bisphosphate. When levels of ATP increase the activity of PFK is allosterically inhibited. ATP binds to an allosteric site on PFK, causing a change in the enzyme's shape. This reduces its affinity for fructose-6-phosphate and ATP at the active site, reducing the rate of glycolysis.

The active and inactive forms of the allosteric enzyme exist in an equilibrium that is dependent on the relative concentrations of substrate and inhibitor. The binding of an allosteric inhibitor causes the enzyme to adopt the inactive conformation and can promote the cooperative binding of a second inhibitor.

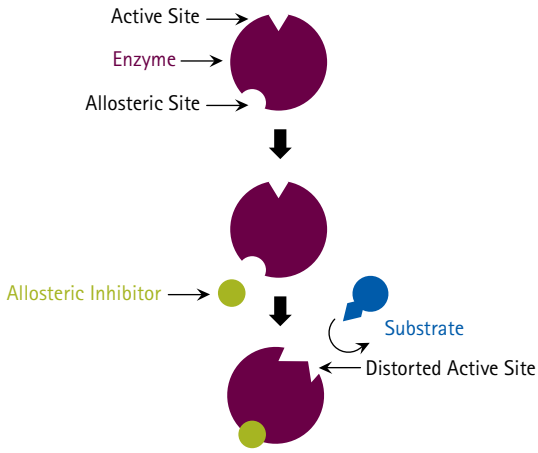


Figure 16.
A model of allosteric inhibition.

1.7.1.6 End product inhibitors

Many enzyme-catalyzed reactions occur sequentially, in a biochemical pathway, in which the product of one reaction becomes the substrate for the next reaction. If the end product of the pathway accumulates in quantities more than needed, then this end product can inhibit the activity of the first enzyme. A pathway is shut down when the end product of the pathway is bound to an allosteric site on the first enzyme of the pathway (Figure 17). Upon this binding, the enzyme undergoes a conformational change and cannot react with the first substrate. This is a form of biological control that prevents excessive buildup of the product. An example is the formation of L-isoleucine from L-threonine, catalyzed by L-threonine dehydratase, which is strongly inhibited by L-isoleucine.

Figure 17.

In end product inhibition, the end product of a pathway (D) can directly bind to (and inhibit) the enzyme that converts A to B, thus shutting down the pathway.

1.7.2 Irreversible inhibitors

Irreversible inhibitors are noncompetitive in nature. They include nonspecific protein denaturing agents, such as acids and alkalis, and specific agents, which attack a specific component of the holoenzyme system. Specific inhibitors can be grouped as: (a) coenzyme inhibitors; (b) inhibitors of specific ion cofactor; (c) prosthetic group inhibitors; (d) apoenzyme inhibitors; and (e) physiological modulators of the reaction, such as the pH and temperature that denature the enzyme catalytic site.

Most irreversible inhibitors interact with functional groups on the enzyme and destroy enzyme activity. These interactions are covalent in nature. These inhibitors are highly useful in studying reaction mechanisms.

1.7.2.1 Suicide inhibitors

A special group of irreversible inhibitors is known as suicide inhibitors. They are relatively unreactive until they bind to the active site of the enzyme. In the first few steps of the reaction it functions like a normal substrate, but then it is converted into a very reactive compound that combines with the enzyme to block its activity. Because they use the normal enzyme reaction mechanism to inactivate the enzyme, they are also known as mechanism-based inhibitors or transition state analogs. Suicide inhibitors that exploit the transition state-stabilizing effect of the enzyme result in higher enzyme binding affinity than do substrate-based inhibitor designs.

This approach is highly useful in developing pharmaceutical agents with minimal side effects. However, designing drugs that precisely mimic the transition state is a real challenge because of the unstable, poorly characterized structure of the transition state. Prodrugs undergo initial reaction(s) to form an overall electrostatic and three-dimensional intermediate transition state complex form with close similarity to that of the substrate. These prodrugs serve as guidelines to further develop transition state molecules with modifications.

A common example of a suicide inhibitor is allopurinol, the anti-gout drug that inhibits xanthine oxidase activity. The enzyme commits suicide by initially activating allopurinol into oxypurinol (a transition state analog) that binds very tightly to the molybdenum-sulfide (Mo-S) complex at the active site of xanthine oxidase.

Acyclovir (acycloguanosine (2-amino-9-((2-hydroxyethoxy)methyl)-1H-purin-6(9H)-one) is one of the most commonly used antiviral agents with very low toxicity. It is selectively converted into acyclo-guanosine monophosphate (acyclo-GMP) by viral thymidine kinase. Acyclo-GMP is further phosphorylated into the active triphosphate form, acyclo-GTP, by cellular kinases. Acyclo-GTP is a very potent inhibitor of viral DNA polymerase with over 100-fold greater affinity for viral than cellular polymerase. It is incorporated into viral DNA, resulting in chain termination.

The suicide inhibitor removes enzyme and reduces the formation of ES complex. The V_{max} value is reduced and inhibition cannot be overcome by adding extra substrate. In this regard, suicide inhibition resembles noncompetitive inhibition.

1.7.2.2 Heavy metal ions as enzyme inhibitors

Heavy metals, such as mercury and lead, can bind tightly to enzymes and inhibit their activity. They exhibit higher affinity for enzymes with sulfhydryl (–SH) groups. When they are present in larger quantities, their action is rather nonspecific and can inhibit multiple enzymes, and it may not be clear which particular enzyme is most affected.

Heavy metal inhibition of critical enzymes may result in poisoning, which can be treated by administering metal ion chelators.

1.7.2.3 Time-dependent inhibitors

Time-dependent inhibitors are those that exhibit slow binding to the enzyme. The observed onset of inhibition is slower. These inhibitors display nonlinear initial velocities and nonlinear recoveries of enzyme activity with slow k_{off} values (rate constant of dissociation between enzyme and inhibitor). Time-dependent inhibition is a severe form of inhibition; overcoming inhibition requires *de novo* enzyme synthesis.

Some time-dependent inhibitors interact with enzymes in a covalent manner. For these inhibitors, the k_{off} value approaches zero, and inhibition is irreversible. These molecules are less useful for most biological research, unless the covalent species provides good information about the reaction mechanism.

Interestingly, many successful therapeutic drugs are time-dependent inhibitors. In these cases with slow k_{off} values, the rate of release of inhibitor from the enzyme-inhibitor complex proceeds independent of the substrate concentration, making them attractive for the drug discovery process.

One attractive therapeutic target of time-dependent inhibition is cytochrome P450 (CYP) 3A, which is responsible for the metabolism of about 60% of currently known drugs. Inhibition of CYP by co-administered drugs can lead to overexposure and has been attributed to the withdrawal of several drugs from the market. Time-dependent inhibition can increase the potency of drugs by blocking their degradation. This is due to either the formation of a tight-binding, quasi-irreversible inhibitory metabolite or by inactivation of CYP enzymes by covalent adduct formation. Some of the inhibitors of CYP include popular antibiotics like azithromycin and antidepressant fluoxetine.

1.7.3 Inhibition by temperature change

Most enzymes are stable over a wide range of temperature. However, they work best in the physiological range. Enzymatic activity can be reduced significantly by lowering the temperature and can be increased by increasing the reaction temperature, but only up to a certain limit. Since enzymes are proteins, they are partially unfolded or denatured at higher temperatures. Hence, reaction can also be terminated by bringing the reaction mixture to a high, denaturing temperature (Figure 18).

When reaction temperature is increased, the rate of reaction increases, based on the principle of Q_{10}. The Q_{10}, or temperature coefficient, is a measure of the rate of change of a biological or chemical system as a consequence of increasing the temperature by 10°C. In biological systems, the rule of thumb is that for every 10°C rise in temperature, the rate of reaction doubles. However, when temperature becomes too high, proteins are denatured, the enzymatic activity is lost and the organism will perish. Q_{10} can be calculated by the following equation, where R_1 and R_2 are reaction rates at temperatures T_1 and T_2, respectively.

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{10/(T_2-T_1)}$$

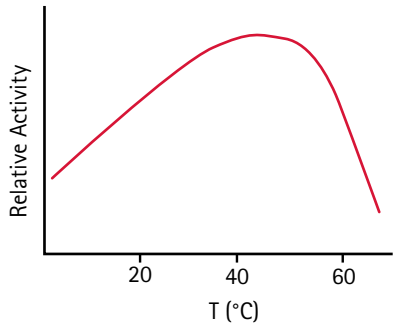


Figure 18.
Effect of temperature on enzyme reactions.

Similarly, lowering the temperature can reduce enzyme activity, because low temperature can change the shape of the enzyme. However, in most cases, when temperature is brought up to physiological range, enzyme activity is restored. It is important to note, however, that enzymes are sensitive to repeated freezing and thawing. Freezing can induce several stresses, such as ice formation, changes in solute concentration due to the crystallization of water, eutectic crystallization of buffer solutes and resulting changes in pH. Hence, it is best to thaw a frozen enzyme only once and then aliquot into single-use vials before refreezing.

Watch Out

Bring the incubation medium to a desired temperature (for example, 37°C) before adding enzyme or inhibitor. Also, if enzyme reaction is to be terminated by increasing reaction temperature to denaturing point, it is best to immerse the reaction tube in a boiling water bath instead of gradually raising the temperature. This is particularly important in experiments with shorter incubation periods.

1.7.4 Inhibition by pH change

pH has a clear effect on rates of enzyme-catalyzed reactions. Each enzyme has a pH optimum above or below which its activity declines or is completely abolished.

Enzymes are active only in a narrow pH range due to:

- pH sensitivity of substrate binding
- Reduced catalytic efficiency of the enzyme
- Ionization of substrate
- Protein structural changes (usually at pH extremes)

Amino acid side chains act as weak acids and bases that perform critical functions in the active site of enzymes. Hence, any change in their ionization state can adversely affect enzyme activity. The pH range over which enzyme activity changes can provide important information about which amino acids are involved in organizing the active site. For example, a change in enzyme activity near pH 7.0 indicates the presence of histidine residue(s) at the active site.

Because enzymes are sensitive to pH changes, most living systems have highly evolved buffering systems to maintain intracellular pH. Although most mammalian cells maintain pH around 7.2, within intracellular compartments or within certain organs, pH can be vastly different. For example, in the stomach, the pH is usually between 1 and 2, which is optimum for pepsin activity. Pepsin activity is rapidly lost when pH is increased to 4 or above (Figure 19). On the other hand, pH in the intestine is slightly alkaline, which is required for optimum chymotrypsin activity. Bicarbonate released from the pancreas contributes to this alkalinity and also neutralizes acidified food entering the duodenum from the stomach. In cells, lysosomal compartments have acidic pH to provide optimum conditions for acid hydrolases, which lose their activity if released into the cytosolic compartment.

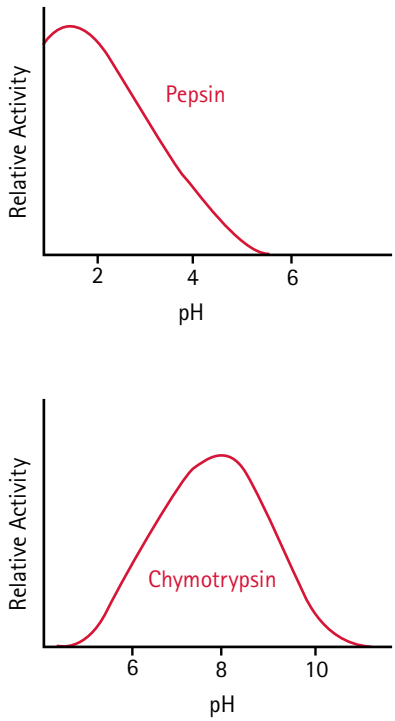


Figure 19.
Effect of pH on the activity of different enzymes that are active in different organs.

Nice to know

pH Measurements: Some Useful Tips

- A pH meter may require a warm up time of several minutes. When a pH meter is routinely used in the laboratory, it is better to leave it "ON" with the function switch at "standby."
- Set the temperature control knob to the temperature of your buffer solution. Always warm or cool your buffer to the desired temperature before checking final pH.
- Before you begin, make sure the electrode is well-rinsed with deionized water and wiped off with a clean absorbent paper.
- Always rinse and wipe the electrode when switching from one solution to another.
- Calibrate your pH meter by using at least two standard buffer solutions.
- Do not allow the electrode to touch the sides or bottom of your container. When using a magnetic bar to stir the solution make sure the electrode tip is high enough to prevent any damage.
- Do not stir the solution while taking the reading.
- Inspect your electrode periodically. The liquid level should be maintained as per the specification provided with the instrument.
- Glass electrodes should not be left immersed in solution any longer than necessary. This is important especially when using a solution containing proteins. After several pH measurements of solutions containing proteins, rinse the electrode in a mild alkali solution and then wash several times with deionized water.
- Water used for preparation of buffers should be of the highest possible purity. Water obtained by a method combining deionization and distillation or reverse osmosis is highly recommended.
- To avoid any contamination, do not store water for longer than necessary. Store water in tightly sealed containers to minimize the amount of dissolved gases.
- One may sterile-filter the buffer solution to prevent any bacterial or fungal growth. This is important when large quantities of buffers are prepared and stored over a long period of time.

1.8 Cell permeability of inhibitors

There is tremendous interest in developing synthetic molecules that can manipulate protein-protein interactions in living cells. This is the basis for many pharmaceutical development programs. However, the plasma membrane is selectively permeable and regulates the entry and exit of most molecules in the cell. The manner in which inhibitor molecules cross the plasma membrane to reach their intracellular targets depends on their size and lipophilic or hydrophobic characteristics. If a molecule is small enough, it can be transported across the membrane via passive diffusion, facilitated diffusion, or active transport. However, larger molecules may move via endocytosis.

Permeability of peptide-based inhibitors

- Peptides with four or more amino acids may not be cell-permeable. However, attaching selective groups to these peptides can make them cell-permeable. For example, the fluoro-methylketone (FMK)-based caspase inhibitors are cell-permeable because the carboxyl groups of aspartic and glutamic acid are esterified, making them more hydrophobic. These inhibitors covalently modify the thiol group of caspases, making them irreversible inhibitors. Also, at the amine end of the inhibitor, a Z group, biotin, or Ac group can be attached. These groups increase hydrophobicity of the molecule, which makes them more cell-permeable. Compared to the inhibitors with an Ac or a biotin group, those inhibitors with a Z group are even more cell-permeable.
- Acyclic peptides are more permeable than corresponding cyclic peptides. This is due to greater conformational freedom of the acyclic peptides.

- Basic peptides, such as HIV-1 Tat-(48-60) and *Drosophila* Antennapedia-(43-58) exhibit high membrane permeability and, when fused to peptide-based inhibitors, can perform as carriers for intracellular delivery.
- A short treatment of cells with digitonin can transiently permeabilize cells and allow peptide-based inhibitors to move into cells.
- Peptides can also be transported into cells by using protein transfection agents, such as ProteoJuice™ Transfection Reagent from Merck Millipore (Cat. No. 71281).

Permeability of small molecule inhibitors

- Some inhibitors are totally impermeable and cannot be used for cell-based studies. However, they are perfectly fine for use with lysates and homogenates.
- Some inhibitor molecules are permeable due to their hydrophobic and lipophilic nature.
- In general, charged molecules are not cell-permeable.
- Most phosphorylated compounds are not cell-permeable. However, modified phosphorylated compounds, such as mono- and dibutyryl-cAMP, are cell-permeable.

1.9 Prozone effect

The prozone effect, also known as high dose-hook phenomenon, is classically used to describe an immunoassay in which adding excess antigen or excess antibody results in false-negative or false-low results. Although originally described in immunometric assays, the prozone effect can also be invoked in biochemical assays where the concentration of an inhibitor or a receptor antagonist far exceeds the concentration of enzyme or receptor. The prozone phenomenon will cause the reaction to be either weak or negative (a false negative) in the first few dilutions, but upon further dilutions the reaction proceeds as expected (Figure 20).

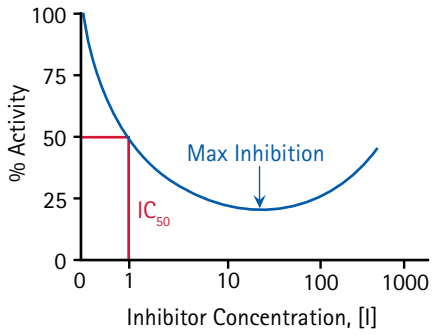


Figure 20.
A graphical representation of the prozone effect.

In a typical immunoassay, antigens and antibodies bind to create a conjugate that can be detected and measured. However, when the prozone effect occurs, excess antigens or antibodies can bind all of the receptor sites, leaving nothing available to become a conjugate. Hence, antibody-antigen conjugate cannot be detected and a false negative result is produced, which can go undetected. In a clinical setting this could lead to misdiagnosis or in case of inhibitors, it can reduce the efficacy of drug or cause toxicity.

Nice to know

The "prozone effect" can be avoided in the following ways: (i) use sequential dilutions of inhibitors (ii) a common experimental observation is that an inhibitor will work well at concentrations that are 5- to 10- fold higher than the reported IC_{50} values. Hence, when an inhibitor fails to perform as expected, it is best to reexamine the IC_{50} values and use an appropriately diluted inhibitor.

1.10 Enzyme inhibitors as therapeutic agents

Most drug therapies are based on inhibiting the activity of overactive enzyme(s). If an overactive enzyme can be inhibited, the progression of disease can be slowed and symptoms can be alleviated. The utility of inhibitors as pharmaceutical agents is based on the concept of competitive enzyme inhibition, where inhibitors are structural analogs of normal biochemical substrates that compete with the natural substrate for the active site of enzyme and block the formation of undesirable quantities of metabolic products.

In addition to active compounds as pharmaceutical agents, prodrugs are preferred in some cases. Prodrugs are not effective until they are metabolized and converted to an active form. Some inhibitors can interfere with the *in vivo* conversion of prodrugs if administered at the same time, reducing the efficacy of the latter. For example, the anticancer prodrug, tamoxifen, requires cytochrome P450 2D6 to become an active drug. However, anti-depressants, such as paroxetine hydrochloride (Paxil®), can inhibit the activity of P450 2D6, thereby severely reducing the efficacy of tamoxifen.

In the drug development process, an initial candidate compound often exhibits merely modest competitive inhibition. Medicinal chemists improve upon the initial candidate by slightly modifying its structure to make it more effective, specific, bioavailable, and less toxic. Inhibitors developed on the basis of structure-based drug design often exhibit poor bioavailability, primarily due to poor solubility. Increasing the solubility helps reduce toxicity of the compound. The effectiveness of the final product depends on the potency, specificity, metabolic pathway, bioavailability, and pharmacokinetic properties of the inhibitor molecule. High specificity for a single reaction can avoid any unwanted side effects and potential toxicity. High specificity can also reduce the depletion of inhibitor by nonspecific pathways.

Common examples of inhibitors used as pharmaceuticals:

- Analgesics: Acetaminophen (Paracetamol)- (COX inhibitor)
- Hypercholesterolemic agents: Lovastatin (HMG-CoA reductase inhibitor)
- Gout control: Allopurinol (xanthine oxidase inhibitor)
- Selected antibiotics: Rifampicin (DNA-dependent RNA polymerase inhibitor)
- Erectile dysfunction: Sildenafil citrate (phosphodiesterase V inhibitor)
- Anti-cancer agents: Doxorubicin (topoisomerase inhibitor)
  - Gleevec® (Imatinib Mesylate: Bcr/Abl tyrosine kinase inhibitor)
  - 5-Fluorouracil (thymidylate synthase inhibitor)
- Blood pressure control: Captopril (angiotensin converting enzyme (ACE) inhibitor)

NOTE: All inhibitors and receptor agonists and antagonists provided by Merck Millipore are for research use only and are not for clinical, diagnostic, or veterinary applications.

1.11 Lipinski's rule of 5

Lipinski et al. (1997)* analyzed the physicochemical properties of more than 2,000 drugs and candidate drugs in clinical trials and concluded that a compound is more likely to be membrane-permeable and easily absorbed by the body if it matches the following criteria:

- Its molecular weight is less than 500.
- The compound's lipophilicity, expressed as a quantity known as logP (the logarithm of the partition co-efficient between water and 1-octanol), is less than 5.
- The number of groups in the molecule that can donate hydrogen atoms to hydrogen bonds (usually the sum of hydroxyl and amine groups in a drug molecule) is less than 5.
- The number of groups that can accept hydrogen atoms to form hydrogen bonds (estimated by the sum of oxygen and nitrogen atoms) is less than 10.

*Lipinski, C.A., et al. (1997). Adv. Drug Delivery Rev. 23, 3-25.

These rules are known as Lipinski's rule of 5, which provide a general guideline to determine the likeliness of a chemical compound to be a successful oral drug. These rules, based on the 90-percentile values of the drugs' property distributions, apply only to absorption by passive diffusion of compounds through cell membranes. Compounds that are actively transported through cell membrane by transporter proteins are exceptions to the rule. Lipinski's criteria are widely used by medicinal chemists to predict not only the absorption of compounds, but also overall drug-likeness.

Although this rule provides a powerful and simple tool to narrow down a pool of potential drugs, it could potentially exclude compounds that could become successful drugs. For example, most TB drugs and anti-bacterials in general do not follow the rule. One concern is that the sharpness of the boundaries can cause one molecule to score a "0" and another extremely similar molecule to score a "4". Another concern is that equal weight is given to each rule. There have been suggestions to modify the rule, particularly to soften sharp boundaries.In "Softening the Rule of Five—where to draw the line" Petit et al. (2012)* propose a new, in-depth approach to both soften the thresholds and assign each rule a specific weight, resulting in improved predictive power. By fine-tuning Lipinski's rule, drug discovery may be improved by avoiding premature and inappropriate discarding of potential drugs.

*Petit, J., et al. (2012). Bioorg. Med. Chem. 20, 5343-5351.

1.12 Pharmacokinetics of inhibitors and drugs

Pharmacokinetics forms the basis of pharmacological effect of a drug (inhibitor) and its toxic effects. It is also a good determinant of dose-efficacy relationships. Pharmacokinetics of a drug are dictated by various physiological and pathological variables. The major parameters are: bioavailability, clearance and volume of distribution. Bioavailability and volume of distribution have been briefly discussed in section 3.4.

In an *in vivo* experimental setup or when an inhibitor is used as a therapeutic agent, it is best to maintain a steady-state concentration of drug in the system. This is achieved when the rate of its clearance and rate of administration are balanced. In pharmacokinetics, the rate of clearance is denoted by CL. Clearance does not reflect how much drug is being removed, but it represents the volume of plasma or other biological fluid that would be completely free of drug to account for its elimination. It is important to note that clearance by various organs is additive. For example:

$$CL_{renal} + CL_{hepatic} = CL_{systemic}$$

1.13 Enzyme inhibitors as metabolic poisons

Many poisonous materials work by inhibiting the action of enzymes. They act as irreversible inhibitors and bind tightly to enzymes. Many animals and plants have evolved to synthesize poisonous products, either for their own protection or to paralyze their prey. For example, some legumes contain trypsin inhibitors to deter seed predators. α-Amanitin, present in some mushrooms, inhibits the activity of RNA polymerase II and blocks DNA transcription.

Naturally occurring poisons are small organic molecules that can block metabolic enzymes, nerve impulses or stop energy production in mitochondria. For example, potassium cyanide is an irreversible inhibitor of cytochrome c oxidase and stops ATP synthesis in mitochondria, causing death in a very short period of time. Malonate binds to the active site of succinic dehydrogenase and blocks the access of succinate to the active site. Oligomycin, an antibiotic, binds to ATP synthase to block oxidative phosphorylation. Insecticides, such as malathion and parathion, contain irreversible inhibitors of acetylcholinesterase (AChE). A few reversible AChE inhibitors are used to treat neurological disorders, such as myasthenia gravis. In the plant kingdom, herbicides, such as glyphosate, inhibit 3-phospho-shikimate-1-carboxyvinyltransferase, preventing plants from synthesizing branched-chain amino acids.

Another role of inhibitors is to function as antidote in certain poisoning incidences. For example, snake venoms have high levels of serine proteases and phospholipase A2 (PLA2). Some snake venoms also contain thrombin-like proteases that coagulate blood. Hence, manoalide and its derivatives can be used to inhibit snake venom PLA2 activity. Natural extracts of several plants in traditional medicine have also been used to neutralize snake venom poisons. For example, *Withania somnifera* (Ashwagandha in Ayurvedic medicine) is used to neutralize *Naja naja* cobra venom, and the ethanol extract of *Acalypha indica* can neutralize Russell viper venom.

1.14 Compound library screening

During the drug development process, most pharmaceutical companies use vast compound libraries to identify potential targets. Searching for an inhibitor that could potentially become a drug target requires thousands of data points. Hence, high-throughput screening (HTS) has become absolutely essential in this process. The cost pressure in screening is enormous. Hence, researchers have opted for miniaturization.

Although 96-well formats are still valid for much research, the drug development process seeks microplates with 384, 1536, or even more wells. To save cost, assay volumes have been scaled down to a few microliters. At the behest of regulatory guidances, drug developers today often complement biochemical assays with cell-based assays. The advantage of cell-based assays is that the target is in an intact cell in its native form, and the data generated may be more predictive of *in vivo* compound performance. However, one drawback of cell-based assays is that many hits are generated, which might include "off-target" hits. This is particularly true in studies involving long, multicomponent signaling pathways. In addition, cell-based assays may be confounded by cytotoxicity problems,which may reduce assay signal even without inhibition of the relevant target. Biochemical assays can be performed at higher inhibitor concentrations than cell-based assays, thereby providing complementary data.

A good HTS program requires three success factors:

- Reproducible, predictive biological assays
- A high-quality HTS compound library
- The ability to test the library in the assay in a timely and cost-effective manner

Of these three success factors, the quality of the compound library is of utmost importance. In the early era of combinatorial chemistry, many compounds were developed at random; several of these were of high molecular weight and were not suited for physiological studies. Besides, these early libraries were limited by poor quality control, lack of solubility data and questionable integrity of compounds upon storage.

Technology Highlight

InhibitorSelect™ Libraries and Panels

Merck Millipore, a world class supplier of high quality inhibitors, has designed and developed multiple libraries and inhibitor panels that meet rigorous criteria of quality and stability of small molecule inhibitors. Recognizing both the tremendous opportunities and critical role of protein kinases and stem cells, we have introduced several Calbiochem® InhibitorSelect™ and StemSelect® Libraries that contain collections of carefully selected, structurally diverse, and potent small molecules targeting members from several families in convenient, cost effective formats.

- **Well-characterized**: Unlike small molecule libraries from other leading suppliers, InhibitorSelect™ and StemSelect® libraries are provided with documented cell permeability, reversibility, potency, published IC_{50}/K_i values, lot-specific data, stability, and HPLC purity for most of the included molecules.
- **Well-documented**: Comprehensive documentation about each inhibitor, such as molecular structure, IC_{50} values, literature citations, and CAS number (where available), is provided at your fingertips.
- **Structurally diverse**: Why is structural diversity so important? Assessing the effect of multiple, structurally diverse inhibitors of the same protein target helps rule out potential non-selective or "off-target" effects of small molecules. Non-selective effects typically are seen when small molecules bind to structurally similar sites on multiple proteins. Inhibitor activity can be considered "on target" if multiple small molecules targeting the same protein, but having distinct chemical structures, all show the same biological activity.

Further, structurally diverse inhibitors can also help researchers to gain insight into mechanism of action of inhibitors. Multiple small molecules with the same biological activity, but possessing distinct chemical structures targeting different functional domains of protein can reveal the biological role of each protein domain. Accordingly, our libraries have been designed to be structurally diverse, so you can obtain the maximum interpretable data for each targeted pathway.

Featured Product

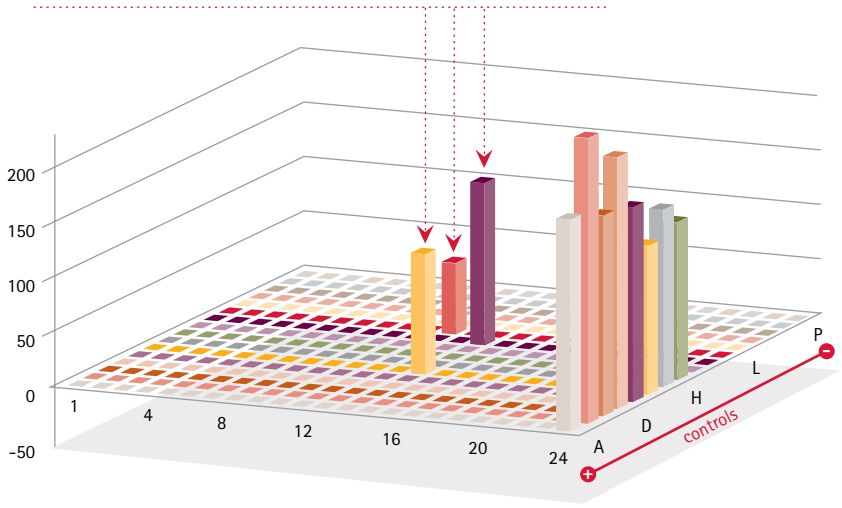
StemSelect® Small Molecule Regulators 384-Well Library I

(Cat. No. 569744)

Comprehensive Documentation includes:

- SD Files
- CAS numbers (where available)
- Concentration
- Target
- Categorical index
- PubChem Substance ID (where available)
- Lot-specific purity
- Molecular formula
- Molecular weight
- Structure
- Web links to catalogue and PubChem for individual small molecule regulators

StemSelect® library molecules affecting cardiomyocyte differentiation



StemSelect®, a library of highly targeted, well-characterized compounds, provide a high hit rate when screening for modulators of cardiomyocyte differentiation. With three molecules (indicated) showing activity, this library proved more effective than doing a large-scale, untargeted compound screen. Data Courtesy of Dr. Mark Mercola, Sanford Burnham Institute

Featured Product

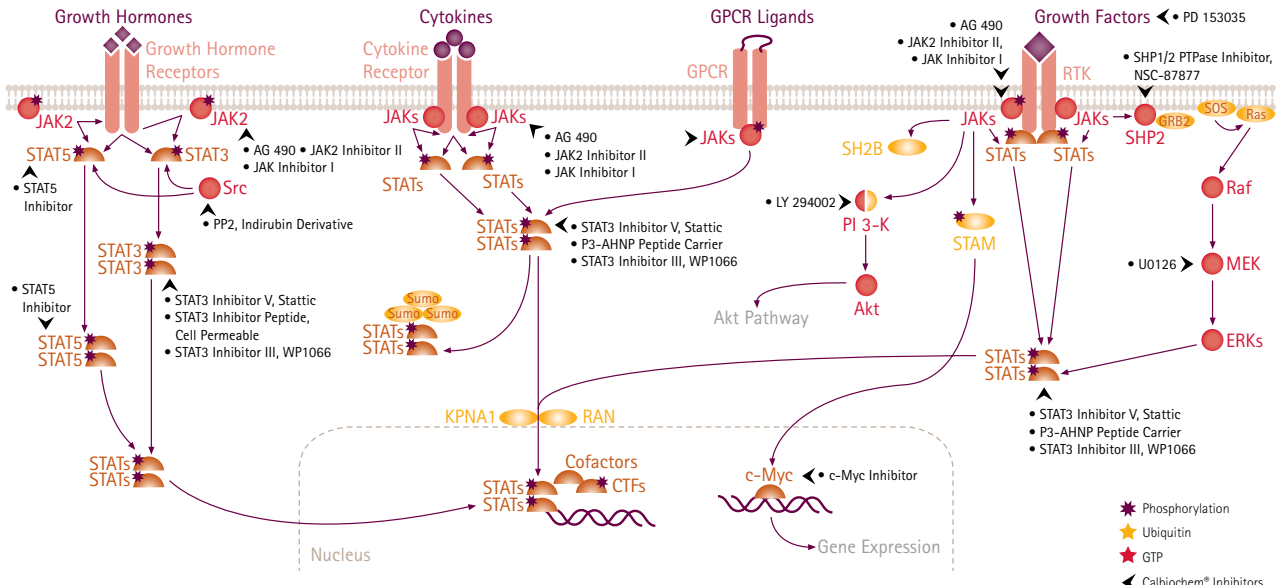
InhibitorSelect™ Signaling Pathway Panels

Merck Millipore's Signaling Pathway Panels contain a collection of carefully selected, cell permeable, and potent small molecules in convenient formats. These panels include inhibitors for each of the major control points in the pathway that allow researchers to zero in on these control points and manipulate them to gain useful information. Currently, Merck Millipore provides the following InhibitorSelect™ panels. In addition to product convenience, these panels also offer substantial cost savings. Please contact your local technical service scientists or your account managers for more information.

- Autophagy Regulators
- Epigenetic Regulators -Acetylation
- Epigenetic Regulators-Methylation
- PI 3-K/Akt/mTOR Signaling
- EGFR Signaling
- JAK/STAT Signaling
- NF-κB Signaling
- TLR Signaling
- mTOR Signaling
- MAP Kinase
- VEGF Signaling
- Wnt Signaling
- IGF Signaling
- FGF Signaling
- p53 pathway regulator
- TGF-βR BMPR signaling

Here is an example of **JAK/STAT Signaling Inhibitor Panel** (Cat. No. 420138). This panel contains 13 potent inhibitors for all major regulatory points in this signaling pathway, and each can be purchased separately as well. A pathway diagram shown below for your reference.

Components	Target	Amount	Cat. No.
PD 153035	EGFR	1 mg	234490
Indirubin Derivative E804	Src, Cdk1/cyclin E, Cdk2/cyclin A, Cdk1/cyclin B	1 mg	402081
JAK Inhibitor I	JAK1, JAK2, JAK3, Tyk2	500 µg	420099
JAK2 Inhibitor II	JAK2	25 mg	420132
LY 294002	PI 3-K	5 mg	440202
PP2	Src	1 mg	529573
SHP1/2 PTPase Inhibitor, NSC-87877	SHP1, SHP2	50 mg	565851
STAT3 Inhibitor Peptide, Cell Permeable	STAT3	1 mg	573096
STAT3 Inhibitor III, WP1066	STAT3	10 mg	573097
STAT3 Inhibitor V, Stattic	STAT3	25 mg	573099
STAT5 Inhibitor	STAT5	10 mg	573108
AG 490	JAK2	5 mg	658401
U0126	MEK1, MEK2	1 mg	662005
DMSO	-	15 mL	KP31817



JAK-STAT signaling pathway

Receptor Agonists & Antagonists

2

2.1 Receptor–ligand interactions

In order to understand the biological and clinical role of agonists and antagonists, it is important to think about the role of receptors in cellular function. Receptors are regulatory proteins embedded in plasma membrane, cytoplasm or on the nuclear surface. The lock-and-key concept of enzyme inhibitors can also be applied to receptors, where certain molecules specifically bind to these receptors. As the name "receptor" implies, they are the recipients of signals that elicit a biological response. These signals may be hormones, selected amino acids, neurotransmitters, toxins or pharmaceutical agents.

The distribution of receptors can be cell-specific, and each cell can have hundreds of different receptors. When a ligand molecule (or an agonist) binds to a receptor, it changes the conformation of the receptor molecule that leads to a specific biological response. As we saw on the preceding pages that inhibitors can reduce enzyme activity, similarly, certain drugs can block the access of ligand or agonist to the receptor site and diminish the biological response.

2.2 Drug receptors and drug:receptor interactions

Although a drug receptor concept was postulated in the early 1900s, solid empirical evidence and subsequent adoption of receptor-mediated drug interactions did not gain wide acceptance until the 1950s (Limbird, 2004). Since that time, multiple models have been proposed to explain the interaction between ligand and receptor, with extended ternary complex models, which also includes resulting molecular changes, such as G-protein interactions (Bridges and Lindsley, 2008).

Researchers have described four basic mechanisms of transmembrane signaling (Figure 21). Each of these mechanisms uses a different strategy to overcome the barrier imposed by plasma membrane. These mechanisms include (a) use of lipid soluble ligand that can easily traverse the membrane (b) use of transmembrane receptor proteins with "built-in" enzyme activity (c) use of ligand-gated channels, which open following ligand binding, and (d) use of transmembrane receptor proteins to stimulate GTP-binding proteins to relay signaling messages.

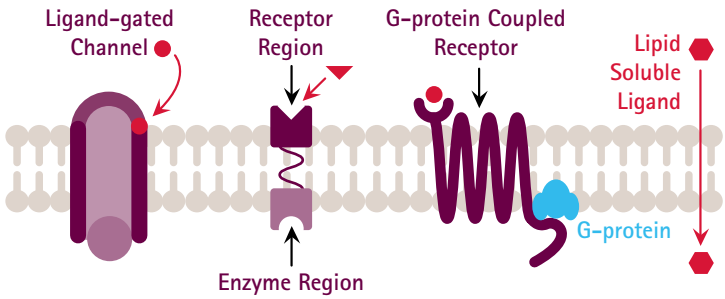
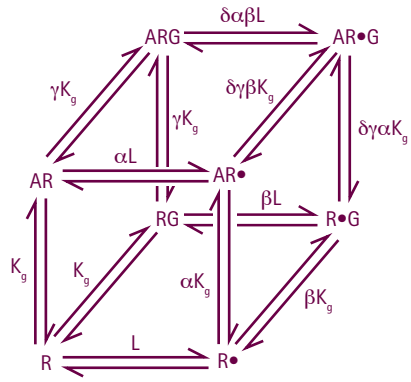


Figure 21.
Four common transmembrane signaling mechanisms.

Most drug receptors are regulatory proteins that exhibit specific modulatory effects, which make then effective therapeutic targets. For example, in the simplest model, a small molecule agonist (A) binds to a receptor (R) with a fairly simple protein binding equilibrium (K_a). This ligand-receptor binding initiates a G-protein (G) engagement, forming the ARG complex, which leads to downstream cascade events.

$$A + R \overset{K_a}{\rightleftharpoons} AR + G \rightleftharpoons ARG \rightarrow Response$$

Extended models, such as various Ternary Complex Models, account for more, complex factors, such as spontaneous isomerization of G-protein coupled receptor (R*), affinity of receptor for G protein, the presence of agonist or antagonist, and inactive ARG complexes (K_a, K_g). A detailed discussion of these factors is beyond the scope of this introduction. However, more information can be obtained from publications by Limbird (2004), Christopoulos and Kenakin (2002), or from a suitable textbook of pharmacology.



Limbird, L.E (2004). Mol. Interv. 4, 326.
Bridges, T.M., and Lindsley, C.W. (2008). ACS Chem. Biol. 3, 530.
Christopoulos, A., and Kenakin, T. G. (2002). Pharmacol. Rev. 54, 323.

2.3 Receptor agonist

A receptor agonist is a molecule that can interact with the receptor and elicit a biological response. In most cases it has a positive modulatory effect. Agonists display both affinity and intrinsic activity. They are mimetics of the natural ligand and produce biological effects that are on par with natural ligands. They bind to the same binding site and generate a complete response (full agonist), partial response (partial agonist) or inverse response (inverse agonist), as shown in Figure 22.

Full agonists display high efficacy for activating receptor function. For example, isoproterenol (Cat. No.420355) mimics the action of adrenaline on β-adrenergic receptors with similar magnitude of action.

Partial agonists do bind to receptor, but elicit only a partial response. For example, buprenorphine does not exhibit efficacy and is used for treatment of opioid dependency. Indeed, partial agonists often do not have reduced affinity for the receptor binding site and can easily act to competitively block the responses produced by full agonists. The molecular mechanism for the partial agonist effect is not exactly clear, but likely the partial agonist causes a different receptor confirmation than full agonist, which results in diminished pharmacological response.

In some cases a co-agonist may be required to produce the desired effect. For example, in NMDA (n-methyl-D-aspartic acid) receptor activation, in addition to glutamate, glycine is required as a co-agonist.

Inverse agonist

These are ligands that bind to the same receptor binding site, but produce an effect that is opposite to that of the full or partial native ligand. Inverse agonists reduce the pharmacological response below the basal, resting level of activity, despite binding to the same receptor site as their full agonist counterpart. Although not common in natural ligand signaling pathways, an inverse agonist effect is sometimes the goal of neural and neuroendocrine drug design to generate either a positive or negative physiological response. An example of inverse agonist is prazosin (Cat. No. 504772), which that acts on α1-adrenergic receptor.

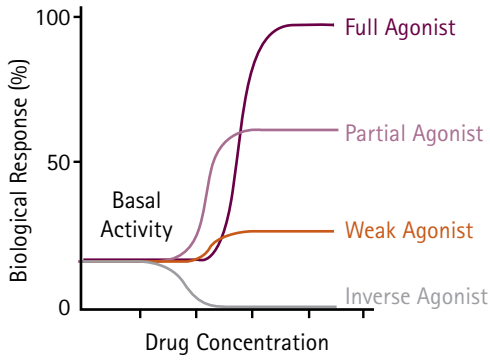


Figure 22.
Action of full, partial, weak, and inverse agonist.

2.4 Receptor antagonist

Antagonists are those compounds that bind to a receptor and prevent the effect of an agonist. Hence, they are also known as receptor blockers. An antagonist can bind to the receptor, but does not initiate a change in cellular function. Hence, they have the affinity, but no biological activity.

Antagonists can act by binding either to the same site as agonists or other unique sites, but they lack any intrinsic activity. Hence, their binding does not result in a biological response. Antagonist receptor binding can have similar kinetics to agonist binding, ranging from weak avidity to irreversible binding. However, most potent pharmacologically active receptor-binding molecules are reversible, display moderate avidity, are competitive with natural ligands, and are antagonistic in nature.

2.4.1 Competitive (reversible) antagonists

Competitive antagonists are those molecules that compete with an agonist for the same binding site, and their blocking effect can be reversed by increasing the amount of agonist. When high concentrations of an agonist results in occupation of all receptor sites, higher amounts of the antagonist will be required to obtain the same degree of occupancy. In functional assays using competitive antagonists, parallel rightward shifts of agonist dose–response curves are observed but with no alteration of the maximal response (Figure 23). The level of activity of the receptor is determined by the relative affinity of each molecule for the site and their relative concentrations.

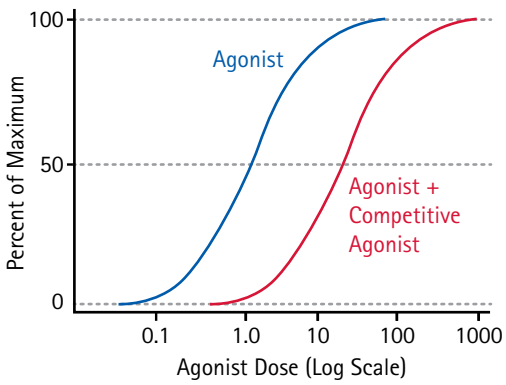


Figure 23.
Effect of a competitive antagonist.

2.4.2 Noncompetitive (irreversible) antagonists

Noncompetitive antagonists are reactive compounds that covalently bind to the receptor at a distinctly separate binding site from the agonist, in close proximity to the active site or to an allosteric site. They do not compete with the agonist for binding. Their binding is often irreversible and they prevent receptors from adopting any conformational change required for their activation. Their binding affinity is high and, at a given time, a few, most, or all receptors can be occupied by the antagonist. Their duration of action is independent of the rate of their elimination and is more dependent on the turnover rate of receptor molecules. Once the receptor is occupied, there is no need for any free antagonist to be present to block agonist responses.

Noncompetitive antagonists reduce the magnitude of the maximum response, which can be attained by any given amount of agonist, and their effects cannot be canceled. For this reason, these molecules are also known as nonsurmountable antagonists. In functional assays with noncompetitive antagonists, a depression of the maximal response of agonist dose-response curves is produced (Figure 24). An example of a noncompetitive antagonist is (+)-MK 801 Maleate (Cat. No. 475878), which irreversibly blocks the NMDA glutamate receptor.

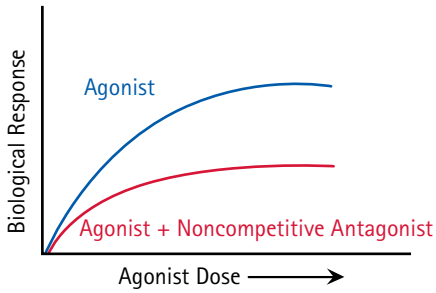


Figure 24.
Effect of a noncompetitive antagonist.

2.4.3 Equilibrium dissociation constant (K_d)

The equilibrium dissociation constant (K_d) is derived from the dose response curve and is defined as the molar concentration of the ligand (L) at which 50% of the receptors (R) are occupied in a ligand-receptor complex (R-L).

$$K_d = \frac{[\text{Receptor}] \times [\text{Ligand}]}{[\text{R–L complex}]}$$

The K_d is a measure of chemical binding and may or may not exactly correlate with the EC_{50} value (which defines pharmacological response), depending on receptor signaling mechanics (as discussed earlier). However, it gives a strong measure of the receptor's affinity to bind a drug molecule, with low K_d indicating high binding affinity and usually indicating a correspondingly low EC_{50} value.

It is important to pay attention to published K_d, EC_{50}/IC_{50} values or determine these values through experimentation. A drug with a picomolar (pM) or nanomolar (nM) dissociation constant binds more tightly to a particular receptor protein than a drug with micromolar (μM) or millimolar (mM) dissociation constant. Solution conditions, such as temperature, pH, ionic strength, can also affect K_d. Choosing appropriate dosing concentrations based on the specific experimental model used will reduce possible data misinterpretation.

2.4.4 Dose–response quantitation

To select a suitable molecule for experimentation or a drug for therapeutic use, the maximum pharmacological effect and maximum efficacy have to be determined. A drug's ability to initiate a pharmacological response depends on multiple factors, such as drug-receptor binding avidity, receptor type or subtype, nature of antagonist, dose and receptor downstream signaling effects. Thus a description of the dose-response relationship needs to be created empirically for proper use of small molecules for their *in vitro* and *in vivo* use. Response to an antagonistic molecule is often complex and nonlinear.

When creating a dose response curve, a wide (log) range of drug concentrations is typically administered and receptor/cellular/organism's response is measured. These response curves are usually sigmoidal in shape (Figure 25) and are derived from the following the equation, where E is the drug effect (response), E_{max} is the maximal response, and C is the drug concentration.

$$E = \frac{E_{max} \times C}{C + EC_{50}}$$

Some drugs may initiate weak responses and have lower efficacy at moderate concentrations. In Figure 25, drug A has the greatest pharmacological activity per dosing equivalent and is the most potent drug; A and C have equal efficacy, and although B is more potent than C, it has lower maximum efficacy. Drug B initiates a response at lower concentrations and has higher potency, despite its lower efficacy even at its highest concentration. Drugs A and C have the same maximum efficacy even though they vary in their potency. For therapeutic use, it is also important to distinguish between potency and efficacy. Potency of a molecule, in part, depends on its affinity for a receptor and efficacy depends on the ability of the drug molecule to reach the site of its action.

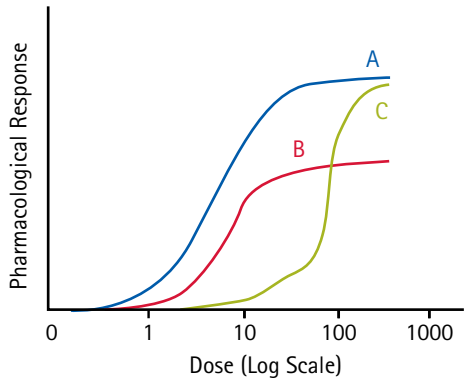


Figure 25.
Comparison of drug–response curves.

Dose response curves are key in determining the right concentrations of small molecule agonists or antagonists in experimental designs to restrict variation, aid in data interpretation, and minimize unnecessary repetitions and deaths of animals under study. Standard nonlinear regression models are used to plot the dose-response curve, measure potency and efficacy, and reveal potential ligand cooperativity.

Once the dose-response curve is generated, the desired concentration for experimentation is selected to produce maximum or partial effects and obtain EC_{50} data. A maximal effective concentration is typically chosen so the curve starts out flat before reaching 100% effect, thus minimizing unnecessary overdosing, which could cause prozone effect or collateral pharmacological responses. Keep in mind that the more potent the drug, the steeper the dose-response curve will be, and the greater will be the chance for under- or overdosing.

Most drugs are reported with K_d or EC_{50}/IC_{50} values, which allows a user to gauge the efficacy of the small molecule. However, it is best to generate a dose-response curve for the particular model system to correctly determine appropriate dosing.

2.4.5 Affinity vs intrinsic activity

Affinity describes the interaction, whether strong or weak, between a drug and the receptor. Higher affinity results in tighter binding. Intrinsic activity, also known as efficacy, describes the ultimate effectiveness of a drug molecule. Years of modeling ligand-receptor pharmacokinetics have shown that agonist or antagonist binding does not equate with functional, physiological or pharmacological response. Although potency, which depends on affinity, and intrinsic activity are closely linked, they may be influenced by variations in reaction time or efficiency, ability of the drug to reach the target site, metabolic disposal rate, etc.

A drug may have high affinity for a receptor and bind maximally even at relatively low concentrations, yet it may produce only a weak physiological or pharmacological response. Such drugs have high affinity but low efficacy. Two agents that interact with equivalent sets of receptors may not produce equal degrees of effect even if they are both administered in maximally effective doses; the agents differ in their intrinsic activities, and the one producing the greater maximum effect is said to have greater intrinsic activity.

The EC_{50} value is a measure of pharmacological response of the drug, because it is proportional to both affinity and efficacy. Since experimental conditions can affect ligand binding kinetics and organismal response, it is not surprising that all drug performance measures, like affinity, efficacy, and EC_{50}, are highly sensitive to assay variations.

2.4.6 Selectivity vs specificity

To be an effective drug or drug candidate, a compound has to offer both selectivity and specificity (Figure 26). Selectivity refers to a drug's ability to preferentially produce a particular effect. It is related to the structural specificity of drug binding to a specific type of receptor. For example, propranolol binds equally well to both β1- and β2-adrenoceptors, whereas metoprolol or atenolol binds selectively only to β1-adrenoceptors. Another common example is salbutamol, which is used to treat asthma. It binds selectively to β2-adrenoceptors and its selectivity is further enhanced by administering it directly to the lung tissue.

In comparison, high specificity indicates that a drug's action results in only a relatively narrow set of cellular responses or downstream signaling paths. Specificity of drug usually relates to the number of different effects a drug may elicit. If a drug has only one effect then it is considered to be specific. It also relates to the number of targets it could interact with or the number of cell populations it will affect. For example, yohimbine is used therapeutically as an α2-adrenoceptor blocker, but at higher concentrations it can also block 5-HT receptors, α1-adrenoceptors, monoamine oxidase, and cholinesterase. activity. Hence it is not considered a very specific drug. On the other hand salbutamol affects β2 receptors in the lung. Hence, it is considered as a specific drug.

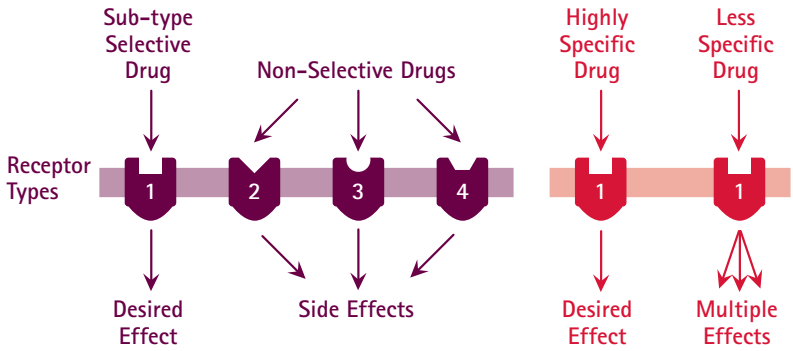


Figure 26.
Selectivity vs. specificity of drugs.

2.3.7 Selective vs nonselective antagonists

As the vast majority of drugs act as receptor antagonists, a brief discussion on the importance of their selectivity is important here. Let us think of ligand-receptor interactions in terms of achieving a single desirable pharmacological response. Centuries of drug design and manufacturing has demonstrated that only a very few drugs have one specific pharmacological effect.

The biological variation seen in macromolecular structure and environmental factors affecting ligand-receptor binding kinetics make it likely that any drug, no matter how carefully designed, will bind under some conditions to multiple targets. Even if a drug was designed to bind to only one receptor, that receptor could be expressed in multiple cell types in an organism, and consequently exhibit different downstream signaling effects. Thus, all antagonists can have qualitative descriptions of selectivity based on their "off-target effects".

A small molecule antagonist that interacts with few receptor or cell types, and has little concomitant pharmacological cross reactivity, is considered to be

selective. Nonselective antagonists have broader actions that may or may not be desirable in experimental or therapeutic settings. In addition, consideration has to be given to potential toxic side effects or otherwise interfering effects, which can make experimental control or interpretation more difficult. It is important to remember that the selectivity of a small molecule inhibitor is often significantly affected by the experimental dosage, because the dose-response curves for both the principle receptor target action and secondary target actions may be considerably different. Figure 27 describes the effects of selective and non-selective adrenergic antagonists.

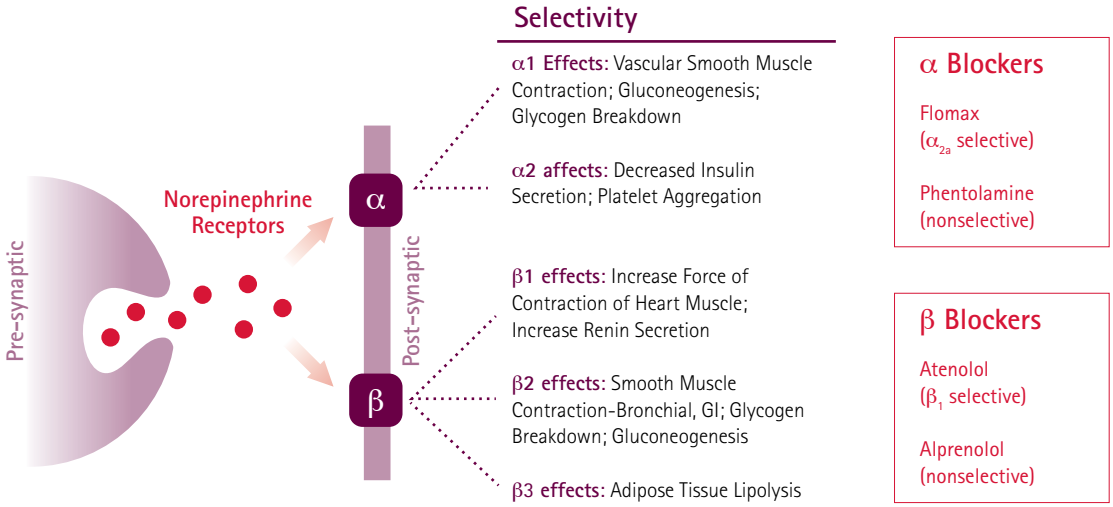


Figure 27.
Importance of antagonist selectivity for receptor subtypes.

2.4.8 ED_{50} and TD_{50}

In pharmacological terms, a drug is applied to a system and a resulting physiological or therapeutic response is measured as described by dose-response curves. From a biological perspective, then, one needs to consider what dose may be the most effective to a given percentage of the target population, as well as toxic and potentially lethal dose thresholds.

Thus, the ED_{50} is defined as the effective dose for 50% of the target population. This measure is specific to the individual experimental design and can be influenced by many chemical and biological factors previously discussed. ED_{50} values are therefore difficult to compare directly across model systems and one can expect some variation, even among experimental replicates.

Converting ED_{50} to specific activity units:

$$\frac{1 \times 10^6}{ED_{50}\ (ng/mL)} = \text{specific activity (units/mg)}$$

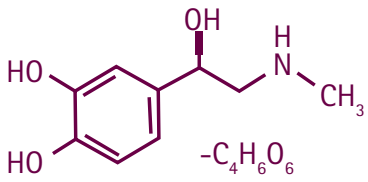
Despite the effect of experimental conditions on dosage effectiveness, ED_{50} values still allow some understanding of efficacy in model systems and serve as a guide to generating dose-response data relevant to the user's experiments.

ED_{50} values also are useful when considering what concentration ranges can be used that do not cause toxicity in the model system. To make that assessment, one needs to determine the median toxic dose data (TD_{50}) and perhaps median lethal dose data (LD_{50}), gathered in a similar fashion as is the ED_{50}. ED_{50} values that are relatively close to TD_{50} for a given system indicate that a narrow range of effective concentrations must be used or that the delivery method (e.g. oral, intravenous or interperitoneal) must be reconsidered. Once again, due to the variability inherent in many model biological systems, LD_{50} values (as with ED_{50}) can be surprisingly variable and unreliable across experimental sets. A low TD_{50} or LD_{50} should be of considerable concern when designing drug-related experiments.

EC_{50} in research: An example

Abstract from:
Shatoori, A. et al. 2012. African J. Pharm. Pharmacol. 6, 1092.

The purpose of this study was to determine the mean EC_{50} of adrenaline on heart rate and force of contraction of isolated New Zealand white rabbit hearts and to determine the concentration of atenolol that completely blocks the effect of adrenaline using one ofa modern physiological acquisition system, PowerLab AD instruments. Twelve Isolated hearts from New Zealand white rabbits were perfused through aorta in a Langendorff mode. Heart rate and contractility were determined for 5 minutes after bolus injection of 5 different concentrations of adrenaline (1.0, 2.5, 5.0, 7.5 and 10 µg/mL). The changes in heart rate and contractility after each treatment were compared with their baseline values. These data were used to calculate the mean EC_{50} of adrenaline on heart rate and force of contraction. This EC_{50} was then used after perfusion of different concentrations of atenolol (1.0, 2.5, 5.0 and 10 µg/mL). Data were collected with the help of PowerLab data acquisition and analyzed by Labchart pro7 software. Adrenaline resulted in a stimulatory effect on the heart rate and the amplitude of the heart contraction. The maximum increases in both heart rate and force of contraction were seen at adrenaline dose of 7.5 µg/mL and the plateau phase was achieved at a dose of 10 µg/mL. The average EC_{50} of adrenaline was 3.5 µg/mL. The positive inotropic effect of adrenaline was antagonized only at atenolol concentrations of 5.0 and 10 µg/mL and complete inhibition of adrenaline effect on heart rate was achieved at atenolol concentrations of 10 µg/mL. These data showed that atenolol must be used at a concentration no less than 7.5 µg/mL to demonstrate if β adrenergic receptors are involved in the mechanism of action of any newly tested positive inotropic or choronotroic drug.



324900 L-(-)-Epinephrine-(+)-bitartrate
1-(3,4-Dihydroxyphenyl)-2-(methylamino) ethanol, Adrenaline Bitartrate

2.4.9 Selected examples of receptor antagonists as drugs

Atropine: Acts as a central and peripheral ACh muscarinic receptor blocker

Phentolamine: α1 and α2 adrenergic receptor blocker

Prazosin: α1-specific adrenergic blocker

Atenolol: β1-specific adrenergic blocker

Naloxone: Opioid receptor antagonist; fast-acting competitive inhibitor of m, k, and s receptors

Candesartan: Angiotensin II type I receptor blocker

Cyclothiazide: A noncompetitive antagonist of metabotropic glutamate receptor 1

Memantine: Uncompetitive antagonist of NMDA glutamate receptor

3

How to use inhibitors: A technical guide

3.1 How to select the right inhibitor

- Determine what molecule(s) is (are) important for disruption of function.
- Determine if a broad spectrum inhibitor or specific inhibitor is suitable for the particular experiment.
- Look at other enzymes potentially inhibited and concentrations at which they are inhibited. For example, H-89 is a good inhibitor for protein kinase A (IC_{50} = 48 nM). However, at much higher concentrations it will also inhibit protein kinase C (IC_{50} =31.7 µM); myosin light chain kinase (K_i = 28.3 µM); Ca^{2+}/calmodulin-dependent protein kinase II (K_i = 29.7 µM); and casein kinase I (K_i = 38.3 µM).
- Consider the IC_{50}. Lower IC_{50} = more potent inhibitor. Is toxicity a concern?
- Determine if a cell-based inhibitor is needed or not. Cell permeability is the primary consideration for cell-based inhibitor studies.
- Determine if the inhibitor should be reversible or not. Cell permeability and reversibility can limit the selection of inhibitors.
- Determine if mechanism of action is of concern—competitive, noncompetitive etc. It may be more important to first understand the mechanism of selected inhibitor.
- How pure is the inhibitor? Purity of 98% by HPLC is much better than 98% purity by TLC.
- Check to see if the reconstitution solvent is compatible with the assay. Most cells will tolerate up to 1% dimethylsulfoxide (DMSO). Solvents such as methanol, acetonitrile, dimethylformamide (DMF) are toxic to cells and will affect their viability.
- Check the vial label to see if it should be protected from light or moisture. In some cases, exposure to light or moisture may damage the molecule.
- Use Merck Millipore's Inhibitor SourceBook™ to locate the appropriate list of inhibitors or contact your local technical service scientist for recommendations.

Here are a few examples relating to selection of inhibitors in various common applications. These are only a few examples, and for your specific experimental models, please contact our technical service scientists or refer to suitable published literature.

An example for selection of a protein kinase C inhibitor:

If one wants to reversibly inhibit a wide range of phosphorylation events affecting serine/threonine residues in live cells, then a broad range, cell-permeable inhibitor, Staurosporine (Cat. No. 569397) can be used. Staurosporine inhibits most of the known serine/threonine protein kinases.

Let us assume that one wants to inhibit all isozymes of protein kinase C in live cells. Then a PKC-specific, cell-permeable inhibitor, such as Bisindolylmaleimide (Cat. No. 203290) should be selected. It has a very low IC_{50} value (low nM range) and will inhibit all PKC isozymes.

In the next step, if one wants to inhibit only the Ca^{2+}-dependent isoforms of PKC in live cells, then Gö6976 (Cat. No. 365250) can be used. It has very low IC_{50} values (2 to 10 nM) for PKC α, β, and γ isozymes and it does not affect non-Ca^{2+}-dependent PKC isozymes.

What criteria should I use when selecting a protease inhibitor?

When processing cells or tissues, one must assume that active proteases are present in the medium or are being secreted. Hence, it is important to include protease inhibitors even in the early steps of sample preparation. For best results, add protease inhibitors to the medium just prior to harvesting. Use of inhibitors in buffers stored over a period of time is not recommended. Different cells and tissue types exhibit different protease profiles. Serine proteases are widely distributed in all

cells, bacterial cells contain higher levels of serine and metalloproteases; animal tissue extracts are rich in serine-, cysteine-, and metalloproteases, and plant extracts contain higher quantities of serine and cysteine proteases. If you are not sure of the type of proteases present in the sample, it is best to use an inhibitor cocktail available from Merck Millipore or customize your own cocktails.

Nice to know

Protease inhibitors classified by their target class:

Protease type	Inhibitor examples
Aspartyl proteases	Acetyl-pepstatin Pepstatin
Cysteine proteases	Antipain Calpastatin Calpeptin Cathepsin inhibitors Chymostatin Cystatin Leupeptin
Metalloproteases	1,10-phenanthroline Bestatin EDTA/EGTA Phosphoramidon
Serine proteases	AEBSF Antichymotrypsin Antipain Antithrombin Antitrypsin Aprotinin Benzamidine Bovine pancreatic trypsin inhibitor (BPTI) DFP Ecotin Leupeptin PMSF TLCK TPCK

How can I determine if a caspase inhibitor is reversible or irreversible?

The C-terminal group determines the reversibility or the irreversibility of any caspase inhibitor. In general, caspase inhibitors with an aldehyde (CHO) group are reversible. The CMK, FMK, and FAOM groups are more reactive and form covalent bonds with the enzyme, creating an irreversible linkage. FMK is slightly less reactive than CMK and therefore is considered more specific for the enzyme site being inhibited.

What are the advantages of using FMK-based caspase inhibitors and how do they differ from CHO-based inhibitors?

The FMK-based caspase inhibitors are cell-permeable because the carboxyl group of aspartic or glutamic acid is esterified. This makes them more hydrophobic. These inhibitors covalently modify the thiol group of the enzyme, making them irreversible inhibitors. Generally, at the amine end of the inhibitor, we have a Z, biotin, or Ac group. These groups also increase hydrophobicity of the molecule, which makes them more cell-permeable. Compared to the inhibitors with an Ac or a biotin group, those inhibitors with a Z group are even more cell-permeable. Inhibitors with a biotin group can serve as a detection tool and are useful in tagging the enzyme's inhibitor binding site.

The CHO-based inhibitors are reversible because the thiol group of the enzyme forms a reversible adduct to the carbonyl group of the aldehyde. As a general rule, CHO-based inhibitors are hydrated and hence are slow-binding. The extent of their reversibility depends on the pH, metal ion concentration, and other conditions. When the aldehyde group is attached to the aspartic acid (D-CHO), the product exists as a pseudo acid aldehyde in equilibrium. This makes the inhibitor somewhat cell-permeable.

3.2 Planning experiments with inhibitors

To quantify enzyme efficiency biochemists perform a series of experiments to monitor initial reaction rate and determine V_{max} of the reaction. In order to reduce the rate of reaction and obtain reliable measurements, one must use reversible or irreversible inhibitors, as discussed on preceding pages. Below are a few recommendations to follow when using inhibitors in a biological or biochemical assay.

Watch Out

You must be decide on your sample preparation method (whole cell, lysate, tissue homogenate, etc.), inhibitor permeability, and potential sensitivity of your sample to toxicity before you plan your experiments. See section above for more details!

- The precise time of inhibitor addition and the duration of treatment are important in the interpretation of data. Inhibitor can be added at time zero along with substrate and enzyme (Figure 28A); it can also be added to an ongoing enzyme-substrate reaction (Figure 28B). Enzyme can also be preincubated with inhibitor for a designated period of time prior to the addition of substrate (Figure 28C). Theoretically, if the enzyme is totally inhibited, no product should be generated. Each of these reaction protocols will give different results and have to be interpreted differently. For any comparison, data should be derived under identical conditions.
- For example, if an inhibitor is added to an ongoing reaction (Figure 28B), the reaction rate will decline. However, some substrate has already been converted to end product. These results cannot be compared to experiment where inhibitor is added at time zero. In the latter case, much less product will be formed than if the inhibitor is added to an ongoing reaction.
- To compensate for nonspecific effects of the inhibitor, it is essential to run a control reaction adding only the solvent used for inhibitor reconstitution. For example, if an inhibitor is dissolved in DMSO, then, with each set of experiments, a DMSO control should be run simultaneously.

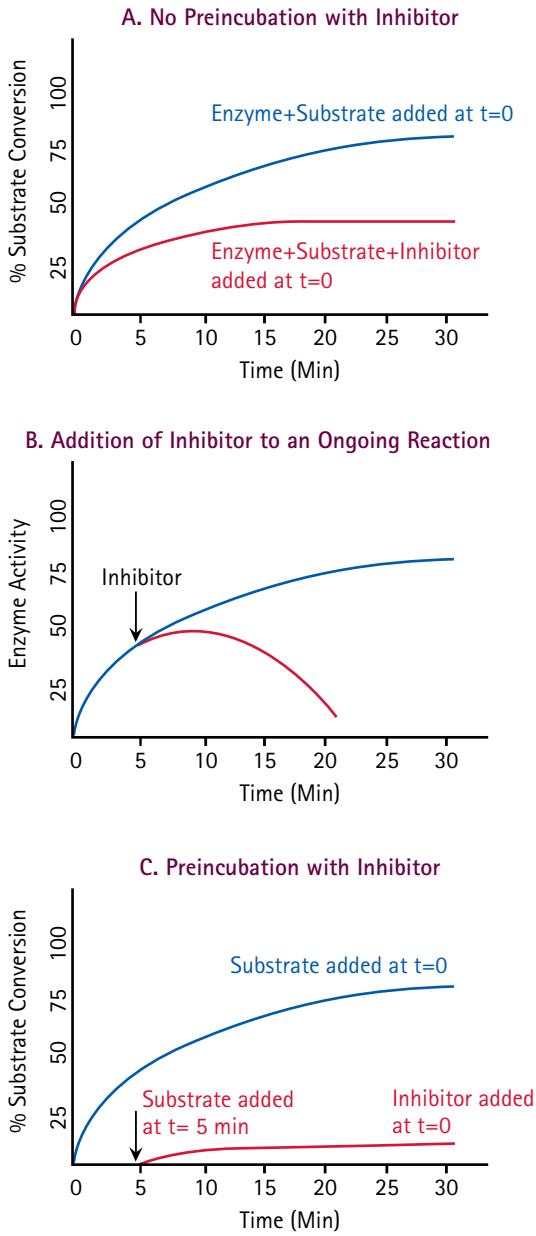


Figure 28.
Effect of timing of inhibitor addition on enzyme activity.

- When multiple samples are run and data are used for comparison, it is important to terminate the reaction at the same time points. For example, if 10 samples are run simultaneously and the incubation period is 10 minute for each sample, it is important to time the reaction in a way that each sample is exposed to inhibitor for exactly 10 minutes. Additional incubation of just one minute may add 10% error to the data. Exact timing is particularly critical with shorter incubations and when studying the time course of any reaction.

- It is possible that, over the course of an experiment, particularly when the incubation period is long, the pH of a weak reaction buffer may change. Hence, it is best to select a buffer with optimum strength to overcome minor changes in pH.
- It is possible that enzyme and reaction product can undergo degradation over time. Hence, extrapolating data may not be appropriate. For example, the outcome of reaction after 60 minutes cannot be deduced from the results of a 5 or 10 minute incubation. In a time course experiment, it is best to run parallel experiments for each time point or pipette out small volumes of the reaction mixture at multiple time points.
- If experiments are being conducted on tissue homogenates or cell lysates, then release of nonspecific proteases can destroy part of the enzyme. This will add experimental error because the amount of active enzyme in the initial phase of reaction (or at early time points) will be greater when compared to later time points. Hence, it is best to add an appropriate protease inhibitor cocktail to the reaction mixture to avoid any nonspecific protease activity. Merck Millipore's ready-to-use Protease Inhibitor Cocktail III (Cat. No. 539134) is highly recommended for use with mammalian cells and tissues.
- To normalize results of an enzyme reaction to a specific amount of protein, it is best to set aside a small volume of sample for protein analysis. Then calculate the reaction as µg or mg substrate converted or product formed per mg protein per unit time.
- The method used to terminate a reaction should be given some consideration, depending on how the reaction product is analyzed. In most cases, perchloric acid (PCA) or trichloroacetic acid can be used. However, in some cases, these acids will interfere with further sample analysis and it may be necessary to neutralize these samples. However, neutralizing with an alkali will generate more salt, which must be removed by an appropriate method. When conducting experiments with substrate labeled with a radioisotope, reaction can be slowed down by adding excessive amount of "cold" non-radioactive substrate, which will reduce the incorporation of radioactivity into the end product. It is best to process samples as soon as possible or freeze them (-20 or -70°C) for later analysis.

How much inhibitor to use?

The amount of inhibitor required depends on various factors, such as accessibility, cell permeability, duration of incubation, type of cells used etc., It is best to survey the literature to determine the initial concentration. If published K_i or IC_{50} values are known, then use 5 to 10 times higher than these values to completely inhibit enzyme activity. If K_i or IC_{50} values are unknown, then try a wide range of inhibitor concentrations and use Michaelis-Menten kinetics to determine the K_i value. When 20-fold or higher concentrations of inhibitors are used, it is not unusual to see either no inhibitory effect or a reverse effect. Always run an appropriate control to compensate for nonspecific effects of solvent used to solubilize the inhibitor.

What is the difference between EC_{50}, ED_{50}, K_i, IC_{50}, and K_d?

In pharmacology and biochemistry, in order to determine the efficacy of a drug or inhibitor, the following terms are commonly used. Sometimes, confusion arises when researchers try to repeat experiments without considering the exact term used by the original investigators.

EC_{50}: Clinical efficacy of a drug expressed as the concentration required to produce 50% of the maximum effect (may be inhibitory or stimulatory effect). This term is usually used with pharmaceuticals.

ED_{50}: Median effective dose (as opposed to concentration) at which 50% of individuals exhibit the specified quantal effect.

IC_{50}: Concentration required to produce 50% inhibition.

K_i: Inhibitor concentration at which 50% inhibition is observed (it is calculated using Michaelis-Menten kinetics).

K_d: An equilibrium constant for the dissociation of a complex of two or more molecules into its components; for example, dissociation of a substrate from an enzyme.

Nice to know

In pharmacology and biochemistry, in order to determine the efficacy of a drug or inhibitor, K_i, IC_{50}, EC_{50} etc are commonly used. Sometimes, confusion arises when researchers try to repeat experiments without considering the exact term used by the original investigators. Information gathered from clinical trials may not directly correlate with *in vitro* applications. Sometimes the working concentration in cultured cells or dose injection into laboratory animals has to be determined empirically.

Technology Highlight

Protease Inhibitor Cocktail Design

Protease inhibitor cocktails are important tools for scientists engaged in a variety of proteomics studies, including protein characterization, biomarker discovery, mapping of post-translational modifications, protein expression profiling, and the quantitative measurement of proteins. The addition of a suitable protease inhibitor cocktail or protease inhibitor ensures the integrity of proteins for downstream analysis and further characterization of samples.

Merck Millipore inhibitor cocktails are available in a variety of formulations and formats to ensure the best results on a wide range of starting materials and applications. For example, inhibitor cocktails are available without EDTA for purification schemes involving metal ion-chelating chromatography or analysis using two dimensional (2D) gel electrophoresis. In select formulations, recombinant aprotinin is included for applications that require the use of animal-free reagents. The table below summarizes the components and application specificity of each protease inhibitor cocktail.

Product	Cat. No.	Recommended For	Components Included
Protease Inhibitor Cocktail Set I	539131	General use; broad specificity	AEBSF, HCl; Aprotinin, Bovine Lung; E-64 Protease Inhibitor; EDTA, Disodium; Leupeptin, Hemisulfate
Protease Inhibitor Cocktail Set I, Animal-Free	535142	General use; broad specificity (animal-free)	AEBSF, HCl; Aprotinin, Bovine, Recombinant; E-64 Protease Inhibitor; EDTA, Disodium; Leupeptin, Hemisulfate
Protease Inhibitor Cocktail Set II	539132	Bacterial cell extracts	AEBSF, HCl; Bestatin; E-64 Protease Inhibitor; EDTA, Disodium; Pepstatin A
Protease Inhibitor Cocktail Set III, EDTA-Free	539134	Mammalian cells and tissue extracts, immobilized metal affinity chromatography	AEBSF, HCl; Aprotinin, Bovine Lung; Bestatin; E-64 Protease Inhibitor; EDTA, Disodium; Leupeptin, Hemisulfate; Pepstatin A
Protease Inhibitor Cocktail Set III, Animal-Free	535140	Mammalian cells and tissue extracts (animal-free)	AEBSF, HCl; Aprotinin, Bovine, Recombinant; Bestatin; E-64 Protease Inhibitor; EDTA, Disodium; Leupeptin, Hemisulfate; Pepstatin A
Protease Inhibitor Cocktail Set IV	539136	Fungal and yeast cell extracts	AEBSF, HCl; E-64 Protease Inhibitor; Pepstatin A; o-Phenanthroline
Protease Inhibitor Cocktail Set V, EDTA Free	539137	Mammalian cells and tissue extracts	AEBSF, HCl; Aprotinin, Bovine Lung; E-64 Protease Inhibitor; Leupeptin, Hemisulfate
Protease Inhibitor Cocktail Set V, Animal-Free	535141	Mammalian cells and tissue extracts	AEBSF, HCl; Aprotinin, Bovine, Recombinant; E-64 Protease Inhibitor; Leupeptin, Hemisulfate
Protease Inhibitor Cocktail Set VI	539133	Plant cell extracts	AEBSF, HCl; Bestatin; E-64 Protease Inhibitor; Leupeptin, Hemisulfate; o-Phenanthroline; Pepstatin A
Protease Inhibitor Cocktail Set VII	539138	Purification of proteins containing His•Tag® sequences	AEBSF, HCl; Bestatin; E-64 Protease Inhibitor; Pepstatin A; Phosphoramidon
Protease Inhibitor Cocktail Set VIII	539129	Broad range cysteine protease inhibition	ALLN; Cathepsin Inhibitor I; E-64 Protease Inhibitor
Serine Protease Inhibitor Cocktail Set I	565000	Broad range serine protease inhibition	AEBSF, HCl; Aprotinin, Bovine Lung; Elastatinal; GGACK

3.3 Preparing your inhibitors

When reconstituting inhibitors in a solvent, short-term and long-term storage and stability should be considered. Some inhibitors may lose their potency quickly in aqueous solutions. In general, most organic molecules, but not all, can be dissolved and stored in dimethylsulfoxide (DMSO). However, always check the product data sheet or contact our technical service scientists for more information. Here are a few steps to consider:

What type of solvent is best suited for dissolving a compound?

In biological experiments, water is the most preferred solvent. However, several organic compounds are either insoluble in water or they degrade rapidly in the presence of moisture. If DMSO is a recommended solvent, it is best to use a fresh stock bottle of DMSO that is deemed free of any moisture. Any contaminating moisture may accelerate the degradation of compound in question or may render it insoluble.

Why can't I make serial dilutions of my DMSO stock solution directly in my buffer?

In some cases, this may not be a problem. However, in most cases, the organic material will precipitate out of the solution. It is best to make the initial serial dilutions only in DMSO and then add the final diluted sample to your buffer or the incubation medium. The compound may be soluble in aqueous medium only at its working concentration.

How can I prevent my inhibitor in DMSO from precipitating in aqueous medium?

Some organic products dissolved in DMSO may fall out of solution when added directly to an aqueous solution, such as buffer or cell culture medium. To avoid this, one should dilute the concentrated stock solution further in DMSO before adding to the aqueous medium. Most cells are able to tolerate up to 0.1% final DMSO concentration. Of course, a control with DMSO alone should always be included in the experiment.

What precautions should be taken during peptide solubilization?

Most peptides, when stored at -20°C, will remain stable for several years. When you are ready to use the peptide, first bring the vial to room temperature in a desiccator. Peptides containing cysteine, methionine, and tryptophan may require special precautions to avoid any oxidation. Peptides should be dissolved in distilled water, dilute acetic acid, or other appropriate solvent stored in a tightly sealed bottle. Most peptides have a limited life in solution and long-term storage should be avoided. Buffer or saline should be added only after the peptide is fully in solution. If complete solubilization is not achieved, solution can be mildly sonicated. Solutions should be aliquoted and stored in the pH range of 5 - 7 at -20°C. Any unused portion of the thawed aliquot should be discarded.

What precautions should be taken during peptide quantitation?

Determining peptide concentrations accurately and quickly can be difficult. Most commonly used methods for peptide quantitation rely on the weight of the lyophilized powder, absorbance of ultraviolet (UV) light or amino acid analysis. Establishing peptide concentration based on the weight of the lyophilized peptide is inaccurate in most cases, because the analyzed powder can contain a significant quantity (10-70%) of bound water, salts or counterions.

Another peptide quantitation method relies on absorbance at 280 nm, and thus can only be used to estimate peptide concentration if tryptophan and tyrosine resides are present in the sequence. Therefore, peptides that do not contain amino acids that absorb light at 280 nm cannot be accurately quantified using this method. While it is possible to determine peptide concentration by measuring absorbance at 205 nm, this measurement is far more sensitive to variations in sample composition, since many solvents and other chemicals will absorb at this wavelength.

Finally, amino acid analysis, recognized as a gold standard in peptide quantitation, delivers possibly the most accurate peptide quantitation; however, it is expensive and requires time-consuming sample manipulation along with specialized equipment.

The Direct Detect® FTIR-based spectrometer (Cat. No. DDHW00010-WW) provides a universal, fast and accurate peptide quantitation method that does not require sample manipulation and only requires single-time calibration curve generation. IR spectroscopy exploits the fact that molecules absorb specific frequencies characteristic of their structure. In order to determine protein and peptide concentration, the Direct Detect® spectrometer uses the intensity of the

Amide I band, which is assigned to C=O stretching vibration of the peptide bond (about 80%) with a minor contribution from C-N stretching vibration (about 20%). The measurement, which requires only 2 µL of peptide sample, is precise and accurate, matching the results of amino acid analysis (Figure 29).

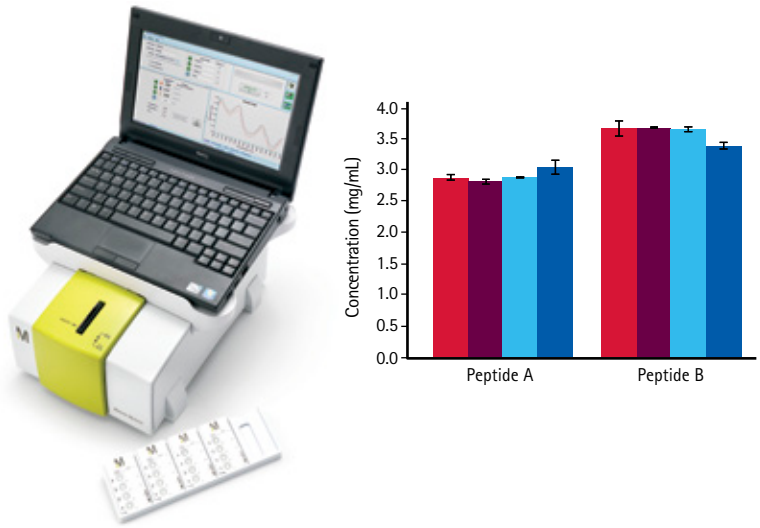


Figure 29.

The Direct Detect® Spectrometer (left) provides universally accurate peptide quantitation that matches the results of amino acid analysis (right). Two peptides were quantitated using the Direct Detect® spectrometer with both protein- and peptide-based calibration and compared to amino acid analysis (labeled AAA).

Why are some small molecules shipped at room temperature when the vial is labeled as 'Refrigerate' or 'Freeze"?

Storage in the refrigerator or freezer is recommended for long-term stability of the product. If the material is shipped at ambient temperature it is considered to be stable for the duration of shipping and normal handling. Upon arrival, one should store it in refrigerator or freezer (as indicated on the label).

How can one calculate concentration by spectrophotometric measurements?

Sometimes it is critical to establish the exact concentration of a molecule in solution. The following example will be helpful not only in determining the concentration, but also the purity of molecules.

The relationship of absorbance to concentration is given by Beer's law,

$$A = abc$$

where:

A = absorbance

a = a proportionality constant defined as absorptivity

b = light path in cm

c = concentration of the absorbing compound

When b is 1 cm and c is expressed in moles/liter, the symbol a is substituted by the symbol ε (epsilon). ε is a constant for a given compound at a given wavelength under prescribed conditions of solvent, temperature, and pH, and is referred to as molar absorptivity. ε is also used to characterize compounds and establish their purity.

Example:

The molar absorptivity (ε) of bilirubin (Mol. Wt. = 584) dissolved in chloroform at 25°C is 60,700.

Hence, 5 mg/L (0.005 g/L) of 100% pure bilirubin analyzed in a 1 cm cuvette should have an absorbance of A = (60,700)(1)(0.005/584) = 0.52 {A = abc} Therefore, a solution of 5 mg/L showing absorbance of 0.49 should have a purity of 94% (0.49/0.52 x 100).

In most biochemical and toxicological work, it is customary to list constants based on concentrations in g/dL rather than mol/L. This is also common when the molecular weight of the substance is not precisely known.

Here, **b = 1 cm; and c = 1 g/dL (1%), and A is written as** $A^{1\%}_{cm}$
This constant is known as absorption coefficient.

The direct proportionality between absorbance and concentration must be established experimentally for a given instrument under specified conditions. Frequently there is a linear relationship up to a certain concentration. Within these limitations, a calibration constant (K) may be derived as follows: **A = abc**. Therefore, **c = A/ab = A x 1/ab**. The absorptivity (a) and light path (b) remain constant in a given method of analysis. Hence, 1/ab can be replaced by a constant (K).

Then, c = A x K and therefore K = c/A. The value of the constant K is thus obtained by measuring the absorbance (A) of a standard of known concentration (c).

3.4 *In vivo* use of inhibitors

The route of inhibitor administration is dependent on its properties and objective of the experiment. Two major routes of inhibitor administration are (a) enteral, which includes oral, sublingual, and rectal and (b) parenteral, which includes intravenous, intraperitoneal, intramuscular and subcutaneous. Sometimes topical and transdermal methods are also used. Oral administration is the most common route for therapeutic use; however, it is also the most complicated pathway for the drug to reach tissues. Parenteral administration is used for those inhibitors that are poorly absorbed from the gastrointestinal tract.

For small animals, due to practical difficulties in oral administration, the intravenous route is the most commonly used method. When injecting an inhibitor into an animal, one important question comes to mind: how much of an inhibitor to inject? There is no simple answer to this question. One must optimize the dose empirically by performing a few preliminary experiments. First, determine if the compound in question is cell-permeable. Also, survey the literature for any reported IC_{50}, ED_{50}, or EC_{50} values. One may follow the sample calculation given below as a general guide:

- H-89, dihydrochloride, a cell-permeable protein kinase A inhibitor, has an IC_{50} of 48 nM. It has a molecular weight of 519.3. If we follow dosing guidelines, then 5 to 10 times the IC_{50} value should be ideal for inhibition. Hence, for H-89, dihydrochloride, the 240 to 480 nM range is sufficient to cause maximal inactivation of protein kinase A. To use it *in vivo* we have to make a few assumptions. Let us assume that we want to inject an inhibitor into a rat weighing about 200 g. We also assume that 70% of its body weight is water; the volume of distribution will be approximately 140 mL. In this case, 240 nM = 240 nmoles/L = 124.63 µg/L. Because the volume of distribution is about 140 mL, 124.63 x 0.140 = 17.45 µg would be the required amount for injection into the rat.

It is important to note that the drug distribution will vary depending on the mode of injection (intravenous, intramuscular, or intraperitoneal), bioavailability, half-life, rates of hepatic and renal clearance, binding to proteins, and tissue-specific distribution and accumulation. The specific tissue uptake may also be limited in whole organs or tissues as compared to isolated cell preparations. In whole animal studies, sometimes a loading dose is required to achieve the target concentration. This may then be followed by a sustained infusion to maintain the drug level in the blood. One must always exercise caution and not overdose the animal.

Here some basic issues to consider when injecting an inhibitor/drug into an animal.

Is the compound cell-permeable?

If yes, proceed with your experiment.

What is the reported or calculated IC_{50} or K_i values?

If K_i, IC_{50}, or EC_{50} values are known, then follow the steps below. Otherwise, search literature or experimentally determine IC_{50} or EC_{50} value.

What is the volume of distribution?

This is an approximate value based on the body weight of the animal. Newborn and younger animals have greater volumes of distribution.

Inhibitor use will also be affected by:

Mode of action:

It is the specific biochemical interaction through which a drug/inhibitor produces its pharmacological effect. A drug may inhibit an enzyme or block a receptor. It could be a reversible effect or irreversible effect. If the drug and substrate are competing, then the excess availability of substrate may reduce the effect of drug.

Bioavailability:

Not everything presented to a cell or an organism is used. For example, if a drug/inhibitor is injected into the bloodstream via injection, most of it is available to the body. However, when it is given orally, some of it may be destroyed by the acid of stomach or alkalinity of intestine. Or, due to physical barriers in the digestive system, only a fraction of it may reach the site of action.

Half-life:

This is the time frame in which 50% of the drug/inhibitor is destroyed in the cell or in the body. A shorter half-life means that you have to administer it more frequently, because it may be destroyed in the liver as it circulates through. Half-life can give a good indication of the time required to reach steady state after a dosing regimen is initiated.

Loading dose (Priming dose):
One or more doses given initially to rapidly achieve target concentration. However, exercise caution that the loading dose does not cause toxicity.

Inhibitor structure:
The chemical nature of the inhibitor strongly influences its ability to cross membranes. Hydrophobic molecules with no net charge readily cross most biological membranes. By contrast, hydrophilic drugs that have a nonuniform electron distribution or have a positive or negative charge do not cross membranes.

Hepatic and renal clearance:
Clearance tells us how quickly or slowly the drug/inhibitor is being metabolized in liver. If the drug is rapidly metabolized by the liver, the amount of drug available for systemic circulation is significantly decreased. If the liver enzymes that metabolize a drug are already activated, then the drug will clear out faster and may not generate its full biological or clinical effects. For example, prior consumption of alcohol will activate the liver enzymes that will cause a faster clearance of some drugs through the system. This will reduce the effectiveness of drug or, in some cases, may cause toxic effects. Similarly, the renal clearance is the volume of plasma from which a substance is completely removed by the kidney in a given amount of time. This may also be affected by drugs that affect glomerular filtration rates.

Binding to proteins:
When circulating in the bloodstream, several drugs/inhibitors can be bound to albumin proteins in the blood, which carry them to the liver for metabolism and clearance. However, if the binding is too tight and the inhibitor or drug is not easily unloaded for further metabolism, then the effect could be delayed. Albumin has strong affinity for anionic and hydrophobic inhibitors. Most neutral and hydrophilic molecules do not bind to albumin.

Tissue-specific distribution:
Most drugs/inhibitors exert their effects not within the plasma compartment, but in defined target tissues into which drugs have to be distributed from the central compartment (blood). Target site drug levels may substantially differ from corresponding plasma levels and a 100% equilibration between blood and tissue cannot always be assumed. Drug distribution processes may be characterized by a high inter-tissue variability. Suboptimal target site concentrations may also have important clinical implications and may indicate therapeutic failure in some cases. Therefore, determination of drug tissue penetration plays an important role in clinical drug development.

Drug/Inhibitor toxicity:
Therapeutic benefits of a drug usually outweigh its risks. However, all drugs are likely to have some side effects. Drug/inhibitor toxicity results when too much of it has accumulated in the bloodstream. Toxicity may result when the dose is too high or when the liver or kidneys are unable to remove the drug from the bloodstream in an effective manner.

Nice to know

When injecting a substance into the tail vein of a small animal (e.g., rat) it is best to submerge the tail in slightly warm water (37°C) for a minute and then wipe with ethanol or isopropyl alcohol. This will cause blood vessel to swell and become visible. Use a smaller gauge needle (e.g., 22-gauge needle) and inject with a slight pressure.

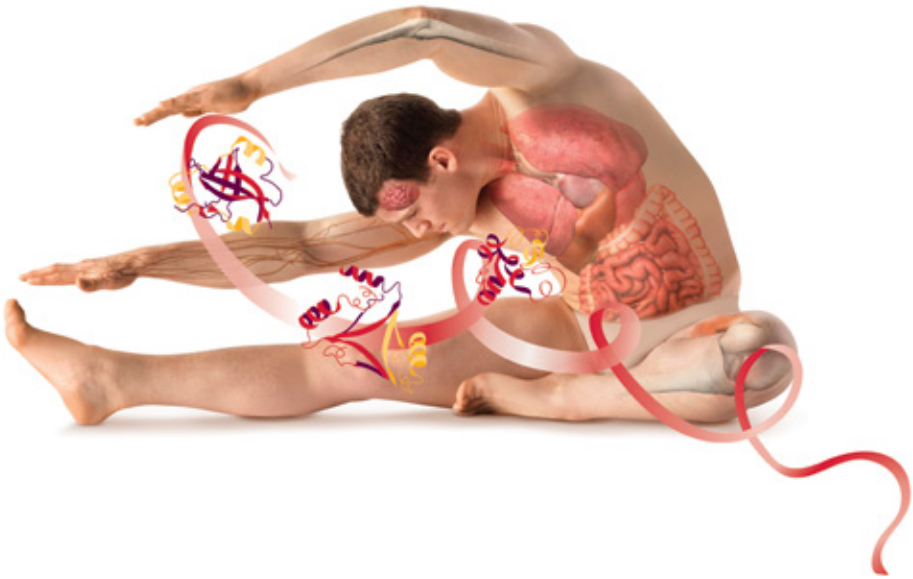
Technology Highlight

MILLIPLEX® MAP Multiplexed Bead-based Detection to Assess Effects of Drug Treatment

Bead-based multiplex immunoassays enable precise, multiparametric analysis of the concurrent processes that take place in response to treatment with small molecule inhibitors. Quantitating multiple biomarkers simultaneously is particularly important to characterize complex effects of inhibitors in clinical or preclinical research samples, where sample volume is limited.

Bring your biomarkers to life with MILLIPLEX® MAP assay kits.

- Based on trusted Luminex® xMAP® technology and 25 years of experience.
- The broadest selection of analytes across a wide range of pathways and disease states, including cellular metabolism, metabolic disease, immunology, neurological disorders, toxicity, intracellular signaling, cancer and more.
- All the components and reagents you need to detect multiple analytes simultaneously in a small sample volume (10-50 µL)—all in a single kit, using a single catalogue number.
- Select a premixed kit or choose analytes within a panel to design a custom kit.
- Quality controls provided to qualify assay performance.
- Analytically validated panels that yield consistent analyte profiles irrespective of plex size.
- Standardized standard curve ranges across analytes and lots to ensure lot-to-lot consistency.
- Panels meet stringent manufacturing criteria to ensure batch-to-batch reproducibility.

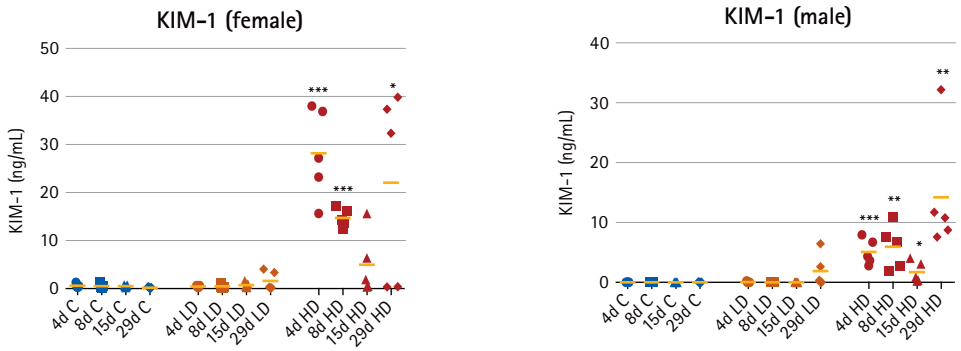


Featured Kits

MILLIPLEX® MAP Rat Kidney Toxicity Magnetic Bead Panels 1 and 2

The kidney is uniquely susceptible to toxicant injury due to several factors, including high blood flow and concentrating mechanisms. The traditional tests to determine kidney toxicity are blood urea nitrogen (BUN) and serum creatinine (SCr), which are not optimal for early detection or localization of kidney damage. As a result, researchers have turned to the recently PSTC-qualified nephrotoxicity biomarkers for more predictive toxicity analysis. Expanding on this set of markers, Merck Millipore developed the MILLIPLEX® MAP Rat Kidney Toxicity Magnetic Bead Panels 1 and 2, containing 14 kidney toxicity biomarkers.

These panels were used to profile response to gentamicin, an aminoglycoside antibiotic and a known nephrotoxicant in rats. By simply measuring biomarker concentrations in urine, kidney toxicity (marked by increased clusterin and KIM-1 levels) was detected in as few as three days.



Kidney toxicity biomarker KIM-1 is elevated in urine samples of rats injected with high doses of vancomycin. Wistar Han Rats, 5 female, 5 male, for each study group, were injected with vancomycin once daily for 28 days. C=Control (0.9% NaCl solution). Low dose (LD)=50 mg/kg vancomycin. High dose (HD)=300 mg/kg vancomycin. KIM-1 levels in urine samples were determined using MILLIPLEX® MAP kidney toxicity panels. High dose groups showed significantly higher KIM-1 levels compared to controls at 4, 8 and 29 days for females, and at 4, 8, 15, and 29 days for males. (data courtesy of EMD Serono) Statistical significance was determined by ANOVA + Dunnett-Test; *p<0.05, **p<0.01, ***p<0.001.

Components	Cat. No.
MILLIPLEX® MAP Rat Kidney Toxicity Magnetic Bead Panel 1	RKTX1MAG-37K
MILLIPLEX® MAP Rat Kidney Toxicity Magnetic Bead Panel 2	RKTX2MAG-37K

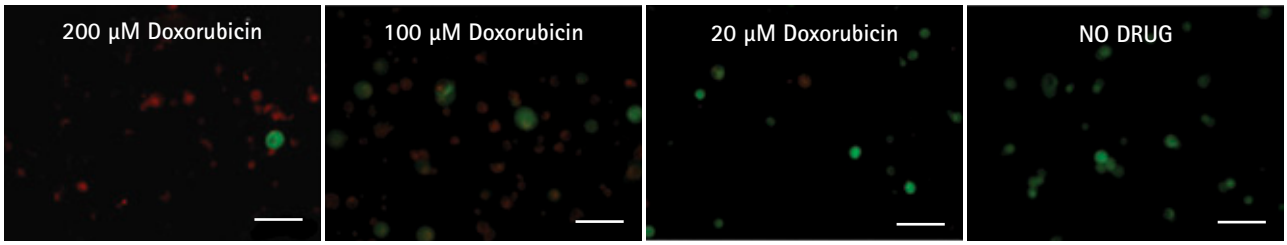
Technology Highlight

Live Imaging of Cellular Response to Inhibitors Using the CellASIC™ ONIX Microfluidic Platform

Automate compound delivery to your cells and image their response immediately with the easy-to-use CellASIC™ ONIX Microfluidic Platform. It delivers precise control for live cell imaging experiments by facilitating long-term perfusion cell culture and enabling automated, preprogrammed changes to flow, gas and temperature. The system integrates with your existing microscope system to enable dynamic time-lapse experiments never before possible.

The platform combines a control system (left), which is the equivalent of 8 syringe pumps, a perfusion chamber apparatus, and a CO_2 incubator, at a fraction of the cost, with greatly improved quality, portability and ease of use with any microscope. A low-profile manifold connects the control system to microfluidic plates (right), which are designed to optimize the health of specific cells during dynamic live cell experiments.

The CellASIC™ platform includes intuitive software with application-specific wizards, minimizing setup time (minutes instead of hours as with traditional protocols). Visit our website to see application data, live cell imaging videos and a video testimonial by a customer who used dynamic live cell analysis to enhance the power of chemical biology: **www.merckmillipore.com/cellasic**



Tracking dose-dependent MCF7 cell death upon doxorubicin treatment using microfluidic live/dead staining of 3D perfusion cultures.

Glossary

Active site: The part of an enzyme or other protein where a reactant or effector can bind and take part in a catalytic transformation.

Agonist: A ligand that binds to a receptor and alters the receptor state, resulting in a biological response.

Affinity: The strength of binding between a ligand and a receptor.

Allosteric effector: A ligand that can bind to a site other than the active site.

Apoenzyme: The polypeptide or protein part of the enzyme is called the apoenzyme and may be inactive in its original synthesized structure. The inactive form of the apoenzyme is known as a proenzyme or zymogen.

Association constant: The association constant (K_a) is the equilibrium constant for the association of a complex from its component parts.

Catalyst: A substance that can speed up a chemical reaction towards equilibrium without itself being consumed.

Catalytic constant: The rate constant that describes the first-order transformation of enzyme-substrate complex into product and free enzyme.

Cofactor: A small molecule required for enzyme activity. It can be organic in nature, like a coenzyme, or inorganic in nature, like a metallic cation.

Competitive inhibitor: A molecule that is structurally similar to the natural substrate of an enzyme, such that it is capable of binding to the active site.

Denaturation: The disruption of the native folded structure of a macromolecule; may be due to heat, chemical treatment, or change in pH.

Dissociation constant: The dissociation constant (K_d) is the equilibrium constant for the dissociation of a complex into its component parts.

Efficacy (or intrinsic activity): A measure of the ligand-receptor complex's ability to produce a maximum physiological response.

End-product inhibition. The inhibition of the first enzyme in a pathway by the end product of that pathway.

Enteral: Pertaining to oral, sublingual, or rectal delivery of drugs.

Enzyme: A protein molecule that behaves as a catalyst.

Equilibrium: The point at which the concentrations of two compounds are such that the interconversion of one compound into the other compound does not result in any change in free energy.

Equilibrium constant: The ratio of the product to the reactants for a chemical reaction at equilibrium.

First-order reaction: Reaction where the reaction rate is a linear function of the concentration of a given reactant.

Half-life: The half-life of a first-order chemical reaction is the time it takes for half the reactant to be consumed.

Hydrophobic: Molecules whose mixing in water is energetically unfavorable.

Induced fit: A change in the shape of an enzyme that results from the initial binding of substrate.

Inhibitor: A molecule that can bind to the enzyme and reduce the rate of reaction.

Inverse agonist: A ligand that, by binding to a receptor, reduces the fraction of receptors in an active conformation, thereby reducing basal activity.

Isoelectric point: The pH at which a protein has no net charge.

Isozymes: Multiple, but related, forms of an enzyme that differ from one another in one or more of their properties.

Loading dose: A larger than normal dose administered as the first in a series of doses to achieve desired level of drug.

Maximum velocity: The maximal velocity, often denoted V_{max}, is the reaction rate that an enzyme-catalyzed reaction achieves at high reactant concentrations. It is the maximal rate that an enzyme catalyzed reaction can reach.

Michaelis-Menten equation: The Michaelis-Menten equation is an equation that describes the rate of an enzyme-catalyzed reaction as a function of the substrate concentration by assuming the rapid-equilibrium assumption.

Noncompetitive inhibition: If an inhibitor can bind to both free enzyme and enzyme-substrate complex then the inhibition is termed noncompetitive inhibition.

Parenteral: Delivery of drug or inhibitor into the body by a route other than the gastrointestinal tract.

Partial agonist: An agonist that produces a maximal response that is less than the maximal response produced by another agonist acting at the same receptors on the same tissue.

Peptide: An organic molecule containing multiple amino acids connected to each other by covalent amide bonds.

Peptide bond: A bond formed between the α-amino group of one amino acid and the α-carboxyl group of another amino acid, with the elimination of a water molecule.

Pharmacokinetics: Study of drug absorption, distribution and the chemical alterations a drug may undergo in the body.

Prodrug: A chemical with little or no pharmacologic activity that undergoes change in the body into a more active material.

Regulatory enzyme: An enzyme in which the active site is subject to regulation by factors other than the enzyme substrate.

Second-order reaction: A second-order reaction is one where the reaction rate is function of the square of the concentration of a given species.

Selectivity: The capacity a drug to affect one receptor or cell population in preference to others.

Side effects: Drug effects that are not desirable and are not part of a therapeutic effect.

Specific activity: The amount of substrate the enzyme converts per mg protein in the enzyme preparation per unit of time.

Specificity: The capacity of a drug to manifest only one kind of action.

Substrate: A molecule that is acted upon, and chemically changed, by an enzyme.

Transition state: The activated state in which reactant(s) have the highest potential energy along a reaction coordinate, and the point at which it is most energetically favorable for the reactant(s) to be converted into product(s).

Turnover number: The maximum number of substrate molecules that can be converted to product per active site per unit time.

Uncompetitive inhibition: Uncompetitive inhibition occurs when an inhibitor only binds when substrate is also bound to the enzyme.

Volume of distribution: The volume, in an organism, throughout which a drug is distributed.

Zero-order reaction: A zero-order reaction is one where the reaction rate is independent of the concentration of a reactant.

Zymogen: An inactive precursor of an enzyme.

Notes

Notes

MERCK MILLIPORE

Reput(Ab)le Antibodies

We're validated.
We're guaranteed.
We're published.
We create the antibodies
most cited by the research community.

Researchers trust our antibodies because we are a thoughtful antibody producer, not a reseller. We're selective about offering the best antibodies based on the expertise of Chemicon® and Upstate®, internal R&D teams and collaborations with leading institutions. We guarantee our antibodies because of a stringent validation process that produces the highest quality antibodies on the market today.

We provide the most reliable, defensible, and publishable antibody performance, because, ultimately, it's not about our reputation. It's about yours.

Put the most reputable antibodies to work for you.

www.merckmillipore.com/Ab

To Place an Order or Receive Technical Assistance

In Europe, please call Customer Service:

France: 0825 045 645
Germany: 01805 045 645
Italy: 848 845 645
Spain: 901 516 645 Option 1
Switzerland: 0848 645 645
United Kingdom: 0870 900 4645

For other countries across Europe, please call: +44 (0) 115 943 0840

Or visit: **www.merckmillipore.com/offices**

For Technical Service visit: **www.merckmillipore.com/techservice**

Get Connected!

Join Merck Millipore Bioscience on your favorite social media outlet for the latest updates, news, products, innovations, and contests!

facebook.com/MerckMilliporeBioscience

twitter.com/Merck4Bio

MERCK MILLIPORE

www.merckmillipore.com/offices

Lit No. PB3375ENEU BS-GEN-13-09324 11/2013 Printed in the USA.