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Winning the Arms Race by Improving Drug Discovery against Mutating Targets

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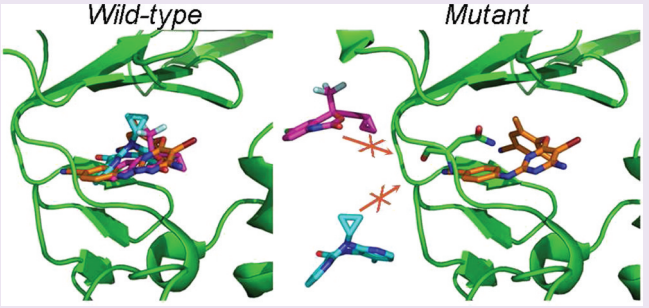
ABSTRACT: Enzymes are often excellent drug targets. Yet drug pressure on an enzyme target often fosters the rise of cells with resistance-conferring mutations, some of which may compromise fitness and others that compensate to restore fitness. This review presents, first, a structural analysis of a diverse group of wild-type and mutant enzyme targets and, second, an in-depth analysis of five diverse targets to elucidate a broader perspective of the effects of resistance-conferring mutations on protein or organismal fitness. The structural analysis reveals that resistance-conferring mutations may introduce steric hindrance or eliminate critical interactions, as expected, but that they may also have indirect effects such as altering protein dynamics and enzyme kinetics. The structure-based development of the latest generation of inhibitors targeting HIV reverse transcriptase, *P. falciparum* and *S. aureus* dihydrofolate reductase, neuraminidase, and epithelial growth factor receptor (EGFR) tyrosine kinase, is highlighted to emphasize lessons that may be applied to future drug discovery to overcome mutation-induced resistance. Successful next-generation drugs tend to be more flexible and exploit a greater number of interactions mimicking those of the substrate with conserved residues.



It has long been recognized that enzymes can be excellent drug targets. In fact, approximately half of all orally dosed clinically used drugs inhibit enzyme targets.[1,2] Over time, however, these drugs, especially when directed against proliferating malignant or pathogenic cell lines, can foster the predominance of a population of resistant cells. While there is a wide range of mechanisms that these cells employ to trigger resistance, frequently the resistant strains have rapidly evolved mutant enzymes that continue to competitively carry out the essential catalytic function while no longer being subject to inhibition by the drug. From a drug discovery perspective, these resistance-inducing mutations are impediments that reduce the efficacy and lifetime of the drug and often force the discovery process to begin anew.

Given the diverse nature of the enzymes that undergo mutation-induced resistance, it is interesting to speculate as to whether there are any consistent patterns in the evolved mutations. For example, do the majority of mutations directly affect inhibitor binding, such as with the introduction of steric interactions or elimination of critical interactions, or do the mutations employ more subtle indirect mechanisms? Are different species of a particular target prone to the same or similar mutations? In the first section of this review, a subset of several enzymes that are targeted by clinically used drugs and are highly prone to mutation were selected for analysis from a diverse group of antibacterial, antifungal, antiprotozoal, antiviral, and anticancer drug targets. Comparisons of the crystal structures of these wild-type and resistant protein enzyme drug targets illuminate the structural mechanisms by which mutations induce resistance.

An extensive body of research has shown that the parent enzyme suffers trade-offs, such as a reduction of catalytic activity or stability, by adopting resistance mutations or other mutations that evolve a new function.[3,4] For example, mutants of TEM-1 β-lactamase that exhibit increased activity toward third-generation cephalosporins show decreased thermodynamic stability and decreased catalytic activity toward penicillins.[5] Interestingly, in several cases, compensatory mutations restore activity or stability.[3] The M182T mutation in β-lactamase is an example of a compensatory mutation that is distant from the active site yet restores protein stability.[5] Therefore, in the second section of this review, detailed discussion of five enzyme targets will feature the structural effects of resistance mutations as well as kinetic and thermodynamic fitness costs and compensation.

It is worthwhile to note that in many cases antibiotics have served as chemical probes to elucidate the details of biological pathways.[6,7] Antibiotics have also been used to investigate the evolution of additive or synergistic mutations to a parent enzyme that reveals a contoured fitness landscape.[8−10] Fitness landscapes can be used to suggest the predictability and/or propensity of particular drug targets to undergo mutations that confer resistance.

Encouragingly, new generations of enzyme inhibitors have been developed to overcome mutation-induced resistance. Examining the latest generation of inhibitors that overcome

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Table 1. Structural Effects of Mutations in Enzyme Drug Targets

target	mutation	inhibitor contact?	effect on structure	reference
HIV RT (NNRTIs)	L100I	yes	steric	15, 16
	K103N	yes	stabilizes closed form of binding site	
	Y181C/I	yes	elimination − reduce favorable $\pi-\pi$ interactions	
	Y188C/L/H	yes	elimination − reduce favorable $\pi-\pi$ interactions	
	V106A	yes	elimination − loss of hydrophobic interactions	
	V108I	yes	steric	
	G190A/S/E	yes	steric	
Abl kinase	T315I	yes	steric	17−19
DHPS (*P. falciparum*, sulfadoxine)	A437G	yes	elimination − loss of hydrophobic interactions	20, 21
	S436F/A	yes	steric (F) or elimination − loss of H-bond (A)	
	K540E	yes	elimination − loss of hydrophobic interactions	
	A581G	yes	elimination − loss of hydrophobic interactions	
	A613T/S	no	unclear	
DHFR (*P. falciparum*, pyrimethamine)	S108N	yes	steric	22−24
	I164L	yes	elimination − loss of van der Waals interactions	
	C59R	no	R59 extends into solvent, perhaps weak electrostatic repulsion	
	N51I	no	elimination − main chain movement	
INH	I21V	yes	elimination − loss of van der Waals interaction	25−27
	S94A	no	elimination − loss of water-mediated H-bond	
neuraminidase (zanamivir)	E119G	yes	elimination − loss of ionic interaction	28−30
neuraminidase (oseltamivir)	H274Y	no	steric − Tyr prevents conformational change associated with E276	31, 32
	R292K	yes	elimination − loss of ionic interaction	
	N294S	no	prevents conformational change associated with E276	
gyrase	G88A	no	elimination − loss of metal ion coordination	37, 42
	A90V	yes	steric	
	D94A/G/N	no	increases or decreases volume of site	
DHPS (*S. aureus*, sulfamethoxazole)	16 possible	no	unclear	38
DHFR (*S. aureus*, TMP)	F98Y	no	elimination − altered conformation of NADPH, loss of H-bond	33, 39−41
	H30N	no	elimination − loss of water-mediated H-bond	
EGFR tyrosine kinase	T790M	yes	enzyme kinetics − increases affinity for ATP	34, 43−49
CYP51	4 hotspots: (1) G464S, G465S R467K; (2) E266D, R267H, D278E and S279F; (3) F72L, F105L, S405F; (4) D116E, F126L, K128T, G129A, Y132H, K143R, F145L, K147R, A149 V and D153E	no	protein dynamics	35, 36

resistance in several different enzyme classes reveals clear strategies for improved drug design. Next generation inhibitors tend to be flexible, interact with conserved residues critical for substrate binding, or in an interesting case, alter enzyme kinetics.

■ ENZYME MUTATIONS EXERT A WIDE RANGE OF STRUCTURAL EFFECTS

An in-depth structural analysis of a broad range of complexes of wild-type and mutant enzymes with clinically used drugs shows that there is a wide range of mechanisms that mutant enzymes employ to generate resistance yet maintain catalytic competency (Table 1). The list in Table 1 is intended to be representative, not exhaustive. In fact, there are additional enzymes that are subject to resistance-conferring mutations, notably HIV protease and the nucleotide binding site of HIV reverse transcriptase that are discussed in recent reviews[11−14] and for which the Stanford HIV database (http://hivdb.stanford.edu/index.html) maintains an updated list of resistance mutations. Surveying the data in Table 1, there are several examples of mutations of residues in the first shell directly contacting the drug that affect drug binding, as one may expect. These resistance-conferring mutations have two primary effects: eliminating critical interactions between the drug and the protein or introducing steric interactions that prevent drug binding. For example, mutations in the first shell of residues of the non-nucleoside binding site of HIV reverse transcriptase cause resistance toward nevirapine, delavirdine, and efavirenz (NNRTIs)[15,16] and in Abl kinase confer resistance for imatinib.[17−19] Additionally, first-shell mutations in *P. falciparum* dihydropteroate synthase (DHPS), *P. falciparum* dihydrofolate reductase, enoyl acyl-carrier reductase (Inh), and neuraminidase affect binding to sulfadoxine,[20,21] pyrimethamine,[22−24] isoniazid,[25−27] and zanamivir,[28−30] respectively.

There are also many examples of enzymes for which resistance mutations are more subtle and affect residues that do not directly contact the drug. These mutations may influence enzyme dynamics, substrate or cofactor equilibrium,

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or solvent networks. For example, Collins *et al.* established that mutations in oseltamivir-resistant neuraminidase (H274Y, N294S, and R292K) primarily influence the capacity of the enzyme to accommodate the pentyloxy chain of oseltamivir near active site residue Glu 276.[31,32] In our own work, we showed that mutations in *S. aureus* DHFR affect the equilibrium of conformations of the cofactor, NADPH.[33] In a third example, Yun *et al.* showed that resistance to erlotinib and gefitinib, conferred by the T790M mutation of the tyrosine kinase domain of epidermal growth factor receptor (EGFR), increases affinity for the substrate, ATP.[34] Neuraminidase, *S. aureus* DHFR and EGFR tyrosine kinase will be discussed in greater detail in the following section. Finally, structures determined by Podust *et al.* reveal that mutations in CYP51, the 14α-demethylase involved in sterol biosynthesis and an important target of the azole antifungal drugs, are also remote from the drug binding site. It appears that the mutated residues in CYP51 are involved in dynamic regions of the protein that may affect the critical conformational changes associated with catalysis.[35,36]

Interestingly, quinolones have acted as chemical probes to reveal important structural features of the enzyme. The structures of gyrase bound to moxifloxacin reveal that resistance mutations arise in proximity to a noncatalytic magnesium ion,[37] explaining structure−activity relationships for the quinolone class of molecules.

It is also valuable to note that mutation-induced resistance mechanisms may differ for the same target in different species, suggesting that a single target does not have a single mechanism by which mutations will incur resistance to a conserved class of drugs. Dihydropteroate synthase (DHPS) from *P. falciparum* and *S. aureus* is inhibited by sulfadoxine and sulfamethoxazole, respectively. In *P. falciparum*, the authors mapped mutations to the active site of DHPS and showed that they affect inhibitor binding directly.[20,21] In *S. aureus*, the authors mapped the 16 known resistance mutations to the surface of the DHPS structure but could not explain the structural basis of resistance.[38] Likewise, several studies have shown that mutations that confer pyrimethamine resistance are located in the first shell of residues in dihydrofolate reductase (DHFR) from *P. falciparum*.[22−24] However, our studies and others show that mutations that confer trimethoprim resistance in *S. aureus* DHFR are located in the second shell of residues and affect cofactor conformation.[33,39−41] Finally, an overall analysis of the mutations in Table 1 shows that most involve amino acid changes that are most likely single nucleotide polymorphisms. Two mutations, however, imply double nucleotide polymorphisms: Arg to Lys (or *vice versa*) occurs in neuraminidase and CYP51 and Asp to Asn occurs in gyrase. It is remarkable that these double nucleotide polymorphisms occur, given the extremely low probability of their occurrence.

DRUG DESIGN TO OVERCOME THE STRUCTURAL AND FITNESS EFFECTS OF RESISTANCE-CONFERRING MUTATIONS IN FIVE ENZYME TARGETS

Mutations that confer resistance have been evolving in enzyme drug targets for as long as enzymes have been targeted for drug pressure. While this problem may appear daunting, there have been significant inroads to understand how to design drugs to overcome resistance mutations in specific enzyme targets. Analyzing a number of these cases of specific targets for which latest generation drugs are clearly less prone to resistance mutations reveals a few principles that may be applicable to a much broader set of enzyme targets. Discovery of drugs with highly improved resistance profiles shows that successful drugs are often more flexible and maintain an interaction profile similar to the substrate. In one interesting case, the improved drug alters enzyme kinetics. Five specific enzyme targets, HIV reverse transcriptase, *P. falciparum* and *S. aureus* DHFR, neuraminidase, and EGFR tyrosine kinase, will be analyzed to illustrate the structural and fitness effects of resistance-conferring mutations. Additionally, each of these enzymes will illustrate one of the concepts for improving drug discovery efforts to overcome resistance mutations.

Along similar lines, Schiffer *et al.* found that resistance mutations in HIV protease typically evolve in residues that contact inhibitors but do not contact substrates.[50] They have proposed that drugs for HIV protease should fit within the volume enclosed by the various substrates of HIV protease, termed the substrate binding envelope, in order to avoid resistance mutations.[50,51] In fact, this group has extended the substrate envelope hypothesis to explain the resistance mutations discovered in hepatitis C viral protease in response to telaprevir, boceprevir, and ITMN-191.[52]

FLEXIBLE DRUGS AVOID RESISTANCE MUTATIONS

HIV Reverse Transcriptase: NNRTIs. Inhibitors of HIV reverse transcriptase (RT), including nucleoside analogues that target the active site and non-nucleoside reverse transcriptase inhibitors (NNRTIs) that target an allosteric site, have been instrumental components of highly active antiretroviral therapy (HAART). Mutations in HIV RT limit the utility of both types of RT inhibitors; the NNRTI binding site will be the focus of this review. Developing successful NNRTIs that are not subject to resistance-conferring mutations is especially difficult because although the allosteric site must adopt the proper shape to maintain catalysis in the active site, it does not have the same strict constraints of maintaining positions of residues poised for catalytic activity. Crystal structures of RT bound to a variety of first-generation NNRTIs such as nevirapine (Figure 1a and b)[53−55] revealed that the binding site is hydrophobic and can be in an open form with a bound NNRTI or a closed form in the absence of an NNRTI.[16] Resistance to nevirapine is primarily conferred by the mutations K103N, Y181C, and/or Y188L in the NNRTI binding site. The Y181C and Y188L mutations interact directly with the NNRTIs by reducing favorable $\pi-\pi$ interactions with the aromatic rings of the inhibitors.[15,16] The K103N mutation acts indirectly by inducing the formation of a hydrogen bond between N103 and Y188, which stabilizes a closed form of the binding site and reduces inhibitor entry.

While viruses with K103N and Y181C NNRTI resistance mutations are equally fit compared to wild-type virus, viruses with mutations V106A, G190A, G190S, and P236L exhibit approximately 50% reduced fitness.[56] Wang *et al.* confirms that both deficiencies in RNase H activity and reduced RT content correlate with the reduction in fitness. Indeed, RT with resistance mutations K103N or Y181C retains wild-type levels of RNase H activity, while RT with mutations V106A or G190S shows decreased RNase H activity and RT with mutation P236L is highly impaired. The content of RT is reduced in all mutant viruses, but the content of RT in viruses with the P236L mutation is higher than that of the G190S or V106A, somewhat compensating for the highly decreased RNase H activity.

The discovery of etravirine (TMC125-R165335; Figure 1a)[16] focused on the development of a potent NNRTI (IC_{50} = 5.4 nM) that maintains affinity for the K103N mutant enzyme (IC_{50} = 3.8 nM).[57] Etravirine incorporates torsional flexibility (Figure 1c)

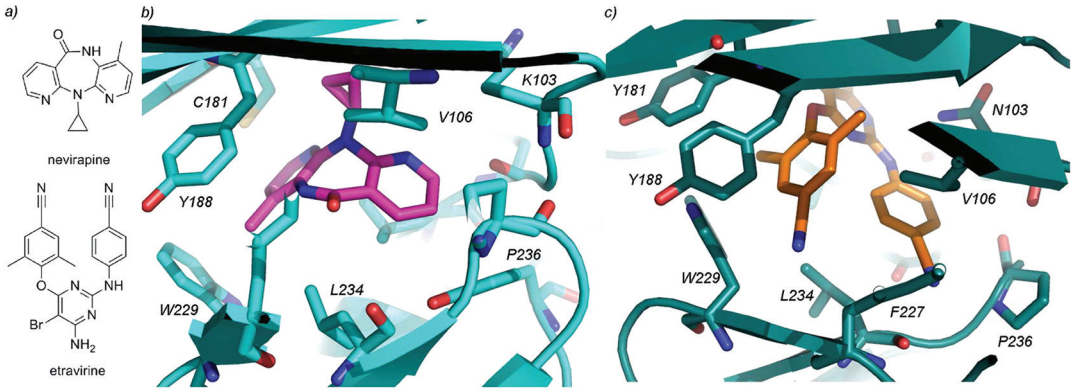

Figure 1. (a) HIV NNRTIs. (b) Nevirapine (magenta) and (c) etravirine (orange) in the non-nucleoside binding pocket of HIV RT from PDB ID 1JLB and 3MED, respectively. Note that PDB ID 1JLB contains a Y181C mutation and 3MED contains a K103N mutant of RT.

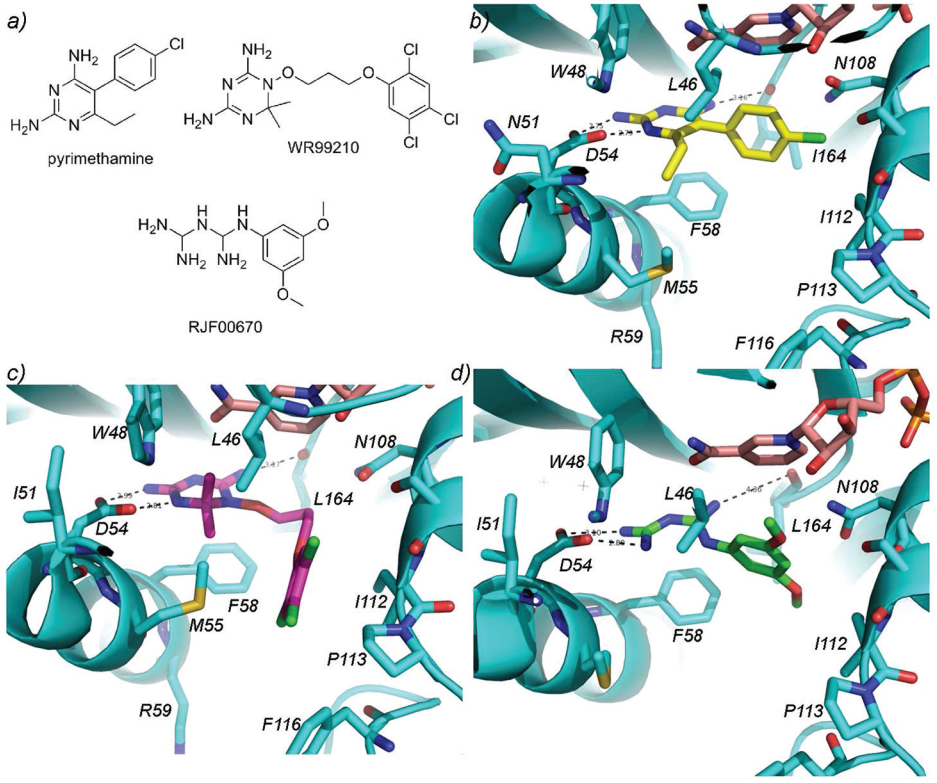

Figure 2. (a) *P. falciparum* DHFR inhibitors. (b) The active site of the double mutant (R59, N108) *P. falciparum* DHFR-TS is bound to pyrimethamine (yellow; PDB ID 1J3J). (c) The quadruple mutant (I51, R59, N108, L164) *P. falciparum* DHFR-TS is bound to WR99210 (magenta; PDB ID 1J3K). (d) The quadruple mutant (I51, R59, N108, L164) *P. falciparum* DHFR-TS is bound to RJF00670 (green; PDB ID 3DG8). The cofactor, NADPH, is shown in salmon.

and multiple binding modes to avoid the steric effects of the resistance mutations.[16] In fact, by adopting a twisted conformation in the K103N mutant enzyme, the inhibitor forms enhanced interactions with Leu 100 and Asn 103. Etravirine has a very high genetic barrier to resistance; it is believed that the enzyme must acquire at least three mutations other than K103N to incur resistance.[57,58]

***P. falciparum* DHFR.** Dihydrofolate reductase (DHFR) catalyzes the reduction of dihydrofolate to tetrahydrofolate, a critical process in the folate biosynthetic pathway. For several decades, pyrimethamine (Figure 2a), a potent DHFR inhibitor, was used in combination with sulfadoxine to treat malarial infections caused by *Plasmodium*. However, owing to widespread increasing resistance, it is no longer recommended for the treatment of uncomplicated malaria.[59] Over time, four mutations in the dihydrofolate reductase domain of the bifunctional *P. falciparum* enzyme (with thymidylate synthase: DHFR-TS) arose in response to treatment with pyrimethamine; the combination of these mutations results in an enzyme that binds pyrimethamine with 1000-fold lower affinity.[22,23] Two of the mutations, S108N and I164L, are in the first shell of residues that contact pyrimethamine (Figure 2b). Using structures of *P. falciparum* DHFR-TS, Yuvaniyama *et al.* discovered that the S108N mutation exerts a primarily steric influence on the *p*-chloro group on pyrimethamine and the I164L mutation slightly opens the site, eliminating interactions for small inhibitors.[24] The remaining two mutations, which are in the second shell, most likely evolved in order to rescue

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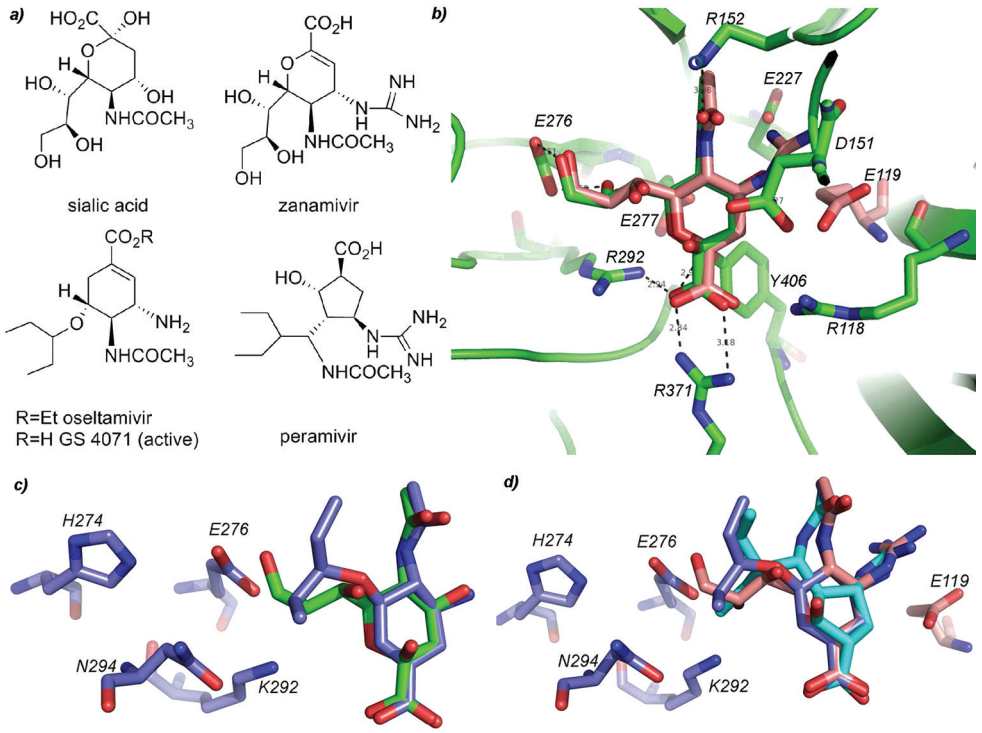

Figure 3. Mutations in neuraminidase cause resistance to zanamivir and oseltamivir. (a) The NA substrate, sialic acid, and NA inhibitors zanamivir, oseltamivir, and peramivir. (b) The crystal structure of N2 neuraminidase bound to sialic acid (PDB ID 2BAT[30]) (green) shown with active site residues and zanamivir (salmon) from the complex with N8 neuraminidase (PDB ID 2HTQ[75]). (c) The complex of the R292K mutant of N9 neuraminidase bound to oseltamivir (purple, PDB ID 2QWH[32]) superimposed with sialic acid from 2BAT (green). The location of additional resistance mutations H274 and N294 are noted, as is the location of E276. (d) The complex of N8 neuraminidase bound to peramivir (cyan, PDB ID 2HTU[75]) superimposed with zanamivir (salmon) and oseltamivir (purple), along with the residues that mutate to cause zanamivir and oseltamivir resistance.

interactions with the glutamate tail of the substrate in the presence of the resistance mutations[24] and to enable a maximal synergistic effect.[23]

Earlier evidence with isolated *P. falciparum* DHFR domains suggested that the mutant enzymes exhibit decreased catalytic efficiency, as measured by k_{cat}/K_m, individually and in combination.[23] More recent evidence, however, shows that activity of the mutant DHFR, when expressed in parallel with the TS domain, is not compromised.[60] In a transgenic *S. cerevisiae* model of antifolate resistance,[61] each of the three predicted evolutionary trajectories that terminate with the quadruple mutant begins with the S108N mutation and a concomitant reduction in growth rate; however, subsequent mutations serve to compensate for the initial reduction in growth rate in order to preserve overall fitness.[61] Hartl *et al.* has analyzed the evolutionary trajectories of DHFR[9] using potent antibiotics such as pyrimethamine as chemical probes to reveal the "fitness landscape". Using pyrimethamine as a probe molecule, it was determined that there are only a few high-probability paths to resistance, which suggests a high degree of predictability for the generation of resistance.[10] These evolutionary traces may also potentially predict the likelihood of successful restoration of the wild-type phenotype after drug removal.

Drug discovery to overcome the effects of the pyrimethamine-induced resistance mutations has focused on the development of more flexible inhibitors that increase interactions and evade the Asn 108 mutation. Specifically, WR99210,[62,63] a dihydrotriazene molecule (Figure 2a), retains potency against the wild-type and resistant strains carrying the mutant enzyme.[64] Crystal structures of *P. falciparum* DHFR-TS bound to WR99210[24] reveal that the flexible propyloxy linker of the compound avoids the steric interference caused by the S108N resistance mutation (Figure 2c). Unfortunately, pharmacokinetic issues have limited the usefulness of WR99210.[65] Recently, a virtual screening effort directed at a nonactive site pocket identified three biguanide compounds (RJF 00719, RJF 01302, and RJF 00670) that show low micromolar activity against the wild-type and drug-resistant enzymes. Co-crystal structures of these biguanide compounds with *P. falciparum* DHFR-TS (Figure 2d) show that while some van der Waals interactions and a conserved hydrogen bond with L164 is lost, these flexible compounds avoid the steric interference from Asn 108.

DRUGS THAT MIMIC INTERACTIONS OF THE SUBSTRATE OVERCOME RESISTANCE MUTATIONS

Neuraminidase. Neuraminidase (NA) is a critical enzyme in the lifecycle of the influenza virus as it cleaves *N*-acetyl neuraminic acid (Neu5Ac, also called sialic acid) from glycoproteins in the mucosal secretions, which degrades the mucous layer and allows the virus to approach host epithelial cells. NA plays a second role in releasing virions from the host cell. Crystal structures of NA bound to sialic acid[30] formed the basis of drug design efforts to develop transition state mimics, including zanamivir (Figure 3a).[28] Zanamivir contains a 4-guanidium group that forms an ionic interaction with Glu 119,[29] which when mutated to glycine (E119G) leads to 1000-fold loss of affinity (Figure 3b).

Efforts to improve the pharmacokinetics of NA inhibitors led to the development of oseltamivir (Figure 3a), a carbocyclic

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analogue with a pentyloxy substituent.[66,67] Upon binding, the pentyloxy substituent induces a 2 Å shift of the carboxylate of active site residue Glu 276 to create hydrophobic interactions with the C_β position of Glu 276. Two prevalent second-shell resistance mutations influence the formation of the hydrophobic interactions.[32] The H274Y mutation employs steric interference to prevent the conformational change in Glu 276 and N294S forms a hydrogen bond with Glu 276, restraining it from the necessary conformational change and eliminating a hydrogen bond that Asn 294 forms with Tyr 347.[31] An additional first shell mutant, R292K, binds the carboxylate of both oseltamivir and the substrate less efficiently, leading to a mutant enzyme with only 20% of the wild-type activity (Figure 3c).

Analysis of the kinetics of oseltamivir and zanamivir binding to wild-type and mutant NA reveals that although the mutant NA retains the same catalytic efficiency as the wild-type, there are significant differences in the association and dissociation rates. For example, the H274Y mutant of NA is resistant to oseltamivir owing to a 10-fold decrease in the association rate and a 25-fold increase in the dissociation rate.[31] From 1999 to 2007, there was a lack of consensus regarding whether viruses containing the H274Y mutation would be clinically relevant. On one hand, studies showed that virus containing H274Y exhibited attenuated fitness in mice, ferrets, and lab strains.[68,69] Alternatively, a Vietnam strain carrying the H274Y mutation retains replication efficiency and pathogenicity.[70] Indeed, while the wild-type and H274Y NA proteins are approximately equally stable, they exhibit differential expression at the cell surface.[71] However, the 2007−2008 flu season revealed that viruses with the H274Y mutation were prevalent,[72] raising questions of whether secondary mutations compensate to restore fitness. Recent work shows that two mutations, R222Q and V234M that do not contact the drug, restore NA surface expression and viral fitness in an H274Y background.[71] The discovery of R222Q and V234M, which are believed to exist before the evolution of H274Y, opens new avenues in our understanding that secondary mutations can precede the predominant resistance-conferring mutation.

New discovery of NA inhibitors has led to the development of peramivir (Figure 3a).[73] Peramivir dissociates from the active site of NA more slowly than zanamivir and oseltamivir; on-site dissociation experiments designed to measure the off-rate of the inhibitor from the enzyme:inhibitor complex show that peramivir has a half-life greater than 24 h, whereas zanamivir and oseltamivir dissociate rapidly with half-lives of approximately 1 h.[74] Crystal structures of peramivir and NA[75] show that the guanidino group binds in a different orientation compared with zanamivir and displaces a bound water molecule observed in the structure with zanamivir. In fact, most likely owing to this altered orientation of the guanidine group, peramivir maintains activity against the E119G mutant enzyme.[73] The hydroxyl group on the cyclopentane of peramivir maintains a hydrogen bond with Asp 151, similar to sialic acid, interactions that are missing in zanamivir and oseltamivir.[75]

Overall, it is clear that the neuraminidase residues that mutate to cause resistance toward zanamivir and oseltamivir form critical interactions with the drugs but do not form critical interactions with the substrate. Zanamivir resistance is promoted by E119G; E119 does not substantially interact with the amino group of sialic acid. In the case of oseltamivir, it might be assumed that Glu 276, which is adjacent to the pentyloxy side chain, could mutate to cause resistance. However, Glu 276 forms a hydrogen bond with the glycerol side chain of sialic acid, thus reducing the probability of mutations at that residue. In response, the mutations that cause oseltamivir resistance, H274Y, N294S and R292K, then employ a more subtle mechanism to prevent the key conformational change of E276 that accommodates the pentyloxy side chain. In fact, it has been noted [32] that NA inhibitors with a glycerol side chain that resemble the substrate have lower losses of affinity for the R292K mutant. The cyclopentyl ring system of the latest inhibitor, peramivir, reduces the projection of the pentyloxy side chain toward E276, relative to oseltamivir, thus reducing the propensity to be affected by mutations in that pocket.

***S. aureus* DHFR.** Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infections are often managed with trimethoprim-sulfamethoxazole, a combination of DHFR and dihydropteroate synthase (DHPS) inhibitors, respectively.[76] Surveys of a collection of strains show that approximately 28% of clinical isolates of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) strains are resistant to trimethoprim[77] and reveal pairs of mutations in DHFR at H30N/F98Y or F98Y/H149R. The F98Y mutation, alone, has been shown biochemically to confer the majority (74-fold) of the loss in affinity.[39] Unlike the examples noted above, the mutant *S. aureus* DHFR catalyzes substrate with approximately the same efficiency as the wild-type enzyme[39] and does not demonstrate a fitness cost *in vitro*.[78] Structures of *S. aureus* DHFR from wild-type and the F98Y mutant bound to trimethoprim suggest that a hydrogen bond between the backbone carbonyl of Leu 5 and the 4-amino group may be eliminated,[39,41] causing the reduction in affinity. Several crystal structures of the propargyl-linked antifolates (Figure 4a) with wild-type and the F98Y mutant DHFR reveal a novel conformation of the cofactor, NADPH, that decreases interactions with the inhibitor and is primarily associated with the mutant enzyme.[33] A crystal structure of a propargyl-linked antifolate with the clinically observed mutant, H30N/F98Y, shows that the solvent network between the 2-amino group on the pyrimidine ring and His 30 is altered in the mutant enzyme, eliminating a hydrogen bond;[40] this mechanism is most likely duplicated for trimethoprim since these inhibitors share the same 2,4-diaminopyrimidine anchor group.

Trimethoprim does not take advantage of all of the interactions that the substrate employs in the active site. In order to generate potent ligands that are less susceptible to resistance-conferring mutations, the development of the propargyl-linked antifolates[33] and iclaprim[79] focuses on increasing interactions with additional conserved residues Leu 20, Leu 28, Val 31, Ile 50, and Leu 54, important for binding substrate. To achieve these extra interactions, the propargyl-linked antifolates extend a biphenyl moiety[80] (Figure 4b), and iclaprim includes a dimethoxy chromene moiety[79] to interact with the hydrophobic pocket (Figure 4c).

DRUGS DESIGNED TO ALTER ENZYME KINETICS OVERCOME RESISTANCE MUTATIONS

EGFR Tyrosine Kinase. The tyrosine kinase domain of epithelial growth factor receptor (EGFR) is a well-recognized target for the development of anticancer therapies owing to the role of EGFR in cellular proliferation and growth.[81−84] Deriving from this, anilinoquinazoline derivatives such as gefitinib and erlotinib (Figure 5a) that compete with ATP were developed during the 1990s for non-small cell lung cancer (NSCLC).

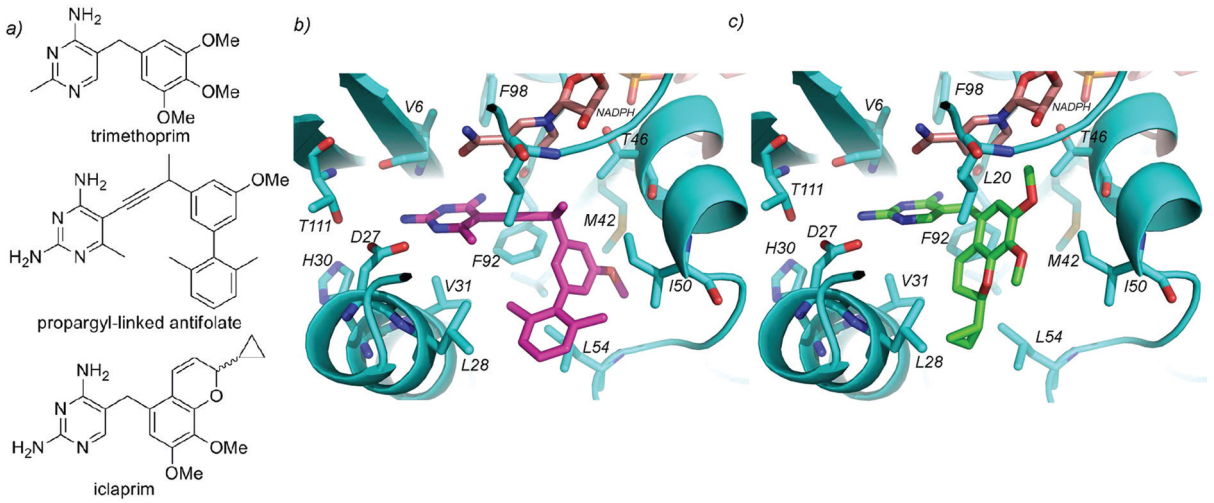

Figure 4. (a) Inhibitors of *S. aureus* DHFR: trimethoprim, propargyl-linked antifolates, and iclaprim. (b) The structure of *S. aureus* DHFR (cyan) bound to NADPH (salmon) and a propargyl-linked antifolate (magenta; PDB ID 3F0Q) with the residues at F98 and H30 shown. (c) The structure of *S. aureus* DHFR bound to NADPH and iclaprim (green; PDB ID 3FRF).

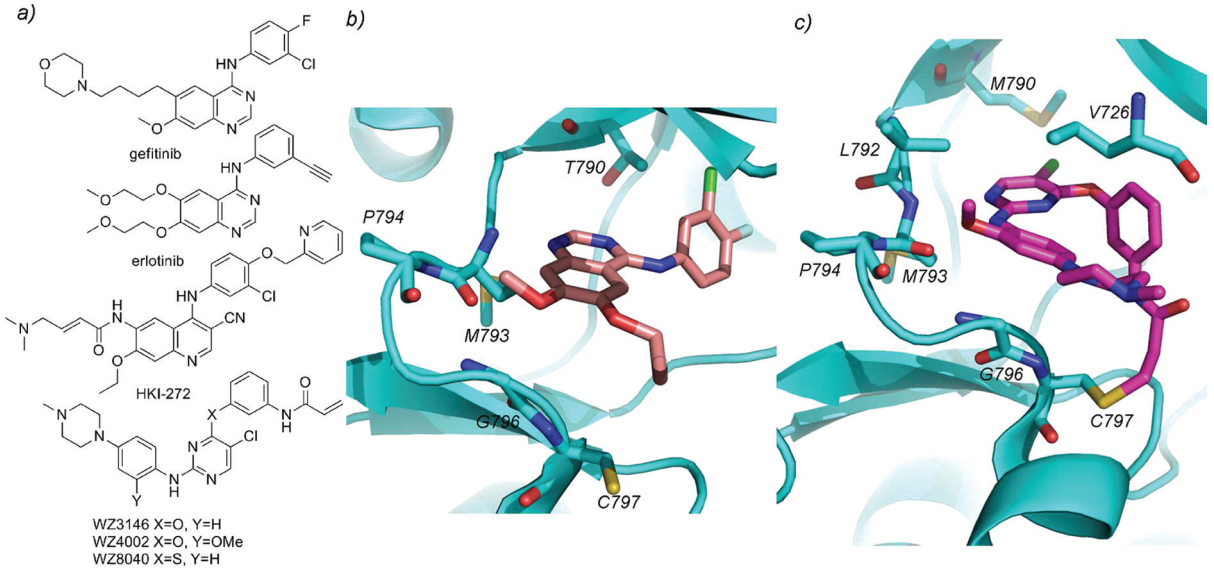

Figure 5. (a) EGFR tyrosine kinase inhibitors gefitinib, erlotinib, HKI-272, and WZ derivatives. (b) The EGFR tyrosine kinase domain bound to gefitinib (salmon) from PDB ID 2ITY[49]. (c) The EGFR tyrosine kinase domain (T790M) bound to WZ4002 (magenta) from PDB ID 3IKA.[95]

After clinical testing of erlotinib, oncogenic mutations such as a deletion in exon 19 of EGFR or point mutations L858R and G719S in the kinase domain were identified in certain populations of patients.[46,85,86] These mutations give rise to a more activated form of EGFR; specifically, L858R and G719S appear to destabilize the inactive form of the kinase.[34] Patients with these activating mutations are more susceptible to gefitinib and erlotinib.[46,85,86]

Mutations to EGFR, especially at T790M, the "gatekeeper residue", arise after clinical use of gefitinib and erlotinib. The T790M mutation was originally assumed to create a steric interaction with the inhibitor, but in fact the affinity of gefitinib for the mutant and wild-type EGFR kinases is practically unchanged,[49] effectively disproving the steric interaction hypothesis. In a rare case, the T790M mutation affects the kinetics of the enzyme by increasing ATP affinity by more than an order of magnitude,[49] thus causing drug resistance by altering the equilibrium of an ATP-competitive inhibitor.

In order to overcome the effects of the T790M mutation, classes of irreversible inhibitors including HKI-272[87] (reviewed in ref [88]; neratinib; Figure 5a), PF-00299804,[89] and CI-1033[90] have been developed to evade the ATP equilibrium. The salient feature of these compounds is an acrylamide moiety that forms a Michael adduct to an active site cysteine, Cys 797.[87,91,92] However, clinical data for these compounds show dose-limiting toxicity that manifests as diarrhea and rash.[93,94] Here we highlight recent attempts to identify new scaffolds that selectively target the mutated EGFR and are more potent than HKI-272. Toward this goal, a library of compounds was screened against cell lines expressing the mutant kinase.[95] Three pyrimidine compounds, WZ3146, WZ4002, and WZ8040 (Figure 5a), were identified and show significantly (~300-fold) lower IC_{50} values against gefitinib-resistant H1975 (L868R/T790M) and Ba/F3 (T790M) cells compared with HKI-272. A crystal structure of EGFR T790M bound to WZ4002 shows that the expected covalent bond is formed between the

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acrylamide and Cys 797 and that the chlorine atom contacts Met 790, explaining the affinity of the compound for the mutant enzyme. Further studies will investigate the clinical utility of this new class of EGFR tyrosine kinase inhibitors effective against the T790M mutant enzyme.[95]

CONCLUSIONS

By incorporating knowledge of drug resistance early in the process of drug discovery, it may be possible to extend the lifetime of new drugs. While there are several possible approaches to understanding resistance, the use of high resolution structural information from a diverse group of enzymes yields a broad perspective of the effects of resistance-conferring mutations and generates hypotheses to guide the successful design of more effective inhibitors. The survey in this review shows that enzymes evolve mutations that employ a wide range of mechanisms to incur resistance including the expected elimination of interactions and introduction of steric hindrance as well as unexpected subtle influence such as elimination of solvent interactions and effects on protein dynamics and enzyme kinetics. Resistance-conferring mutations often affect enzyme kinetics; examples include decreased activity for HIV RT and *P. falciparum* DHFR and an increased inhibitor off-rate (NA). Structural information has proven invaluable in the design of the latest generation of inhibitors incorporating flexibility to accommodate mutated residues in HIV reverse transcriptase and *P. falciparum* dihydrofolate reductase. A structure-based strategy that has proven successful for the design of new inhibitors of neuraminidase and *S. aureus* DHFR is one that primarily targets substrate-binding residues, as the mutation of these residues may increase the fitness cost to a level that precludes the viability of the mutation. New designs for EGFR tyrosine kinase inhibitors incorporate both structural and enzyme kinetic studies to target the mutant enzyme. Lessons for overcoming resistance-conferring mutations should have broad application in early drug discovery programs.

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KEYWORDS

Resistance: decreased or eliminated drug efficacy; Mutation-induced resistance: drug resistance owing to the alteration of the drug target by mutation(s); Fitness: the capacity of a resistant organism to survive; Enzyme fitness: the capacity of a mutated enzyme to stably carry out catalytic function; Non-nucleoside reverse transcriptase inhibitor (NNRTI): a drug that binds an allosteric site on the enzyme to target HIV reverse transcriptase; Antifolate: a compound that acts by inhibiting folate biosynthesis; EGFR TKI: a molecule that inhibits the intracellular tyrosine kinase domain of epithelial growth factor receptor; Neuraminidase: the influenza viral enzyme that cleaves sialic acid from glycoproteins, allowing viral entry and exit from the host cell

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