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Divergent Modes of Enzyme Inhibition in a Homologous Structure−Activity Series

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Abstract: A docking screen identified reversible, noncovalent inhibitors (e.g., **1**) of the parasite cysteine protease cruzain. Chemical optimization of **1** led to a series of oxadiazoles possessing interpretable SAR and potencies as much as 500-fold greater than **1**. Detailed investigation of the SAR series subsequently revealed that many members of the oxadiazole class (and surprisingly also **1**) act via divergent modes of inhibition (competitive or via colloidal aggregation) depending on the assay conditions employed.

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Small-molecule aggregation leading to promiscuous inhibition of enzymes is now well recognized as a common source of false positives in high-throughput screening.[1−4] While these phenomena are not thoroughly understood at the molecular scale, common characteristics of aggregates can be useful in their identification, for example, their capacity to inhibit unrelated enzymes[5] and the ability of nonionic detergents to disrupt them and reverse enzyme inhibition.[6,7] Aggregation is viewed by many medicinal chemists as an all-or-nothing phenomenon of primary concern in high-throughput screening and hit validation. That nonspecific modes of inhibition could emerge in the course of a standard hit-to-lead optimization campaign is not generally appreciated. Even less appreciated is the notion that promiscuous inhibition could be responsible for multiple logs of apparent (interpretable) SAR. Recently, we uncovered exactly these effects while optimizing a novel class of reversible, nonelectrophilic inhibitors of the trypanosome cysteine protease cruzain. Here we describe aspects of this work that bear consideration by any group engaged in chemical optimization guided by biochemical assay data.

Cruzain is the major cysteine protease of the protozoan parasite *Trypanosoma cruzi*, the causative agent of Chagas' disease, which affects 16−18 million people in Latin America. The importance of cruzain as a target has been demonstrated through the efficacy of inhibitors of this enzyme in mouse[8] and dog[9] models of the disease. Several efforts have been conducted to find cruzain inhibitors, and diverse classes of compounds have been reported but mostly covalent warhead-based chemotypes.[10,11]

Motivated to find novel inhibitors with a reversible, noncovalent mode of inhibition, we turned to virtual screening of commercially available leadlike[12,13] compounds that could subsequently be optimized by medicinal chemistry. Publically available structures of cruzain[14,15] were interrogated computationally with half a million compounds from the leadlike subset of the ZINC database[16] using DOCK 3.5.54 (see Supporting Information for details). Following visual inspection of the 500 top-ranked docked molecules, 17 were selected for purchase and testing in an enzyme assay. Among these, glycolamide ester **1** inhibited cruzain with an IC_{50} of 77 μM (Figure 1).

Inhibition of cruzain by **1**, though relatively modest, was independent of incubation time and was competitive, with a K_i of 32 μM (Figure 2). Both observations were consistent with a reversible, noncovalent mechanism of inhibition. The assays were conducted in the presence of 0.01% of the detergent Triton X-100 (a control for colloidal aggregation[2,17]), and close congeners of **1** such as **2** and **3** were also competitive inhibitors of cruzain under similar conditions. Thus, the initial compounds in this series (**1**−**3**) were deemed "clean" as far as aggregation and reversibility was concerned, and chemistry was initiated on the series.

Preliminary SAR profiling around the glycolamide scaffold was carried out with commercially available and synthesized analogues (Figure 1, Table 1, and Supporting Information). The dichloroaniline ring in **1** could be replaced by aromatic, benzylic, or aliphatic ring systems without substantially impacting potency (e.g., **2** and **3**). In contrast, the SAR of the aminopyridine ring was much less forgiving. For example, an aniline ring could be substituted for aminopyridine (compare **1** and **6**, Table 1) but only if the amine was positioned in the ortho position. Analogues lacking an ortho amino group or having the amino group in another position on the ring were essentially inactive (e.g., **4** and **5**, Figure 1).

With an aminopyridine (or aniline) ring established as a putative pharmacophore, we set about replacing the labile ester function in **1** with more druglike surrogates. The most straightforward modification (ester to amide) uniformly produced less potent analogues (e.g., analogues **7** and **8**, Table 1). We next examined 1,2,4-oxadiazole and related heterocycles as ester bioisosteres.[18] Gratifyingly, oxadiazole analogues (e.g., **9**−**11**) were found to be at least as potent as the esters-based progenitors **1**−**3**. Both aliphatic and aromatic "C-rings" were tolerated in the oxadiazole series (e.g., **10** and **11**).

With more leadlike scaffolds in hand, we sought to expand our survey of C-ring variants in the oxadiazole series. An established method[18] for preparing the oxadiazole ring system proved well suited for library generation (see Supporting Information). The end result of the C-ring survey was ∼50 new analogues, prepared over three cycles of synthesis, with potencies ranging from >100 μM to 200 nM and with clear and interpretable SAR (Table 2 and Supporting Information). Hence, analogues bearing basic, amine containing rings (**17**, **18**) and analogues bearing heterocycles linked by aliphatic chains of more than one carbon atom (**19**) were generally without measurable activity ($IC_{50} > 100$ μM). Consistent with the preliminary SAR profile, analogues bearing aliphatic, benzylic, or aryl/heteroaryl C-rings generally possessed mid−low micromolar potencies against cruzain. Most exciting was the

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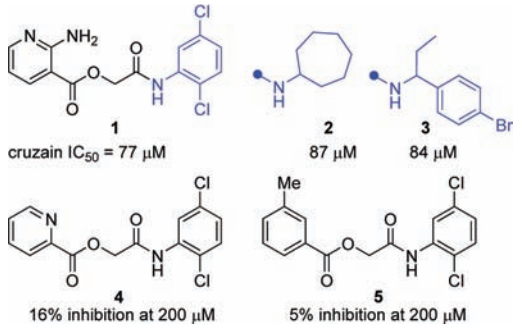


Figure 1. Structures and cruzain inhibition data for original screening hit **1** and structurally related analogues obtained from commercial sources.

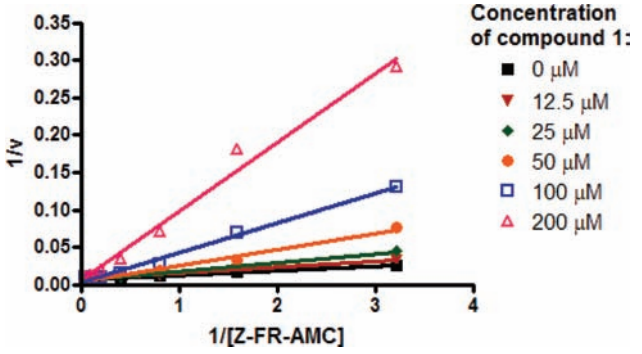
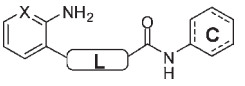
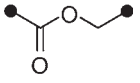
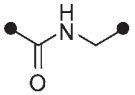
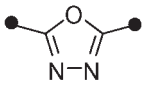


Figure 2. Lineaweaver–Burk plot indicating competitive inhibition of cruzain by **1** (K_i = 32 μM).

Table 1. SAR for Cruzain Inhibitors Bearing Ester, Amide, or Heterocyclic Linkers



Compd	Linker (L)	X	C-Ring	cruzain IC_{50} (μM)
1		N	DiCl-Ph[a]	77
6		CH	DiCl-Ph[a]	58
2		N	cycloheptyl	87
7		N	cyclohexyl	16% at 200 μM
8		CH	cyclohexyl	15% at 200 μM
9		CH	cyclohexyl	78
10		CH	cycloheptyl	25
11		CH	DiMeOPh[b]	27

[a] DiCl-Ph = 2,5-dichlorophenyl. [b] DiMeOPh = 2,4-dimethoxyphenyl.

finding that substituted pyrazole C-ring analogues inhibited cruzain with submicromolar potencies (e.g., cruzain $IC_{50} \approx 200$ nM for analogue **21**). Further exploration of the pyrazole series produced additional interpretable SAR. Hence, aryl (**20**, **21**, **22**), heteroaryl (**23**), or bulky alkyl (**24**) substitution on the pyrazole ring conferred superior potency than did small alkyl (e.g., Me, **25**) substituents. Strikingly, methylation of the pyrazole N–H function completely abrogated activity (analogue **26**; IC_{50} > 100 μM), implying that the enhanced potency of the pyrazole series might result in part from a hydrogen bonding interaction not present in the other aryl and benzylic C-rings analogues studied.

With ~50 oxadiazoles synthesized, we were in possession of three logs of consistent, intepretable SAR and had identified analogues that were nearly 500-fold more potent than **1**. We were troubled, however, that few of the oxadiazole analogues exhibited measurable activity against cultured *T. cruzi* or *Trypanosoma brucei* parasites. This discrepancy was initially rationalized as arising from poor cell permeability or active efflux from parasite, but a close inspection of the enzyme dose–response curves, many of which turned out to have unusually high Hill slopes, suggested a second possibility: that the inhibitors were acting by superstoichiometric mechanisms.[1,19] At this juncture we also determined that the analogues from the C-ring survey had inadvertently been assayed at a 10-fold lower detergent concentration (0.001% Triton X-100) than was employed in the original profiling of analogues **1**–**11** (0.01%).

Concerned, we repeated the IC_{50} determinations for selected oxadiazole and glycolamide analogues at low and high detergent concentrations (Table 2, Figure 3).[6,7] Significantly, each of the oxadiazoles examined showed no measurable dose–response or a significantly higher IC_{50} when tested at the higher Triton concentration. Only one analogue (**16**) exhibited potency comparable to the early oxadiazole leads **9**–**11** under high Triton conditions. In contrast, the IC_{50} values of glycolamide analogues **1**–**3** were unchanged or only modestly altered (~3-fold in the case of **1**, essentially unaltered for **2** and **3**) at the different detergent concentrations. Also consistent with aggregation, some oxadiazoles were sensitive to preincubation with bovine serum albumin (BSA[a]), which at high concentration competes with enzyme for colloid particles (aggregates), preventing or reducing inhibition of the target enzyme.[20] Thus, oxadiazoles **14** and **23** showed, respectively, 10- and 100-fold increases in IC_{50} in the presence of 1 mg/mL BSA, while analogue **16** was unaffected by BSA pretreatment.

Detergent-reversible inhibition of AmpC β-lactamase is another marker of promiscuous aggregation, and so this was examined next. Four of five oxadiazoles tested did indeed inhibit β-lactamase in a detergent-reversible fashion at relevant compound concentrations, the exception being analogue **25**. Quite surprisingly, the original glycolamide lead **1** (but not **2** or **3**) was also found to inhibit AmpC, and its inhibition was reversed by 0.01% Triton. Thus, glycolamide **1** might also act as an aggregator under certain assay conditions, although clearly this was not true of **1** in its inhibition of cruzain where competitive inhibition (at 0.01% Triton) had already been established (Figure 2).

Next, we sought direct evidence of particle (aggregate) formation by dynamic light scattering (DLS)[21] and flow cytometry.[22] We studied suspected aggregators and analogues that fail to inhibit enzyme under any conditions, as the latter compounds presumably do not aggregate (an thus do not inhibit). At relevant inhibitory concentrations for low detergent conditions (0.001% Triton X-100), suspected oxadiazole

[a] Abbreviations: DLS, dynamic light scattering; BSA, bovine serum albumin.

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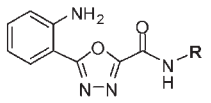
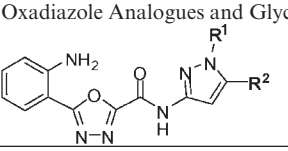
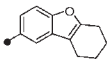
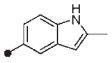
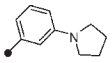
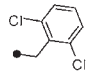
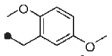
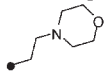
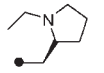
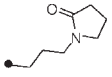
Table 2. Results of Enzyme Inhibition Assays and Secondary Assays of Aggregation for Selected Oxadiazole Analogues and Glycolamide **1**





		cruzain IC_{50} (μM)[a]						cruzain IC_{50} (μM)[a]				
cpd	R =	0.001 % triton	0.01% triton	0.001 % + BSA	inhibits AmpC ?[b]	particles? (μM)[c]	cpd	R^1 = R^2 =	0.001 % triton	0.01% triton	0.001 % + BSA	inhibits AmpC ?[b]	particles? (μM)[c]
12		2.0	>100	---	yes 50 μM	>100	**20**	H 4-MePh	0.50	>100	---	---	---
13		6.0	>100	---	---	---	**21**	H 4-MeOPh	0.20	>100	---	yes 13 μM	3.1
14		7.0	>100	99	yes 50 μM	50	**22**	H Ph	0.8	>100	---	---	---
15		8.0	>100	---	---	---	**23**	H 2-thioph.	1.0	82.4	102	---	---
16		8.0	44	45	---	---	**24**	H *t*-Bu	2.0	>50	---	yes 13 μM	3.1
17		>100	>100	---	---	>200	**25**	H Me	11	>100	---	no 50 μM	50
18		>100	>100	---	---	>200	**26**	Me H	>100	>100	---	---	200
19		>100	>100	---	---	>200	**1**	---	27	77	116	yes 100 μM	25

[a] Cruzain IC_{50} values were determined in 0.01% or 0.001% Triton, and in the later case with or without preincubation with BSA. [b] Compounds were tested against β-lactamase in the absence or in the presence of 0.01% Triton, at the concentrations noted, and classified as aggregators if inhibition was detergent-sensitive. [c] Particle formation was evaluated by flow cytometry and in some cases examined and confirmed by DLS. The concentrations noted are the lowest evaluated concentration at which particle formation was observed.

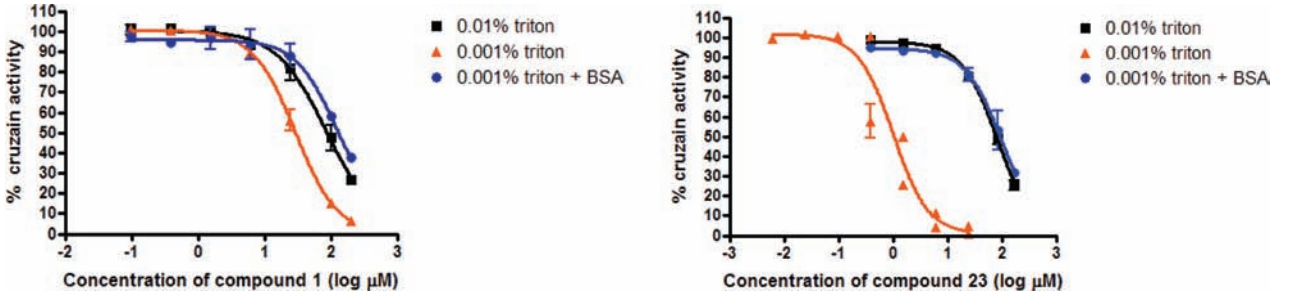


Figure 3. IC_{50} curves for original lead compound **1** (top) and oxadiazole **23** (bottom) at various concentrations of Triton X-100 and with or without preincubation with BSA. The oxadiazole shows a much more significant dose−response shift at low detergent concentrations, likely owing to the inadvertent optimization of this series under low Trion X-100 conditions.

aggregators and also **1**−**3** were above their critical aggregation concentrations (CACs)[22] and formed colloidal aggregates (the exception being **12**) of size 250−400 nm. In contrast, inactive analogues (e.g., **17**−**19**) did not show particle formation even at high concentration.

These studies provided strong evidence for a nonspecific mode of inhibition by oxadiazoles under low Triton (0.001%) assay conditions. Not all the secondary assay data were in perfect agreement however, and the DLS and AmpC inhibition data were puzzling, as they suggested that at least some of the original glycolamide analogues could also form aggregates under low Triton conditions. We therefore re-evaluated the nature of cruzain inhibition at high and low Triton concentrations for glycolamide **1** and a representative oxadiazole (**23**). Under low Triton conditions, oxadiazole **23** (at 5 μM) was a noncompetitive inhibitor of cruzain, consistent with aggregation-based inhibition. In contrast, under high Triton conditions, **23** (at 50 μM) was a competitive inhibitor of cruzain. Similarly, glycolamide **1** was found to be noncompetitive under low Triton conditions and at a relevant inhibitor concentration (25 μM). Hence, in glycolamide **1** and oxadiazole **23**, divergent modes of enzyme inhibition are operating under the two different assay conditions. Even so, there are subtle differences between these two chemotypes. Members of the oxadiazole class, having been unwittingly optimized for aggregation (driven by an assay condition favoring aggregation) can be >100-fold more potent as aggregators than as competitive inhibitors (e.g., **20**−**23** Table 2, Figure 3). In contrast, glycolamide **1** confers similar potencies by the two modes of inhibition. Thus, the

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differences in detergent effects (Figure 3) and initially surprising results concerning AmpC inhibition by **1** can be reconciled.

Two important conclusions can be drawn from the studies described here. First, divergent modes of inhibition can exist in a homologous SAR series, and these divergent mechanisms are only discernible by careful consideration of assay conditions. We argue that such a possibility should be considered as the default position, since the migration from competitive to noncompetitive regimes will not usually be obvious from structure alone. The ester bioisostere approach described here is a standard one and could not have been predicted a priori to have led to a series of aggregators with nanomolar potencies. That this in fact occurred owes to the use of an assay condition favoring the noncompetitive mode of inhibition.

The second important finding is that interpretable SAR spanning a range of potencies can originate from a wholly nonspecific mode of inhibition. Thus, the presence of SAR does not necessarily imply a specific binding interaction. Unfortunately, the medicinal chemist's natural aptitude for pattern recognition and hypothesis generation works against them here. The poor activity of analogues such as **17**, **18**, and **26** was easily rationalized in structural terms (e.g., poor tolerance of a charged substituent in **17**, entropic effects in **18**, loss of a hydrogen bond in **26**) when in fact the failure of these analogues to inhibit cruzain owes to a failure to aggregate. The danger for the medicinal chemist lies in interpreting SAR in terms of a specific binding interaction when, as shown here, such patterns of varying potency (SAR) may derive from a relative likelihood of aggregation (i.e., an SAR of aggregation). This is a humbling realization and emphasizes the peril of interpreting SAR derived from biochemical assays without considering the possibility of promiscuous inhibition and more generally emphasizes the importance of understanding molecular mechanism throughout a series.[23] Fortunately, experiments to exclude aggregation-based inhibition are straightforward.[24] Docking methods may also be helpful in spotting problematic scaffolds; a retrospective DOCK ranking of oxadiazole analogues revealed no correlation with enzyme inhibition data obtained under low Triton conditions (see Supporting Information). The cautionary contribution of this study is to point out that even within a clear SAR series, one is never entirely free from the concern that nonstoichiometric, artifactual mechanisms are contributing to the inhibition one observes.

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Supporting Information Available: Experimental details for the DOCK screen (including docking pose for **1**) and for all biochemical assays and secondary assays of aggregation, synthetic procedures and spectroscopic characterization data for all new compounds, table of cruzain inhibition data at 0.001% Triton for the full set of ~50 oxadiazole analogues synthesized, retrospective comparison of DOCK scores and biochemical inhibition data for oxadiazole analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

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