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Bioorganic & Medicinal Chemistry Letters 15 (2005) 121–123
Bioorganic & Medicinal Chemistry Letters

# Discovery of potent thiosemicarbazone inhibitors of rhodesain and cruzain

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Received 10 September 2004; revised 7 October 2004; accepted 7 October 2004
Available online 28 October 2004

**Abstract**—Herein we report the synthesis and evaluation of a series of thiosemicarbazones as potential inhibitors of cysteine proteases relevant to parasitic diseases. Derivatives of thiosemicarbazone **1** were discovered to be potent inhibitors of cruzain and rhodesain, crucial proteases in the life cycles of *Trypanosoma cruzi* and *T. brucei rhodesiense*, the organisms causing Chagas' disease and sleeping sickness. However, the entire series had only modest potency against falcipain-2, an essential protease for *Plasmodium falciparum*, the organism causing malaria. Among the active inhibitors, several potently inhibited proliferation of cultures of *T. brucei*. However, only modest activity was observed in inhibition of proliferation of *T. cruzi* or *P. falciparum*.
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Unicellular protozoal parasites of humans cause several of the major neglected diseases of the world including malaria, Chagas' disease, and sleeping sickness. For the latter two diseases, all existing drugs have inadequate efficacy and/or unacceptable toxicity profiles. While useful drugs do exist for malaria, worldwide resistance has emerged for many of the available therapeutics. Therefore, there is an urgent need for new clinical candidates for these diseases.

Malaria is one of the most profound human health problems, with several hundred million cases worldwide.[1] Most morbidity can be attributed to *Plasmodium falciparum*. Unfortunately, cheap, effective drugs such as chloroquine and Fansidar now suffer from worldwide resistance problems.[2] This problem is made worse by slow anti-malarial drug development. One reason for the paucity of new drugs is the lack of validated targets.[3] Among targets of recent interest have been falcipain cysteine proteases, which appear to be required for hemoglobin hydrolysis and possibly other processes.[4,5]

Chagas' disease, the leading cause of heart disease in Latin America,[6] is transmitted to humans by fecal to wound contact during feeding of the 'kissing beetle' host, as an infectious trypomastigote form of the protozoan parasite *Trypanosoma cruzi*. Cruzain (also referred to as cruzipain) is the major cysteine protease of *T. cruzi*. The protease is expressed in all life cycle stages of the parasite. It may function in nutrition, remodeling of the mammalian cell, or evasion of host defense mechanisms. Addition of a cruzain inhibitor to cultures of mammalian cells exposed to trypomastigotes or to mammalian cells already infected with *T. cruzi* amastigotes blocks replication and differentiation of the parasite, thus arresting the parasite life cycle.[7] Therefore, cruzain is essential for replication of the intracellular parasite.

Sleeping sickness, which in its late stages induces a terminal somnolent state, is transmitted to humans by the bite of the tsetse fly as an infectious form of the protozoan parasite *Trypanasoma brucei*. There are two dominant subspecies of *T. brucei: gambiense and rhodesiense*. Sleeping sickness is widespread in sub-Saharan Africa. The disease is fatal if untreated and no agents

*Keywords*: Protease inhibitor; Thiosemicarbazone; Malaria; Chagas; Sleeping sickness.
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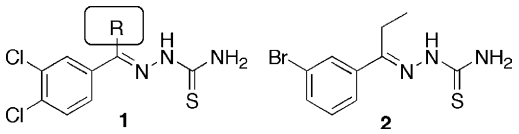
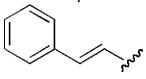
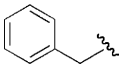
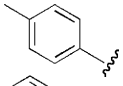
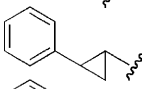
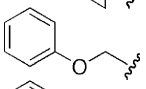
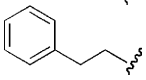
doi:10.1016/j.bmcl.2004.10.023

**Table 1.** Chemical structures and yields of thiosemicarbazone cysteine protease inhibitors **1a–j** and **2**




| # | R | Yield[a] (%) | |
|---|---|---|---|
| | | Step (a) | Step (b) |
| **1a** |  | — | — |
| **1b** |  | 40 | 59 |
| **1c** |  | 22 | 36 |
| **1d** |  | 42 | 35 |
| **1e** |  | 12 | 88 |
| **1f** |  | 13 | 27 |
| **1g** |  | 20 | 19 |
| **1h** |  | 45 | 22 |
| **1i** |  | 6 | 48 |
| **1j** |  | 33 | 49 |

[a] Isolated, not optimized. See Figure 1.

exist that are efficacious, nontoxic, and affordable in the developing world. Rhodesain is a cysteine protease expressed in *T. brucei rhodesiense* that regulates replication of the parasite. Inhibitors of rhodesain block the parasite life cycle in infected mammalian cells.

During our continuous effort to find mechanism-based small molecule inhibitors for these parasite proteases, Du and co-workers discovered thiosemicarbazone derivatives as promising lead compounds against cruzain.[8,9] In early structure–activity relationship (SAR) studies, they explored thiosemicarbazone derivatives of aromatic aldehydes, acetophenones, and propiophenones, finding that 3,4-dichlorophenyl- or 3-trifluoromethylphenyl thiosemicarbazones such as **1a** were potent inhibitors of cruzain.

However, these studies did not investigate thiosemicarbazones possessing larger alkyl groups than ethyl at the adjoining alkyl position (R in **1**, Table 1). Therefore, we began optimizing **1a** by chemical modification of its ethyl side chain to obtain new SAR data (Table 1). Here we report potent inhibitors having significantly improved activity against both cruzain and rhodesain relative to the prior best inhibitor, **1a**.

To produce diverse substituents (R in **1**, Table 1), we needed a reliable protocol to synthesize ketones having a 3,4-dichlorophenyl group. A straightforward method to synthesize diverse ketones is the palladium-catalyzed coupling of acid chlorides and organometallic reagents such as organoboranes and organostannanes. Organoboronic acids are most useful reagents for chemical library synthesis because of commercial availability and lower toxicity of any residual starting materials. However, couplings of boronic acids are generally carried out under basic aqueous conditions, thus giving hydrolytic decomposition of the acid chlorides. We found that 3,4-dichlorobenzeneboronic acid **3** coupled with diverse acid chlorides using potassium phosphate monohydrate in nonaqueous conditions.[10] However, a decarbonylative coupling product was produced as a significant side product along with the desired ketone. We have optimized the reaction conditions and found that 80 °C is the appropriate temperature to give ketones **4**. The ketones were converted to thiosemicarbazones **1b–j** (Table 2) in the conventional manner (Fig. 1). Because of the difficult separation of **4** from the decarbonylative coupling products and incomplete conversion to thiosemicarbazones, overall chemical yields were generally modest.

Compound series **1** was characterized for activity against the cysteine proteases cruzain, falcipain-2, and

**Table 2.** Inhibition of cruzain, rhodesain, and falcipain enzymes and *T. brucei* and *P. falciparum* growth by thiosemicarbazone derivatives **1**

| Compounds | Enzymatic activity inhibition | | | Proliferation inhibition | | |
|---|---|---|---|---|---|---|
| | Cruzain $IC_{50}$ (nM[a]) | Rhodesain $IC_{50}$ (nM[a]) | Falcipain-2 $IC_{50}$ (nM[a]) | *T. cruzi* $ED_{50}$ (nM[a]) | *T. brucei rhodesiense* $ED_{50}$ (nM[a]) | *P. falciparum W2* $ED_{50}$ (nM[a]) |
| **1a** | 30 | 110 | nt | nt | nt | nt |
| **1b** | **19** | **60** | 620 | 3000 | 130 | nd |
| **1c** | >10,000 | 3330 | nd | nt | nt | nd |
| **1d** | 3000 | 1100 | 14,000 | nt | nt | 6000 |
| **1e** | 30 | **38** | 470 | nd | 72 | 9000 |
| **1f** | 80 | **80** | 8400 | nd | nt | 19,000 |
| **1g** | 40 | **38** | 1800 | nd | nt | nd |
| **1h** | 30 | **60** | 640 | nd | 570 | nd |
| **1i** | 30 | **70** | 375 | nd | 1500 | nd |
| **1j** | 40 | **90** | 2600 | nd | 69 | nd |
| **2** | 310 | 230 | nt | nt | nt | nt |

Untested compounds are designated 'nt'; Compounds with no detectable activity are designated 'nd'. Activities stronger than **1a** are bolded.

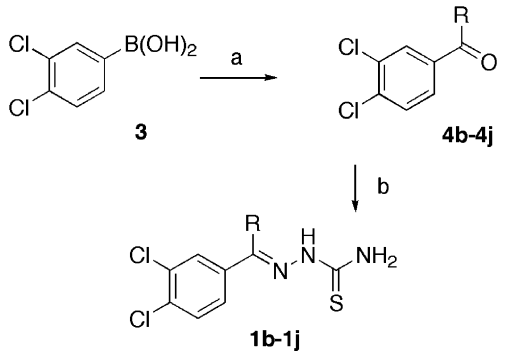



**Figure 1.** Synthesis of thiosemicarbazone **1b**–**j**. Reagents and conditions: (a) RCOCl, $PdCl_2(PPh_3)_2$, $K_3PO_4$-monohydrate, toluene, 80 °C; (b) $H_2NNHC(S)NH_2$, EtOH, AcOH, $H_2O$, 70 °C, 3 days.

rhodesain. Recombinantly expressed cruzain,[8] falcipain-2,[4] or rhodesain[11] was incubated with inhibitors **1a**–**j** and **2** in sequential dilution followed by addition of a fluorescent indicator of protease activity (e.g., *Z*-Phe-Arg-AMC). The increase in fluorescence produced by cleavage of the substrate allows determination of protease inhibition.[9] $IC_{50}$s were determined in the linear portion of a plot of inhibition versus log of inhibitor concentration.

The results are summarized in Table 2. For inhibition of cruzain, simple extension of the ethyl of **1a** to *n*-butyl gives two times improvement of activity (**1b**), however, incorporating bulkier alkyl substituents having a branch drastically reduces the potency (**1c**,**d**). Interestingly, a compound with a small branched alkyl (cyclopropyl) group having an aromatic ring (**1h**) possesses the same potency as **1a**. This led us to investigate compounds having aromatic rings connected with several linkages to the 3,4-dichlorobenzoyl moiety. These compounds (direct bond: **1g**; one methylene: **1f**; two methylenes: **1j**; alkene: **1e**; ether: **1i**) generally possess potency equivalent to **1a**.

Next, we tested these compounds against rhodesain. As expected, all of the compounds except **1c**,**d** gave potent inhibition, which was stronger than that of **1a** (Table 2). It is noteworthy that both *n*-butyl and bulkier arylalkyls possess the same level of potency, while bulkier alkyl substituents drastically reduce the potency. These inhibitors represent the most potent inhibitors discovered against rhodesain to date.

Finally, the compounds were tested against falcipain-2. All of the compounds were significantly less active inhibitors of this protease, with none having potencies less than 100 nM.

A subset of the active compounds were also evaluated in whole cell assays for inhibition of proliferation of *T. brucei rhodesiense*, *T. cruzi*, and *P. falciparum*. As is common with protease inhibitors, the overall activities were lower than in the enzymatic assays, presumably due to limitations in transport. Nevertheless, a number of the compounds were highly potent against *T. brucei*. Although most were inactive, several of the compounds had inhibitory activities in the 10 μM concentration range against a drug resistant strain of *P. falciparum*.

Finally, in attempts to discern activity against *T. cruzi* cultured in human macrophages, it was discovered that the majority of the compounds were toxic to macrophages at dosages higher than 10 μM, before they arrested parasite growth. However, one compound, **1b**, completely cured the infected macrophages at a dosage of 3 μM.

In this study, we intended to expand the SAR of thiosemicarbazone derivatives for inhibition of cysteine proteases of protozoans. The SAR presented in this report finishes the preliminary definition of functional groups from this scaffold that define activity against these proteases. It will facilitate the design of lead optimization libraries of thiosemicarbazone inhibitors having potent parasiticidal activities. In particular, it points out the value of fully exploring the benzophenone series of thiosemicarbazones.

## Acknowledgements

The Sandler Center for Basic Research in Parasitic Diseases and the National Institutes of Health.

## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2004.10.023.

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