Effect of Detergent on "Promiscuous" Inhibitors

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Abstract: The term "promiscuous" inhibitors has been coined for compounds whose inhibition mechanism involves the interaction of aggregates of many compound molecules with the target protein, rather than the binding of individual molecules. This paper demonstrates that promiscuous inhibitors can be differentiated from classical 1:1 inhibitors by the judicious use of detergents, making it possible to configure assays that significantly reduce this undesirable mechanism of inhibition without compromising assay performance.

High throughput screening is often a vital component in drug discovery programs. It is important for the success of the program to identify those hits that are most tractable for optimization and most likely to lead to a molecule with "drug-like" properties.[1−3] The selection process is often very subjective, but there are a number of compound properties that are generally considered as undesirable. One undesirable property is chemical reactivity, as this may lead to problems with specificity. Other undesirable properties relate more to how the target and ligand interact. One such mechanism, recently described by McGovern et al.,[4,5] involves the formation of ligands into aggregates of ∼30−400 nm in diameter. These aggregates were proposed to inhibit either by absorption onto the surface of enzymes or by incorporating enzymes within them. Inhibitors acting in this manner were termed "promiscuous" inhibitors, as they appeared to inhibit a number of unrelated enzymes, although it is likely that there are other mechanisms by which compounds may inhibit numerous enzymes. McGovern et al.[4,5] identified and characterized a number of these "promiscuous" inhibitors by testing for properties that distinguished them from classical 1:1 reversible inhibitors. For example, the compounds showed marked increases in potency on 5 min preincubation.

Compounds with this undesirable mode of action are unlikely to make good starting points for drug discovery programs, and it is advantageous to remove them from consideration at the earliest opportunity. "Promiscuous" inhibition involves the interaction between protein and aggregate surfaces. Detergents modulate surface properties and are often used to improve the robustness of assays. This paper therefore explores the effect of detergents on the inhibition profile of three "promiscuous" compounds, characterized by McGovern et al.[4] (compounds A−C in Figure 1) and two standard reversible 1:1 inhibitors (compounds X and Y in Figure 1). (Ampicillin (compound X) is a competitive substrate that we ensured acted as a competitive inhibitor in the assay employed by using conditions under which negligible turnover occurred.) It was found that these two classes of inhibitor exhibit different behavior in the presence of detergents, allowing them to be differentiated and thereby giving a simple way of rapidly identifying potential promiscuous inhibitors.

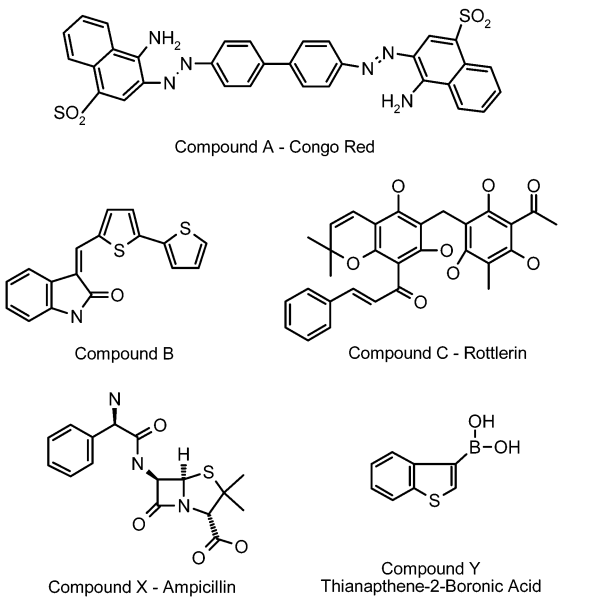

Figure 1. Structures of three promiscuous inhibitors: Compounds A, B, and C, and two β-lactamase standard inhibitors, compounds X and Y.

Originally, the promiscuity of the three inhibitors (A, B, C) was established using an *E. coli* AmpC β-lactamase assay.[4] In this paper, *Enterobacter cloacae* P99 AmpC β-lactamase[6−8] has been used. Hence, it was first necessary to show that the standard and promiscuous inhibitors behaved in a manner similar to that described with the *E. coli* AmpC enzyme. This was confirmed by examining the effects of preincubation.

Assays were performed using 1nM *E. cloacae* P99 β-lactamase, 100 μM nitrocefin, 25 mM PIPES/KOH, pH 7, 10% (v/v) glycerol, 1 mM dithiothreitol, and 2 mM $MgCl_2$. Glycerol was included to enhance enzyme stability under assay conditions. The enzyme activity was measured using the initial linear rate of hydrolysis of nitrocefin at 492 nm. All experiments were performed on 96-well plastic microtiter plates in a Wallac Envision reader. All assays measuring compound inhibition were performed in duplicate or triplicate, and averaged points are shown in the graphs. The results of testing the two standard inhibitors and three promiscuous inhibitors are shown in Figure 2.

As expected, preincubation with enzyme did not cause any change in the IC_{50} of either of the standard compounds, X and Y (Figure 2). In contrast, preincubation affected the dose−response curves of compounds A, B, and C (Figure 2). This behavior is consistent with one of the properties described for their interaction with the *E. coli* AmpC enzyme which was considered indicative of being a promiscuous inhibitor.

Thus, the *E. cloacae* β-lactamase would appear to be a suitable enzyme for further study of such compounds.

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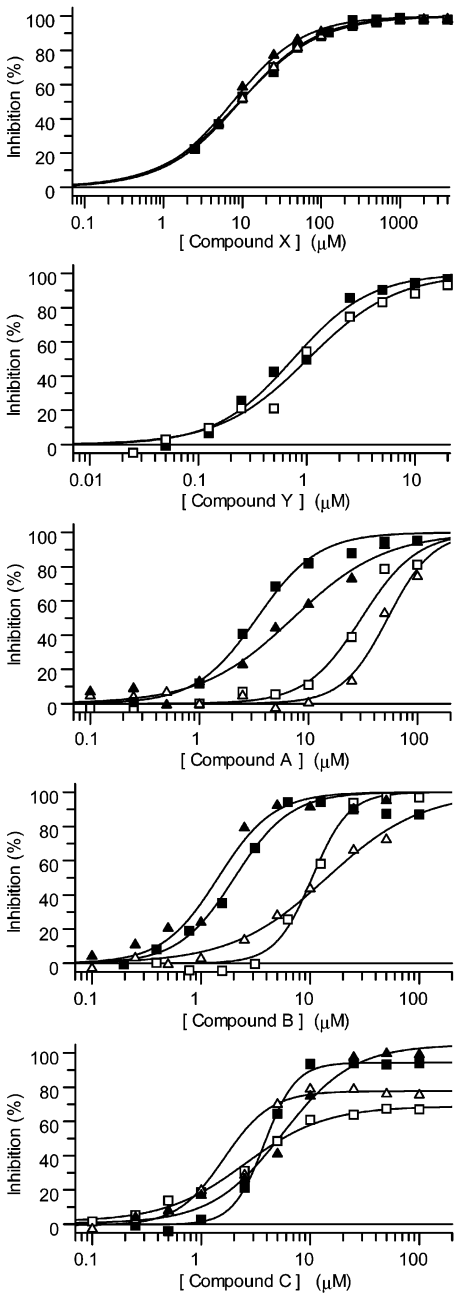

Figure 2. The effect of preincubation with enzyme on the dose−response curves of the standard 1:1 reversible inhibitors, X and Y, and compounds A, B, and C. Open symbols (□, △) are without preincubation (final addition was enzyme), and filled symbols (■, ▲) are with 5 min preincubation of compound and enzyme (final addition was nitrocefin). Where two repetitions are shown, repetition 1 is shown with squares (□, ■), while repetition 2 is in triangles (△, ▲).

It was noted in detail that the three compounds showed some difference in behavior. Preincubation with compounds A and B resulted in about a 10-fold decrease in IC_{50}, whereas with compound C there was an increase in the maximum inhibition.

Figure 2 shows the results of independent replicate experiments on the compounds. No significant variation in IC_{50} curve is apparent for the classical inhibitors, but considerable variability was observed for the promiscuous compounds. On each occasion, preincubations with the promiscuous compounds showed the same qualitative effect, either an IC_{50} decrease or increase in the maximum inhibition. However, the precise shape and position of the IC_{50} curves were found to be quite variable for the promiscuous compounds. This may be a consequence of their mechanism of inhibition and probably reflects minor changes in the degree of aggregation between experiments.

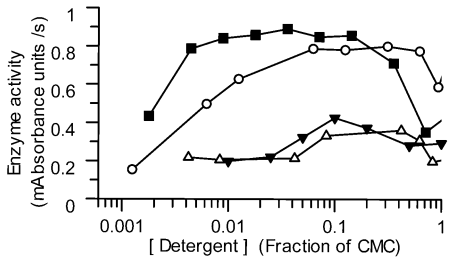

Figure 3. The effect of varying detergent concentration on enzyme activity. Data for four detergents expressed as a fraction of their CMCs (given in brackets) are shown. ○ CHAPS (8 mM); ■ Tween-20 (59 μM); △ cholic acid (12 mM); ▲ Triton X-100 (0.5 mM). Enzyme activity with no detergent was 0.19 mAbsorbance units/s.

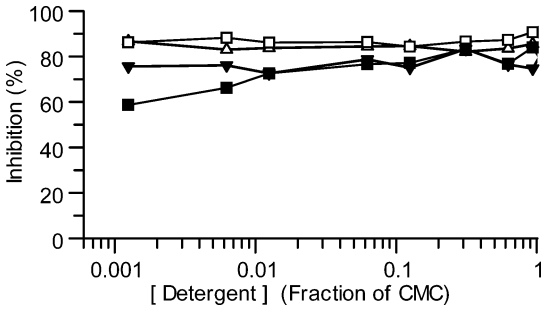

Figure 4. The effect of detergent concentration upon the inhibition of β-lactamase activity by standard inhibitors. Inhibition of catalytic activity by 80 μM compounds X (filled ■, ▲) or by 5 μM compound Y (unfilled □, △) was measured without preincubation in the presence of the indicated concentrations of CHAPS (■, □) and Tween-20 (▲, △). In the absence of detergent, compounds X and Y gave 76% and 80% inhibition, respectively.

Initially, the effect of four detergents (CHAPS, cholic acid, Triton X-100, and Tween-20) on enzyme activity was determined. The catalytic activity was measured in the presence of concentrations of detergent up to their critical micellar concentrations[9] (CMC) (Figure 3).

Inclusion of low concentrations of Tween-20 or CHAPS increased the enzyme catalytic activity, probably due to the detergent causing a reduction in nonspecific protein binding onto the plastic plates. With increasing concentrations of these detergents, the enzyme activity plateaus and remains at this optimal level until close to the detergents' CMCs whereupon the enzyme activity declines. Addition of Triton X-100 or cholic acid, however, does not allow the enzyme activity to reach this optimal level, and these detergents were therefore excluded from further study.

The inhibition profiles of the two standard compounds, X and Y, over the same range of CHAPS and Tween-20 concentrations are shown in Figure 4. There was no significant effect on the inhibition caused by these two standard inhibitors.

The inhibition profiles of the three promiscuous inhibitors in CHAPS and Tween-20 is given in Figure 5. The effect on the inhibition of this class of compounds is clearly very different from the standard compounds.

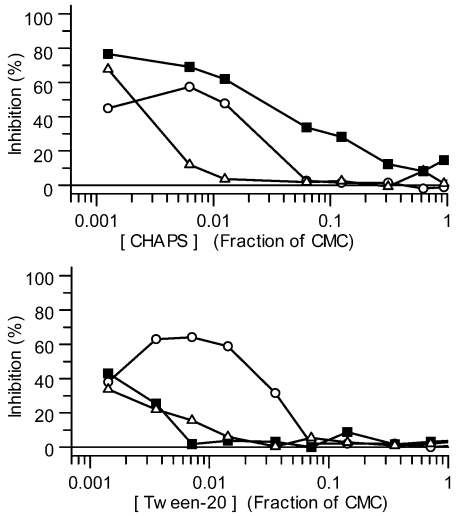

Figure 5. The effect of detergent concentration upon the inhibition of β-lactamase activity by promiscuous inhibitors. Inhibition of catalytic activity of the compounds was measured without preincubation in the presence of the indicated concentrations of CHAPS or Tween-20. The concentrations used were 30 μM compound A (○), 50 μM compound B (■), and 10 μM compound C (△). In the absence of detergent, the compounds gave 56%, 73%, and 82% inhibition, respectively.

Their inhibition is dramatically reduced by the addition of detergents at concentrations well below their CMC.

The degree to which promiscuous inhibition is suppressed is dependent on the nature of the detergent, its concentration, and the compound itself. For example, at 0.02 CMC CHAPS, compound C is no longer inhibitory and compounds A and B retain inhibitory activity, whereas at the same fraction of the CMC of Tween-20, compounds B and C are not inhibitory and compound A retains some inhibitory effect (Figure 5).

It is convenient to divide the inhibition profiles shown in Figure 5 into three detergent regimes: high, intermediate, and low. In the high detergent regime there is total suppression of the promiscuous inhibition. In the intermediate region, the inhibition is reduced but not eliminated. Finally, at low detergent levels the detergent has minimal effect on the inhibitory potency of promiscuous inhibitors.

An assay configured with high detergent concentrations, e.g., 0.625 CMC CHAPS or 0.36 CMC Tween-20 would have the dual benefit of preventing protein adhesion onto the plates and removing the effects of the three promiscuous inhibitors. Figure 6 illustrates this. At these concentrations of the two detergents, the standard compound X has the same IC_{50} as in the absence of detergents. but the inhibition of the promiscuous inhibitor A is almost completely abrogated. Compounds B and C (data not shown) showed the same dramatic reduction in inhibitory potency in the presence of these concentrations of the two detergents.

Detergents can therefore be used to differentiate promiscuous inhibitors from classical 1:1 inhibitors if used at an appropriate concentration. Although it is difficult, without determining the inhibition profile of all promiscuous inhibitors, to make a universal judgment of where the boundaries of this high detergent regime lie, our results suggest that it may be possible to chose a detergent concentration that significantly

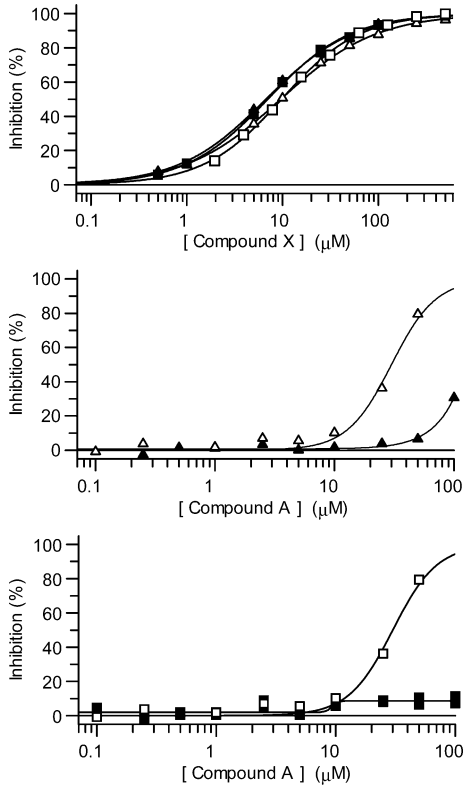

Figure 6. The effect of "high" detergent concentrations on the dose−response curves for compounds X and A. IC_{50} curves show the effect of 0 (△) and 0.36 CMC (▲) of Tween-20 and 0 (□) and 0.625 (■) CMC of CHAPS on the inhibitory activity of compound X (uppermost graph) and compound A (lower two graphs).

reduces the number of compounds showing inhibition by this mode of action.

If an intermediate detergent concentration had been chosen for the comparison, differentiation between promiscuous and classical inhibition is more difficult, as more subtle shifts in the IC_{50} curves occur. However, the drop in potency observed may be sufficient to highlight the potential of this undesirable mechanism and prompt further investigation.

In the low concentration regime, e.g., 0.0072 CMC Tween-20, which may have been typically chosen if the only detergent effect desired was elimination of protein binding to the plastic plates, no change in inhibition occurs on addition of detergent to the promiscuous inhibitor A. However, this level of detergent still affects another property of promiscuous inhibitors, namely the effect of preincubation. Thus, in this detergent regime, IC_{50} curves for compounds determined with and without 5 min compound preincubation are found to be within experimental error of one another (data for compound A shown in Figure 7).

This is in stark contrast to the behavior in the absence of detergents, shown in Figure 7 and in ref 4, where differences on preincubation were used to characterize promiscuous behavior. The 5 min preincubation test thought to be diagnostic of this mode of action can therefore not be used to discriminate between standard and promiscuous compounds in the presence of detergents.

It is interesting to hypothesize why detergents affect the behavior of promiscuous inhibitors. A simple hy-

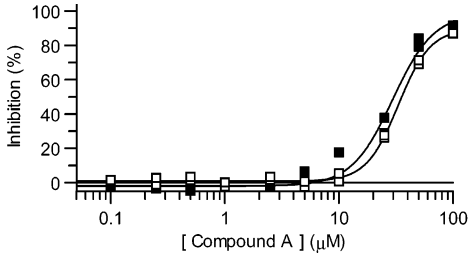

Figure 7. The effect of preincubation with enzyme in the presence of detergents on the dose−response curve of compound A. IC_{50} curves for compound A in the presence of 0.0072 CMC Tween-20 without (□) and with 5 min compound preincubation with enzyme (■).

pothesis would be that the detergents affected the aggregation state of these compounds. Preliminary attempts to analyze this by dynamic light scattering gave data that was difficult to interpret due to high levels of scattering being observed in some detergent solutions even in the absence of compound.

The precise mechanism by which the aggregated compounds inhibit a variety of unrelated enzymes remains unclear. A given promiscuous inhibitor can exhibit a range of potencies against different enzymes,[4] e.g., compound A against chymotrypsin (40 μM), β-lactamase (3.9 μM), β-galactosidase (100 μM), dihydrofolate reductase (0.4 μM). There appears to be some variation in the strength or nature of the surface interactions. Therefore, the formation of a specific concentration of aggregate is not the only determinant of inhibitory activity. It is interesting to note that β-lactamase, an enzyme sensitive to promiscuous inhibition, is also an enzyme we observed to be quite "sticky". It appears to bind readily to plastic surfaces, thereby losing its activity; detergents probably block this effect. This loss in activity is most likely caused by a surface denaturation phenomenon. This stickiness of β-lactamase may extend to compound aggregates. Thus, surface denaturation may also be the basis of promiscuous inhibition.

In conclusion, this paper demonstrates that promiscuous inhibitors can be discriminated from classical 1:1 inhibitors by a judicious use of detergents and that it is possible to design assays that significantly reduce this undesirable mechanism of inhibition without compromising the assay performance. The results in this Letter also highlight that the 5 min compound preincubation test considered to be diagnostic for promiscuous inhibitors cannot be used in the presence of detergents. It would be possible to use this detergent test to identify such compounds from a screening compound collection. However, further analysis is required to establish whether such compounds should be eliminated, as it has been shown that "promiscuous" inhibitors against one target can be classical inhibitors of another target.[5]

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References

(1) Muegge, I.; Heald, S. L.; Brittelli, D. Simple selection criteria for drug-like chemical matter. *J. Med. Chem.* **2001**, *44*, 1841−1816.
(2) Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development setting. *Adv. Drug. Discovery Rev.* **1997**, *23*, 3−25.
(3) Walters, W. P.; Ajay; Murcko, M. A. Recognizing molecules with drug-like properties *Curr. Opin. Chem. Biol.* **1999**, *3*, 384−387.
(4) McGovern, S. L.; Caselli, E.; Grigorieff, N.; Shoichet, B. K. A Common Mechanism Underlying Promiscuous Inhibitors from Virtual and High-Throughput Screening. *J. Med Chem.* **2002**, *45*, 1712−1722.
(5) McGovern, S. L.; Shoichet, B. K. Kinase Inhibitors: Not Just for Kinases anymore. *J. Med. Chem.* **2003**, *46*, 1478−1483.
(6) Joris, B.; De Meester, F.; Galleni, M.; Reckinger, G.; Coyette, J.; Frere, J−M. The β-lactamase of *Enterbocter cloacae* P99. *Biochem. J.* **1985**, *228*, 241−248.
(7) Lobkovsky E.; Billings E M.; Moews P C.; Rahil J.; Pratt R F.; Knox, J. R. Crystallographic structure of a phosphonate derivative of the Enterobacter cloacae P99 cephalosporinase: mechanistic interpretation of a beta-lactamase transition-state analogue. *Biochemistry* **1994**, *33*, 6762−6772.
(8) Lobkovsky, E.; Moews, P C.; Liu, H.; Zhao, H.; Frere, J. M.; Knox, J. R. Evolution of an enzyme activity: crystallographic structure at 2-A resolution of cephalosporinase from the ampC gene of *Enterobacter cloacae* P99 and comparison with a class A penicillinase. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 11257−11261
(9) Neugebauer, J. M. Detergents: An Overview. *Methods Enzymol.* **1990**, *182*, 239−253.

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