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Mechanistic Basis of Enzyme-Targeted Drugs

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ABSTRACT: Enzymes offer unique opportunities for drug design that are not available to cell surface receptors, nuclear hormone receptors, ion channels, transporters, and DNA. Here, we review the variety of inhibition mechanisms for enzyme-targeted drugs, and establish an enzyme target database for drugs currently marketed in the United States. From an analysis of the FDA Orange Book, there are 317 marketed drugs that work by inhibiting an enzyme. These drugs inhibit 71 enzymes, including 48 human, 13 bacterial, five viral, four fungal, and one protozoal enzyme. Among the 317 drugs, 65% either undergo reactive chemistry in the active site of the target enzyme or contain a structural motif related to the substrate. Among the 71 enzyme targets, 25 are irreversibly inhibited by drugs, and 19 of the 25 irreversibly inhibited enzymes are covalently modified by the drug. In two additional cases, the drug forms a covalent complex with the substrate, and in three more cases, the drug traps a covalent enzyme−substrate intermediate. Four of the 71 enzymes are inhibited by transition-state analogues. Moreover, advanced methods for determining transition-state structure now offer the opportunity for direct drug design without resorting to expensive random testing campaigns. A full appreciation of enzyme mechanisms sets enzymes apart as a specialized class of targets for highly directed drug design.

The sequencing of the human genome has promised a revolution in medicine. The genome encodes ~20000−25000 human genes, and thousands more proteins as a result of alternative gene splicing (*1*). Many of these will hold the keys to treating disease. Nevertheless, the academic and pharmaceutical communities face enormous challenges in translating the genomic revolution into useful medicines. Currently, a major pharmaceutical company might perform as many as 50 million biological assays a year, up from 0.2 million more than a decade ago (*2*), yet despite these advances in genomics and assay technology, there has not been a corresponding increase in new drugs reaching the market (*3*).

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Drug discovery generally focuses on six major categories of drug targets: enzymes, cell surface receptors, nuclear hormone receptors, ion channels, transporters, and DNA. In drug discovery, target classes influence everything from automation engineering to the design of chemical libraries. However, business models and the inexorable push of technology often blur the distinctions between target classes, and as a result, various misconceptions that are counterproductive to drug design take root. Here, we will take a close look at one target class, enzymes, and provide an assessment of what kinds of enzyme inhibitors succeed in the marketplace, and an assessment of what makes enzymes different from other target classes.

Enzyme Drug Targets

Various compendiums hold the information on drugs that target enzymes (*4*−*7*), but there appears to be no single

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source that provides a concise, authoritative, and current list of enzymes targeted by marketed drugs. To establish this list, each of the drugs in the FDA[1] Orange Book was reviewed systematically to identify its mechanism of action. The FDA Orange Book lists 1278 unique chemical entities that are approved for therapeutic use in the United States (*8*). Each of these was cross referenced in four authoritative texts (*4*−*7*), as well as in the primary literature, and those drugs that target enzymes were identified.

From this analysis, there are 317 drugs that target an enzyme as their primary or most probable mode of action. There are 71 enzyme targets for this pharmacopia, including 48 human, 13 bacterial, five viral, four fungal, and one protozoal enzyme. Table 1 provides the full list. A few of the drugs inhibit more than one enzyme, and thus, the total drug count in Table 1 is 333 since 12 drugs are counted twice and two are counted three times. Prodrugs are included in the total count. Also, isozymes are counted as a single enzymatic activity. These include amine oxidase, commonly termed MAO A and MAO B, prostaglandin-endoperoxide synthase, also known as COX-1 and COX-2, and iodide deiodinase, which exists in three isoforms known as type I, type II, and type III.

Several of the targets listed are less than certain, but have been included for completeness. These include dolichyl phosphatase as the target for bacitracin, and histone acetyltransferase as the target for valproic acid. Bacitracin is well-known as a protease inhibitor, but here we list the original reference. Valproic acid has been used for 35 years as an antiepileptic, but no single mechanism of action accounts for all its effects. Here we suggest that histone acetyltransferase is an important target for valproic acid.

Substrate Structure in Enzyme-Targeted Drugs

The first lesson to be gleaned from Table 1 is that the majority of marketed enzyme-targeted drugs are related to enzyme substrate structure. Of the 317 drugs that target the enzymes in Table 1, 205, or 65%, either undergo catalysis in the active site of an enzyme, chemically react with an enzyme cofactor, or contain a structural motif related to the substrate. There are 52 antibiotics, including penicillins, cephalosporins, and carbenapems, that target serine type D-Ala-D-Ala carboxypeptidase, and they all bear some structural similarity to the terminal D-Ala-D-Ala of bacterial peptidoglycan (*9*). In addition, they all undergo catalysis on the enzyme and acylate the active site serine. Similarly, the three inhibitors of β-lactamase, which are used to overcome β-lactamase resistance, acylate the β-lactamase active site serine.

Purine- and pyrimidine-based inhibitors are another good example. Ten enzymes in Table 1 are inhibited by a total of 41 drugs that contain purine- or pyrimidine-related core structures. These include the DNA and RNA polymerases, the phosphodiesterases, ribonucleoside-diphosphate reductase, adenosine deaminase, IMP dehydrogenase, xanthine oxidase, and thymidylate synthase. For the DNA and RNA polymerases, several deoxynucleoside-based drugs are recognized as substrates for the enzymes, but lack the necessary ribityl hydroxyl and therefore terminate polynucleotide strand extension. Thus, they compete against the natural nucleotide and waste catalytic cycles. The 17 sulfonamide inhibitors of dihydropteroate synthase are yet another example. The sulfonamides are structural analogues of *p*-aminobenzoic acid, the substrate of dihydropteroate synthase, and act as competitive inhibitors of PABA.

As a further example, the six statin inhibitors of HMG-CoA reductase all retain a 3,5-dihydroxyvaleric acid motif that is a close analogue of the HMG portion of HMG-CoA. In 2001, crystal structures of HMG-CoA reductase complexed with each of the marketed statins were determined, and the structures showed that, indeed, the HMG-like moieties of the statins bind in the same pocket as the HMG portion of HMG-CoA (*10*).

Many of the substrate-like drugs belong to the major groups of cellular metabolites. Steroids inhibit 3(or 17)-β-hydroxysteroid dehydrogenase, 3-oxo-5-α-steroid 4-dehydrogenase, and an unspecific monooxygenase. A long chain lipid inhibits triacylglycerol lipase. An amino acid inhibits tyrosine 3-monooxygenase. Naphthoquinones mimic vitamin K and inhibit vitamin-K-epoxide reductase. Sugars inhibit α-amylase, α-glucosidase, sucrose α-glucosidase, glucan 1,4-α-glucosidase, and exo-α-sialidase. Phosphate mimetics inhibit farnesyl-diphosphate farnesyltransferase. Synthetic peptides, peptide mimetics, and natural peptides inhibit peptidyl dipeptidase A, HIV-1 retropepsin, and plasma kallikrein. Substrates belong in the cell, and hence, it is not surprising that inhibitors with substrate character are therapeutically effective.

Transition-State Inhibitors as Enzyme-Targeted Drugs

In the early 1970s, investigators began to popularize the idea that transition-state analogues function as tight binding enzyme inhibitors (*11*). Through the 1970s and 1980s, most of the known examples were natural products (*12*). In the 1990s, this changed, and synthetic inhibitors became the predominate examples of transition-state inhibitors (*13*). As of 1995, there were transition-state analogues for at least 132 enzymes (*13*).

Four of the 71 enzymes in Table 1 are inhibited by drugs that function as transition-state analogues. The natural product pentostatin, produced by *Streptomyces*, is a transition-state inhibitor of adenosine deaminase. It binds to the human erythrocyte enzyme with a K_d of 2.5 pM (*14*). Captopril, the first rationally designed enzyme-targeted drug, can be considered a transition-state inhibitor of peptidyl-dipeptidase A, also known as ACE (*15*). The design of captopril was based on the structure of a byproduct analogue inhibitor of carboxypeptidase A (*16*), and incorporates many of the features that would be expected to participate in reaction chemistry. For HIV retropepsin (HIV protease), a variety of structure-assisted drug design techniques were successful in developing inhibitors. The initial design of

[1] Abbreviations: FDA, Food and Drug Administration; MAO, monoamine oxidase; COX, cyclooxygenase; IMP, inosine monophosphate; HMG-CoA, hydroxymethylglutaryl coenzyme A; PABA, *p*-aminobenzoic acid; HIV, human immunodeficiency virus; ACE, angiotensin converting enzyme; UDP, uridine diphosphate; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate; TS, thymidylate synthase; THF, tetrahydrofolate; DFMO, α-difluoromethylornithine; PLP, pyridoxal phosphate; ATP, adenosine triphosphate; PTU, propylthiouracil; SDS, sodium dodecyl sulfate; NADP, nicotinamide adenine dinucleotide phosphate; NADH, reduced nicotinamide adenine dinucleotide; XMP, xanthine monophosphate; NAD, nicotinamide adenine dinucleotide.

Table 1: Database of Enzyme Targets for Marketed Drugs

entry	enzyme common name	EC no.[a]	example drug	indication	no. of drugs	organism[b]	ref[c]
1	1,3-β-glucan synthase	2.4.1.34	caspofungin	antifungal	1	F	*87*
2	3(or 17)-β-hydroxysteroid dehydrogenase	1.1.1.51	trilostane	breast cancer	1	H	*88*
3	3′,5′-cyclic-GMP phosphodiesterase	3.1.4.35	sildenafil	ED[d]	3	H	*89*
4	3′,5′-cyclic-nucleotide phosphodiesterase	3.1.4.17	theophylline	asthma	11	H	*90*
5	4-hydroxyphenylpyruvate dioxygenase	1.13.11.27	nitisinone	tyrosinemia	1	H	*91*
6	3-oxo-5-α-steroid 4-dehydrogenase	1.3.99.5	finasteride	BPH[e]	2	H	*92*
7	acetylcholinesterase	3.1.1.7	pyridostigmine	MG[f]	11	H	*93*
8	adenosine deaminase	3.5.4.4	pentostatin	HCL[g]	2	H	*94*
9	alanine racemase	5.1.1.1	cycloserine	tuberculosis	1	B	*95*
10	alcohol dehydrogenase	1.1.1.1	fomepizole	alcoholism	1	H	*96*
11	aldehyde dehydrogenase (NAD)	1.2.1.3	disulfiram	alcoholism	1	H	*97*
12	α-amylase	3.2.1.1	acarbose	diabetes	1	H	*98*
13	α-glucosidase	3.2.1.20	miglitol	diabetes	1	H	*99*
14	amine oxidase (flavin-containing)	1.4.3.4	tranylcypromine	depression	5	H	*100*
15	arabinosyltransferase	2.4.2.34	ethambutol	antibacterial	1	B	*101*
16	arachidonate 5-lipoxygenase	1.13.11.34	zileuton	inflammation	4	H	*102*
17	aromatic L-amino acid decarboxylase	4.1.1.28	carbidopa	Parkinson's	1	H	*103*
18	β-lactamase	3.5.2.6	tazobactam	antibacterial	3	B	*104*
19	carbonate dehydratase[m]	4.2.1.1	acetazolamide	glaucoma	6	H	*105*
20	catechol *O*-methyltransferase	2.1.1.6	entacapone	Parkinson's	2	H	*106*
21	ceramide glucosyltransferase	2.4.1.80	miglustat	Gaucher's	1	H	*107*
22	D-alanine-D-alanine ligase	6.3.2.4	cycloserine	tuberculosis	1	B	*108*
23	dihydrofolate reductase	1.5.1.3	methotrexate	leukemia	7	H	*109*
24	dihydroorotate dehydrogenase	1.3.99.11	leflunomide	inflammation	1	H	*110*
25	dihydropteroate synthase	2.5.1.15	dapsone	antifungal	17	F	*111*
26	DNA topoisomerase	5.99.1.2	topotecan	ovarian cancer	2	H	*112*
27	DNA topoisomerase (ATP-hydrolyzing)	5.99.1.3	ciprofloxacin	antibacterial	18	B	*113*
28	DNA-directed DNA polymerase	2.7.7.7	acyclovir	herpes	11	V	*114*
29	DNA-directed RNA polymerase	2.7.7.6	rifapentine	antibacterial	3	B	*115*
30	dolichyl phosphatase	3.1.3.51	bacitracin	antibacterial	1	B	*116*
31	enoyl-[acyl carrier protein] reductase	1.3.1.9	isoniazid	tuberculosis	1	B	*117*
32	exo-α-sialidase	3.2.1.18	oseltamivir	influenza	2	V	*118*
33	factor Xa	3.4.21.6	fondaparinux	thrombosis	2	H	*119*
34	farnesyl-diphosphate farnesyltransferase	2.5.1.21	alendronate	osteoporosis	6	H	*120*
35	fatty acid synthase	2.3.1.85	pyrazinamide	tuberculosis	1	B	*121*
36	glucan 1,4-α-glucosidase	3.2.1.3	miglitol	diabetes	1	H	*122*
37	histone acetyltransferase	2.3.1.48	valproic	seizures	1	H	*123*
38	HIV-1 retropepsin	3.4.23.16	nelfinavir	AIDS[h]	8	V	*124*
39	hydrogen/potassium-exchanging ATPase	3.6.3.10	esomeprazole	GERD[i]	5	H	*125*
40	HMG-CoA reductase (NADPH2)	1.1.1.34	atorvastatin	hyperlipodemia	6	H	*126*
41	IMP dehydrogenase	1.1.1.205	mycophenolate	IS[j]	2	H	*127*
42	iodide peroxidase	1.11.1.8	propylthiouracil	hyperthyroid	2	H	*128*
43	isoleucine tRNA ligase	6.1.1.5	mupirocin	antibacterial	1	B	*129*
44	membrane dipeptidase	3.4.13.19	cilastatin	resistance	1	H	*130*
45	ornithine decarboxylase	4.1.1.17	eflornithine	trypanosomes	1	P	*131*
46	orotidine-5′-phosphate decarboxylase	4.1.1.23	allopurinol	gout	1	H	*132*
47	peptidyl-dipeptidase A	3.4.15.1	captopril	hypertension	12	H	*133*
48	phosphoribosylglycinamide formyltransferase	2.1.2.2	pemetrexed	cancer	1	H	*134*
49	plasma kallikrein	3.4.21.34	aprotinin	thrombosis	1	H	*135*
50	plasmin	3.4.21.7	aminocaproic	thrombosis	3	H	*136*
51	prostaglandin-endoperoxide synthase	1.14.99.1	etodolac	inflammation	30	H	*137*
52	proteasome endopeptidase complex	3.4.25.1	bortezomib	myeloma	1	H	*138*
53	protein-tyrosine kinase	2.7.1.112	imatinib	leukemia	3	H	*139*
54	ribonucleoside-diphosphate reductase	1.17.4.1	gemcitabine	cancer	4	H	*140*
55	RNA-directed RNA polymerase	2.7.7.48	ribavirin	pneumonia	1	V	*141*
56	RNA-directed DNA polymerase	2.7.7.49	abacavir	AIDS	13	V	*142*
57	serine-type D-Ala-D-Ala carboxypeptidase	3.4.16.4	cefonicid	antibacterial	52	B	*143*
58	sodium/potassium-exchanging ATPase	3.6.3.9	digitoxin	CHF[k]	3	H	*144*
59	squalene monooxygenase	1.14.99.7	butenafine	antifungal	3	F	*145*
60	sterol 14-demethylase	1.14.13.70	itraconazole	antifungal	11	F	*146*
61	sucrose α-glucosidase	3.2.1.48	miglitol	diabetes	1	H	*147*
62	thrombin	3.4.21.5	lepirudin	thrombosis	10	H	*148*
63	thymidylate synthase	2.1.1.45	floxuridine	cancer	6	H	*149*
64	thyroxine 5′-deiodinase	1.97.1.10	propylthiouracil	hyperthyroid	1	H	*150*
65	triacylglycerol lipase	3.1.1.3	orlistat	obesity	1	H	*151*
66	tyrosine 3-monooxygenase	1.14.16.2	metyrosine	PC[l]	1	H	*152*
67	UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase	2.5.1.7	fosfomycin	antibacterial	1	B	*153*
68	unspecific monooxygenase	1.14.14.1	aminoglutethimide	breast cancer	5	H	*154*
69	urease	3.5.1.5	acetohydroxamic	gastritis	1	B	*155*
70	vitamin-K-epoxide reductase (warfarin-sens.)	1.1.4.1	dicumarol	thrombosis	5	H	*156*
71	xanthine oxidase	1.17.3.2	allopurinol	gout	1	H	*157*

[a] EC numbers are from IUBMB Enzyme Nomenclature (www.chem.qmw.ac.uk/iubmb/enzyme). [b] Organism is the target organism: H, human; B, bacterial; V, viral; F, fungal; P, protozoal. [c] The reference is a general reference for the target. [d] Erectile dysfunction. [e] Benign prostatic hyperplasia. [f] Myasthenia gravis. [g] Hairy cell leukemia. [h] Acquired immunodeficiency syndrome. [i] Gastroesophageal reflux disease. [j] Immunosuppression. [k] Congestive heart failure. [l] Pheochromocytoma. [m] Several enzymes are better known by their more popular names: 3-oxo-5-α-steroid 4-dehydrogenase as 5-α-reductase, carbonate dehydratase as carbonic anhydrase, DNA topoisomerase as DNA gyrase, DNA-directed DNA polymerase as DNA polymerase, DNA-directed RNA polymerase as RNA polymerase, HIV-1 retropepsin as HIV protease, peptidyl-dipeptidase A as angiotensin-converting enzyme, RNA-directed DNA polymerase as reverse transcriptase, ribonucleoside-diphosphate reductase as ribonucleotide reductase, serine-type D-Ala-D-Ala carboxypeptidase as DD transpeptidase, and unspecific monooxygenase as microsomal P_{450} or aromatase.

saquinavir, the first marketed HIV retropepsin inhibitor, involved hydroxyethylamine isosteres that function as transition-state analogues (*17*). In a similar combination of mechanistic and structural analysis, the nature of the transition state for exo-α-sialidase was deduced from kinetic isotope effects (*18*). Using the crystal structure of the enzyme and the model of the transition state, chemists explicitely designed two potent transition-state inhibitors (*19*), oseltamivir and zanamivir, that are now used to treat influenza.

The design of transition-state inhibitors is likely to become more frequent in the future as the theory and technology for understanding enzyme transition states become more widespread (*20*). This method has been used to design immucillin-H, a 56 pM inhibitor of human purine nucleoside phosphorylase that is in phase IIa clincal trials for T cell leukemia (www.biocryst.com). It also has been used to develop picomolar (pM) inhibitors of 5′-methylthioadenosine phosphorylase (*21*) and femtomolar (fM) inhibitors of 5′-methylthioadenosine nucleosidase (*158*).

Irreversible Inhibitors as Enzyme-Targeted Drugs

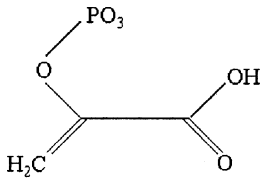
Drug discovery programs never set out to make irreversible inhibitors. However, 25 of the 71 targets in Table 1 are irreversibly inhibited by marketed drugs. This belies the general prejudice against irreversible inhibitors. Most of the enzymes that are irreversibly inhibited by drugs are covalently modified by the respective drug. In other cases, binding is so tight that the inhibitor remains bound for hours or even days, and binding can be considered functionally irreversible. The following 25 enzymes are irreversibly inhibited by drugs. The first 19 in the list are covalently modified by the drug. Two enzymes, 3-oxo-5-α-steroid 4-dehydrogenase and enoyl-[acyl carrier protein] reductase, are irreversibly inhibited by covalent complexes between the inhibitor and substrate.

(1) Serine Type D*-Ala-*D*-Ala Carboxypeptidase.* Serine type D-Ala-D-Ala carboxypeptidase is perhaps the most thoroughly studied example of a covalently inactivated enzyme drug target. All β-lactam antibiotics acylate the active site serine of the carboxypeptidase (*22*, *23*). This is generally a stable acylation that is resistant to hydrolysis, and therefore, it effectively eliminates all transpeptidase activity in the bacterium.

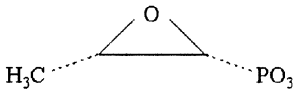
(2) β-Lactamase. The major form of resistance to β-lactam antibiotics is hydrolysis of β-lactams by β-lactamase. While the acyl−enzyme intermediate in D-Ala-D-Ala carboxypeptidase is stable for hours to days, the β-lactamase acyl−enzyme intermediate hydrolyzes at rates of 100−4000 s^{-1} (*24*). However, several naturally occurring β-lactams, including clavulanate, tazobactam, and sulbactam, form kinetically stable acyl−enzyme intermediates and inactivate β-lactamase. These drugs are used to overcome β-lactamase resistance.

(3) Acetylcholinesterase. Acetylcholinesterase is another serine hydrolase characterized by drug-targeted covalent active site modification (*5*). Several anti-cholinesterase agents, such as pyridostigmine, act as alternative substrates and undergo attack by the active site serine. This leads to pyridostigmine-induced carbamoylation of the serine. Methylcarbamoyl or dimethycarbamoyl intermediates are far more stable than the acetyl−enzyme intermediate generated with acetylcholine, and thus, in vivo, the carbamoylating inhibitors may yield covalent enzyme inhibition for 3−4 h.

(4) UDP-N-acetylglucosamine 1-Carboxyvinyltransferase. Fosfomycin, a phosphoenolpyruvate substrate analogue, is another example of an antibiotic that covalently modifies the target enzyme. Fosfomycin was discovered in fermentation broths of *Streptomyces* (*25*), and later was demonstrated to acylate an active site cysteine in UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase (*26*). The epoxide ring of fosfomycin presents a latent warhead to the enzyme that is unmasked by the catalytic mechanism and subsequently covalently modifies the active site.



Phosphoenolpyruvate



Fosfomycin

(5) Prostaglandin-Endoperoxide Synthase. Aspirin is perhaps the most highly consumed drug in the United States, and is an irreversible covalent inactivator of prostaglandin-endoperoxide synthase, also called cyclooxygenase, or COX. Incubation of purified prostaglandin-endoperoxide synthase with [*acetyl*-^{3}H]aspirin leads to inactivation and covalent modification of a single serine residue, which can be recovered as the [^{3}H]acetylserine in proteolytic digests of the enzyme (*27*, *28*).

(6) Unspecific Monooxygenase. Aromatase, or unspecific monooxygenase, catalyzes the conversion of androgens to estrogens, and was identified as a target for anti-estrogen therapy in 1975 (*29*). Exemestane, which has a 1,2-unsaturated bond in the A ring of the steroid nucleus, causes time-dependent inactivation of aromatase, and has been hypothesized to redirect the enzyme's aromatization reaction to covalent modification of the enzyme. On the basis of the similarity of exemestane to the substrate of the reaction, it has been concluded that exemestane is an irreversible mechanism-based inactivator of aromatase (*30*).

(7) Amine Oxidase (Flavin-Containing). Mitochondrial amine oxidase, commonly called monoamine oxidase, or MAO, is a flavin-containing enzyme that catalyzes the oxidative deamination of catecholamines and serotonin in the central nervous system. Selegiline (or L-deprenyl) contains an acetylenic function that covalently modifies the reactive N5 atom of the flavin cofactor (*31*). The drug binds in reversible equilibrium with the enzyme, undergoes catalytic oxidation to a reactive intermediate, and then yields time-dependent covalent modification of the flavin (*5*, *32*).

(8) Thymidylate Synthase. Thymidylate synthase catalyzes the reductive methylation of dUMP to dTMP, and is the sole de novo means for synthesizing dTMP. Floxuridine, a fluorine-containing substrate analogue, acts as a potent thymidylate synthase inhibitor by stabilizing the covalent TS−dUMP−CH_2THF ternary complex, with the inhibitor covalently bound to the enzyme through the active site cysteine (*5*, *33*). Since the C−F bond stabilizes the C5-methylene bridge, it also freezes the chemical mechanism in a transition state.

(9) Ornithine Decarboxylase. Ornithine decarboxylase catalyzes the conversion of ornithine to putrescine, and provides the entry point for amino acids into polyamine synthesis. The marketed drug that targets ornithine decarboxylase, α-difluoromethylornithine, is a substrate for the enzyme and undergoes PLP-dependent decarboxylation in the active site (*65*). This generates a reactive intermediate that then may attack either a lysine or a cysteine residue to form a covalently inhibited complex (*34*). The X-ray crystal structure of *Trypanosoma brucei* ornithine decarboxylase shows that DFMO covalently modifies cysteine 360 in the trypanosomal enzyme (*35*).

(10) Alanine Racemase. Bacteria exclusively use D-Ala in peptidoglycan, and express alanine racemase to synthesize it. Alanine racemase was first thought to be reversibly, competitively inhibited by D-cycloserine, but later it was shown that D-cycloserine causes time-dependent inhibition (*36*). Recently, the X-ray crystal structure of the inhibited complex was determined, and it was shown that D-cycloserine covalently modifies PLP in the enzyme (*37*).

(11) H^+/K^+ ATPase. The H^+/K^+ ATPase of gastric parietal cells is responsible for pumping HCl into the extracellular lumen, and is the source of acid that causes gastroesophageal reflux disease (*38*). The marketed inhibitors of H^+/K^+ ATPase, omaprazole, esmoprazole, and lanoprazole, react with cysteines on the extracellular loops of the H^+/K^+ ATPase. Once covalently inactivated, the H^+/K^+ ATPase may be neutralized for 24−48 h, and will only be regenerated by synthesis of new enzyme (*5*).

(12) Triacylglycerol Lipase. Triacylglycerol lipase catalyzes the interfacial hydrolysis of triacylglycerol on the surface of emulsified lipid droplets, and is the critical enzyme involved in processing dietary fats. The one marketed lipase inhibitor, orlistat, or tetrahydrolipstatin, forms an ester with Ser152 in porcine triacylglycerol lipase, with the incorporation of 1 mol of tetrahydrolipstatin/mol of enzyme (*39*). Formation of the *β*-lactone-derived ester at Ser152 in the porcine enzyme suggests an identical mechanism in the human enzyme, since the catalytic triad in the X-ray crystal structure of the human lipase, including Ser152, can be superimposed with the catalytic triad of other serine proteases (*40*).

(13) Ribonucleoside-Diphosphate Reductase. Ribonucleoside-diphosphate reductases catalyze the formation of new deoxyribonucleotides for incorporation in DNA. Several 2′-substituted nucleotides have been shown to be time-dependent, mechanism-based inactivators of ribonucleoside-diphosphate reductase (*41*). One particular inhibitor, gemcitabine, a 2′-difluoronucleoside, has been approved for use in pancreatic and non-small cell lung cancer. Though gemcitabine inactivation is irreversible, the presumed site of covalent modification has not been identified.

(14) Iodide Peroxidase. In the United States, propylthiouracil and methimazole are the two drugs used to treat hyperthyroidism. Both drugs inhibit iodide peroxidase (*42*, *43*), and both contain an essential thiol. Enzyme inhibition appears to involve reactions with both iodide and the heme center of the enzyme. In vitro, enzyme inhibition by methimazole is not reversed by dialysis, and therefore, methimazole is considered to be an irreversible inhibitor (*42*), though under some conditions inactivation is not complete and the enzyme appears to recover iodination activity (*43*).

(15) Thyroxine 5′-Deiodinase. Thyroxine 5′-deiodinase is a family of three isozymes encoded by three distinct genes (*44*). Propylthiouracil inhibits type I deiodinase (*45*), a selenoenzyme, and is thought to form a covalent enzyme−Se−S−PTU adduct through the thiol on PTU (*45*). Type I deiodinase from rat liver cytosol can be recovered as a complex with [^{35}S]PTU, and 25% of the [^{35}S]PTU remains bound after dialysis, precipitation with trichloroacetic acid, and multiple extractions with ethanol, methanol, and chloroform (*46*).

(16) Aldehyde Dehydrogenase. Aldehyde dehydrogenases catalyze the oxidation of a wide variety of endogenous and exogenouse aldehydes to water soluble carboxylic acids. Disulfiram is a time-dependent irreversible inhibitor of aldehyde dehydrogenase (*47*), and is used to treat alcoholism. In vitro, *S*-methyl-*N*,*N*-diethylthiocarbamoyl sulfoxide, the principle metabolite of disulfiram, carbamoylates Cys302 in purified human aldehyde dehydrogenase (*48*). The carbamoylated enzyme also can be recovered from animals treated with disulfiram (*49*).

(17) Thrombin. Thrombin is a serine protease that activates platelet receptors and initiates intracellular signaling events that transform the platelet into an active clot-forming cell. Antithrombin is a 58 kDa serine protease inhibitor that reacts with and covalently inactivates thrombin (*50*, *51*). Heparin, one of the most widely used anticoagulants, accelerates the thrombin−antithrombin reaction by ∼10000-fold, and works by holding thrombin and antithrombin in proximity (*52*). Direct thrombin inhibitors also have been introduced, the most potent of which is recombinant hirudin, which has a K_i of 231 fM and a k_{off} of 3.17×10^{-5}, which translates to a $t_{1/2}$ for dissociation of 6 h (*53*). The slow dissociation means that inhibition by the recombinant inhibitor is functionally irreversible.

(18) Factor Xa. Antithrombin also is an inactivator of factor Xa, and inactivates Xa in the same way that it inactivates thrombin. As in the case of thrombin, heparin also accelerates the factor Xa−antithrombin reaction by ∼10000-fold (*52*). As in the case of thrombin, inactivation is irreversible, due to the covalent modification of factor Xa and the enhanced proteolysis of the inactivated species.

(19) 4-Hydroxyphenylpyruvate Dioxygenase. Hydroxyphenylpyruvate dioxygenase is an Fe(II)-dependent, nonheme oxygenase that catalyzes the conversion of 4-hydroxyphenylpyruvate to homogentisate. In 1991, it was discovered that inhibition of hydroxyphenylpyruvate dioxygenase with nitisinone blocks tyrosine degradation at the top of the tyrosine catabolic pathway, and that this treatment lowers the incidence of liver cancer in children with tyrosinemia (*54*). Nitisinone is an irreversible triketone inhibitor that forms a stable complex with the Fe(II) center in the enzyme. In rat liver extract, the $t_{1/2}$ for dissociation of the inhibitor was estimated to be 101 h. In enzyme purified from *Streptomyces avermitilis*, the complex was stable for at least 2 days (*55*).

(20) Vitamin K Epoxide Reductase. The enzymes involved in the carboxylation reactions that produce active factor II, factor VII, factor IX, and factor X also are targets for anticoagulants. In particular, vitamin K epoxide reductase is the target of warfarin, an oral anticoagulant. Warfarin inhibits the disulfide form of the reductase activity (*56*). Warfarin-treated microsomes do not recover vitamin K

epoxide reductase activity after repeated dilution and centrifugation, and for this reason, it has been concluded that warfarin inhibition is irreversible (*57*).

(21) Ile tRNA Synthetase. Aminoacyl-tRNA synthetases catalyze the ligation of amino acids and their cognate tRNAs. Mupirocin is a competitive inhibitor of *Escherichia coli* Ile tRNA synthetase, with a K_i of 2.5 nM against isoleucine aminoacylation and a K_i of 6 nM against pyrophosphate exchange into ATP (*58*). Mupirocin is an irreversible inhibitor, as demonstrated by the recovery of [^{3}H]mupirocin complexed with the enzyme after gel filtration (*58*, *59*). The bound complex is stable to dialysis for up to 18 days (*59*). However, inhibition is not covalent, and mupirocin can be released from the enzyme by boiling in SDS (*58*) or by HPLC under reverse phase conditions (*59*).

(22) DNA-Directed DNA Polymerase. The DNA-directed polymerases have been pursued as important drug targets because of their critical role in replication. Deoxynucleotide-based inhibitors are recognized as substrates for the enzyme, and terminate strand extension because they lack the ribityl hydroxyl necessary for ligation to the next nucleotide. In this sense, the inhibitory consequence is irreversible. In addition, acyclovir, which targets herpes virus DNA-directed DNA polymerase, does produce irreversible enzyme inactivation in addition to nucleotide incorporation and strand termination (*60*). Acylcovir produces time-dependent inactivation during catalytic turnover, and the inactivated enzyme is not reactivated after desalting on a gel filtration column (*60*). It has been suggested that the enzyme undergoes an irreversible conformational change that locks the misincorporated nucleotide-containing strand in place (*60*).

(23) 3-Oxo-5-α-steroid 4-Dehydrogenase. The enzyme 3-oxo-5-α-steroid 4-dehydrogenase catalyzes the reduction of testosterone to dihydrotestosterone. Finasteride, prescribed for the treatment of benign prostatic hyperplasia, is an analogue of testosterone that acts as a substrate for 3-oxo-5-α-steroid 4-dehydrogenase (*61*). Catalytic turnover of finasteride proceeds through an enzyme-bound NADP-dihydrofinasteride adduct, which, once formed, serves as an extremely potent bisubstrate analogue inhibitor with a K_i of $\leq 1 \times 10^{-13}$ M. The partition ratio for catalysis to inactivation is ~1, which means that every catalytic turnover of finasteride is lethal for the enzyme. The release of [^{3}H]dihydrofinasteride from the enzyme occurs with a half-life of 1 month at 37 °C (*61*).

(24) Enoyl-Acyl Carrier Protein Reductase. Isoniazid, the frontline treatment for tuberculosis, forms an enzyme-bound NADH adduct with long-chain enoyl-acyl carrier protein reductase (*62*). Gel filtration of the enzyme−inhibitor complex demonstrates that [^{14}C]isoniazid remains tightly bound to the enzyme (*63*). This indicates that inhibition is irreversible, though the inhibitor is covalently bound to NADH and not to the enzyme.

(25) Xanthine Oxidase. Xanthine oxidase is a complex molybdoflavoenzyme that catalyzes the oxidation of purines to uric acid (*64*, *65*). The primary treatment for gout is to lower plasma uric acid concentrations by inhibiting xanthine oxidase, most effectively with allopurinol, a stoichiometric inactivator of xanthine oxidase, with a K_i of 630 pM (*66*, *67*). Under catalytic conditions, allopurinol undergoes enzyme-catalyzed oxidation to alloxanthine, and formation of alloxanthine traps the enzyme in a partially reduced state where the molybdenum center remains reduced. Alloxanthine remains tightly bound to the partially reduced enzyme, and by this measure, inhibition is functionally irreversible.

Reaction Intermediate Traps as Enzyme-Targeted Drugs

Reaction intermediate trapping is another mechanism for the formation of covalent inhibitory complexes, though this does not involve covalent bonds between the enzyme and inhibitor. Two enzymes fall into this category, inosine monophosphate dehydrogenase and DNA topoisomerase. Inosine monophosphate dehydrogenase catalyzes the oxidation of IMP to XMP through the formation of a covalent cystinyl intermediate at C2 of the purine ring (*68*, *69*). Mycophenolic acid, an uncompetitive inhibitor, binds in the NAD cofactor site, and prevents the hydrolysis reaction, thus trapping and stabilizing the covalent E−XMP complex (*68*). The structure of the trapped complex shows that the bicyclic ring system of mycophenolic acid packs underneath the hypoxanthine ring of XMP, thereby preventing release of the intermediate (*68*).

Mammalian and bacterial DNA topoisomerases catalyze the topological rearrangement of supercoiled and concatenated DNA, and serve as targets for the antitumor agents topotecan and irinotecan, and as targets for the fluoroquinoline antibiotics (*70*−*72*). In human topoisomerase I, the enzyme catalyzes single-strand cleavage and formation of a phosphodiester bond between Tyr^{723} and the DNA 3′-phosphate (*73*). Biochemical evidence indicates that camptothecin, the parent compound for the marketed drugs irinotecan and topotecan, stabilizes the covalent complex, and several models suggest that it binds near the DNA cleavage site (*73*, *74*). In topoisomerase II, the enzyme cuts two DNA strands and generates four free ends. The enzyme covalently links both of the 5′-ends to catalytic residues in the active site (*75*), in effect prying open the DNA on the ends of a molecular fork and opening a gate through the DNA (*76*). The quinolone antibiotics are thought to stabilize this covalent intermediate (*70*).

Boronic Acid-Based Inhibitors as Enzyme-Targeted Drugs

Boronic acids display the important property of acting as strong Lewis acids because of their open boron shell (*77*). They can be converted from a trigonal planar sp^2 geometry to a tetrahedral sp^3 geometry by substitution at boron. This ready interconversion is remarkably similar to the formation of tetrahedral intermediates in many hydrolytic enzymes, and allows boronic acids to act as reaction intermediate analogues with potent inhibitory properties in the femtomolar range (*78*). Bortezomib, the first FDA-approved boronic acid-based inhibitor, reached the market in 2003. Bortezomib inhibits proteasome degradation of intracellular proteins (*79*), and has shown efficacy in solid and hematological cancers. Because of the chemical interaction with active site catalytic residues, boronic acid inhibitors often display slow-binding inhibition, and this is true of bortezomib, which is a slow-binding inhibitor with a K_i of 600 pM (*80*).

Noncompetitive Inhibitors as Enzyme-Targeted Drugs

While most enzyme-based drugs are active site inhibitors, some are noncompetitive inhibitors that do not bind in the active site. Nonnucleoside reverse transcriptase inhibitors,

including nevirapine, are a good example. Analysis by pre-steady-state kinetics has shown that nonnucleoside inhibitors do not interfere with nucleotide binding or the change in conformation induced by nucleotide binding (*81*). Rather, they bind at an allosteric site, and this slows the rate of chemical catalysis, making chemical catalysis the slowest step in the reaction. In contrast, in the uninhibited enzyme, the conformational change due to nucleotide binding is the slowest step.

This offers a lesson in drug design, and argues that knowledge of the microscopic rate constants provides a competitive advantage to enzymologists developing non-competitive inhibitors as drugs. Two inhibitors might have the same K_i, but if one slows the rate of catalysis 10-fold more than the other, all else being equal, it will be a more potent drug.

Activators as Enzyme-Targeted Drugs

In principle, there are always two possibilities for enzyme-targeted drug design: inhibition or activation of an enzyme. In the sense of a pure k_{cat} or k_{cat}/K_m activator, there are no pure enzyme activators on the market. However, in time, this approach may succeed. Investigators at Roche have now discovered activators of glucokinase that increase k_{cat} and decrease the $S_{0.5}$ for glucose, and these may offer a treatment for type II diabetes (*82*). The activators bind in a glucokinase regulatory site that originally was discovered in patients with persistent hyperinsulinemic hypoglycemia (*83*, *84*). This result may suggest that the best strategy for developing activators is to focus on highly regulated enzymes, or cooperative enzymes such as glucokinase, where nature has provided binding sites that are designed to modulate catalysis.

Enzymes Are a Distinct Target Class

Enzymes are catalysts. They make and break specific covalent chemical bonds. Cell surface receptors, ion channels, transporters, nuclear hormone receptors, and DNA do not. Though this is axiomatic, surprisingly, it is not well appreciated. In drug discovery, the focus is always on the binding event, and the vocabulary of binding dominates perceptions, even for enzyme targets.

Thus, it should be stated again. Enzymes are catalysts, and the catalytic event is at least as important if not more important than the binding event. Enzyme catalysis progresses through binding events, conformational changes, one or more transition states, or reaction intermediates, and product release, and all of these steps occur with defined rate constants. The rate constants define a thermodynamic profile that can be used for drug design, and this differentiates enzymes from all other target classes.

The most powerful use of the thermodynamic profile of an enzyme reaction is in deciphering the transition state through kinetic isotope effects, and using this information to design potent inhibitors, as has been noted above (*20*). In addition, purely kinetic information can be used to predict potential drug toxicity. As an example, a detailed pre-steady-state kinetic analysis of human mitochondrial DNA polymerase has been used to predict the potential toxicity of marketed antiviral drugs.

A toxicity scale was developed by determining the kinetic specificity constants (k_{pol}/K_d), discrimination factors, and exonuclease rates for various nucleoside-based drugs as compared to the corresponding natural nucleotides. With this information, it is possible to calculate the "relative increase in the time required to replicate the mitochondrial genome" in the presence of antiviral nucleoside analogues (*85*). The larger the increase in the replication time, the more toxic the drug. This type of analysis, based on purely kinetic information, does, in fact, identify the drugs known to be toxic in clinical practice.

Similarly, it is possible to compute a therapeutic index for nucleoside analogues, wherein the discrimination factors for DNA polymerase and reverse transcriptase are compared. The most therapeutically effective drugs are predicted to have a high rate of incorporation by viral reverse transcriptase (i.e., good enzyme inhibition due to viral enzyme utilization), and a low rate of incorporation into human DNA by human DNA polymerase (i.e., low toxicity) (*85*). The ratio of inhibition to toxicity is a measure of the therapeutic usefulness. The therapeutic index, again, predicted from purely kinetic analysis, also agrees with the known efficacy of the antiviral drugs. This type of analysis now provides an in vitro method for predicting potential nucleoside analogue toxicity, and complements animal experiments, which sometimes fail to predict toxicity (*86*).

This kinetic approach logically might be extended to drug inhibition of other enzymes that are not related to the therapeutic target. For instance, cytochrome P_{450}'s are always potential sites of drug inhibition due to the primary role of P_{450}'s in xenobiotic metabolism. However, most attempts to generate predictive scales of P_{450} inhibition based on drug structures do not include any information about the mechanism of inhibition, and rarely attempt to correlate inhibition rates with toxicity. This would appear to be an area ripe for exploration.

Summary

This short review provides a current list of known enzyme targets for marketed drugs, and highlights the variety of successful approaches to enzyme-targeted drug design. One of the major lessons to be learned from marketed drugs is that nature has designed enzymes to perform a selective chemical reaction, and therefore, it is likely that the most potent drugs to be discovered or designed will be related to the substrate structure, reactivity, or electrostatic potential surface of intermediate(s) or transition state(s). In contrast, the expectation of serendipitously finding potent druglike inhibitors that never would have been predicted or designed is statistically less probable, since these types of molecules are in the minority of marketed drugs. The future holds more than 71 enzyme targets for treating disease, and applying the lessons of the first 71 will speed the development of the next generation.

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