The Challenge of Developing Robust Drugs to Overcome Resistance

Celia Schiffer, Celia.Schiffer@umassmed.edu, Amy C. Anderson, Michael Pollastri and Norton P. Peet, npeet@microbiotix.com

Drug resistance is problematic in microbial disease, viral disease and cancer. Understanding at the outset that resistance will impact the effectiveness of any new drug that is developed for these disease categories is imperative. In this Perspective, we detail approaches that have been taken with selected drug targets to reduce the susceptibility of new drugs to resistance mechanisms. We will also define the concepts of robust drugs and resilient targets, and discuss how the design of robust drugs and the selection of resilient targets may lead to successful strategies for combating resistance.

Introduction

Drug resistance is defined, in a clinical setting, as the point at which administration of the drug can no longer safely treat the disease state due to an induced change in the drug target or an inability of the drug to reach the target. With an anti-microbial agent, clinical resistance occurs when the minimum inhibitory concentration (MIC) of the drug, for a given microbial strain, exceeds that concentration of drug that can safely be administered. Resistance to a drug can arise by (1) mutation of the gene (or gene cluster); (2) by acquisition of extrachromosomal DNA, or a transposable plasmid, that carries the resistance gene or genes; (3) upregulation of the target; or (4) upregulation of an efflux mechanism.

We have recently suggested [1] an approach to combating drug resistance that involves the selection of resilient drug targets [2] that are evolutionally constrained and the development of robust drugs [3] that are less susceptible to the development of resistance. This Perspective will focus on selected, potentially resilient drug targets and describe efforts to produce drugs with various degrees of robustness for these targets in the disease areas of cancer, bacterial and viral infections. The highlighted drug targets include: BCR-ABL kinase; epithelial growth factor receptor (EGFR) kinase, and platelet-derived growth factor (PDGFR) kinase in cancer; dihydrofolate reductase (DHFR) and
DNA gyrase/topoisomerase IV in bacteria; and human immunodeficiency virus (HIV) protease and hepatitis C (NS3/4A) protease in viruses. As enzymes are evolutionally constrained to catalyze a chemical reaction, they have the potential of being resilient targets; however, properly identifying robust inhibitors remains a challenge for the field.

Drug resistance in enzyme targets in cancer

Drug resistance is now a widespread problem in cancer and is particularly problematic with kinase inhibitors, proteasome inhibitors [4,5] and monoclonal antibodies [6]. Of these, kinase inhibitors comprise the largest class of anticancer agents where drug resistance has significantly limited treatment. In this Perspective the focus will be on the challenges of developing robust drugs to overcome resistance to inhibitors of the kinase domains of BCR-ABL, EGFR and PDGF.

BCR-ABL inhibitors

Gleevec (imatinib) shows remarkable efficacy by achieving clinical remission in chronic myeloid leukemia (CML). Imatinib inhibits the constitutively active kinase domain, c-Abl, of the fusion protein, Bcr-Abl [7] existing in CML patients. Evidence from biochemical and crystallographic studies [8] shows that imatinib selectively binds a unique conformation of the activation loop in which a conserved phenylalanine, a member of the Asp-Phe-Gly (DFG) trio of amino acids, undergoes an extensive 10Å conformational change, into what is called the DFG-out conformation [9,10]. Imatinib binding freezes the kinase in the inactivated state. Interestingly, many kinases cannot adopt an inactive conformation [11]; therefore, targeting this state yields inhibitors with selectivity over other kinases. Crystal structures of other kinases in the DGF-out conformation include: KIT (mast/stem cell growth factor receptor; Aurora A; EGFR (covered in this perspective); p38 or MAPK14; KDR; and BRAF [9].

However, the success of imatinib was limited due to the selection of resistant mutants. Specifically, the Bcr-Abl variant with a T315I gatekeeper mutation in the ATP binding site was observed in 10-20% of CML patients after failure of imatinib [12,13]. Consequently, there are approximately 200 follow-on inhibitors of BCR-ABL kinase, [14-16]. Unfortunately, it has been difficult to develop robust inhibitors against the mutant variant of Bcr-Abl. Two recently approved agents, dasatinib and nilotinib (Figure 1), have shown success against imatinib-resistant CML in clinical trials.
However, the BCR-ABL1 T315I variant also confers resistance to both nilotinib and dasatinib [17-21]. Thus, the problem of resistance to this class of inhibitors is still unsolved. Approaches that are being taken to develop robust drugs effective against resistant Bcr-Abl include (1) the design of compounds to inhibit the mutated enzyme; (2) allosteric inhibitors that bind at a site different from the ATP binding site; (3) inhibitors that target other tyrosine kinases and simultaneously inhibit the T315I variant of BCR-ABL; and (4) development of other dual or multi-kinase inhibitors that simultaneously inhibit the ABL and SFK (SRC family of kinases) families [15]. The SFKs are thought to be involved in the proliferation of BCR-ABL1-expressing cell lines [15,16,22]. The outcomes of these approaches will be critical to the successful management of CML.

Epithelial growth factor receptor (EGFR) inhibitors

Since epithelial growth factor receptor (EGFR), a receptor tyrosine kinase, plays a critical role in cellular signaling leading to growth, proliferation and metastasis, [23-30] some investigators believed that inhibitors of its function would serve as leads for antineoplastic agents. Gefitinib (Figure 2) was approved in 2003 for the treatment of non-small cell lung cancer (NSCLC) after failure of platinum-based and docetaxel therapies, but did not significantly improve survival [31]. In 2004 Erlotinib (Figure 2) was also approved for NSCLC (after failure of one other agent) and in 2005 for pancreatic cancer in combination with gemcitabine. Both compounds are anilinoquinazolines that compete with ATP to bind the active site of the EGFR tyrosine kinase; a co-crystal structure of erlotinib bound to the EGFR tyrosine kinase [32] reveals the details of that interaction (Figure 3a).

Genetic evidence shows that resistance occurs in response to gefinitib and erlotinib in EGFR through mutation of the “gatekeeper” residue T790M [33,34], similar to mechanisms of resistance that occur with mutants T315I (BCR-ABL/Imatinib [35]), T674I (PDGFR/Imatinib [36]) and T670I (c-KIT/Imatinib [37]). Interestingly, there is evidence that the T790M mutation exists before the initiation of therapy [34] and that cells with the mutation are then selected during therapy. Originally, it was believed that the T790M mutation introduced steric bulk that interfered with the binding of the inhibitors [38]. However, crystal structures and biochemical experiments with the EGFR T790M and T790M/L858R variants bound to inhibitors reveal that the T790M mutation increases ATP affinity, especially in the context of the L858R mutation that
activates the kinase [39]. By increasing affinity for ATP, the T790M mutation is considered to be a generic mutation that will reduce the potency of any ATP-competitive inhibitor.

The development of irreversible inhibitors such as HKI-272 (Figures 2 and 3b), which is now in Phase II trials [33,40,41], may overcome the effects of the T790M mutation. The irreversible inhibitors have a Michael acceptor that forms a covalent bond with a cysteine thiol in the active site pocket, thus eliminating the equilibrium condition with ATP. HKI-272 has been shown to inhibit EGFR function even in the presence of the T790M mutation, but may be subject to resistance via a C797 mutation [40].

**Platelet-derived growth factor (PDGFR) inhibitors**

PDGFR tyrosine kinase inhibitors have been investigated as antineoplastic agents to inhibit angiogenesis. PDGFR inhibitors are also critical in treating hypereosinophilic syndrome (HES), a disease in which the patient has a prolonged state of eosinophilia that can lead to organ dysfunction and death [36]. Some patients with this disease respond favorably to treatment with imatinib, as they harbor a fused Fip1-like 1 (FIP1L1)-PDGFRα gene that generates an activated kinase. After initial treatment with imatinib, resistance is once again selected with the mutation of the gatekeeper residue T674I in the kinase domain of PDGFR. Interestingly, in an analogous fashion as EGFR, the gatekeeper mutation in many kinases, including PDGFR, does not exert a steric effect, but has a role in favoring an activated kinase [42]. Several compounds in different stages of development are successful in overcoming the T674I variant (Figure 4). Ki11502 [43] is a potent multi-kinase inhibitor of PDGFRα/β and inhibits T674I as well as KIT, KDR and FLT3. Nilotinib [44] and EXEL0862 [45] also inhibit the growth of cells transfected with FIP1L1-PDGFRα harboring the T674I mutation. PKC412 (Midostaurin) [36], a natural product isolated from *Streptomyces*, is reported to be an effective treatment for FIP1L1-PDGFRα-induced disease and of imatinib-induced resistance. While still ATP-competitive, PKC412 may have a different binding mode than imatinib, making it less susceptible to imatinib-specific resistance. Sorafenib is an inhibitor of the RAF-1, B-RAF, VEGFR and PDGFR tyrosine kinases and is also active against cells harboring the T674I mutation [46]. The inhibitor, HG-7-85-01, is reported to bind kinases with the T674I mutation [47]. A co-crystal structure of HG-7-85-01 with Src kinase shows that the compound forms a hydrogen bond with a neighboring
methionine (Met 341 in Src), possibly explaining how it overcomes the effects of the mutation.

Drug resistance in enzyme targets in antibiotics

Antibacterial agents, like antineoplastic agents, target fast-growing proliferative cells. While the targets differ, the occurrence of resistance is common to both classes of drugs and the development of robust drugs to overcome resistant variants is an active area of research. In this Perspective, we highlight efforts to develop robust drugs against dihydrofolate reductase (DHFR) and gyrase/topoisomerase IV.

Dihydrofolate reductase (DHFR) and trimethoprim (TMP) resistance

Trimethoprim (TMP) is a potent inhibitor of bacterial species of the essential enzyme dihydrofolate reductase (DHFR), which plays a critical role in the folate biosynthetic pathway. Trimethoprim, co-administered with sulfamethoxazole (SMZ), is an effective therapeutic agent used to treat community-acquired methicillin-resistant Staphylococcus aureus (CA-MRSA). However, epidemiological surveys show that approximately 28% of MRSA strains are TMP-resistant [48-50]. A common resistance mechanism involves the acquisition of one of two sets of chromosomal mutations in the DHFR gene, either H30N/F98Y or F98Y/H149R. Structures of wild-type and mutant (F98Y and H30N/F98Y) Staphylococcus aureus DHFR with several novel propargyl-linked antifolates show that the structural effects of the resistance mutations are subtle [51,52].

Development of compounds that have more interactions within the DHFR active site has been the predominant strategy to overcome trimethoprim resistance. Iclaprim, which underwent Phase III trials but did not continue further development, achieved nanomolar affinity for the wild-type and mutant enzymes and showed a lower propensity for resistance [53]. Crystal structures of iclaprim bound to the wild-type and mutant enzymes show additional hydrophobic interactions in a highly conserved area of the substrate binding site [54]. Reported propargyl-based inhibitors also interact more substantially with the active site [51,52] than TMP and are less affected by the mutations that cause trimethoprim resistance; further experiments to assess their propensity for resistance are ongoing.

DNA gyrase and topoisomerase IV inhibitors
In Gram-negative and Gram-positive bacteria, gyrase and topoisomerase IV, respectively, are important drug targets [55] that serve parallel functions and are often targeted with the same compounds. To perform the processes of transcription, replication, repair, and recombination, topoisomerases catalyze either a transient single strand break (Type I) or a double strand break (Type II) in supercoiled DNA. In bacteria, gyrase introduces negative supercoils for the entire chromosome, creating condensed packages of genetic material that can be divided correctly during cell division [56,57].

Fluoroquinolones are an established class of drugs (Figure 5) that stabilize the covalent DNA-gyrase complex and the homologous topoIV-DNA complex. The prototypical quinolone antibiotic nalidixic acid was discovered in the early 1960s and has since been replaced by compounds with higher potency and improved dosing modalities such as moxifloxacin and ciprofloxacin.

Resistance has emerged to the quinolone antibiotics in both Gram-positive and Gram-negative strains. A summary of hospital surveys from 1997-2001 showed an expanding rate of resistance across infectious species [58]. Resistance mechanisms include increasing drug efflux or mutating the genes that code for DNA gyrase and topoisomerase IV. Structural studies of a DNA-topoIV cleavage complex with bound moxifloxacin (Figure 6) reveal the effects of amino acid mutations in the quinolone-resistance determining region (QRDR), located in the GyrA subunit [59]. Other drug-binding models attempt to explain the effects of other resistance-conferring mutations [60,61]. Compounds that inhibit DNA cleavage via a new binding mechanism that evades the fluoroquinolone resistance mutations and compounds that simultaneously inhibit GyrB/ParE ATPase function represent promising new approaches to overcome resistance.

The topoIV/GyrA subunit inhibitor GSK299423 (Figure 7) inhibits the turnover of the DNA cleaved complex by stabilizing the enzyme-DNA complex prior to cleavage and inhibiting separation of the DNA strands. In this way, GSK299423 employs a mechanism that is significantly different from that of the fluoroquinolones [62]. The binding site for GSK299423 is highly conserved in bacteria and does not overlap with the known binding sites for fluoroquinolones, creating a potent inhibitor that shows antibacterial activity against a range of resistant strains with mutations in DNA gyrase and topoisomerase IV.
Using the topology of the binding sites of DNA gyrase (GyrB) and topoisomerase IV (ParE), (reviewed in [63]), novel classes of inhibitors of the ATPase activity have been designed to circumvent the resistance observed with quinolones. Interestingly, these compounds also overcome the resistance associated with the coumarins and cyclothialidines [64,65] that bind in a region that overlaps the ATP binding site [66,67]. These ATPase inhibitors inhibit both gyrase and topoisomerase IV simultaneously, thus yielding an advantage in that the emergence of resistance would require the unlikely occurrence of simultaneous mutations. Combinations of virtual and high-throughput screening led to compound A (Figure 8), which was of modest potency. Compound A was optimized using structure-based design to arrive at B, a compound that displayed nanomolar Ki values against S. aureus and E. coli gyrase and topoisomerase IV, and impressive MIC₉₀ values of 0.03-0.12 µg/mL against a range of bacteria. This compound has since spawned a wide variety of related chemotypes from a number of other companies [65,68-70], such as compound C, recently reported by Pfizer [69].

Drug resistance in enzyme targets induced by antiviral agents

Much of anti-viral therapy is rightly focused on vaccine development. However, for a growing number of viruses, vaccines have remained elusive, or the virus evolves so quickly that their scope is limited. Although some successful antiviral agents target viral entry, several target the viral enzymes that include: proteases, transcriptases, integrases and neurominidases. As with all quickly evolving diseases there are challenges in developing inhibitors that are robust; one strategy is to target enzymes that are evolutionarily constrained. The viral proteases, which cleave a number of diverse substrates, represent an example of such a class, and to retain activity, the protease must cleave all the substrates.

Human immunodeficiency virus (HIV) protease inhibitors

HIV protease, which cleaves Gag and Gag-Pro-Pol polyproteins at ten varied sites necessary for the maturation of virus [71], is a major therapeutic target for antiviral drugs. In the last twenty years, structure-based drug discovery efforts have led to the development of nine approved competitive active site protease inhibitors (PIs). These inhibitors are the most potent anti-HIV drugs and essential components of the highly active antiretroviral therapy (HAART) [72,73].
The development of drug resistance is a major reason for the failure of protease inhibitor therapy. The virus accumulates many mutations within the protease that prevent PIs from binding to the protease. More than half the residues within the protease mutate in different combinations and lead to drug resistance [74] [75]. Drug resistance is a subtle change in the balance of recognition events: the protease is still able to recognize and process the natural substrate sites in the Gag and Gag-Pro-Pol polyprotein, while no longer being effectively inhibited by the competitive inhibitor. When resistance emerges, the interactions of the protease with an inhibitor are significantly altered while the interactions with a natural substrate are maintained.

Crystallographic studies of the wild-type protease bound to inhibitor molecules have shown that most of the PIs occupy a similar volume and contact similar residues within the active site of the protease. Drug resistance occurs where inhibitor atoms protrude beyond the substrate envelope and contact protease residues [76]. Thus, mutations at these sites would specifically impact inhibitor binding while maintaining substrate cleavage. The observation that many of the drug resistant mutations in the active site do not contact the substrates led to the development of the substrate envelope hypothesis: inhibitors that fit well within the substrate envelope would be less susceptible to drug resistance as a mutation that affects inhibitor binding would simultaneously impact the recognition and processing of the majority of the substrates [76]. Of the currently prescribed inhibitors the most potent is darunivar (DRV) and although not designed with the substrate envelope constraint, DRV fits well within this volume [2,77]. Darunavir, in combination with ritinovir, has a high genetic barrier to resistance [78]

Developing robust HIV-1 PIs that avoid drug resistance has proven a challenging task, and the substrate envelope hypothesis provides an approach to solving this problem. A survey of five approved drugs using quantitative measures of the bound inhibitor outside the substrate envelope indicated that the exterior volume of the inhibitors correlated with the loss of affinity to mutant proteases [79]. The ability of the substrate envelope to correlate with resistance mutations prompted the prospective design of new inhibitors with a lower likelihood to develop resistance [3,80-83]. These studies assist in validating the use of the substrate envelope hypothesis [84] in structure-based drug design of novel HIV protease inhibitors and have yielded several leads for potential new drugs.
Hepatitis C NS/3A Protease Inhibitors

Drug resistance is also a major obstacle in the treatment of hepatitis C virus (HCV). HCV infects an estimated 180 million people worldwide [85]. The essential HCV NS3/4A protease is an attractive therapeutic target responsible for cleaving at least four sites along the viral polyprotein. Many protease inhibitors are currently in clinical trials; however, multi-drug resistance is widespread and arises very quickly. A recent study [86] compares the co-crystal structures of substrate structures with co-crystal structures of inhibitor complexes and shows that, as in the case of HIV-1 protease [77,79,81-83,87,88], primary drug resistance occurs in HCV NS3/4A where the inhibitors protrude away from the substrate envelope.

These findings suggest a general model for using the substrate envelope approach to predict patterns of drug resistance in other quickly evolving diseases. For drug resistance to occur, mutations must selectively weaken enzyme affinity for an inhibitor without significantly altering its activity. Mutations occur outside the substrate envelope to achieve this effect, as these molecular changes can selectively alter inhibitor binding without compromising enzyme activity. Whenever the interaction of a drug target with its biological substrates can be structurally characterized, we predict that drugs designed to fit within the substrate envelope will be less susceptible to resistance. Structure-based design strategies can incorporate this as an added constraint to develop inhibitors that fit within the substrate envelope. As a general paradigm, design efforts incorporating the substrate envelope approach can lead to the development of more robust inhibitors that are less susceptible to resistance.

Conclusion

Drug resistance negatively impacts the lives of millions of patients and costs our society billions of dollars by limiting the longevity of many of our most potent drugs. Drug resistance develops so rapidly that new drugs quickly become less effective, as witnessed by the evolution of resistance to existing anti-cancer and antimicrobial agents (antibiotics, antivirals, antifungals and antiprotozoals). A recent survey of European intensive care doctors indicated that “50% have treated at least one patient with a gram-negative pathogen that was totally or almost totally resistant to all antibiotics during the previous six months.” [89]
Drug resistance research has been very much a disease specific endeavor, which has limited intellectual progress and breakthroughs. However, as shown in this perspective, we have highlighted many parallels among quickly evolving diseases, such as cancer and infectious disease.

A drug target is likely to be resilient to resistance if it cannot easily tolerate change and maintain function. Many drug targets are enzymes. As these enzymes perform highly constrained and critical chemical reactions, they may represent resilient targets that are less susceptible to drug resistance. However, disrupting the therapeutic target activity is necessary but not sufficient for developing a drug that avoids resistance.

A robust inhibitor is one that successfully inhibits a resilient target, and one that does not quickly lose effectiveness due to resistance. Such an inhibitor may bind only to critical regions within the target that would be essential for function and thus intolerant to change. The use of high resolution structures and evolutionary constraints aids the design of robust inhibitors. By choosing resilient targets and designing robust inhibitors, the Institute for Drug Resistance (IDR) [1] proposes to put drug resistance first in drug design strategies, and develop a new generation of more effective therapeutics.

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Celia Schiffer\textsuperscript{1,2}  
Amy C. Anderson\textsuperscript{1,3}  
Michael Pollastri\textsuperscript{1,4}  
Norton P. Peet\textsuperscript{1,5}

\textsuperscript{1}Institute for Drug Resistance, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01605-2324
\textsuperscript{2}Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01605-2324
\textsuperscript{3}Department of Pharmaceutical Sciences, University of Connecticut, 69 N. Eagleville Road, Storrs, CT 06269
\textsuperscript{4}Northeastern University, 417 Egan Research Center, 360 Huntington Avenue, Boston, MA 02115
\textsuperscript{5}Microbiotix, Inc., One Innovation Drive, Worcester, MA 01605-4306

Corresponding authors  
Celia.Schiffer@umassmed.edu (Celia Schiffer)  
npeet@microbiotix.com (Norton P. Peet)
Figure 1.
Structures of First and Second Generation BCR-ABL Kinase Inhibitors.

Figure 2.
 Structures of EGFR Tyrosine Kinase Inhibitors.
Figure 3.
Crystal structures of EGFR (green) with bound inhibitors (purple or magenta). (a) erlotinib competes with ATP in the active site of EGFR; (b) HKI-272 forms a covalent link with Cys 797 to eliminate the equilibrium between inhibitor and ATP.

Figure 4.
Structures of PDGFR Inhibitors.

- KI1502
- nilotinib
- PCK412 (Midostaurin)
- sorafenib
- HG-78501
Figure 5.
Inhibitors that Target the GyrA/ParA Subunit of Gyrase and/or TopoIV.

naldixic acid
diprofloxacin
moxifloxacin
GSK 299423

Figure 6.
Crystal structure of moxifloxacin bound to GyrA [59].

Figure 7.
Crystal structure of GSK299423 bound to ParB [62].

Figure 8.
Benzimidazole GyrB/ParE inhibitors.