Kinase Inhibitors: Not Just for Kinases Anymore

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Kinase inhibitors are widely employed as biological reagents and as leads for drug design. Their use is often complicated by their lack of specificity. Although binding conserved ATP sites accounts for some of their nonspecificity, some compounds inhibit proteins not known to bind ATP. It has been found that promiscuous hits from high-throughput screening may act as aggregates. To explore whether this mechanism might explain the action of widely used nonspecific kinase inhibitors, 15 such compounds were studied. Eight of these, rottlerin, quercetin, K-252c, bisindolylmaleimide I, bisindolylmaleimide IX, U0126, indirubin, and indigo, inhibited three diverse non-kinase enzymes. Inhibition was time-dependent and sensitive to enzyme concentration; by light scattering, the compounds formed particles of 100−1000 nm diameter. These observations suggest that these eight kinase inhibitors, at least at micromolar concentrations, are promiscuous and act as aggregates. Results obtained from the use of these compounds at micromolar or higher concentrations against individual enzymes should be interpreted cautiously.

Introduction

Kinases play a central role in signal transduction and disease and are widely studied.[1−3] Small-molecule kinase inhibitors are the subject of much interest, both as potential therapeutics and as experimental tools for understanding the physiological roles of these enzymes. Accordingly, much research over the past 2 decades has been devoted to the identification and development of such compounds; currently, 20−30% of pharmaceutical discovery programs are focused on kinases.[3−6]

A drawback of many kinase inhibitors is their lack of specificity.[1] This is often explained by the common fold and similar ATP-binding site that many kinases share.[7] Whereas this enzyme similarity undoubtedly explains much of the promiscuity of these inhibitors, some kinase inhibitors, such as bisindolylmaleimide I, quercetin, and rottlerin, can modulate the activity of receptors and enzymes other than kinases.[8−12] These observations have raised some questions about the use of these compounds as kinase-directed ligands.[8−14]

We recently found that many nonspecific hits from drug discovery screening projects shared several peculiar, nonclassical behavioral features that appeared to be related to their promiscuity.[15] This broad class of small molecules inhibited not only a particular reported target but many other enzymes as well, including several model enzymes (e.g., β-lactamase and chymotrypsin) that bore no relation to their original targets. These molecules showed steep IC_{50} curves, with time-dependent and noncompetitive behavior. They were also very sensitive to ionic strength, the presence of urea or guanidinium HCl, and the concentration of target enzyme. By dynamic light scattering (DLS) and transmission electron microscopy, these "inhibitors" were observed to form aggregates of 30−400 nm in diameter. These observations suggested that it was the formation of aggregates in solution that explained the inhibitory behavior of these molecules and not the formation of classical inhibitor−enzyme complexes. Rather, the aggregates were proposed to either adsorb or absorb enzymes, thereby inhibiting them.

To determine if an aggregate model could also describe the behavior of nonspecific kinase inhibitors, we first asked whether 15 such compounds, each of which was commercially available, inhibited three well-studied enzymes that share no obvious similarity with kinases or each other: β-lactamase, chymotrypsin, and malate dehydrogenase (MDH). We then asked if the compounds showed unusually steep IC_{50} curves and if inhibition was time-dependent. We also investigated the effect of enzyme concentration and ionic strength on inhibition. All of these features correlate with an aggregation-based mechanism of inhibition. Finally, we studied these compounds by DLS, which gives a direct indication of the presence of aggregate particles in solution. Surprisingly, this simple physical model appears to account for the activity, at least at micromolar concentrations, of eight widely used kinase inhibitors.

Results

To evaluate their specificity, we first tested 15 kinase inhibitors against a panel of model enzymes: β-lactamase, α-chymotrypsin, and MDH (Figure 1 and Table 1). These 15 compounds inhibit various kinases with nanomolar to micromolar IC_{50} values. For instance, rottlerin is marketed as a PKCδ inhibitor with an IC_{50} of 3 μM,[16] and quercetin is advertised as a PI3-K inhibitor with an IC_{50} of 3.8 μM.[17] Eight of the 15 compounds inhibited the model non-kinase enzymes with micromolar IC_{50} values. For instance, rottlerin

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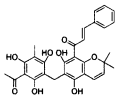
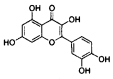
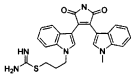
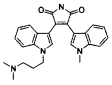
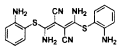
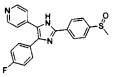
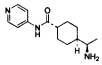
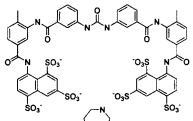
Table 1. Specificity of 15 Kinase Inhibitors

Compound	Structure	IC$_{50}$ (μM) Original Targets		β-lactamase	Chymo	MDH	# of PubMed references
Rottlerin		3 PKCδ[16]	1.9 PRAK[1]	1.2	2.5	0.7	133
Quercetin		3.8 PI3-K[17]	22.8[a] GLUT2[9]	4	100	6	2878
K-252c		2.45 PKC[31]	25.7 PKA[31]	8	10	8	363
Bisindolyl-maleimide IX		0.033 PKCα[1]	1.25 Na$^+$ channel[8]	5	20	45	606
Bisindolyl-maleimide I		0.020 PKCα[32]	< 10 Na$^+$ channel[8]	60	200	> 400	950
U0126		13 MKK1[1]	50 PRAK[1]	30	120	100	517
Indirubin		2.2 CDK2[33]	18 c-Src kinase[33]	20	100	30	66
Indigo		70 CDK2[33]	28 c-Src kinase[33]	30	90	60	59
PD98059		2 MKK1[34,35]	1 COX-1[36]	> 400			2695
SC68376		5 p38[37]		> 400			2
SB203580		0.050 p38[1]	2 COX-1[36]	> 400			1221
Y-27632		0.7 ROCKII[38]	0.6 PRK2[1]	> 400			261
Suramin		29 PKC[39]	1.7 DNA-PK[40]	> 400			2654
HA-1077		1.7 PRK1[41]	1.9 ROCKII[1]	> 400			114
Wortmannin		0.005 PI3-K[42]	1.9 SmMLCK[43]	> 400			2859

[a] K_i. Chymo, chymotrypsin.

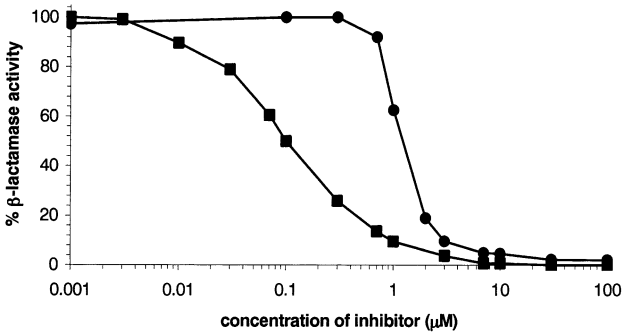


Figure 1. Representative dose−response curves showing inhibition of β-lactamase by rottlerin (●), a nonspecific kinase inhibitor, and by BZBTH2B (■), a specific and competitive β-lactamase inhibitor.

inhibited β-lactamase with an IC$_{50}$ of 1.2 μM, and quercetin inhibited β-lactamase with an IC$_{50}$ of 4 μM. Inhibition of β-lactamase by these eight compounds yielded steep IC$_{50}$ curves compared to inhibition by a specific, well-behaved, and competitive inhibitor (Figure 1). Surprisingly, suramin, which is active against many different enzymes[18] and is structurally similar to Congo Red, another promiscuous compound,[15] did not inhibit the model enzymes (Table 1).

Inhibition by nonspecific, aggregate-forming small molecules occurs in a time-dependent manner that is sensitive to the concentration of the target enzyme.[15] The eight nonspecific kinase inhibitors compounds behaved similarly. The apparent IC$_{50}$ values of the compounds against β-lactamase decreased (improved) 2-fold to over 50-fold when each was preincubated with

Table 2. Effect of Incubation or a 10-Fold Increase in Enzyme Concentration on Inhibition of β-Lactamase

compd	increasing IC_{50} with incubation	decreasing IC_{50} vs 10× enzyme
BZBTH2B[a]	no change	no change
rottlerin	>50-fold	6-fold
quercetin	2-fold	>50-fold
K-252c	8-fold	>50-fold
bisindolylmaleimide IX	5-fold	>50-fold
bisindolylmaleimide I	2-fold	>50-fold
U0126	39-fold	19-fold
indirubin	>50-fold	4-fold
indigo	>50-fold	>50-fold

[a] A specific inhibitor of AmpC β-lactamase.[28]

Table 3. Some Nonspecific Kinase Inhibitors Form Particles Detectable by Dynamic Light Scattering

compd	IC_{50} vs β-lactamase (μM)	DLS concn[a] (μM)	intensity (kcps[b])	diameter (nm)
50 mM KP_i			0.4 ± 0.1	no particles
BZBTH2B[c]	0.1	100	0.9 ± 0.2	no particles
ANS[d]	>1600	1000	0.5 ± 0.1	no particles
suramin	>400	400	0.4 ± 0.02	no particles
PD98059	>400	100	0.4 ± 0.1	no particles
rottlerin	1.2	15	11.8 ± 1.2	99.0 ± 6.7
quercetin	4	100	65.0 ± 11.1	>1000[e]
K-252c	8	10	13.5 ± 1.7	780.9 ± 65.7
bisindolylmaleimide IX	5	60	25.6 ± 3.0	578.6 ± 66.5
bisindolylmaleimide I	60	400	2.9 ± 0.5	287.1 ± 7.7
U0126	30	80	53.7 ± 9.9	432.2 ± 42.1
indirubin	20	10	62.5 ± 6.9	>1000[e]
indigo	30	20	32.1 ± 1.2	>1000[e]

[a] DLS performed in 50 mM KP_i at the concentration given under "DLS concn". [b] kcps: kilocounts per second. [c] A specific, competitive, and reversible inhibitor of AmpC β-lactamase.[28] [d] ANS is a dye that is known not to aggregate.[27] [e] Particles appear to be greater than 1000 nm in diameter.[19]

the enzyme before addition of substrate (Table 2). Conversely, the apparent IC_{50} values increased (worsened) 4-fold to over 50-fold when tested against a 10-fold increase in the concentration of β-lactamase (Table 2). In control experiments, benzo[*b*]thiophene-2-boronic acid (BZBTH2B), a competitive and specific inhibitor of β-lactamase, was not affected by incubation or enzyme concentration (Table 2).

To determine if the nonspecific kinase inhibitors formed aggregates in solution, DLS was used to analyze aqueous mixtures of 12 compounds (Figure 2 and Table 3). Most of the nonspecific inhibitors showed high-intensity, well-defined autocorrelation functions that decayed on the 100−10000 μs time scale, consistent with the presence of 100−800 nm particles. Three nonspecific inhibitors, quercetin, indigo, and indirubin, showed high-intensity scatter that decayed on the 1000−100000 μs time scale. This suggested that particles larger than 1 μm in diameter were present. Since this is outside the reliable sizing range of the DLS instrument used, we do not report a definite particle diameter for these compounds. This size is also supported by earlier findings, in a different context, that indigo formed particles of about 0.5−2 μm diameter in solution.[19] In a control experiment, suramin, a promiscuous inhibitor that did not inhibit our model enzymes, yielded a low-intensity, poorly defined autocorrelation function, consistent with the absence of particles in this solution (Figure 2C).

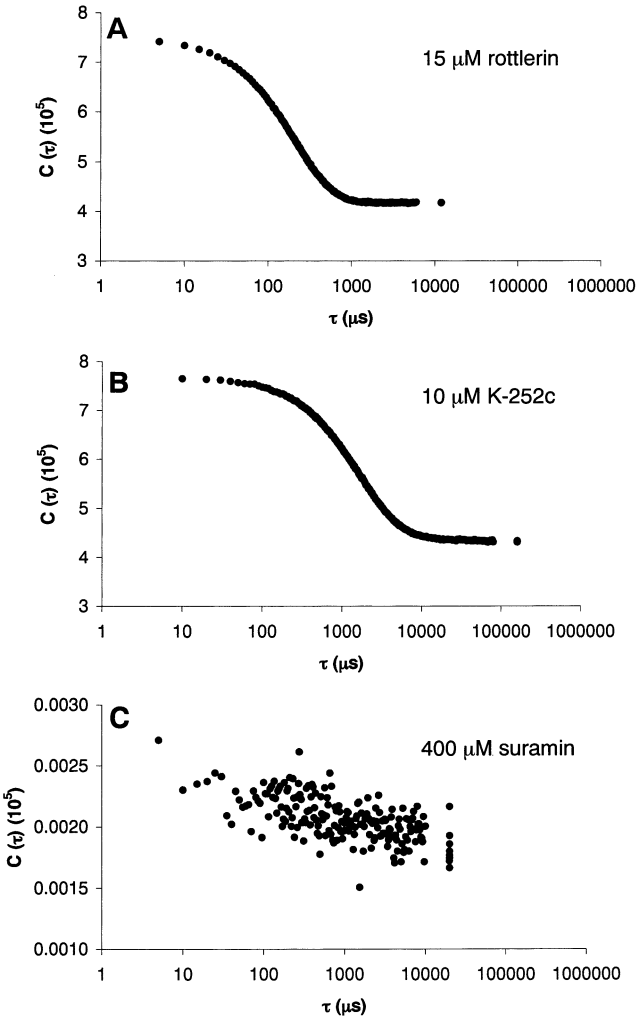
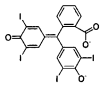
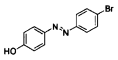
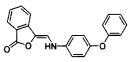
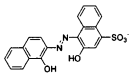
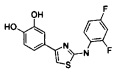


Figure 2. Representative autocorrelation functions from dynamic light scattering: (A) 15 μM rottlerin in 50 mM KPi; (B) 10 μM K-252c in 50 mM KP_i; (C) 400 μM suramin in 50 mM KP_i. The laser power and integration times for the experiments in panels A−C are comparable. Note the scale difference for the suramin experiment in panel C.

Similar to the promiscuous screening hits studied earlier,[15] the apparent IC_{50} values of the eight kinase inhibitors monotonically increased (worsened) as the ionic strength of the assay buffer increased (data not shown). Concomitantly, the average size of the particles formed by these compounds increased as the concentration of the buffer increased (data not shown). The IC_{50} of a specific and classically behaved β-lactamase inhibitor was not significantly affected by the changes in ionic strength.

If aggregation was the mechanism responsible for enzyme inhibition, it seemed likely that aggregate-forming molecules previously uncharacterized as kinase inhibitors would be active against kinases. To investigate this, five aggregate-forming inhibitors that had been shown to inhibit several enzymes, including β-lactamase, chymotrypsin, dihydrofolate reductase, and β-galactosidase,[15] were tested against Abl1 kinase (Table 4). These five compounds inhibited the kinase with IC_{50} values of 25−120 μM, consistent with the hypothesis that kinases are also susceptible to inhibition by promiscuous molecules.

Table 4. Inhibition of Abl1 Kinase by Promiscuous Enzyme Inhibitors

Structure	IC_{50} (μM)		
	β-lactamase[15]	Chymotrypsin[15]	Abl1
	3	11	25
	5	25	30
	5	15	120
	10	90	50
	18	100	25

Discussion

Many screening hits are nonspecific. This is a serious problem in early drug discovery, and much work has been devoted to understanding these peculiar inhibitors.[20−24] At least some of these promiscuous molecules share a common, nonclassical mechanism of action: the formation of large aggregates that appear to be the inhibitory species. The striking result from this study is that some kinase inhibitors, even those that are widely used and sold as biological reagents, also appear to share this mechanism. Compounds such as rottlerin and K-252c have the following characteristic properties: time-dependent behavior, steep IC_{50} curves, extreme sensitivity to enzyme concentration, and decreasing inhibition with increasing ionic strength. Finally, dynamic light scattering experiments suggest that the compounds form micrometer and submicrometer particles in aqueous solution. These results are consistent with the hypothesis that these compounds form aggregates in solution, and it is the aggregate that is the active inhibitory species.[15]

Six of the eight nonspecific kinase inhibitors were active against β-lactamase, chymotrypsin, and MDH at concentrations similar to those at which these compounds inhibit their kinase targets (Table 1). Intriguingly, three of the most potent against non-kinase targets, rottlerin, quercetin, and K-252c, were among the least specific against kinases themselves.[1] When these compounds are used as biological reagents in an in vivo experiment, they are often used at micromolar concentrations to overcome poor cellular permeability, high intracellular ATP concentrations, and high kinase concentrations.[1,8] Although we have not experimentally tested the behavior of these compounds in whole-cell experiments, our in vitro results suggest that at these concentrations, the specificity of several of these compounds becomes questionable. We note that compounds that form aggregates, and hence act promiscuously, at micromolar concentrations might well be specific at nanomolar concentrations, where aggregation is less likely. Thus, although bisindolylmaleimide IX inhibits nonspecifically at micromolar concentrations, its activity against PKCα at 33 nM may well reflect specific, classical binding. Indeed, at least one of these promiscuous inhibitors, quercetin, has been crystallographically observed in complex with two kinases, phosphatidylinositol-3-kinase[25] and the tyrosine kinase Hck,[26] suggesting that monomeric binding by quercetin can occur under certain conditions.

Key questions about the mechanism of aggregate-forming inhibitors remain outstanding, such as how they interact with enzymes.[15] The current work does shed some light on the chemical space of aggregate-forming inhibitors. For instance, suramin has long been described in the literature as a promiscuous enzyme inhibitor.[18] It is structurally similar to Congo Red, another promiscuous small molecule that is known to form aggregates and appears to inhibit many enzymes as an aggregate species.[15,27] But suramin does not inhibit our model enzymes (Table 1) and it does not form detectable particles at 400 μM. In a separate comparison, PD98059 is structurally similar to quercetin, an aggregate-forming promiscuous inhibitor (Tables 1 and 3). Yet PD98059 does not inhibit the model enzymes or form aggregates in solution (Tables 1 and 3). It may be that fairly subtle structural differences can dramatically change the behavior of individual molecules. Since aggregation involves several hundred to thousands of molecules and may resemble a phase transition, it is reasonable that small chemical differences might lead to large changes in the behavior of the system.

These eight promiscuous compounds have appeared in tens to thousands of PubMed references, attesting to the widespread interest in these small-molecule kinase inhibitors (Table 1). The formation of aggregates in aqueous solution appears to explain their inhibition of non-kinase enzymes such as β-lactamase, chymotrypsin, and MDH at micromolar concentrations. Consequently, the meaning of their apparent inhibition of kinases at similar concentrations may need to be reconsidered. More broadly, a nonspecific, aggregation-based mechanism may be involved in the activity of other classes of biological reagents.

Experimental Section

Materials. AmpC β-lactamase was purified from *E. coli* as described.[28] α-Chymotrypsin, MDH from *Thermus flavus*, succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, reduced β-nicotinamide adenine dinucleotide (NADH), rottlerin, indigo, and quercetin were purchased from Sigma-Aldrich (St. Louis, MO). PD98059, bisindolylmaleimide IX (Ro-31-8220), bisindolylmaleimide I (GF 109203X), U0126, SB203580, suramin, and wortmannin were purchased from Alexis Biochemicals (San Diego, CA). K-252c, SC68376, and Y-27632 were purchased from Calbiochem (San Diego, CA). Indirubin was purchased from Apin Chemicals Ltd. (Abingdon, Oxon, U.K.). HA-1077 was purchased from Toronto Research Chemicals (North York, Ontario, Canada). Oxaloacetate was purchased from Fluka (Milwaukee, WI). Nitrocefin was purchased from Oxoid (Ogdensburg, NY). Cephalothin-G-ester was a gift from Eli Lilly (Indianapolis, IN). All materials were used as supplied without further purification.

Enzyme Assays. Kinase inhibitors were tested for inhibition of β-lactamase, α-chymotrypsin, and MDH. Unless otherwise stated, assays were performed in 50 mM potassium phosphate (KP_i) buffer, pH 7.0, at 25 °C. Stocks of inhibitors were typically prepared at 10 mM in dimethyl sulfoxide (DMSO). No more than 5% DMSO was present in any assay, and results were controlled for the effect of DMSO. All reactions were monitored on an HP8453 spectrophotometer.

For most β-lactamase assays, inhibitor and 1 nM enzyme were incubated for 5 min and the reaction was initiated with

200 μM nitrocefin. Nitrocefin was prepared as a 20 mM stock in DMSO. For β-lactamase assays without incubation, inhibitor and 200 μM nitrocefin were mixed, and the reaction was initiated with 1 nM enzyme. For assays with a 10-fold increase in β-lactamase, inhibitor and 10 nM enzyme were incubated for 5 min, and the reaction was initiated with 100 μM cephalothin-G-ester.[15] Cephalothin-G-ester was prepared as a 10 mM stock in DMSO. Hydrolysis was monitored at 265 nm for cephalothin-G-ester and at 482 nm for nitrocefin.

For chymotrypsin assays, inhibitor and 28 nM enzyme were incubated for 5 min, and the reaction was initiated with 200 μM succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide. Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide was prepared as a 20 mM stock in DMSO. Reaction progress was monitored at 410 nm. For MDH assays, inhibitor and 2 nM enzyme were incubated for 5 min and the reaction was initiated with 200 μM oxalacetate and 200 μM NADH; progress was monitored at 340 nm.[29] Oxalacetate and NADH were each prepared as 20 mM stocks in 50 mM KP_i buffer, and the NADH stock contained 2 mM DTT.

Five compounds previously shown to act as promiscuous enzyme inhibitors[15] were tested for inhibition of Abl1 kinase with the Z′-lyte β kit from PanVera (Madison, WI) and a PC1 fluorimeter from ISS (Champaign, IL) (Table 4). Inhibitors were dissolved to 10 mM in DMSO. A 1.2 nM Abl1 kinase was mixed with 2 μM peptide substrate and then incubated with inhibitor for 5 min. The reaction was initiated by 10 μM ATP. After 1 h, 440 nM chymotrypsin was added to cleave unphosphorylated peptide. The peptide contained a coumarin label (FRET donor) and a fluorescein label (FRET acceptor). FRET between these labels was disrupted by proteolysis, whereas phosphorylated peptide was not cleaved and retained FRET after excitation at 400 nm.[30] The ratio of unphosphorylated to phosphorylated peptide was calculated as the ratio of coumarin emission (445 nm) to fluorescein emission (520 nm).[30] Emission and excitation bandwidths were 8 nm. All incubations and reactions took place at room temperature, and no reaction mixture contained more than 5% DMSO.

Dynamic Light Scattering (DLS). Compounds were generally dissolved to 10 mM in DMSO and diluted with filtered 50 mM KP_i. All compounds were analyzed with a 3 W argon ion laser at 514.4 nm with optical systems from Brookhaven Instrument Corporation. Most of the compounds were analyzed without an incubation period; quercetin was incubated at room temperature for 30 min, over which time scattering intensity increased. The laser power and integration times were comparable for all experiments. Calculation of the mean particle diameter was performed by the cumulant analysis tool of a 400-channel BI9000AT digital autocorrelator, with the last eight channels used for baseline calculation. The detector angle was 90°. Each diameter and intensity value represents four or more independent measurements at 25 °C.

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Appendix

Abbreviations. ANS, 8-anilino-1-naphthalenesulfonic acid; BZBTH2B, benzo[*b*]thiophene-2-boronic acid; CDK2, cyclin-dependent kinase 2; COX-1, cyclooxygenase-1; DLS, dynamic light scattering; DNA-PK, DNA-dependent protein kinase; FRET, fluorescence resonance energy transfer; GLUT2, glucose transporter isoform 2; KP_i, potassium phosphate; MDH, malate dehydrogenase; MKK1, MAPK kinase I (MAP kinase kinase I, MEK1); PKA, protein kinase A; PKC, protein kinase C; PI3-K, phosphatidylinositol-3-kinase; PRAK, p38-regulated/activated kinase; PRK1, protein kinase C-related protein kinase 1; ROCK, Rho-dependent protein kinase; SmMLCK, smooth muscle myosin light chain kinase.

Supporting Information Available: Structures of the compounds in Table 1 in .sdf format. This material is available free of charge via the Internet at http://pubs.acs.org.

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