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## Identification of non-peptidic cysteine reactive fragments as inhibitors of cysteine protease rhodesain



Danielle McShan<sup>a</sup>, Stefan Kathman<sup>b</sup>, Brittiney Lowe<sup>a</sup>, Ziyang Xu<sup>b</sup>, Jennifer Zhan<sup>b</sup>, Alexander Statsyuk<sup>b</sup>, Ifedayo Victor Ogunjibe<sup>a,\*</sup>

<sup>a</sup> Department of Chemistry and Biochemistry, Jackson State University, Jackson, MS 39217, United States

<sup>b</sup> Department of Chemistry, Center for Molecular Innovation and Drug Discovery, Chemistry of Life Processes Institute, Northwestern University, Evanston, IL 60208, United States

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## ABSTRACT

Rhodesain, the major cathepsin L-like cysteine protease in the protozoan *Trypanosoma brucei rhodesiense*, the causative agent of African sleeping sickness, is a well-validated drug target. In this work, we used a fragment-based approach to identify inhibitors of this cysteine protease, and identified inhibitors of *T. brucei*. To discover inhibitors active against rhodesain and *T. brucei*, we screened a library of covalent fragments against rhodesain and conducted preliminary SAR studies. We envision that in vitro enzymatic assays will further expand the use of the covalent tethering method, a simple fragment-based drug discovery technique to discover covalent drug leads.

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Fragment-based drug discovery (FBDD) is a powerful method to discover drug leads and has been widely adopted in both academia and industry.<sup>1</sup> FBDD can be used to explore chemical diversity space with libraries which are smaller in size, producing drug leads with high ligand-binding efficiency.<sup>2</sup> It also provides a rational path to high-affinity lead compounds that possess drug-like properties.<sup>3</sup> The fragment-based approach is particularly well-suited for target-based drug discovery because the structural requirements for inhibition or inactivation can be used to guide the choice of pharmacophore and other structural motifs during drug design.<sup>4</sup>

The human African trypanosomiasis (sleeping sickness), a disease caused by the kinetoplastid protozoans *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*, is one of the neglected tropical diseases (NTDs). The disease is fatal if untreated, and current treatment options available for the disease are ineffective and have well-documented adverse effects. It is important to note that the incidence of sleeping sickness has decreased in the last decade, although the need to develop new and effective drugs remains a key objective in controlling and eradicating the disease.<sup>5</sup> A promising drug candidate in clinical trials for human African trypanosomiasis is the nitroimidazole fexinidazole. Fexinidazole is also being developed as a potential treatment for Chagas Disease.<sup>6,7</sup> The major cathepsin L-like cysteine protease in *T. brucei rhodesiense*, rhodesain, is a validated drug target. The cysteine protease is essential for the survival and infectivity of the parasite. Its

important role in the ability of the parasite to proliferate has been investigated by several groups.<sup>8–10</sup>

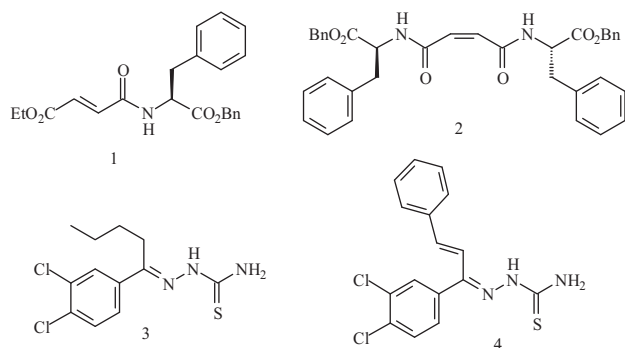
Steverding et al. have also shown that pharmacological inhibition of rhodesain is lethal to *T. brucei*.<sup>11</sup> A number of potent inhibitors of rhodesain that also have antitrypanosomal activity have been reported.<sup>8</sup> Among them are peptide-based covalent inhibitors with Michael acceptors such as 1 and 2 and thiosemicarbazone based inhibitors for example 3 and 4 (Fig. 1). Furthermore, a peptide derived covalent inhibitor of its homologue in *Trypanosoma cruzi*, K777, was recently a promising pre-clinical drug candidate for the treatment of Chagas disease (American trypanosomiasis), which highlights the importance of rhodesain as a drug target.<sup>12,13</sup>

This provides the rationale that covalent or non-covalent inhibitors of rhodesain may be advanced into the drug development pipeline against African sleeping sickness. To discover rhodesain inhibitors, we employed the irreversible tethering method to discover covalent inhibitors of cysteine proteases.<sup>14</sup> In this method, a mixture of cysteine reactive electrophilic fragments were incubated with the cysteine protease, allowing the best binding fragments to covalently and irreversibly modify the catalytic cysteine of the protease, and the covalent cysteine protease-inhibitor complexes were subsequently detected using mass spectrometry methods. The fragments can subsequently be elaborated into drug leads while retaining the original Michael acceptor electrophile. The originally developed method requires mass spectrometry to identify fragment hits, and this requirement limits the widespread use of this technology. We thought to expand the method and

\* Corresponding author.

**Table 1**  
Antitrypanosomal activity, cytotoxicity and protease inhibitory data of active acrylates and their vinyl sulfone analogues

Compound	Rhodesain $k_{\text{inact}}/K_{\text{I}}$ ( $\text{M}^{-1} \text{s}^{-1}$ )	<i>T. brucei</i> $\text{IC}_{50}$ ( $\mu\text{M}$ )	Hep G2 $\text{IC}_{50}$ ( $\mu\text{M}$ )
<b>5</b>	18.32	$30.31 \pm 1.92$	>150
<b>6</b>	3.47	$30.16 \pm 0.86$	$69 \pm 13.68$
<b>7</b>	13.22	$43.36 \pm 2.50$	>150
<b>8</b>	Inactive	$29.52 \pm 2.83$	$76 \pm 13.17$
<b>9</b>	Inactive	>50	>150
<b>10</b>	Inactive	>50	>150



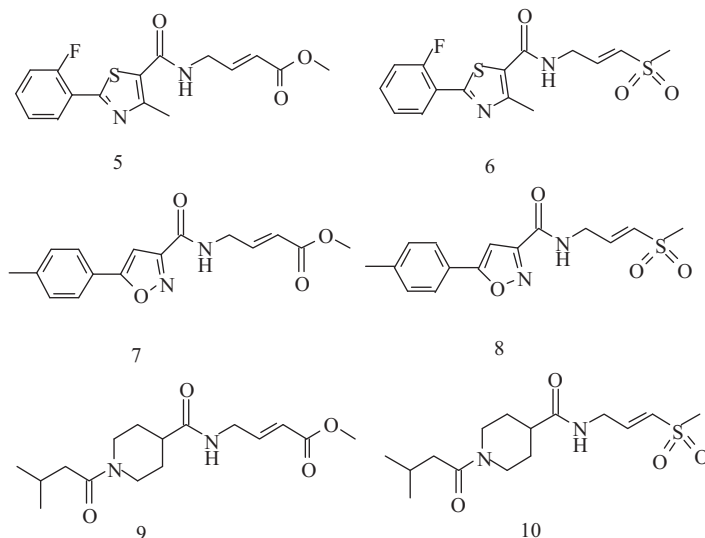
**Figure 1.** Inhibitors of rhodesain that have antitrypanosomal activity.

asked if the electrophilic fragments can be screened individually in enzymatic assays to identify weak and irreversible fragment inhibitors. Notably, such an approach would contradict current practices in academia and industry, in which reactive compounds are removed from compound screening collections. We envisioned that if successful, this strategy would substantially expand the use of irreversible tethering in laboratory settings in which mass spectrometry services are not available. Thus, a previously made library of 200 electrophilic fragments was screened for inhibitor activity against rhodesain in *in vitro* enzymatic assays.<sup>14,15</sup> The active hits were then investigated for their antitrypanosomal activity and cytotoxicity to human Hep G2 cells. The initial screen of the fragment library (10  $\mu\text{M}$ ) against rhodesain in 384-well assay plates led to the identification of seven positive hits (SI Fig. 1). These seven compounds caused  $\geq 85\%$  inhibition of rhodesain. A

follow-up assay using 200  $\mu\text{L}$  reaction mixtures in 96-well plates to confirm the activity of the active compounds led to the identification of compounds **5** and **7** as hits, the other five compounds caused <40% inhibition of rhodesain at 10  $\mu\text{M}$  (Table 1, Fig. 2). Fragments **5** and **7** were recently reported as inhibitors of papain, a cysteine protease structurally and biochemically related to rhodesain.<sup>14</sup> Fragments **5** and **7** seem to be better inhibitors of rhodesain than papain judging by the  $k_{\text{inact}}/K_{\text{I}}$  values, however, it is important to note that the optimal assay conditions for the two cysteine proteases are different, and the assay conditions may influence the observed inactivation constants. Since K777 has a vinyl sulfone electrophile as a Michael acceptor, we investigated if vinyl sulfone analogues of **5** and **7** were capable of inhibiting rhodesain.<sup>16</sup> We have found that compound **6**, which is the vinyl sulfone analogue of **5**, was also active in our assays, but it was less reactive towards the cysteine protease than the acrylate (**5**) (Table 1). However, compound **8**, the vinyl sulfone analogue of **7**, was inactive in the enzymatic assay, suggesting that unique structural features of **7** drive its activity against rhodesain.

In addition, we investigated if the previously reported<sup>14</sup> papain inhibitor **9** can inhibit rhodesain, and found that it was not active at inhibiting rhodesain. The vinyl sulfone analogue of **9** (**10**) was then synthesized and tested, and it was also inactive towards rhodesain. We then determined the time dependent inhibition constant  $k_{\text{inact}}/K_{\text{I}}$  of compounds **5**, **7** and **6**, which were 18.32, 13.22 and 3.47  $\text{M}^{-1} \text{s}^{-1}$ , respectively (Table 1, Fig. 3). Taken together, our preliminary SAR analysis indicates that a combination of specific structural features of the fragment and the electrophile make inhibitors of rhodesain. Crystal structure analysis of the fragment-rhodesain complexes is underway, and it should provide a structural rationale for extensive SAR studies. Although the identified covalent inhibitors of rhodesain are fragments, and therefore need to be optimized further to increase their potency and selectivity, we asked if any of those compounds have antitrypanosomal activity. We rationalized that those fragments that display antitrypanosomal activity, yet are not toxic to human hepatocellular carcinoma (Hep G2), could be further optimized into inhibitors that display selective toxicity to *T. brucei* but not to human cells.

Therefore, compounds **5–10** were tested for growth inhibitory activity on *T. brucei* as well as their cytotoxicity on Hep G2 cells. Compounds **5** and **7** have emerged as promising lead structures, since both compounds were potent at inhibiting rhodesain *in vitro* ( $k_{\text{inact}}/K_{\text{I}}$  values 18.32 and 13.22, respectively), and



**Figure 2.** Inhibitors of rhodesain from this study.

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