Chemistry & Biology
Article

A Small Molecule Discrimination Map of the Antibiotic Resistance Kinome

Tushar Shakya,[1] Peter J. Stogios,[2,3] Nicholas Waglechner,[1] Elena Evdokimova,[2,3] Linda Ejim,[1] Jan E. Blanchard,[4] Andrew G. McArthur,[5] Alexei Savchenko,[2,3] and Gerard D. Wright[1,*]
[1]M.G. DeGroote Institute for Infectious Disease Research, Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, ON, L8S 3Z5, Canada
[2]Department of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, ON, M5G 1L6, Canada
[3]Center for Structural Genomics of Infectious Diseases (CSGID), Toronto, ON, M5G 1L6
[4]High-Throughput Screening Lab, Centre for Microbial Chemical Biology, McMaster University, Hamilton, ON, L8S 3Z5, Canada
[5]Andrew McArthur Consulting, Hamilton, ON L8S 3P6, Canada
*Correspondence: wrightge@mcmaster.ca
DOI 10.1016/j.chembiol.2011.10.018

SUMMARY

Kinase-mediated resistance to antibiotics is a significant clinical challenge. These enzymes share a common protein fold characteristic of Ser/Thr/Tyr protein kinases. We screened 14 antibiotic resistance kinases against 80 chemically diverse protein kinase inhibitors to map resistance kinase chemical space. The screens identified molecules with both broad and narrow inhibition profiles, proving that protein kinase inhibitors offer privileged chemical matter with the potential to block antibiotic resistance. One example is the flavonol quercetin, which inhibited a number of resistance kinases in vitro and in vivo. This activity was rationalized by determination of the crystal structure of the aminoglycoside kinase APH(2'')-IVa in complex with quercetin and its antibiotic substrate kanamycin. Our data demonstrate that protein kinase inhibitors offer chemical scaffolds that can block antibiotic resistance, providing leads for co-drug design.

INTRODUCTION

Resistance to antibiotics is a problem of global scope with a massive impact on clinical treatment of infectious diseases (Choffnes et al., 2010). The emergence of resistance to all classes of currently used antibiotics and the accumulation of multiple resistance mechanisms in individual pathogens has resulted in strains impervious to all available drugs. This regression toward the pre-antibiotic era continues unabated as efforts to bring new antibiotics to market are diminishing (Cooper and Shlaes, 2011; Gwynn et al., 2010). New drugs and antibiotic strategies are urgently needed to fill this growing gap in infectious disease control.

One of the challenges facing the drug discovery community is the lack of new chemical scaffolds with antibiotic activity. This has led to the open question of whether all easily implementable antibiotic chemical scaffolds have already been exploited over the last 50 years: the so-called "low hanging fruit" (Baltz, 2006; Fischbach and Walsh, 2009). One option to meet the challenge of new scaffold discovery is to expand antibiotic chemical space using combinations of antibiotics and other bioactive compounds (Ejim et al., 2011; Spitzer et al., 2011). A subset of this strategy is to rescue "old" or orphaned antibiotics by inhibiting the resistance-conferring enzymes (Kalan and Wright, 2011). This has been highly effective in the β-lactam field, where synthetic and natural product inhibitors of β-lactamases have proven to be highly successful β-lactam antibiotic adjuvants (Testero et al., 2010). Examples of such combinations include Augmentin (amoxicillin-clavulanate) (Leigh et al., 1981), Zozysn (piperacillin-tazobactam) (Akova et al., 1990), and Unasyn (ampillicin-sulbactam) (Labia et al., 1986). New combinations continue to be actively pursued; for example, meropenem and clavulanate have been recently shown to have potent activity against *Mycobacterium tuberculosis* (Hugonnet et al., 2009), and the novel β-lactamase inhibitor NXL104 (avibactam), in combination with the cephalosporin ceftazidime, is active against several *Enterobacteriaceae* harboring extended-spectrum β-lactamases and carbapenemases (Livermore et al., 2008). The success of these combination therapies in combating β-lactam resistance via β-lactamase inhibition suggests that the same strategy could be applied to other resistance mechanisms. Aminoglycosides and macrolides are clinically important antibiotics that are susceptible to inactivation by phosphorylation catalyzed by drug modifying kinases and are thus amenable to such an approach.

The antibiotic resistance kinome represents a set of enzymes found at the cross-section of the microbial kinome (Kannan et al., 2007) and antibiotic resistome (Wright, 2007). This subset of microbial kinases is prevalent both in clinical and environmental isolates and confers resistance to several classes of antibiotics, in particular the aminoglycosides (e.g., gentamicin, amikacin, tobramycin, and streptomycin) and macrolides (e.g., erythromycin, azithromycin, and telithromycin) (Wright and Thompson, 1999). These kinases show only modest primary sequence similarity (pairwise sequence identities typically less than 35%) forestalling accurate prediction of their specific activity and of their substrate profile; however, structural analysis of several members of this group demonstrate that they all adopt the canonical Ser/Thr/Tyr protein kinase three-dimensional fold (Fong et al., 2010; Hon et al., 1997; Nurizzo et al., 2003; Stogios et al., 2011; Toth et al., 2010; Young et al., 2009). Furthermore,

these enzymes share similarity in chemical mechanism of phosphate transfer (Boehr et al., 2001b; Thompson et al., 2002) and cryptic ability to phosphorylate peptides and proteins (Daigle et al., 1999). The antibiotic binding site shows a higher degree of structural dissimilarity than does the nucleotide-binding site, the latter of which preserves an overall conserved architecture and several conserved key catalytic residues. These observations suggest that kinase inhibitors, many of which target the nucleotide-binding site yet are capable of remarkable specificity (reviewed in refs. [Dar and Shokat, 2011; Liao, 2007]), have the potential to also block antibiotic kinase (AK) activity. Indeed, we have previously demonstrated the inhibition of the aminoglycoside kinases APH(3′)-IIIa and APH(2″)-Ia by sulfonamide, flavonoids, and the natural product wortmannin (Boehr et al., 2001a; Daigle et al., 1997).

Human protein kinases are primary targets for the treatment of cancer and have been extensively explored in drug discovery (Knight et al., 2010; Zhang et al., 2009). To this end the pharmaceutical industry has significantly invested in the design and synthesis of large libraries of compounds that target kinases. These are an untapped resource of small molecule scaffolds with the potential of blocking AK activity and through this overturning antibiotic resistance in bacteria. Testimony to the utility of such libraries is the report that pyridopyrimidine kinase inhibitors have unanticipated antibiotic activity through the inhibition of bacterial biotin carboxylase, a critical enzyme in fatty acid biosynthesis (Miller et al., 2009).

Susceptibility to protein kinase inhibitors has been previously tested using a high-throughput platform to quantitatively explore the relationship among human protein kinases (Fabian et al., 2005; Fedorov et al., 2007; Karaman et al., 2008). These efforts provide a quantitative map of inhibitor-kinase space that can inform drug discovery. This approach allows simultaneous evaluation of the spectrum of kinase activity among a panel of enzymes and facilitates selection of compounds based on downstream requirements as probes of biology or as drug leads. Here we report a screen of a library of well-characterized kinase inhibitors against a panel of fourteen AKs and show that these inhibitors can be used to qualitatively and quantitatively discriminate between different antibiotic resistance kinases. We identified specific and general inhibitors of AKs, including pyridopyrimidines for the selective inhibition of APH(3′) enzymes and flavonoids as general inhibitors of APH enzymes, and determined the crystal structure of an inhibitor-kinase pair. This screening and structural analysis revealed AK active site features that can be exploited in the identification of molecules that reverse antibiotic resistance.

RESULTS

Functional and Phylogenetic Analyses of AKs

Antibiotic kinase enzymes vary in their antibiotic and nucleotide triphosphate substrate specificities and regiospecific sites of phosphorylation (Thompson et al., 1994). To ensure that this diversity is adequately represented in our kinase inhibitor chemical-genetic interaction study, we initiated a thorough analysis of relatedness among AK sequences. Previously, such analyses had been conducted using classical phylogenetic reconstruction methods, such as neighbor joining (Thompson et al., 1994; Wright and Thompson 1999). This methodology is particularly useful in phylogenetic analysis of high similarity sequences. However, because of the significant sequence diversity among AKs, especially in the C-terminal subdomain of the APH structure (Stogios et al., 2011), this approach does not provide sufficient rigor for classification of the diverse candidates for our analysis of kinase inhibitor chemical-genetic interactions. We therefore turned to a Bayesian analysis (Huelsenbeck and Ronquist, 2001; Scheeff and Bourne, 2005) to construct a statistically robust tree of 34 AKs (Figure 1), whose antibiotic kinase activity has been validated by either in vivo and/or in vitro activity. The topology of this tree is consistent with empirically determined enzyme functional data in that enzymes with similar phosphorylation site regiospecificity and/or antibiotic substrate specificity grouped together in the same clade. Consistent with their broad antibiotic specificity and distribution among many genera, the APH(3′) clade demonstrates the largest phylogenetic diversity. This tree provided the basis for collection of a group of representative AKs to conduct the inhibitor screen.

We selected 14 AKs that represent the phylogenetic distribution, range of antibiotic specificity, source organism including important pathogens, and localization of genes in the chromosome or in mobile genetic elements (Table S1 available online). This subset of enzymes provides a broad cross-section of currently known resistance kinase genetic space suitable for our interrogation with protein kinase inhibitors. All fourteen enzymes were expressed, purified and analyzed for antibiotic substrate specificity and for nucleotide triphosphate selectivity (Table S2).

Protein Kinase Inhibitor Screen of AKs

The 14 AKs were screened against the Screen-Well protein kinase inhibitor library (http://www.enzolifesciences.com/BML-2832/kinase-inhibitor-library/) to discover and assess chemo-selective relationships across AKs. This library was chosen as it includes broad kinase inhibitor chemical diversity and provides a tractable number of compounds (80) for multiple enzyme screens. The primary screens (Figure 2) were consistent with previous results of similar screens conducted with a small number of kinase inhibitors (Daigle et al., 1997), which provided the first evidence that these compounds would serve as privileged scaffolds for AK inhibition. Screens were conducted in duplicate and at two compound concentrations (10 μM and 50 μM), facilitating validation by two-point dose response for inhibitor-AK pairs.

Our primary screen demonstrated that several compounds (Figure 3), such as flavonoid natural products apigenin (**1**) and quercetin (**2**) and the anthraquinone damnacanthal (**3**), possess broad AK inhibitor activity. The screen also identified inhibitors with narrow specificity, such as the trisubstituted imidazole MAP kinase inhibitor SB-203580 (**4**) that only affected APH(3′)-Va and the isoquinoline sulfonamides H8 (**5**) and H9 (**6**) that only inhibit APH(3′)-IIIa.

In reciprocal analysis, we found that some AK enzymes, such as APH(3′)-Va from the neomycin producer *Streptomyces fradiae,* were highly sensitive to many compounds. On the other hand, the macrolide antibiotic kinases were conspicuously unaffected by most of the inhibitors in this library, suggesting these enzymes have significant structural differences with APHs.

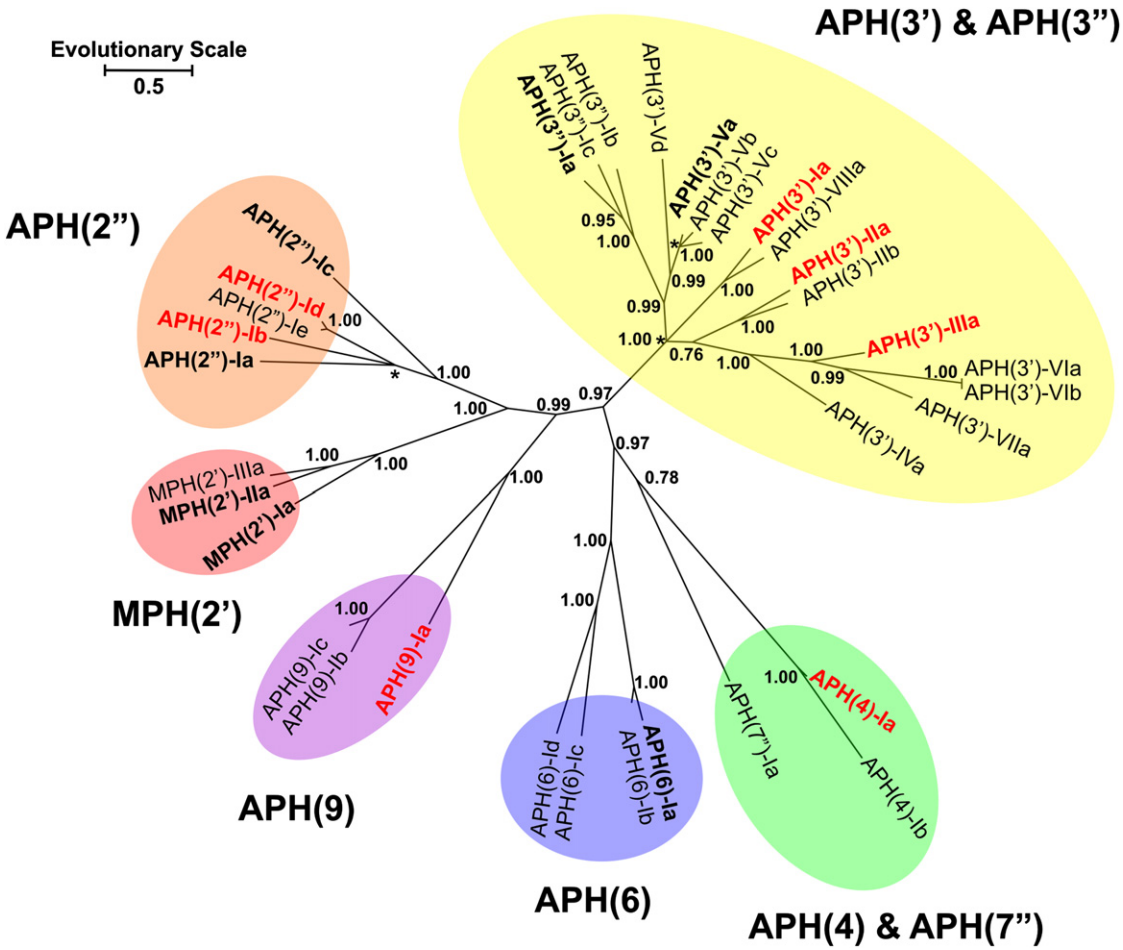

Figure 1. Phylogenetic Analysis of Antibiotic Resistance Kinases

A phylogenetic analysis of 34 bona fide AKs, which have been grouped into six major groups: APH(2"), orange; APH(3′) and APH(3"), yellow; APH(4) and APH(7"), green; APH(6), blue; APH(9), purple; MPH(2′), red. Enzymes listed in bold were screened against the Screen-Well library, and those in red represent enzymes with structural data. The evolutionary distances are to scale. A star (*) represents a branch point in which a polytomy was observed. See also Tables S1 and S2.

The primary screening data were used to select candidate compounds for further study based on the following criteria: representative chemical scaffolds, range of specificity of the inhibitors for AKs, and compound potency. Using this selection criteria, 18 inhibitors were selected for follow-up studies (Figure 4): apigenin (**1**), quercetin (**2**), genistein (**7**), LY294002 (**8**), Tyrphostin 25 (**9**), Tyrphostin 47 (**10**), Tyrphostin AG1478 (**11**), GW5074 (**12**), indirubin-3′-monooxime (**13**), Ro-31-8220 (**14**), olomoucine (**15**), PP1 (**16**), KN-93 (**17**), SB-202190 (**18**), BML-259 (**19**), LFM-A13 (**20**), SP600125 (**21**), and ZM449829 (**22**).

The in vitro dose dependence (IC_{50}) of these compounds was first established. Compound-enzyme pairs that showed reversible inhibition behavior (dose dependence, slope factor ~1) were further analyzed by determination of the K_i versus ATP (or GTP) to provide a robust and quantitative measure of inhibitor affinity (Table S3). The majority of inhibitors were competitive with NTP with the exception of mixed inhibition behavior noted for GW5074 (**12**) versus MPH(2′)-Ia and MPH(2′)-IIa; Ro-31-8220 (**14**) versus APH(2")-IIIa and APH(3′)-Ia; and ZM449829 (**22**) versus APH(4)-Ia and APH(9)-Ia. K_i values determined for the selected inhibitor scaffolds varied over four orders of magnitude, reflecting the significant diversity in their chemical structure and their interactions with AK enzymes. These results supported our hypothesis that protein kinase inhibitor compounds are discriminating probes of antibiotic inactivating enzymes and represent privileged chemical matter suitable for inhibition of AKs.

Quantitative Analysis of AK Inhibition

The wide range in inhibitor affinity for AKs suggested that these compounds could fingerprint specific AKs and associated families. We therefore correlated inhibition data with AK phylogeny by mapping the potency of each compound on the phylogenetic tree (Figure 5) (Fabian et al., 2005). Inhibitors, such as quercetin (**2**), Tyrphostin 47 (**10**), and GW5074 (**12**), showed broad specificity across clades, whereas others, such as genistein (**7**), PP1 (**16**), and Tyrphostin AG1478 (**11**), showed distinct clade specificity.

Inhibitor-enzyme specificity varied significantly, even among compounds with similar chemical scaffolds, paralleling analogous studies with eukaryotic kinases (Fabian et al., 2005). For example, four flavonoid natural products were surveyed in the

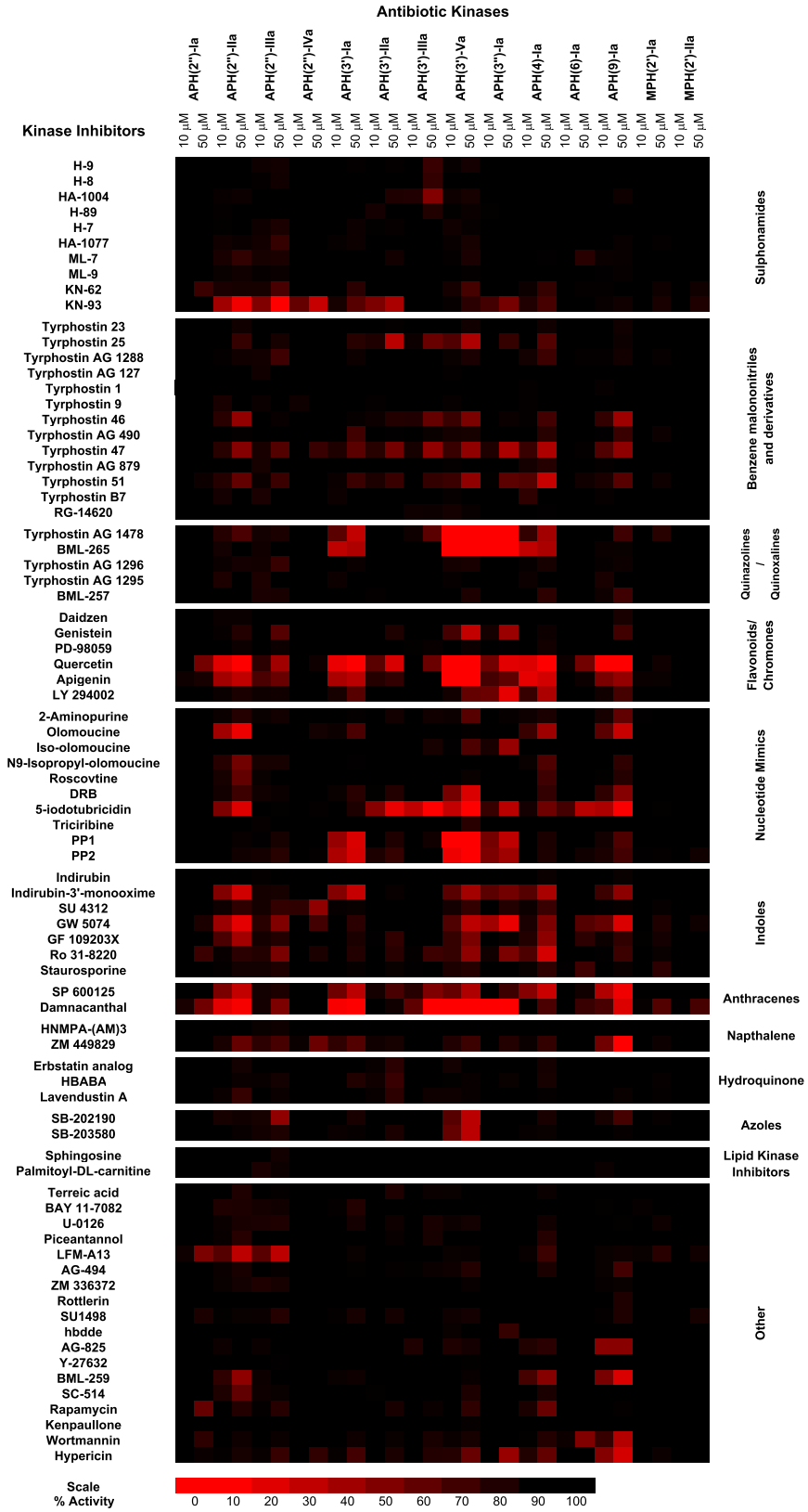

Figure 2. Primary Screen of AK against Protein Kinase Inhibitors

A 2D array of the 14 AKs against the 80 kinase inhibitors tested from the Screen-Well kinase inhibitor library. The average of the residual activities, at both 10 μM and 50 μM, were plotted and assigned a color based on the potency of the inhibitor. Red denotes complete inhibition, and black is no effect. The compounds have been ordered based on relatedness by chemical substructure.

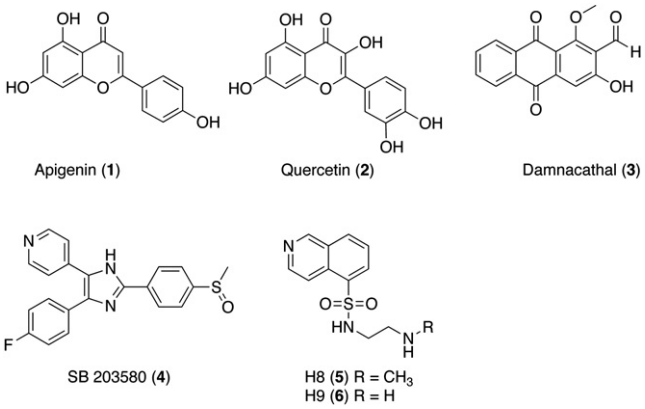

Figure 3. Screening Compounds 1
Chemical structures of screening compounds 1–6.

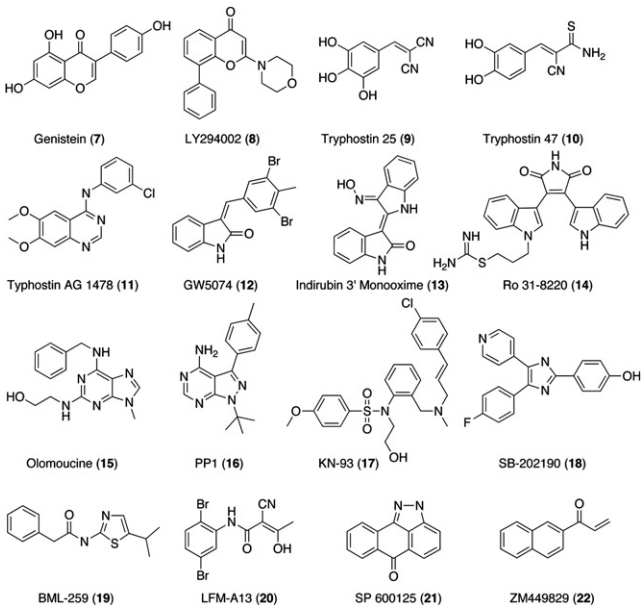

Figure 4. Screening Compounds 2
Chemical structures of screening compounds 7–22.

screen: the flavone apigenin (**1**), the flavonol quercetin (**2**), the isoflavone genistein (**7**), and the flavan-like chromone LY294002 (**8**). These compounds are highly bioactive with well-known antioxidant properties. Genistein (**7**), an inhibitor of the Tyr kinase epidermal growth factor receptor (EGFR) (Traxler et al., 1999), was the most selective flavonoid inhibitor of AKs with activity versus APH(3′)-Va, APH(3″) Ia and, to a lesser extent, APH(2″)-IIIa but showed no activity against any other APH. LY294002 (**8**), an inhibitor of phosphoinositide 3-kinases (Vlahos et al., 1994), showed modest affinity for members of the APH(3′), APH(9), and APH(4) clades but was a potent (2.4 μM) inhibitor of the streptomycin modifying enzyme APH(3″)-Ia. Similarly, apigenin (**1**), a regioisomer of genistein (**7**), displayed enzyme selectivity analogous to LY294002 but with improved affinity (sub μM K_i). On the other hand, quercetin (**2**), which differs from apigenin (**1**) by the presence of hydroxyl groups at position 3 of the C ring and at position 3′ of the B ring, was the most promiscuous inhibitor in the screen. This compound targeted 12 of the 14 enzymes tested and was active against all of the aminoglycoside kinases with affinities spanning three orders of magnitude (K_is, ranging from 70 nM [APH(3′)-Va] to 25.1 μM [APH(2″)-IVa]).

Several inhibitors demonstrated AK clade specificity. Both the Src kinase inhibitor PP1 (**16**) (Hanke et al., 1996) and the EGFR inhibitor Tyrphostin AG1478 (**11**) (Ward et al., 1994) were specific for APH(3′)-Ia, APH(3′)-IVa and APH(3″)-Ia. The Bruton's tyrosine kinase inhibitor LFM-A13 (**20**) (Mahajan et al., 1999) was only active against representatives of the APH(2″), APH(4), and MPH(2′) groups. Notably, all these enzymes are able to utilize GTP, preferentially or in addition to ATP, as a phosphate donor. LFM-A13 (**20**) also inhibited the APH(4)-Ia enzyme, which is consistent with our structural analysis of this APH enzyme that revealed significant active site similarity with APH(2″)-IIa in the nucleotide binding region (Stogios et al., 2011). The isoquinoline sulfonamide KN-93 (**17**) also showed moderate activity against two of the four APH(2″) enzymes and both MPH(2′) enzymes tested, in addition to APH(3′)-Ia and APH(3′)-IIa.

Chemically related Tyr kinase inhibitors Tyrphostin-25 (**9**) and Tyrphostin-47 (**10**) (Gazit et al., 1989) showed contrasting effects against AK enzymes. Tyrphostin-47 (**10**) showed broader AK inhibition, notably, in its affinity for APH(2″) enzymes. With both of these compounds containing the same benzylidenemalonitrile core, this difference in specificity may reflect the subtle differences in their chemical composition, such as the absence of the C2-OH or the presence of a thioamide group. Notably, the K_i values for these compounds showed that they are competitive inhibitors of ATP binding to AKs, in contrast with their activity against protein tyrosine kinase targets in which they have been shown to be competitive with the non-ATP substrate by mimicking the target tyrosine residue (Gazit et al., 1989).

The remaining molecules showed little clade specificity. However, SP600125 (**21**), indirubin-3′-monooxime (**13**), olomoucine (**15**), and BML-259 (**19**) all share the ability to block APH(2″)-IIa, APH(4)-Ia, and APH(9)-Ia, suggesting active site structural similarities that were not apparent from the phylogenetic analysis.

Structure of the APH(2″)-IVa-Quercetin Complex

To further understand the molecular mechanism behind the broad inhibition of AKs by the flavonol quercetin (**2**), we determined the crystal structure of *Enterococcal* enzyme APH(2″)-IVa in apo form, as well as in complex with antibiotic substrate kanamycin and in complex with both kanamycin and quercetin (Figure 6). The latter crystal was obtained with the inhibitor soaked into pre-grown co-crystals of enzyme with substrate. The apo and kanamycin-bound structures were solved by the single anomalous dispersion technique using Se-Met derivatized protein, and this structure was used for determining the quercetin-soaked structure by molecular replacement (Supplemental Experimental Procedures and Table S4).

The 3D structure of the protein shows the canonical APH fold (Figure 4A) and closely resembled the structure of the apo form of this enzyme reported by Toth et al. (form I; Toth et al., 2010) with RMSD of 0.7 Å over 281 of the 299 residues. Quercetin

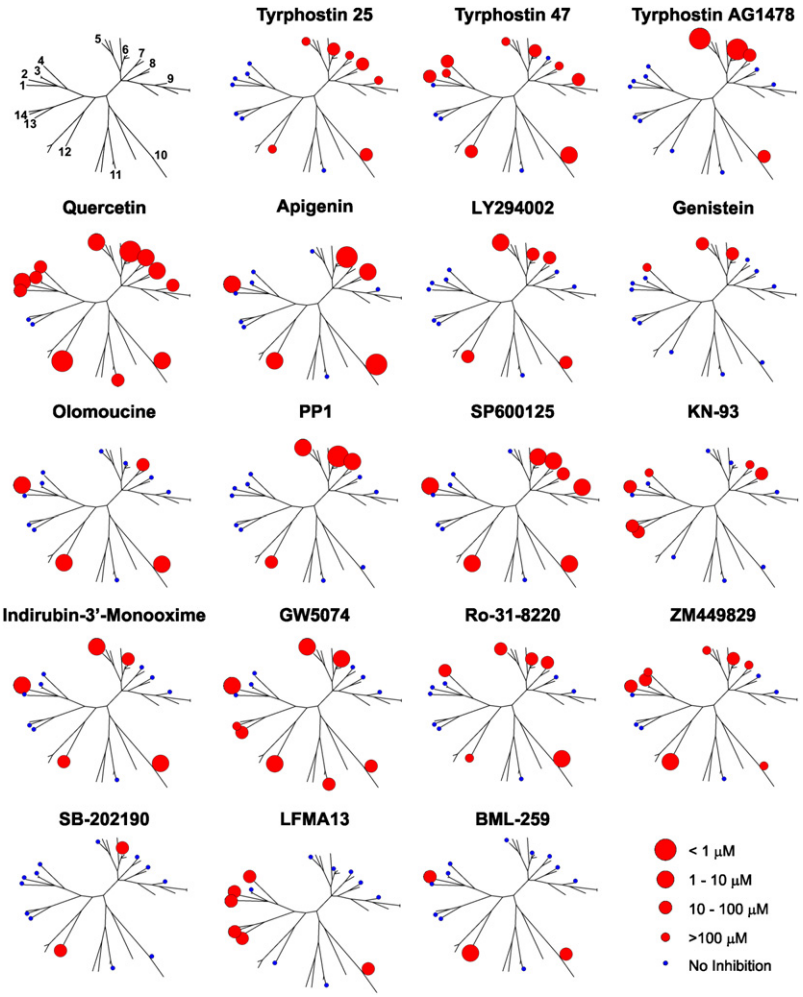

Figure 5. Specificity Profiles of Kinase Inhibitors versus AKs

The K_i of each compound was plotted against each enzyme on the phylogenetic tree. The K_is are graphically represented with a red circle to represent potency; the larger the circle, the lower the associated K_i value; a small blue circle denotes no inhibition. Complete quantitative data can be found in Table S3.

molecules were successfully built into positive F_o-F_c density in each nucleotide binding site of both APH(2'')-IVa chains in the asymmetric unit. Quercetin binding did not introduce any significant conformational changes compared to the apo or kanamycin-bound structures of this enzyme.

The quercetin molecule interacted with the hinge region of the enzyme, partially mimicking interactions and the shape complementarities between ATP and APH enzymes (Figure 4B). Comparing the conformation of the inhibitor to that of ATP bound to the closely related APH(2'')-IIa structure (Young et al., 2009; Figure S1), the central ring of the 3,5,7-trihydroxychromen-4-one moiety of the inhibitor roughly superimposed with the adenine moiety of ATP. The 5-OH and 4-ketone groups of quercetin superimposed well with the N6 and N1 groups of the purine ring; these groups all formed hydrogen bond interactions with the hinge region of the enzyme. The 3-OH of quercetin formed a hydrogen bond with the enzyme that mimics the weak hydrogen bond between the C2-H atom of the purine ring of ATP and the enzyme. The inhibitor also formed van der Waals interactions with Ile44, Phe95, Leu204, and Ile216, residues that are also involved in hydrophobic interactions with the adenine ring of ATP. The A ring of quercetin extends into the phosphate binding pocket, as the 7-OH formed hydrogen bonds to the side chains of Lys46 and Asp217, two residues involved in contacting the phosphate groups of ATP. The B ring of the inhibitor accesses a region of the enzyme that ATP does not and formed one hydrogen bond between the 3′-OH and the enzyme. The extent of the enzyme-quercetin interaction network and its K_i value compared favorably with the extent of the enzyme-ATP interaction network and its K_m value (six compared to four hydrogen bonds and 25.1 μM compared to 11.3 μM for quercetin and ATP, respectively). The broad spectrum of inhibition of APH enzymes by quercetin could be rationalized by the observation that most of the interactions observed involved backbone residues and amino acid side chains that are universally conserved across all APH enzymes because of their involvement in ATP/GTP binding.

Structure-Activity Relationship of Flavonoids with APH(2'')-IVa

Based on the obtained structural and inhibition data, a structure-activity relationship analysis was conducted to identify the determinants of flavonoid binding to APH(2'')-IVa. A spectrum of molecules was surveyed to explore flavonoid chemical space, including flavans ((+)-catechin, (±)-naringenin), flavones (apigenin, baicalein, and luteolin), flavonols (3-hydroxyflavone, kaempferol, quercetin, morin, and myricetin), and the isoflavone genistein (Table S5).

The most potent inhibitory activity against APH(2'')-IVa was seen with the flavonols. Myricetin showed activity against APH(2'')-IVa compared to that of quercetin. Myricetin differs from quercetin by an additional hydroxyl group attached to the C5′ of the B ring. Based on our structure of APH(2'')-IVa, this additional group would not make interactions with the enzyme, and therefore the similar inhibition potency was expected.

Luteolin was the only flavone to show activity against APH(2'')-IVa. This compound is also highly similar to quercetin, differing only in the absence of a hydroxyl on C3. In the crystal structure of APH(2'')-IVa in complex with quercetin, this hydroxyl formed an interaction with the backbone carbonyl of Ile98 (interaction E in Figure 4C); consequently the absence of this group in luteolin results a 3-fold drop in K_i. Similarly, the flavonol kaempferol is highly similar to quercetin but lacks the hydroxyl on C3′, which interacts with the backbone carbonyl of Gly100 (interaction E in Figure 4C). This difference resulted in a 4-fold decrease in

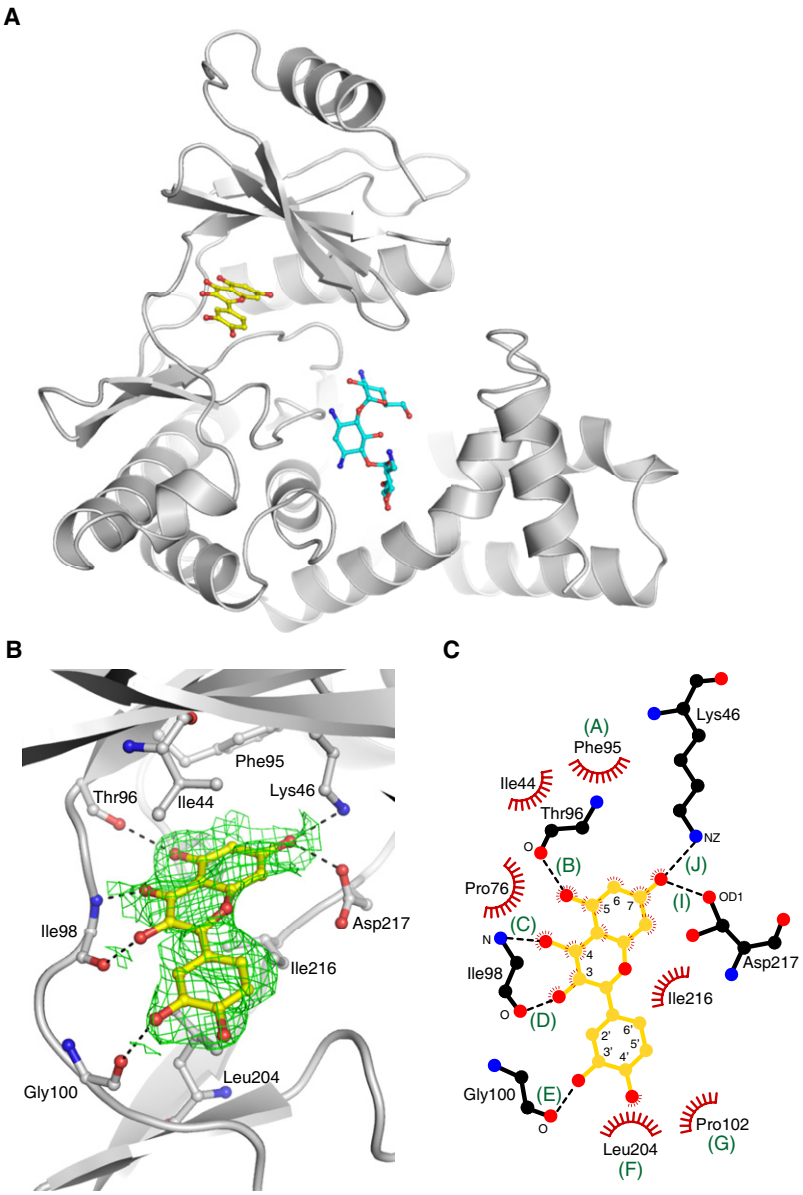

Figure 6. Crystal Structure of the APH(2")-IVa-Kanamycin-Quercetin Complex

(A) Structure of the APH(2")-IVa-kanamycin-quercetin complex, showing one of the two complexes in the asymmetric unit.

(B) Quercetin molecules bound to chain A of APH(2")-IVa, showing simulated annealing omit F_o-F_c density at 1.0σ (green). Side chains forming interactions with the inhibitor are labeled and hydrogen bonds are indicated with dashed red lines.

(C) Mapping of interactions between quercetin and APH(2")-IVa. Interaction sites in which the distance between the flavonoid and APH enzyme are within 4 Å are labeled with letters A through J, with H-bonds indicated by dashed black lines, and van der Waals interactions indicated by red fans. Also see Figures S1 and S3; Tables S4 and S5.

K_i. Along the same lines, apigenin, differing from quercetin by lacking both the C3 and C3′ hydroxyls, showed no affinity against APH(2")-IVa (highest concentration tested 50 μM), indicating the importance of at least one of interactions D or E. Morin, on the other hand, is a structural isomer of quercetin and might be expected to have similar binding kinetics to that of kaempferol. However, the swap of the hydroxyl from C3′ to C2′ resulted in a complete loss in inhibitory activity; it is possible that the C2′ hydroxyl produced a steric hindrance with the enzyme as the B ring would adopt a flipped orientation, playing the C2′ hydroxyl 3.3 Å from the Cγ1 group of Ile216. Finally, the flavone baicalein had no activity against APH(2")-IVa, consistent with the absence of functional groups to take part in the essential contacts D and E. In addition, baicalein has a hydroxyl moiety on C6, which may have produced a steric clash with the Phe95 (interaction A).

The lack of inhibition of APH(2")-IVa by apigenin was unexpected because the closest structural homolog of this enzyme, APH(2")-IIa, showed a high level of sensitivity to this flavone. Superposition of the 3D structures of APH(2")-IVa and APH(2")-IIa revealed that Arg92 in APH(2")-IIa, which corresponds to Pro102 in APH(2")-IVa, could be capable of forming an interaction "G" with the C4′-OH of apigenin. The lack of interactions D and E in apigenin could be compensated by the presence of interaction G with the C4′-OH in APH(2")-IIa.

Of the remaining flavonoids, none of the flavans (catechin and naringenin) showed any activity against APH(2")-IVa. This indicates that the carbonyl on C4 absent in these compounds is essential to flavonoid binding to this enzyme. In addition, the presence of a chiral center at C2 in naringenin may have created an unfavorable conformation of the flavonoid, disallowing binding in the hinge region. 3-hydroxyflavone also did not inhibit this enzyme; it contains the 4-carbonyl oxygen but lacks the C5 and C7 hydroxyls, suggesting one or both of interactions B and J were critical for inhibition. The isoflavone genistein also showed no interaction against APH(2")-IVa, which is likely due to the positioning of ring B at C3 versus C2 as seen in quercetin. The 3′-hydroxyphenyl ring at this position would sterically interfere with the hinge region of the enzyme.

Impact of Kinase Inhibitors on Antibiotic Resistance

The protein kinase inhibitors identified in these screens having in vitro inhibitory activity against AKs were examined for their ability to attenuate resistance in vivo of bacteria harboring resistance genes. While several compounds were highly bioactive and significantly reduced the activity of resistance elements, none of the inhibitors was sufficiently potent to completely block resistance. To quantify this intermediate bioactivity, we developed a measure that we term the antibiotic rescue factor (ARF; Supplemental Experimental Procedures and Figure S2). The

Table 1. Antibiotic Rescue Factors

Antibiotic Resistance Enzyme	Antibiotic	MIC (μg/mL)	Kinase inhibitor	K_i (μM)	ARF[1]
APH(2")-Ia	Kanamycin A	128	Quercetin	25	0.45
APH(2")-IVa	Kanamycin A	128	Quercetin	25	0.10
APH(3′)-Ia	Kanamycin A	512	Apigenin	5.3	0.49
APH(3′)-Ia	Kanamycin A	512	Quercetin	1	0.19
APH(3′)-IIa	Kanamycin A	256	Quercetin	8.1	0.12
APH(3′)-IIIa	Kanamycin A	128	Quercetin	24	0.30
APH(3′)-Va	Neomycin	32	Apigenin	0.18	0.30
APH(3′)-Va	Neomycin	32	Quercetin	0.07	0.05
APH(3′)-Va	Neomycin	32	Genistein	21	0.25
APH(4)-Ia	Hygromycin B	512	Apigenin	0.86	0.09
APH(4)-Ia	Hygromycin B	512	Quercetin	1.2	0.17
APH(9)-Ia	Spectinomycin	64	Apigenin	5.9	0.56
APH(9)-Ia	Spectinomycin	64	Quercetin	0.56	0.01

1-ARF was calculated with antibiotics at one-fourth MIC and kinase inhibitor at 5xK_i following 16 hr of growth at 37°C. See also Figure S2.

ARF was measured at one-fourth of the antibiotic minimal inhibitory concentration (MIC) at an inhibitor concentration equal to 5x the in vitro K_i. The ARF is a normalized value that indicated the increase in cell death resulting from the combination of the inhibitor and the antibiotic in the presence of a resistance enzyme; a value of 0 indicates no effect while a positive number reflects inhibition of the resistance mechanism. We explored the ARF values with the flavonoids quercetin, apigenin, and genistein against a representative panel of APHs (Table 1). In several cases a positive ARF value was measured, correlating well with K_i and demonstrating that protein kinase inhibitors can suppress the activity of antibiotic resistance enzymes.

DISCUSSION

The global spread of antibiotic resistance has created an urgent need for novel antimicrobial therapies. There is significant concern that identifying novel chemical scaffolds suitable for new antibiotics will be extremely challenging. This notion is in line with only a few examples of truly novel compounds recently approved for use or in late-stage clinical trials. It is therefore imperative to investigate novel ways to re-deploy known antibiotics in a fashion that minimizes resistance. One approach to extend the efficacy of existing antibiotics is their co-administration with inhibitors of resistance elements, a clinically proven method to overcome β-lactam antibiotic resistance caused by β-lactamases (Kalan and Wright, 2011). Antibiotic kinases are another suitable target for such inhibitors as they are common sources of resistance to aminoglycoside and, to a lesser extent, macrolide antibiotics in pathogens. As well, they share elements of protein structure, including a "druggable" active site and mechanism that could be targeted by small molecules. The link between AKs and Ser/Thr/Tyr protein kinases (Daigle et al., 1999; Hon et al., 1997) offers an opportunity to repurpose protein kinase inhibitors as leads for molecules that block AKs.

The relationship between AKs and protein kinases was exploited to establish a quantitative small molecule-AK interaction map using a library of structurally diverse and well-characterized protein kinase inhibitors. This analysis confirmed that these molecules are suitable for AK inhibition and identified both highly promiscuous (e.g., quercetin) and clade-specific inhibitors. Examples of the later included LFM A13, which targets the GTP-utilizing APH(2") and MPH enzymes, and PP1 and AG1478 that primarily inhibit APH(3′) enzymes.

An ideal AK inhibitor would efficiently block the activity of most AKs and in our screen; the flavonol quercetin was the best candidate, inhibiting all of the tested APHs (but not MPHs). This flavonol blocked antibiotic modification by all of the twelve tested APH with K_i values spanning four orders of magnitude. Flavonoids are ubiquitous in nature and have been sourced from a number of plants, including grasses, trees, fruits, and vegetables. They are among the active agents in many traditional medicines and are known for their antioxidative (Woodman and Chan, 2004), antihypertensive (Perez-Vizcaino et al., 2009), anticancer (Teillet et al., 2008), and antimicrobial properties (Cushnie and Lamb, 2011). Quercetin and its glycoconjugates are widely distributed in plants and well tolerated in humans (Bischoff, 2008; Kelly, 2011).

We determined the crystal structure of APH(2")-IVa in complex with its substrate kanamycin and the inhibitor quercetin. It revealed several key contacts required for quercetin binding to the APH(2")-IVa active site and allowed rationalization of inhibitory action of other flavonoid molecules. Quercetin occupied the ATP binding site and interacted with the enzyme through a series of hydrogen bonds, involving hydroxyls 3,5,7, and 3′, plus the ketone on position 4 (interactions D, B, I-J, E, and C in Figure 4C). The importance of the C3′-OH group was inferred from the 4-fold increase in K_i with the 3-deoxy-analog kaempferol. Analogues lacking 3, 5, or 7 hydroxyls are either weak or noninhibitory against APH(2")-IVa (Table S5). Notably, apigenin (lacking hydroxyls at position 3 and 3′ and therefore unable to make contacts D and E) did not affect APH(2")-IVa but did inhibit the closely related enzyme APH(2")-IIa. A structural comparison (Figure S3) between APH(2")-IIa and APH(2")-IVa enzymes revealed that APH(2")-IIa may form a novel contact G between the C4′-OH and Arg-92, which could override the necessity of

contacts D and E. Taken together, these findings validated that quercetin is a bona fide inhibitor of AKs, and the inhibition observed by quercetin is not the result of the formation of nonspecific aggregates as demonstrated by other studies (McGovern and Shoichet, 2003).

We observed that several compounds partially blocked resistance enzyme activity in vivo as evidenced by increased antibiotic potency by decrease of culture survival of drug-resistant bacteria. Unlike the precedent setting co-drug inhibitors of β-lactamases, none of the resistance kinase inhibitors identified in this study irreversibly blocked enzyme activity. Rather, all were reversible inhibitors with no evidence of slow onset or tight binding inhibition that is characteristic of highly potent in vivo activity, perhaps due to the fact that these compounds were not optimized and/or selected from a commercial library designed to target eukaryotic kinases. To take this reversible incomplete inhibition into account, we established a quantitative measure of this activity in the form of an antibiotic rescue factor (ARF). Using the ARF measure, quercetin was shown to be significantly bioactive against isolates expressing a number of aminoglycoside resistance kinases. This demonstrated that kinase inhibitors in general, and the flavanoid scaffold in particular, can be used successfully as lead molecules to potentiate antibiotics in resistant bacteria. Further optimization of the identified scaffolds is warranted in order to improve their efficacy.

In this study we have developed a quantitative interaction map of antibiotic kinase genetic and protein kinase inhibitor space. This work unequivocally demonstrated that these compounds represent privileged chemical matter with affinity for antibiotic resistance kinases that can also potentiate antibiotics in vivo. In an era of increasing antibiotic resistance and a commensurate clinical need for new anti-infective agents, inhibiting resistance to rescue our existing arsenal of drugs is an attractive strategy. Given significant advancement in composition of diverse libraries of kinase inhibitors and in their optimization currently present in industrial and academic laboratories, this work opens a new application for these compounds by harnessing their potential as antibiotic co-drugs.

SIGNIFICANCE

Antibiotic resistance kinases reduce the efficacy of aminoglycoside and macrolide antibiotics and are a growing problem in the successful management of bacterial infections. These resistance kinases share structure, mechanism, and function with protein kinases that are prized targets for medicines in many human diseases. We screened a chemically diverse library of protein kinases against a panel of 14 resistance kinases that sample the broad genetic diversity of these enzymes in the clinic and the environment. We showed that protein kinase inhibitors offer privileged scaffolds with affinity for resistance kinases. This work conclusively shows that the repurposing of chemical libraries well developed in pharma for campaigns in cancer and other diseases, involving protein kinases, can identify molecules with orthogonal ability to inhibit antibiotic resistance kinases. Such compounds could be formulated as co-drugs to overcome antibiotic resistance.

EXPERIMENTAL PROCEDURES

Pyruvate Kinase/Lactate Dehydrogenase Coupled Enzyme Assay

The phosphorylation of antibiotics was monitored by coupling the release of ADP or GDP with pyruvate kinase/lactate dehydrogenase (PK/LDH; Pon and Bondar, 1967). The oxidization of NADH (ε = 6220 M^{-1} cm^{-1}) was monitored at 340 nm using a SpectraMax Plus^{384} microtiter plate reader in a 96-well format. A typical reaction contained 250 μl of reaction contained 50 mM HEPES (pH 7.5), 40 mM KCl, 10 mM $MgCl_2$, 0.3 mM NADH, 3.5 mM phosphoenolpyruvate, 0.00125 units PK/LDH, 1% DMSO (v/v), antibiotic kinase, antibiotic substrate, and nucleotide. The reaction was allowed to incubate for 5 min at room temperature in the absence of nucleotide and subsequently initiated with nucleotide and monitored for 5 min at 340 nm.

Initial rates were determined by utilizing the linear portion of the progress curve and analyzed by nonlinear least-squares fitting of Equation 1,

$$v = \frac{V_{max} S}{(K_m + S)} \quad (1)$$

or Equation 2 for substrate inhibited reactions,

$$v = \frac{V_{max} S}{(K_m + S + S^2/K_i)}. \quad (2)$$

Kinase Inhibitor Screening Conditions

The assay conditions described previously were scaled down 100 μl to accommodate 384-well plate screening; the nucleotide concentration was held at a concentration equal to the K_m, antibiotic concentration was kept at 2–10 times K_m, and the amount of enzyme added was determined empirically such that the reaction was in the linear range for 10 min at the fixed drug and nucleotide concentration (Table S2). Reagent transfers to assay plates were made with a Biomek FX liquid handler (Beckman Coulter Inc., Fullerton, CA). A Z′-score (Zhang et al., 1999) was determined for each of the screening conditions, with the high and low controls, where the low control is an absence of enzyme, are compared statistically by Equation 3,

$$Z' = 1 - \left| \frac{(3\sigma_{HIGH} + 3\sigma_{Low})}{(\mu_{High} - \mu_{Low})} \right|. \quad (3)$$

A Z′-score greater than 0.5 was considered acceptable for screening purposes. A summary of the Z′-score data and screening conditions is found in Table S2. The screen was conducted in duplicate at two concentrations (10 μM and 50 μM) of kinase inhibitor from the Screen-Well Kinase Inhibitor library (version 2.1; Enzo Life Sciences/BIOMOL; Figure 1). Hits were assessed by residual activity, which is calculated by Equation 4, where v is the velocity of the reaction (Table S5):

$$\%\text{Residual Activity} = \frac{(v - \mu v_{Low})}{(\mu v_{High} - \mu v_{Low})} * 100. \quad (4)$$

Inhibition Kinetics

A secondary analysis was conducted on hits by assessing the IC_{50} of each compound against each enzyme (data not shown). If the IC_{50} showed dose-response against the activity of the enzyme, a K_i against ATP was conducted in triplicate. The K_i data were fit to multiple models of reversible enzyme inhibition and best fit assessed by chi-square values and F-test comparisons in GraFit 4.0.21 (Erithacus Software).

Phylogenetic Analysis of AKs

A preliminary alignment of 34 AKs (see Supplemental Experimental Procedures) was produced using the phosphotransferase enzyme model from Pfam (Pf01636, Pfam database at the Welcome Trust Sanger Institute) in order to align all of the core APH motifs with HMMER 3.0 (Eddy, 2009) using default parameters. It was followed by five rounds of refinement with MUSCLE v3.7 (Edgar, 2004) and manual adjustment of APH(4)-Ia, APH(6)-Id, and APH(9)-Ia to ensure alignment in the conserved regions.

The tree was estimated with two runs of 5 million generations of a Metropolis-coupled Markov chain Monte Carlo (MCMCMC) algorithm (Altekar

et al., 2004) with four chains per run in MrBayes (v3.1.2; Ronquist and Huelsenbeck, 2003) using a gamma model of among-site variation and estimating a proportion of invariant sites. A fixed Whelan and Goldman (WAG) model was used after previous runs with mixed substitution models converged to 100% posterior probability for WAG (Whelan and Goldman, 2001). Random topology trees were sampled every 100 generations. The most probable tree and associated posterior probabilities were determined using a burn-in of 1,000 sampled generations using the 50% majority rule.

ACCESSION NUMBERS

Structures of the APH(2")-IVa apo, kanamycin-bound, and kanamycin and quercin-bound complexes were deposited to the Protein Data Bank with the accession codes 3R7Z, 3R81, and 3R82, respectively.

SUPPLEMENTAL INFORMATION

Supplemental information includes three figures and five tables and can be found with this article online at doi:10.1016/j.chembiol.2011.10.018.

ACKNOWLEDGMENTS

We acknowledge the generous gift of the plasmids for the templates of *aph(2")-IIa*, *aph(2")-IIIa*, *aph(2")-Iva,* and *aph(3′)-IIa* from Dr. Sergei Vakulenko and Dr. Shahriar Mobashery; the *aph(9)-Ia* expression plasmid from Dr. Albert Berghuis; and the *mph(2′)-Ia* and *mph(2′)-IIa* from Dr. Norihisa Noguchi. We thank Dr. Tomohiro Tamura for all strains and plasmids required for protein expression in *Rhodococcus*. We would like to acknowledge Kemin Tan, Jurek Osipiuk, and Bogi Nocek at beamline 19-ID, Structural Biology Centre, Advanced Photon Source, Argonne National Laboratory for X-ray diffraction data collection; Aiping Dong at the Structural Genomics Consortium for X-ray diffraction data collection; Rosa Di Leo in the Savchenko lab for cloning; and Nadine Elowe for preliminary work in establishing high-throughput screening conditions. This research was supported by the Canadian Institutes of Health Research (MT-13536 to G.D.W.) and the Canada Research Chairs program (G.D.W.). The structures presented in this paper were solved by the Center for Structural Genomics of Infectious Diseases (http://csgid.org), funded by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services (under Contract No. HHSN272200700058C to A.S.).

Received: September 19, 2011
Revised: October 21, 2011
Accepted: October 24, 2011
Published: December 22, 2011

REFERENCES

Akova, M., Yang, Y., and Livermore, D.M. (1990). Interactions of tazobactam and clavulanate with inducibly- and constitutively-expressed Class I beta-lactamases. J. Antimicrob. Chemother. *25*, 199–208.

Altekar, G., Dwarkadas, S., Huelsenbeck, J.P., and Ronquist, F. (2004). Parallel Metropolis coupled Markov chain Monte Carlo for Bayesian phylogenetic inference. Bioinformatics *20*, 407–415.

Baltz, R.H. (2006). Marcel Faber Roundtable: is our antibiotic pipeline unproductive because of starvation, constipation or lack of inspiration? J. Ind. Microbiol. Biotechnol. *33*, 507–513.

Bischoff, S.C. (2008). Quercetin: potentials in the prevention and therapy of disease. Curr. Opin. Clin. Nutr. Metab. Care *11*, 733–740.

Boehr, D.D., Lane, W.S., and Wright, G.D. (2001a). Active site labeling of the gentamicin resistance enzyme AAC(6′)-APH(2″) by the lipid kinase inhibitor wortmannin. Chem. Biol. *8*, 791–800.

Boehr, D.D., Thompson, P.R., and Wright, G.D. (2001b). Molecular mechanism of aminoglycoside antibiotic kinase APH(3′)-IIIa: roles of conserved active site residues. J. Biol. Chem. *276*, 23929–23936.

Choffnes, E.R., Relman, D.A., and Mack, A. (2010). Antibiotic Resistance: Implications for Global Health and Novel Intervention Strategies (Washington: National Academy of Sciences).

Cooper, M.A., and Shlaes, D. (2011). Fix the antibiotics pipeline. Nature *472*, 32.

Cushnie, T.P., and Lamb, A.J. (2011). Recent advances in understanding the antibacterial properties of flavonoids. Int. J. Antimicrob. Agents *38*, 99–107.

Daigle, D.M., McKay, G.A., and Wright, G.D. (1997). Inhibition of aminoglycoside antibiotic resistance enzymes by protein kinase inhibitors. J. Biol. Chem. *272*, 24755–24758.

Daigle, D.M., McKay, G.A., Thompson, P.R., and Wright, G.D. (1999). Aminoglycoside antibiotic phosphotransferases are also serine protein kinases. Chem. Biol. *6*, 11–18.

Dar, A.C., and Shokat, K.M. (2011). The evolution of protein kinase inhibitors from antagonists to agonists of cellular signaling. Annu. Rev. Biochem. *80*, 769–795.

Eddy, S.R. (2009). A new generation of homology search tools based on probabilistic inference. Genome Inform. *23*, 205–211.

Edgar, R.C. (2004). MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics *5*, 113.

Ejim, L., Farha, M.A., Falconer, S.B., Wildenhain, J., Coombes, B.K., Tyers, M., Brown, E.D., and Wright, G.D. (2011). Combinations of antibiotics and nonantibiotic drugs enhance antimicrobial efficacy. Nat. Chem. Biol. *7*, 348–350.

Fabian, M.A., Biggs, W.H., 3rd, Treiber, D.K., Atteridge, C.E., Azimioara, M.D., Benedetti, M.G., Carter, T.A., Ciceri, P., Edeen, P.T., Floyd, M., et al. (2005). A small molecule-kinase interaction map for clinical kinase inhibitors. Nat. Biotechnol. *23*, 329–336.

Fedorov, O., Marsden, B., Pogacic, V., Rellos, P., Müller, S., Bullock, A.N., Schwaller, J., Sundström, M., and Knapp, S. (2007). A systematic interaction map of validated kinase inhibitors with Ser/Thr kinases. Proc. Natl. Acad. Sci. USA *104*, 20523–20528.

Fischbach, M.A., and Walsh, C.T. (2009). Antibiotics for emerging pathogens. Science *325*, 1089–1093.

Fong, D.H., Lemke, C.T., Hwang, J., Xiong, B., and Berghuis, A.M. (2010). Structure of the antibiotic resistance factor spectinomycin phosphotransferase from *Legionella pneumophila*. J. Biol. Chem. *285*, 9545–9555.

Gazit, A., Yaish, P., Gilon, C., and Levitzki, A. (1989). Tyrphostins I: synthesis and biological activity of protein tyrosine kinase inhibitors. J. Med. Chem. *32*, 2344–2352.

Gwynn, M.N., Portnoy, A., Rittenhouse, S.F., and Payne, D.J. (2010). Challenges of antibacterial discovery revisited. Ann. N Y Acad. Sci. *1213*, 5–19.

Hanke, J.H., Gardner, J.P., Dow, R.L., Changelian, P.S., Brissette, W.H., Weringer, E.J., Pollok, B.A., and Connelly, P.A. (1996). Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. J. Biol. Chem. *271*, 695–701.

Hon, W.C., McKay, G.A., Thompson, P.R., Sweet, R.M., Yang, D.S., Wright, G.D., and Berghuis, A.M. (1997). Structure of an enzyme required for aminoglycoside antibiotic resistance reveals homology to eukaryotic protein kinases. Cell *89*, 887–895.

Huelsenbeck, J.P., and Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics *17*, 754–755.

Hugonnet, J.E., Tremblay, L.W., Boshoff, H.I., Barry, C.E., 3rd, and Blanchard, J.S. (2009). Meropenem-clavulanate is effective against extensively drug-resistant *Mycobacterium tuberculosis*. Science *323*, 1215–1218.

Kalan, L., and Wright, G.D. (2011). Antibiotic adjuvants: multicomponent anti-infective strategies. Expert Rev. Mol. Med. *13*, e5.

Kannan, N., Taylor, S.S., Zhai, Y., Venter, J.C., and Manning, G. (2007). Structural and functional diversity of the microbial kinome. PLoS Biol. *5*, e17.

Karaman, M.W., Herrgard, S., Treiber, D.K., Gallant, P., Atteridge, C.E., Campbell, B.T., Chan, K.W., Ciceri, P., Davis, M.I., Edeen, P.T., et al. (2008). A quantitative analysis of kinase inhibitor selectivity. Nat. Biotechnol. *26*, 127–132.

Kelly, G.S. (2011). Quercetin. Monograph. Altern. Med. Rev. *16*, 172–194.

Knight, Z.A., Lin, H., and Shokat, K.M. (2010). Targeting the cancer kinome through polypharmacology. Nat. Rev. Cancer *10*, 130–137.

Labia, R., Morand, A., Lelievre, V., Mattioni, D., and Kazmierczak, A. (1986). Sulbactam: biochemical factors involved in its synergy with ampicillin. Rev. Infect. Dis. *8* (*Suppl 5*), S496–S502.

Leigh, D.A., Bradnock, K., and Marriner, J.M. (1981). Augmentin (amoxycillin and clavulanic acid) therapy in complicated infections due to beta-lactamase producing bacteria. J. Antimicrob. Chemother. *7*, 229–236.

Liao, J.J. (2007). Molecular targeting of protein kinases to optimize selectivity and resistance profiles of kinase inhibitors. Curr. Top. Med. Chem. *7*, 1332–1335.

Livermore, D.M., Mushtaq, S., Warner, M., Miossec, C., and Woodford, N. (2008). NXL104 combinations versus *Enterobacteriaceae* with CTX-M extended-spectrum beta-lactamases and carbapenemases. J. Antimicrob. Chemother. *62*, 1053–1056.

Mahajan, S., Ghosh, S., Sudbeck, E.A., Zheng, Y., Downs, S., Hupke, M., and Uckun, F.M. (1999). Rational design and synthesis of a novel anti-leukemic agent targeting Bruton's tyrosine kinase (BTK), LFM-A13 [alpha-cyano-beta-hydroxy-beta-methyl-N-(2, 5-dibromophenyl)propenamide]. J. Biol. Chem. *274*, 9587–9599.

McGovern, S.L., and Shoichet, B.K. (2003). Kinase inhibitors: not just for kinases anymore. J. Med. Chem. *46*, 1478–1483.

Miller, J.R., Dunham, S., Mochalkin, I., Banotai, C., Bowman, M., Buist, S., Dunkle, B., Hanna, D., Harwood, H.J., Huband, M.D., et al. (2009). A class of selective antibacterials derived from a protein kinase inhibitor pharmacophore. Proc. Natl. Acad. Sci. USA *106*, 1737–1742.

Nurizzo, D., Shewry, S.C., Perlin, M.H., Brown, S.A., Dholakia, J.N., Fuchs, R.L., Deva, T., Baker, E.N., and Smith, C.A. (2003). The crystal structure of aminoglycoside-3′-phosphotransferase-IIa, an enzyme responsible for antibiotic resistance. J. Mol. Biol. *327*, 491–506.

Perez-Vizcaino, F., Duarte, J., Jimenez, R., Santos-Buelga, C., and Osuna, A. (2009). Antihypertensive effects of the flavonoid quercetin. Pharmacol. Rep. *61*, 67–75.

Pon, N.G., and Bondar, R.J. (1967). A direct spectrophotometric assay for pyruvate kinase. Anal. Biochem. *19*, 272–279.

Ronquist, F., and Huelsenbeck, J.P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics *19*, 1572–1574.

Scheeff, E.D., and Bourne, P.E. (2005). Structural evolution of the protein kinase-like superfamily. PLoS Comput. Biol. *1*, e49.

Spitzer, M., Griffiths, E., Blakely, K.M., Wildenhain, J., Ejim, L., Rossi, L., De Pascale, G., Curak, J., Brown, E., Tyers, M., and Wright, G.D. (2011). Cross-species discovery of syncretic drug combinations that potentiate the antifungal fluconazole. Mol. Syst. Biol. *7*, 499.

Stogios, P.J., Shakya, T., Evdokimova, E., Savchenko, A., and Wright, G.D. (2011). Structure and function of APH(4)-Ia, a hygromycin B resistance enzyme. J. Biol. Chem. *286*, 1966–1975.

Teillet, F., Boumendjel, A., Boutonnat, J., and Ronot, X. (2008). Flavonoids as RTK inhibitors and potential anticancer agents. Med. Res. Rev. *28*, 715–745.

Testero, S.A., Fisher, J.F., and Mobashery, S. (2010). Lactam Antibiotics. In Burger's Medicinal Chemistry, Drug Discovery and Development, D.J. Abraham and D.P. Rotella, eds. (New York: Wiley & Sons), pp. 259–404.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. *22*, 4673–4680.

Thompson, P.R., Boehr, D.D., Berghuis, A.M., and Wright, G.D. (2002). Mechanism of aminoglycoside antibiotic kinase APH(3′)-IIIa: role of the nucleotide positioning loop. Biochemistry *41*, 7001–7007.

Toth, M., Frase, H., Antunes, N.T., Smith, C.A., and Vakulenko, S.B. (2010). Crystal structure and kinetic mechanism of aminoglycoside phosphotransferase-2″-IVa. Protein Sci. *19*, 1565–1576.

Traxler, P., Green, J., Mett, H., Séquin, U., and Furet, P. (1999). Use of a pharmacophore model for the design of EGFR tyrosine kinase inhibitors: isoflavones and 3-phenyl-4(1H)-quinolones. J. Med. Chem. *42*, 1018–1026.

Vlahos, C.J., Matter, W.F., Hui, K.Y., and Brown, R.F. (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J. Biol. Chem. *269*, 5241–5248.

Ward, W.H., Cook, P.N., Slater, A.M., Davies, D.H., Holdgate, G.A., and Green, L.R. (1994). Epidermal growth factor receptor tyrosine kinase. Investigation of catalytic mechanism, structure-based searching and discovery of a potent inhibitor. Biochem. Pharmacol. *48*, 659–666.

Whelan, S., and Goldman, N. (2001). A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. Mol. Biol. Evol. *18*, 691–699.

Woodman, O.L., and Chan, E.Ch. (2004). Vascular and anti-oxidant actions of flavonols and flavones. Clin. Exp. Pharmacol. Physiol. *31*, 786–790.

Wright, G.D. (2007). The antibiotic resistome: the nexus of chemical and genetic diversity. Nat. Rev. Microbiol. *5*, 175–186.

Wright, G.D., and Thompson, P.R. (1999). Aminoglycoside phosphotransferases: proteins, structure, and mechanism. Front. Biosci. *4*, D9–D21.

Young, P.G., Walanj, R., Lakshmi, V., Byrnes, L.J., Metcalf, P., Baker, E.N., Vakulenko, S.B., and Smith, C.A. (2009). The crystal structures of substrate and nucleotide complexes of Enterococcus faecium aminoglycoside-2″-phosphotransferase-IIa [APH(2″)-IIa] provide insights into substrate selectivity in the APH(2″) subfamily. J. Bacteriol. *191*, 4133–4143.

Zhang, J.H., Chung, T.D., and Oldenburg, K.R. (1999). A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J. Biomol. Screen. *4*, 67–73.

Zhang, J., Yang, P.L., and Gray, N.S. (2009). Targeting cancer with small molecule kinase inhibitors. Nat. Rev. Cancer *9*, 28–39.