Synthesis of a sugar-based thiosemicarbazone series and structure-activity relationship versus the parasite cysteine proteases: rhodesain, cruzain and Schistosoma mansoni cathepsin B1

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The pressing need for better drugs against Chagas disease, African sleeping sickness and schistosomiasis motivates the search for inhibitors of cruzain, rhodesain and SmCB1, the major cysteine proteases from *Trypanosoma cruzi*, *Trypanosoma brucei* and *Schistosoma mansoni*, respectively. Thiosemicarbazones and heterocyclic analogues have been shown to be both antitrypanocidal and inhibitory against parasite cysteine proteases. A series of compounds was synthesized and evaluated against cruzain, rhodesain and SmCB1 through biochemical assays to determine their potency and structure-activity relationships (SAR). This approach led to the discovery of 6 rhodesain, 4 cruzain and 5 SmCB1 inhibitors with IC$_{50} \leq$ 10 µM. Among the compounds tested, the thiosemicarbazone derivative of peracetylated galactoside (4i) was discovered as a potent rhodesain inhibitor (IC$_{50}$ = 1.2 ± 1.0 µM). The impact of a range of modifications was determined: removal of thiosemicarbazone or its replacement by semicarbazone resulted in virtually inactive compounds and modifications in the sugar also diminished potency. Compounds were also evaluated *in vitro* against the parasites *T. cruzi*, *T. brucei* and *S. mansoni*, revealing active compounds among this series.

Key words: thiosemicarbazone series, rhodesain, cruzain, *Schistosoma mansoni* cathepsin B1.
INTRODUCTION

New drugs for parasitic diseases are urgently needed but these globally important infections are often “neglected” because they most commonly afflict poor and marginalized communities. Current therapies are limited by poor efficacy, toxicity, high costs and parasite resistance. Chagas disease, African sleeping sickness and schistosomiasis are examples of diseases for which new therapies are needed (1, 2). Among the most studied and exploited molecular targets for these diseases are cysteine proteases. These enzymes have essential roles in parasite nutrition, immune evasion, host cell invasion and metacyclogenesis (3–6). Indeed, the cysteine proteases cruzain, rhodesain and SmCB1 from Trypanosoma cruzi, Trypanosoma brucei and Schistosoma mansoni, respectively, are validated molecular targets and have been the subject of numerous medicinal chemistry projects (7–17) that have yielded trypanocidal inhibitors, both in parasite culture and in animal models of infection (13, 15, 18–21).

The diverse inhibitors against these enzymes comprise compound classes which bind non-covalently (11, 12) and scaffolds containing a warhead that binds covalently to the catalytic cysteine. Within the latter category, vinylsulfones (8, 22–25), oxy-methyl ketones (7, 26), nitriles (16), epoxides and thiosemicarbazones (13–15, 27–29) have been described. Thiosemicarbazones present as advantages their low molecular weight, low cost of synthesis and their nonpeptidic nature (27). Greenbaum and co-workers (13) synthesized and evaluated the cysteine protease inhibitory and anti-parasitic activities of a library of thiosemicarbazones, with promising results. According to these authors, the thiosemicarbazones are regarded as validated drug leads capable of killing different species of protozoan parasites (T. cruzi, P. falciparum, and T. brucei) via inhibition of cysteine proteases.

Heterocyclic thiazole derivatives are also of great importance in medicinal chemistry due to their broad spectrum of biological activities (30–34). Also, many cysteine proteases inhibitors bearing thiazole or isothiazolone ring systems have been described as promising compounds against parasitic diseases (35, 36). Because of the versatile approach to the synthesis of the thiazole scaffold in
thiosemicarbazones, we synthesized and evaluated a series of thiazole analogues as potential inhibitors of cysteine proteases. The covalent attachment of the thiosemicarbazone or thiazole unit and a carbohydrate moiety was also designed to modulate solubility and interaction properties (for example, by hydrogen bonding) with the molecular target (cysteine protease).

Here, we screened a series of thiosemicarbazones and cyclic analogues against rhodesain and discovered an acetylated derivative of galactose as a potent inhibitor (IC$_{50} = 1.2 \pm 1.0$ µM). This is the first case of a sugar moiety being present in an inhibitor from this chemical class, encouraging further SAR (structure-activity relationship) studies on these series. Herein we report their synthesis and evaluation against the proteases, cruzain, rhodesain and SmCB1, and their $in vitro$ bioactivities against $T. cruzi$, $T. brucei$ and $S. mansoni$.

MATERIALS AND METHODS

Chemistry. All melting points were determined on a Microquímica MQAPF 301 apparatus. The IR spectra were recorded using a PerkinElmer Spectrum One infrared spectrometer and absorptions are reported as wave numbers (cm$^{-1}$). The NMR spectra were recorded on a Bruker AVANCE DRX200 or Bruker AVANCE DRX400 instrument, using tetramethylsilane (TMS) as the internal standard. Chemical shifts are given in $\delta$ (ppm) scale and $J$ values are given in Hz. All reagents of analytical grade were obtained from commercial suppliers and used without further purification. Compounds $4c$, $4h$, $4n-q$ and $6a-f$ were synthesized according to the published procedure (36).

General procedure A, for the synthesis of aryl glycosides bearing a formyl group (1-3). To a solution of vanillin (3 equiv.) in water containing 2.8 equimolar amounts of lithium hydroxide was added the corresponding peracetylglycosyl bromide (1 equiv.) dissolved in acetone. The reaction mixture was stirred at room temperature for 2h. The progress of the reaction was followed by thin-layer chromatography (TLC) (1:1 hexane:ethyl acetate). The mixture was concentrated to remove acetone.
and then diluted with water (10 mL) and washed with dichloromethane. The organic layer was separated, and washed with 10 % (w/v) NaOH aqueous solution and water until pH 7. The resulting organic phase was dried over sodium sulfate, filtered, and concentrated to dryness under reduced pressure.

4-Formyl-2-methoxyphenyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside (1). Obtained from the general procedure A as a white solid, yield 60 %; mp 123.1-123.8 °C (123-124 °C(37)); [α]₀ -8.1 (c 0.49, CH₂Cl₂); IR (υ/cm⁻¹): 2988, 2901 (C-H sp³), 1752, 1740 (C=O), 1693 (C=O), 1590, 1514 (C=C), 1370 (C-H sp³); ¹H NMR (400 MHz; CDCl₃) δ 9.89 (s, 1H, CHO); 7.43-7.40 (m, 2H); 7.25 (d, 1H, J = 8.4 Hz); 5.55 (t, 1H, J = 8.0 Hz); 5.46 (d, 1H, J = 2.8 Hz); 5.12 (dd, 1H, J = 8.0 Hz, J = 2.8 Hz); 5.05 (d, 1H, J = 8.0 Hz); 4.23 (dd, 1H, J = 11.8 Hz, J = 6.8 Hz); 4.16 (dd, 1H, J = 11.8 Hz, J = 6.4 Hz); 4.07-4.03 (m, 1H); 3.90 (s, 3H, OCH₃); 2.17-2.02 (4s, 12H, COCH₃); ¹³C NMR (100 MHz; CDCl₃) δ 190.89 (CHO); 170.33-169.35 (4C OCOCH₃); 151.29; 150.97; 132.77; 125.39; 117.99; 110.78; 100.35; 71.28; 70.60; 68.48; 66.82; 61.31; 56.13 (OCH₃); 20.69-20.58 (4C, COCH₃).

4-Formyl-2-methoxyphenyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside (2). Obtained from the general procedure A as a white solid, yield 57 %; mp 136.1-137.3 °C (135-137 °C(38)); [α]₀ -39.2 (c 0.51, CH₂Cl₂); IR (υ/cm⁻¹): 2988, 2901 (C-H sp³), 1753, 1737 (C=O), 1694 (C=O), 1591, 1510 (C=C), 1378 (C-H sp³); ¹H NMR (400 MHz; CDCl₃) δ 9.89 (s, 1H, CHO); 7.43-7.40 (m, 2H); 7.21 (d, 1H, J = 8.0 Hz); 5.34-5.28 (m, 2H); 5.13 (t, 1H, J = 6.8 Hz); 5.09 (d, 1H, J = 6.4 Hz); 4.27 (dd, 1H, J = 12.4 Hz, J = 5.2 Hz); 4.18 (dd, 1H, J = 12.4 Hz, J = 2.4 Hz); 3.86 (s, 3H, OCH₃); 3.85-3.70 (m, 1H); 2.07-2.04 (4s, 12H, COCH₃); ¹³C NMR (100 MHz; CDCl₃) δ 190.89 (CHO); 170.52-169.25 (4C OCOCH₃); 151.11; 151.03; 132.86; 125.34; 118.23; 110.85; 99.73; 72.41; 72.28; 71.06; 68.28; 61.90; 56.12 (OCH₃); 20.67-20.59 (4C, COCH₃).
4-Formyl-2-methoxyphenyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-O-acetyl-β-D-glucopyranoside (3). Obtained from the general procedure A as a white solid, yield 52%; mp 120.0-122.1 °C (121-124 °C(39)); [α] D -12.5 (c 0.48, CH₂Cl₂); IR (υ/cm⁻¹): 2942 (C-H sp³), 1741 (C=O), 1687 (C=O), 1592, 1507 (C=C), 1424, 1370 (C-H sp³); ¹H NMR (400 MHz; CDCl₃) δ 9.88 (s, 1H, CHO); 7.42-7.39 (m, 2H); 7.17 (d, 1H, J = 8.0 Hz); 5.35 (d, 1H, J = 8.0 Hz); 5.32 (t, 1H, J = 8.8 Hz); 5.22 (t, 1H, J = 8.8 Hz); 5.14-5.08 (m, 2H); 4.97 (dd, 1H, J = 12.0 Hz, J = 3.2 Hz); 4.53-4.51 (m, 2H); 4.17-4.06 (m, 3H); 3.93-3.89 (m, 2H); 3.88 (s, 3H); 3.79-3.75 (m, 1H); 2.15-1.97 (s, 21H); ¹³C NMR (100 MHz; CDCl₃) δ 190.89 (CHO); 170.38-169.10 (7C COCH₃); 151.16; 150.93; 132.72; 125.37; 117.87; 110.77; 101.13; 99.35; 76.05; 73.04; 72.47; 71.33; 70.94; 70.79; 69.14; 66.64; 61.84; 60.84; 56.11 (OCH₃); 20.79-20.50 (7C, COC₃H₃).

General procedure B, for the synthesis of thiosemicarbazones. To a suspension of 1 equiv. of thiosemicarbazide and 1 equiv. of the corresponding aldehyde or ketone in ethanol were added 3 drops of glacial acetic acid. The reaction mixture was kept under reflux and magnetic stirring for 2 hours. Then, the resulting suspension was vacuum filtered and washed with cold distilled water.

2-Phenylmethylenehydrazinecarbothioamide (4a). Obtained from the general procedure B as a white solid (91% yield). Mp 157.3-158.6 °C (lit. 157-159 °C(40)). IR, (υ/cm⁻¹): 3418 (NH), 1589 (C=N), 1539, 1447 (C=C aromatic). ¹H NMR (200 MHz, DMSO-d₆), δ/ppm: 11.43 (1 H, s, NH); 8.20 (1 H, s, NH₂); 8.05 (1 H, s, CH=N); 7.99 (1 H, s, NH₃); 7.78 (2 H, m, ArH); 7.39 (3 H, m, ArH).

2-[(4-methylphenyl)methylene]hydrazinecarbothioamide (4b). Obtained from the general procedure B as a white solid (82% yield). Mp 168.5-169.8 °C (lit. 162-163 °C(40)). IR, (υ/cm⁻¹): 3398 (NH), 1596
2-(4-pyridinylmethylene)hydrazinecarbothioamide (4d). Obtained from the general procedure B, as a pale yellow solid (80% yield). Mp 235-236 °C (lit. 240 °C(41)). IR, (ῡ/cm–1): 3420 (NH), 1591 (C=N), 1536 (C=C aromatic). ¹H NMR (200 MHz, DMSO-d₆), δ/ppm: 11.69 (1 H, s, NH); 8.58 (2 H, d, H-2 pyridine); 8.04 (1 H, s, NH₂); 8.21 (1 H, s, NH₂); 8.00 (1 H, s, CH=N); 7.76 (2 H, d, H-3 pyridine).

2-(1H-pyrrol-2-ylmethylene)hydrazinecarbothioamide (4e). Obtained from the general procedure B, as a solid (66% yield). Mp 191.9-193.6 °C (lit. 195-197 °C(42)). IR, (ῡ/cm –1): 3445 (NH), 1583 (C=N), 1530, 1550 (C=C aromatic).

1-Cyclopentylidenethiosemicarbazide (4f). Obtained from the general procedure B, as a solid (51% yield). Mp 155.3-157 °C (lit. 152-154 °C(43)). IR, (ῡ/cm–1): 3375 (NH), 1586 (C=N), 1508, 1448 (C=C aromatic). ¹H NMR (200 MHz, CDCl₃), δ/ppm: 8.61 (1 H, s, NH); 7.33 (1 H, s, NH₂); 6.67 (1 H, s, NH₂); 2.36 (4 H, m, CH₂); 1.85 (4 H, m, CH₂).

1-Cyclohexylidenethiosemicarbazide (4g). Obtained from the general procedure B, as a solid (65% yield). Mp 160.2-161.1 °C (lit. 154-155 °C(43)). IR, (ῡ/cm–1): 3375 (NH), 1583 (C=N), 1505, 1461 (C=C). ¹H NMR (200 MHz, CDCl₃), δ/ppm: 8.93 (1 H, s, NH); 7.30 (1 H, s, NH₂); 6.60 (1 H, s, NH₂); 2.32 (4 H, m, CH₂); 1.67 (6 H, m, CH₃).

2-[3-methoxy-4-[(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)oxy]phenyl]methylene] hydrazinecarbothioamide (4i). Obtained from the general procedure B as a solid, yield 76%; mp 127.2-129.9 °C; [α]D -18.9 (c 0.53, EtOH); IR (ῡ/cm–1): 3454 (NH), 1743 (C=O), 1597 (C=N), 1504, 1450 (C=C), 1068 (C-O); ¹H NMR (200 MHz; CDCl₃) δ/ppm: 10.27 (1H, s, NH); 7.90 (1H, s, CH=N); 7.27-10.27 ppm.
7.12 (4H, m, ArH); 6.64 (1H, s, NH₂); 5.57-5.44 (m, 2H); 5.13 (1H, dd, J = 10.4 Hz, J = 3.2 Hz); 4.97 (1H, d, J = 8.0 Hz); 4.29-4.00 (3H, m); 3.86 (3H, s, OCH₃); 2.17-2.02 (12H, 4s, COCH₃); 13C NMR (50 MHz; CDCl₃) δ/ppm: 177.55 (C=S); 170.02-169.09 (4C, OCOCH₃); 150.38; 147.96; 143.33; 128.81; 121.22; 118.59; 109.59; 100.33; 70.65; 70.19; 68.10; 66.40; 60.86; 55.73 (OCH₃); 20.34-20.25 (4C, COCH₃); HRMS (m/z) 556.1590 [M+H]⁺, calcd 556.1596 C₂₃H₃₀N₃O₁₁S⁺.

2-[[3-methoxy-4-[(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)oxy]phenyl)methylene]hydrazinecarbothioamide (4j). Obtained from the general procedure B as a solid, yield 84%; mp 119.4-122.9 ºC; [α]D -28.6 (c 0.28, MeOH); IR (ῡ/cm –1): 3278 (NH), 1739 (C=O), 1597 (C=N), 1504, 1450 (C=C), 1030 (C-O); 1H NMR (200 MHz; CDCl₃) δ/ppm: 10.37 (1H, s, NH); 7.91 (1H, s, CH= N); 7.21-7.10 (4H, m, ArH); 6.72 (1H, s, NH₂); 5.31-5.00 (m, 4H); 4.32-4.15 (2H, m); 3.84 (4H, m, OCH₃ + H-5); 2.07-2.04 (12H, 4s, COCH₃); HRMS (m/z) 556.1591 [M+H]⁺, calcd 556.1596 C₂₃H₃₀N₃O₁₁S⁺.

2-[[3-methoxy-4-[(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-O-acetyl-β-D-glucopyranosyl)oxy]phenyl)methylene]hydrazinecarbothioamide (4k). Obtained from the general procedure B as a solid, yield 71%; mp 220.1-212.8 ºC; [α]D -12.0 (c 0.5, MeOH); IR (ῡ/cm –1): 3469 (NH), 1739 (C=O), 1594 (C=N), 1528, 1505 (C=C), 1046 (C-O); 1H NMR (200 MHz; CDCl₃) δ/ppm: 10.0 (1H, s, NH); 7.86 (1H, s, CH=N); 7.27-7.08 (4H, m, ArH); 6.60 (1H, s, NH₂); 5.37-4.98 (7H, m); 4.53 (2H, d, J = 7.8 Hz); 4.16-3.77 (8H, m); 2.16-1.97 (s, 21H); 13C NMR (50 MHz; CDCl₃) δ 178 (C=S); 170.33-169.05 (7C COCH₃); 150.72; 148.20; 143.60; 129.08; 121.63; 118.88; 109.92; 100.99; 99.75; 75.97; 72.84; 72.36; 71.25; 70.85; 70.62; 69.01; 66.53; 61.72; 60.72; 56.07 (OCH₃); 20.74-20.45 (7C, COCH₃); HRMS (m/z) 844.2423 [M+H]⁺, calcd 844.2441 C₃₅H₄₆N₃O₁₉S⁺.

**General procedure C, for the synthesis of thiazole derivatives.** To a solution of 1 equiv. of thiosemicarbazone in isopropyl alcohol was added 1 equiv. of 2-bromoacetophenone, and the resulting
mixture was kept under reflux and magnetic stirring. The completion of the reaction was monitored by TLC (approximately 2 hours). After cooling to room temperature, the formed precipitate was filtered, washed with saturated solution of NaHCO₃ followed by cold distilled water. The final product was recrystallized in ethanol.

Benzaldehyde-2-(4-phenyl-2-thiazolyl)hydrazone (5a). Obtained from the general procedure C as a pale solid, yield 63%; mp 187.6-188.8 °C (lit. 186-187 °C(44)). IR, (ῡ/cm–1): 3306 (NH), 1557 (C=N), 1482, 1428 (C=C). ¹H NMR (200 MHz, DMSO-d₆), δ/ppm: 12.16 (1 H, s, NH); 7.88 (2 H, broad s., ArH); 7.61-7.08 (9 H, m, 8 x ArH and CH=N); 6.85 (1 H, s, H-thiazole).

4-Methylbenzaldehyde 2-(4-phenyl-2-thiazolyl)hydrazone (5b). Obtained from the general procedure C as a pale solid, yield 73%; mp 192.4-194.3 °C (lit. 195-196 °C(44)). IR, (ῡ/cm–1): 3279 (NH), 1553 (C=N), 1509, 1480 (C=C).

4-N,N-dimethylbenzaldehyde-2-(4-phenyl-2-thiazolyl)hydrazone (5c). Obtained from the general procedure C as a solid, yield 81%; mp 203.4-204.4 °C (lit. 207-208 °C(45)). IR, (ῡ/cm–1): 3288 (NH), 1603 (C=N), 1520, 1480 (C=C). ¹H NMR (200 MHz, DMSO-d₆), δ/ppm: 11.85 (1 H, s, NH); 7.92-7.86 (3 H, m, ArH); 7.48-7.24 (6 H, m, ArH and CH=N); 6.74 (2 H, broad s, ArH and H-thiazole).

4-Pyridinylcarbaldehyde-2-(4-phenyl-2-thiazolyl)hydrazone (5d). Obtained from the general procedure C as an orange solid, yield 53%; mp 240.1-242.2 °C (lit. 250-252 °C (46)). IR, (ῡ/cm–1): 3450 (NH), 1571 (C=N), 1482, 1441 (C=C). ¹H NMR (200 MHz, DMSO-d₆), δ/ppm: 12.57 (1 H, broad s., NH); 8.60 (1 H, broad s., ArH); 8.00 (1 H, s, CH=N); 7.85 (2 H, d, ArH); 7.61 (2 H, broad s., ArH); 7.38 (4 H, m, ArH and H-thiazole).
Pyrrole-2-carboxaldehyde-(4-phenyl-1,3-thiazol-2-yl)hydrazone (5e). Obtained from the general procedure C as a dark solid, yield 99%; mp 126 °C (lit. 125 °C(47)). IR, (GHz/cm⁻¹): 3304 (NH), 1623 (C=N), 1498, 1422 (C=C).

2-[(2-Cyclopentylmethylene)hydrazino]-4-phenyl-thiazole (5f). Obtained from the general procedure C as a solid, yield 98%, mp 170.2-172.4 °C (lit. 156-157 °C(48)). IR, (GHz/cm–1): 3438 (NH), 1626 (C=N), 1560, 1481 (C=C). 1H NMR (200 MHz, CDCl₃), δ/ppm: 7.73 (2 H, dd, J = 7.8 Hz; J = 2.0 Hz, ArH); 7.51-7.36 (3H, m, ArH and NH); 6.73 (1H, s, H-thiazole); 2.61-2.48 (4H, m, CH₂); 1.99-1.79 (4H, m, CH₂).

2-[(2-Cyclohexylmethylene)hydrazino]-4-phenyl-thiazole (5g). Obtained from the general procedure C as a solid, yield 51%, mp 149-151 °C (lit. 148-149 °C(48)). IR, (GHz/cm –1): 3050 (NH), 1610 (C=N), 1476, 1431 (C=C).

2-[(6,6-dimethylbicyclo[3.1.1]hept-2-en-2-yl)methylene]hydrazino-4-phenyl-1,3-thiazole (5h). Obtained from the general procedure C as a solid, yield 29 %, mp 152.4-154.2 °C. IR, (GHz/cm⁻¹): 3363 (NH), 1615 (C=N), 1495, 1470 (C=C). 1H NMR (200 MHz, DMSO-d₆), δ/ppm: 7.87-7.69 (3H, m, ArH); 7.47-7.29 (4H, m, ArH, CH=N and NH); 7.26 (1H, s, H-thiazole); 5.97 (1H, s, C=CH); 2.93-2.85 (1H, m, CH); 2.48-2.33 (3H, m, CH₃, CH); 2.14 (1H, m, CH); 1.33 (3H, s, CH₃); 1.13-1.03 (1H, m, CH₂); 0.78 (3H, s, CH₃). HRMS (m/z) 324.1530 [M+H]+, calcd 324.1529 C₁₉H₂₂N₃S⁺.

2-[[3-methoxy-4-[(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)oxy]phenyl]methylene]hydrazino-4-phenyl-thiazole (5i). Obtained from the general procedure C as a solid, yield 41%; mp 111.4-113.7 °C; [α]D -36 (c 0.5, MeOH); IR (GHz/cm⁻¹): 2952 (NH), 1748 (C=O), 1602 (C=N), 1567, 1510 (C=C), 1071 (C-O); 1H NMR (200 MHz; CDCl₃) δ/ppm: 7.80 (2H, d, J = 7.0 Hz, ArH); 7.44-7.29 (4H, m, ArH and
Enzyme expression and purification. Recombinant enzymes cruzain, rhodesain and SmCB1 have been expressed and purified as previously described (11, 49–51).

Assay against cruzain, rhodesain and SmCB1. Cruzain, rhodesain and SmCB1 activity were measured by monitoring the cleavage of the fluorogenic substrate Z-Phe-Arg-aminomethylcoumarin (Z-FR-AMC) in a Synergy 2 (Biotek), from the Center of Flow Cytometry and Fluorimetry at the Biochemistry and Immunology Department (UFMG). All assays were performed in triplicate using 0.1M sodium acetate, pH 5.5, in the presence of 1 mM beta-mercaptoethanol and 0.01% Triton X-100.

The final concentrations of cruzain and rhodesain were 0.5 nM, and the substrate concentration was 2.5 μM (K_m = 1 μM). For assays with SmCB1, the enzyme concentration was 8 nM and substrate concentration was 5 μM. Enzyme kinetic was followed by continuous reading for 5 min at 12s intervals, in the case of cruzain and rhodesain, and for 30 min at 23s intervals, in the case of SmCB1. Activity was calculated based on initial velocity rates, compared to a DMSO control. For evaluation of time-dependent inhibition, percentages of enzyme inhibition by a compound with or without pre-incubation with enzyme for 10 min were compared. Firstly, the inhibitory activity for all enzymes were screening at 100 μM of compound. When the inhibition was higher than 80%, the IC_{50} was determined based on at least two IC_{50} curves. Each curve was determined based on at least seven compound concentration, each in triplicate, and the data analyzed with GraphPad Prism 5.0, employing a nonlinear regression analysis of “log (inhibitor) vs response with variable slope – four parameters” (Figure S1). The reported values on Tables 1-3 refer to the average and standard deviation between the values obtained for at least two curves.
T. brucei brucei 221 maintenance. The parasites were cultured in HMI-9 medium supplemented with 20% of heat-inactivated Fetal Bovine Serum (FBS Gibco) starting at a density of $2 \times 10^4$ parasites/ml and sub-culturing every other day.

Compound plates preparation. All compounds were stored in powder. Solutions at 10 mM in neat DMSO were prepared a few hours before assay experimentation, and seeded in the row A of 384 wells plate (Greiner 784201). Serial dilution with two-fold factor was prepared until the row P (from 10mM to 305nM). The compounds were pinned and 50nl of each well was transferred to the assay plate (Greiner 781091) containing 25µl of HMI-9 media supplemented with 20% FBS.

T. brucei brucei screening assay. Five thousand ($5 \times 10^3$) parasites were seeded in a volume of 25 µl in the 384 wells plate already containing the compounds in serial dilution, being the highest concentration tested 10 µM. After 72 hours incubation at 37°C and 5% CO$_2$, each well received 12.5 µl of Sybr Green in lysis solution (30 mM Tris pH 7.5, 7.5 mM EDTA, 0.012% saponin, 0.12% Triton X-100 and 0.3μl/ml of Sybr Green). After addition of lysis solution the plates were sealed with plastic film and the mixture was vortexed for 45 seconds at 1700 rpm (MixMate). The mixture was incubated for 1 hour at room temperature and the plate was read in Flexstation (Molecular Devices) to detect the fluorescence signal corresponding to parasite viability (Ex 485nm/Em 530nm). Raw viability data consists of values of relative fluorescence unit (R.F.U.) obtained from the reading of Sybr Green that binds to the viable parasite's DNA, and includes max and min controls and measured values. Thymerasol (2 μM) was used as the reference drug EC$_{100}$. The activity normalization was done based on the non-treated (negative control, 0% activity) and the reference drug at the EC$_{100}$ concentration (positive control, 100% activity), with at least 16 wells for each control per plate.

T. cruzi screening assay. The assay was performed using T. cruzi (Tulahuen strain) expressing the...
Escherichia coli β-galactosidase as reporter gene (52, 53). Infective trypomastigote forms were obtained through culture in monolayers of mouse L929 fibroblasts in RPMI-1640 medium (pH 7.2-7.4) without phenol red (Gibco BRL) plus 10% fetal bovine serum and 2 mM glutamine. For the bioassay, 4,000 L929 cells in 80 μl of supplemented medium were added to each well of a 96-well microtiter plate. After an overnight incubation, 40,000 trypomastigotes in 20 μl were added to the cells and incubated for 2 h. The medium containing extracellular parasites was replaced with 200 μl of fresh medium and the plate was incubated for an additional 48 h to establish the infection. For IC₅₀ determination, the cells were exposed to active samples at serial decreasing dilutions starting at 1000 μM in DMSO (less than 1% in RPMI-1640 medium), and the plate was incubated for 96 h. After this period, 50 μl of 500 μM chlorophenol red β-D-galactopyranoside (CPRG) in 0.5% Nonidet P40 was added to each well, and the plate was incubated for 16 to 20 h, after which the absorbance at 570 nm was measured. Controls with uninfected cells, untreated infected cells, infected cells treated with benznidazole at 1 μg/ml = 3.8 μM (positive control) or DMSO 1% was used (Faria J et al, manuscript in press). The results were expressed as the percentage of T. cruzi growth inhibition in compound-tested cells as compared to the infected cells and untreated cells. IC₅₀ values were calculated by linear interpolation. Tetraplicates were run in the same plate, and the experiments were repeated at least once.

**In vitro assay for analysis of cell viability.** The active compounds were tested in vitro for cytotoxicity versus L-929 cells using the alamarBlue® dye. Were used the same cell number, time of the cell development and time of compound exposure used for the beta-galactosidase assay. The cells were exposed to compounds at increasing concentrations starting at the IC₅₀ value of T. cruzi. The compounds were tested in tetraplicate. After 96 hours of compound exposure, alamarBlue® was added and the absorbance at 570 and 600 nm measured after 4-6 h. The cell viability was expressed as the percentage of difference in the reduction between treated and untreated cells (53). IC₅₀ values were calculated by linear interpolation and the Selectivity Index (SI) was determined by the ratio between...
the cytotoxic concentration (CC50) and the IC50 value against the parasite for each compound.

**S. mansoni screening assay.** The acquisition, preparation and *in vitro* maintenance of newly transformed *S. mansoni* schistosomula (derived from infective stage cercariae) and adult parasites have been described by us (54, 55). We employ a Puerto Rican isolate of *S. mansoni* that is cycled between *Biomphalaria glabrata* snails and female Golden Syrian hamsters (infected at 4-6 weeks of age) as intermediate and definitive hosts, respectively. Maintenance and handling of small mammals are carried out in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California San Francisco. For schistosomula, 200-300 parasites are incubated in 96w flat-bottomed plates (Corning Costar 3599) containing 200 µL in Basch medium 169 (56) supplemented with 2.5% FBS and 1x penicillin-streptomycin solution in a 5% CO2 atmosphere at 37 °C. For adult parasites, 5 male worms are incubated in 2 ml of the above medium in 24w flat-bottomed plates (Corning Costar 3526) under the same conditions. Compound, 100% DMSO, is added at the final concentrations indicated in Table 4. Controls employ the equivalent volume of DMSO at a final concentration never exceeding 0.5%. Parasite responses to chemical insult are adjudicated visually each day using a constrained descriptive nomenclature (54, 57). The types and number of phenotypic responses recorded are then converted into a ‘severity score’ ranging from 0 (no effect) to 4 (severely compromised). Thus, for schistosomula and adults, alterations in shape (e.g., ‘rounding’), motility (‘slow’ or ‘overactive’) and density (‘darkening’) are each awarded a score of 1 up to the maximum of 4. In addition, for adults, the inability to adhere to the bottom of the well is awarded a score of 1: damage to the integrity of the outer surface (tegument) is considered lethal to the parasite and is awarded the maximum score of 4.

**RESULTS AND DISCUSSION**

**Chemistry**
The sugar derivatives 1, 2 and 3 were synthesized by reaction of vanillin with the corresponding peracetylglycosyl bromide using lithium hydroxide as a base, according to a method previously described (Figure 1) (58). A series of thiosemicarbazones was synthesized by classical methods from an aldehyde or ketone and thiosemicarbazide with yields in the range of 51-95 %. Then, the thiosemicarbazones obtained were subjected to reaction with α-bromo-acetophenone, giving the corresponding thiazole heterocycles (yield 29-98 %) (Figure 2). The stereochemistry at the C=N bond of the thiosemicarbazone derivatives was established by $^1$H NMR spectroscopy. The value of the chemical shift of the NH (9–12 ppm) is indicative of (E) configuration (59). Some semicarbazones were also synthesized according to a previously described procedure (60), for comparison of the activity of semicarbazones with that of the thiosemicarbazones.

Fifteen thiosemicarbazones and nine thiazole analogues were prepared containing an aryl or heteroaryl, and/or cycloalkyl or glycosyl moieties (Figure 3). The introduction of the carbohydrate moiety (compounds 4i-4k and 5i) was designed to modulate solubility and interaction properties (for example, by hydrogen bonding) with the molecular target (cysteine protease).

**Discovery of a potent rhodesain and SmCB1 inhibitor**

An initial screen of 18 compounds, mostly thiosemicarbazones, was performed against rhodesain. Compounds were screened in two conditions, that is with and without a 10 min pre-incubation with the enzyme (Table 1). A clear time-dependence was observed for the active compounds, as expected based on the formation of a covalent bond between rhodesain and the inhibitor. To verify if the differences in percentage of inhibition observed were statistically significant, we have applied an unpaired t-test comparing the results of assays with and without pre-incubation. This analysis reveals that, for all compounds which inhibited rhodesain by more than 80% in the screen, the percentage of inhibition is higher if the enzyme is pre-incubated with the compound, and this difference is statistically significant, with p-values <0.0002 (Table S1). Therefore all subsequent
assays were performed with a 10 minute pre-incubation against the enzymes; the SAR discussion refers to the inhibition observed under this condition.

Eleven compounds inhibited rhodesain by at least 50% at 100 µM; IC₅₀ values were determined for five of them. Several trends were observed based on this initial screen. Comparison between 4a and 4g indicates the importance of the aromatic ring. Addition of a methyl substituent at the para position does not affect inhibition (4b), whereas a dimethylamine at the same position results in a more potent compound (4c, IC₅₀ = 3.0 ± 0.8 µM), as does the replacement of the phenyl by a pyridine (4d, IC₅₀ = 3.3 ± 0.9 µM) or by an imidazol (4e, IC₅₀ = 4.0 ± 1.8 µM). Three cyclic analogues were less soluble, and in a few cases could not be evaluated at 100 µM. They were therefore assayed at 50 µM (5c) or 75 µM (5a and 5b). Assay results for several pairs of compounds suggested that replacement of the thiosemicarbazones by a cyclized analogue decreases potency against the enzyme (4d vs. 5d, 4h vs. 5h, 4e vs. 5e, 4j vs. 5i), except for 4a vs. 5a, which showed similar potency. This initial screen resulted in the discovery of a potent inhibitor, 4i, with an IC₅₀ of 1.2 ± 1.0 µM. Comparison to 4j shows the importance of the sugar, since replacement of the acetylated galactose by an acetylated glucose, representing a difference in only one chiral center, results in a 20-fold decrease in potency (4j, IC₅₀ = 26.2 ± 1.5 µM).

The compounds were also evaluated against the T. cruzi and S. mansoni cysteine proteases, cruzain and SmCB1. Like rhodesain, cruzain is a cathepsin L-like protease, and only two active residues are different between both proteins. Similarity between the cathepsin B-like SmCB1 and rhodesain active sites is lower, however common inhibitors have been reported for these enzymes (19). The SAR for the three enzymes showed several similarities and also interesting differences. The importance of the aromatic ring was confirmed (4a vs. 4g), and as observed for rhodesain both the addition of a dimethylamine in this ring and its replacement by a pyrol increased potency against cruzain (4c, IC₅₀ = 6.6 ± 3.2 µM, and 4e, IC₅₀ = 9.7 ± 5.2 µM) and SmCB1 (4c, IC₅₀ = 1.5 ± 0.4 µM, and 4e, IC₅₀ = 6.8 ± 2.1 µM). However, in contrast to what was measured for rhodesain, against cruzain and
SmCB1, the pyridine analogue (4d) was not as potent as the compound containing a phenyl ring (4a). Interestingly, although addition of the methyl substituent neither influenced the inhibition of cruzain nor rhodesain, potency against SmCB1 was increased ten-fold (4a, IC<sub>50</sub> = 22.4 ± 3.1 μM vs 4b, IC<sub>50</sub> = 2.5 ± 1.9 μM). Overall, the thiosemicarbazones 4c and 4e were the most potent inhibitors of the three enzymes.

The most significant difference in potency was observed for 4i. Although this compound had low micromolar potency against rhodesain (IC<sub>50</sub> = 1.2 ± 1.0 μM), it was approximately 35-fold less potent against cruzain (IC<sub>50</sub> = 37.7 ± 9.8 μM) and essentially inactive against SmCB1 (IC<sub>50</sub> could not be determined). Despite the similarity of the active sites of cruzain and rhodesain, the bottom of the S2 pocket in cruzain and SmCB1 contains a glutamate (Glu208 and Glu316, respectively), whereas rhodesain has an alanine in the equivalent position. The S2 pocket is therefore considerably more open in rhodesain, possibly providing an explanation for the ability of this enzyme to bind larger scaffolds.

The epimer 4j showed lower potency against cruzain and SmCB1, when compared to rhodesain. Nevertheless, against these two enzymes we observed that both compounds (4i and 4j) were only modest inhibitors.

**SAR**

The SAR for 4i was exploited based on the impact of removing or modifying the sugar (Table 2), and removing the thiosemicarbazone or modifying it to a semicarbazone (Table 3). Significant differences were observed in SAR for the three enzymes regarding modifications in the sugar. Removal of the sugar moiety decreased potency against rhodesain by at least 5-fold (4l, IC<sub>50</sub> = 7.3 ± 4.0 μM; 4m, IC<sub>50</sub> = 6.2 ± 2.7 μM) or more so depending on the pattern of phenyl substitution (4n, 4o). On the other hand, against cruzain this modification increased potency by 6-fold, and both 4l and 4m had IC<sub>50</sub> values of approximately 10 μM. It is worth noting that in the analogues which do not contain a sugar (4l-4o) the potencies against cruzain and rhodesain are similar.
Despite the sugar not being essential for binding, it could drastically affect inhibition. For example, addition of another sugar monomer (4k) resulted in a compound inactive against both cruzain and rhodesain, but active against SmCB1 (4k, IC$_{50}$ = 7.7 ± 1.7 μM).

Removal of the thiosemicarbazone (galactosyl 1 and lactosyl 3) or its replacement by semicarbazone (6a vs. 4n, 6b vs. 4m, 6c vs. 4l, 6d vs. 4o, 6f vs. 4h) resulted in compounds inactive against the three enzymes, the only exception being 6f against SmCB1 (IC$_{50}$ = 5.2 ± 2.8 μM). This effect has also been reported for a related compound series (27) and can be explained by the more electrophilic nature of the thiosemicarbazones, and the mechanism of cysteine protease inhibition by these compounds.

**Assays against parasites in vitro**

Compounds were evaluated against *T. brucei*, *T. cruzi* and *S. mansoni*. To assess the anti-parasitic activity of the 24 compounds against the bloodstream form of *T. b. brucei*, we used a viability assay based on the fluorescence of the parasite’s nucleic acid (Faria J et al, manuscript in press). Parasites were co-incubated for 72 h with compound two-fold serially diluted from 10 μM to 305 pM. At 10 μM, none of the 24 tested compounds showed more than 60% bioactivity, defined as the reduction in parasite number compared to untreated control (Table S2).

For *T. cruzi*, compounds were tested against both amastigote and trypomastigote forms of the Tulahuen strain (52). Weak trypanocidal activity was observed for this class and five compounds generated IC$_{50}$ values under 100 μM. Based on the difference between the IC$_{50}$ values for parasites and the L929 mouse fibroblast cell line, a Selectivity Index (SI) of each compound could be determined. The SI ranged from 1.3 to 10.7 (Table S2). Importantly, even though the trypanocidal IC$_{50}$ values were high, the trypanocidal concentration was not toxic to fibroblasts, and in the case of compounds 4b and 4h, the IC$_{50}$ values for fibroblasts were an order of magnitude greater than those for *T. cruzi* (Table S2).
Although those compounds with SI values $\geq 10$ might be considered prototypes for new trypanocidal drugs, they are not recommended for \textit{in vivo} tests as the SI values do not cross a decision gate threshold of 50 (53).

For \textit{S. mansoni}, screens were performed against post-invasive larvae (schistosomula) and adult parasites, as previously described (54, 55). Parasite responses to chemical insult were adjudicated visually each day using a constrained nomenclature (54, 57) that accounts for changes in shape, motility and density. The types and number of phenotypic responses recorded are then converted into a ‘severity score’ ranging from 0 (no effect) to 4 (severely compromised). Five compounds (5a, 5b, 5c, 5d, 4h) showed activity against schistosomula. Among these compounds only 5d was active against adult worms.

**CONCLUSION**

Here we report the discovery of 4i, a sugar-containing thiosemicarbazone which showed low micromolar potency against rhodesain (IC$_{50}$ = 1.2 ± 1.0 $\mu$M) and modest potency against cruzain (IC$_{50}$ = 37.7 ± 9.8 $\mu$M). Synthesis of a series of analogues allowed determination of the SAR in this series, and resulted in six rhodesain, four cruzain and five SmCB1 inhibitors with IC$_{50}$ $\leq$ 10 $\mu$M. Only three thiosemicarbazones (4c, 4e and 4m) showed similar potencies against the rhodesain, cruzain, and SmCB1, a result that demonstrates that considerable differences in the SAR for the three enzymes exist.

In a few cases, using larger scaffolds, higher potency was observed against rhodesain. Direct assays of the most potent inhibitors against the parasites \textit{T. cruzi}, \textit{T. brucei} and \textit{S. mansoni} showed some antiparasitic activity but also suggested that further SAR modifications will be needed to produce lead compounds.

**ACKNOWLEDGMENTS**

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Biocomputacional AUXPE 3379/2013) and FAPEMIG (Grant PPM-00357-14) and RBO acknowledges CNPq (Grant 4041130/2012-0) for financial support. GANP acknowledges a postdoctoral fellowship from CAPES Grant A118/2013. JHM and JLdeSN received funding from the European Community’s 7th Framework Programme (602777) Project Kindred. Research by CRC is supported in part by NIH-NIAD R21AI107390 and R01AI089896 awards. The authors also thank the Center of Flow Cytometry and Fluorimetry at the Biochemistry and Immunology Department (UFMG) and the Program for Technological Development of Tools for Health-PDTIS-FIOCRUZ for use of its facilities and Plataforma de Bioprospecção RPT10A-PDTIS-CPqRR-Fiocruz for HRMS measurements.
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60. De Oliveira RB, de Souza-Fagundes EM, Soares RPP, Andrade AA, Krettli AU, Zani CL.
FIGURE LEGENDS

Figure 1 – Synthesis of the glycosides 1, 2 and 3.

Figure 2 – General synthetic route for preparation of thiosemicarbazones and their corresponding thiazole heterocycles.

Figure 3 – Chemical structure of the thiosemicarbazones 4a-o and cyclic analogues 5a-i synthesized.
Table 1. Inhibition of rhodesain, cruzain and SmCB1 by a series of thiosemicarbazones and cyclic analogues.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>Rhodesain</th>
<th>Cruzain</th>
<th>SmCB1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>inhibition (%)</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (μM)</td>
<td>inhibition (%)</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (μM)</td>
</tr>
<tr>
<td>4a</td>
<td>Ph</td>
<td>72.5 ± 12.7</td>
<td>ND</td>
<td>53.7 ± 1.2</td>
</tr>
<tr>
<td>4b</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;C-</td>
<td>77.6 ± 2.7</td>
<td>ND</td>
<td>41.9 ± 1.1</td>
</tr>
<tr>
<td>4c</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;C-N-</td>
<td>100.0 ± 0.0</td>
<td>ND</td>
<td>91.9 ± 0.2</td>
</tr>
<tr>
<td>4d</td>
<td>N-</td>
<td>81.7 ± 2.1</td>
<td>3.3 ± 1.0</td>
<td>73.1 ± 2.4</td>
</tr>
<tr>
<td>4e</td>
<td>N-</td>
<td>100 ± 0.0</td>
<td>97.2 ± 0.1</td>
<td>86.1 ± 4.4</td>
</tr>
<tr>
<td>4f</td>
<td>0.0 ± 0.0</td>
<td>ND</td>
<td>0.0 ± 0.0</td>
<td>ND</td>
</tr>
<tr>
<td>4g</td>
<td>0.0 ± 0.0</td>
<td>ND</td>
<td>0.0 ± 0.0</td>
<td>ND</td>
</tr>
<tr>
<td>Compound</td>
<td>Structure</td>
<td>Percentage Inhibition (%)</td>
<td></td>
<td></td>
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<tr>
<td>----------</td>
<td>-----------</td>
<td>---------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4h</td>
<td><img src="image" alt="Structure 4h" /></td>
<td>69.6 ± 7.0 ND 32.7 ± 2.8 ND 56.1 ± 3.0 ND</td>
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<td></td>
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<tr>
<td>4i</td>
<td><img src="image" alt="Structure 4i" /></td>
<td>96.9 ± 0.6 1.2 ± 1.0 71.4 ± 1.7 ± 9.8 10.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4j</td>
<td><img src="image" alt="Structure 4j" /></td>
<td>95.4 ± 1.2 26.2 ± 1.5 70.3 ± 4.9 ND 52.6 ± 5.3 ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5a**</td>
<td><img src="image" alt="Structure 5a" /></td>
<td>Ph 72.0 ±13.3 ND 74.6 ± 2.6 ND 9.3 ± 3.8 ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5b*</td>
<td><img src="image" alt="Structure 5b" /></td>
<td>H₂C⁻ 64.6 ± 3.6 ND 39.4 ± 6.1 ND 53.6 ± 16.0</td>
<td></td>
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<tr>
<td>5c**</td>
<td><img src="image" alt="Structure 5c" /></td>
<td>H₂C⁻N⁺ 65.3 ± 2.0 ND 80.9 ± 0.2 ± 1.6 0.0 ± 0.0 ND</td>
<td></td>
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<tr>
<td>5d</td>
<td><img src="image" alt="Structure 5d" /></td>
<td>13.6 ± 5.4 ND 37.3 ± 5.7 ND 0.0 ± 0.0 ND</td>
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<td></td>
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<tr>
<td>5e</td>
<td><img src="image" alt="Structure 5e" /></td>
<td>30.2 ± ND 27.5 ± 5.4 ND 41.0 ± 2.7 ND</td>
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<tr>
<td>5f</td>
<td><img src="image" alt="Structure 5f" /></td>
<td>0.0 ± 0.0 ND 16.9 ± 4.8 ND 0.0 ± 0.0 ND</td>
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<tr>
<td>5g</td>
<td><img src="image" alt="Structure 5g" /></td>
<td>5.1 ± 8.7 ND 23.7 ± 1.0 ND 38.0 ± 9.8 ND</td>
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<tr>
<td>5h</td>
<td><img src="image" alt="Structure 5h" /></td>
<td>34.7 ± ND 47.8 ± 3.4 ND 53.7 ± ND</td>
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<td></td>
</tr>
</tbody>
</table>

*Percentages of inhibition are reported as the average and standard deviation of at least two independent experiments, each performed in triplicate. IC₅₀ values represent the average and standard deviation of at least two independent experiments. ND = not determined. *Compound evaluated at 75 μM; ** Compound evaluated at 50 μM.
Table 2. Inhibition of rhodesain, cruzain and SmCB1 by 4i analogues modified in the sugar moiety.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>Inhibition at 100 μM</th>
<th>IC₅₀ (μM)</th>
<th>Inhibition at 100 μM</th>
<th>IC₅₀ (μM)</th>
<th>Inhibition at 100 μM</th>
<th>IC₅₀ (μM)</th>
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<tbody>
<tr>
<td>4k</td>
<td></td>
<td>37.6 ± 8.7 (ND)</td>
<td>7.3 ± 1.7 (ND)</td>
<td>30.9 ± 2.1 (ND)</td>
<td>14.1 ± 0.7 (ND)</td>
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<tr>
<td>4l</td>
<td></td>
<td>85.6 ± 1.5 (4.0)</td>
<td>7.3 ± 1.7 (ND)</td>
<td>80.4 ± 0.8 (11.0)</td>
<td>11.0 ± 1.5 (ND)</td>
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<tr>
<td>4m</td>
<td></td>
<td>89.8 ± 0.7 (2.7)</td>
<td>7.3 ± 1.7 (ND)</td>
<td>89.9 ± 1.5 (9.0)</td>
<td>9.0 ± 1.5 (ND)</td>
<td></td>
<td></td>
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<tr>
<td>4n</td>
<td></td>
<td>60.6 ± 6.4 (ND)</td>
<td>7.3 ± 1.7 (ND)</td>
<td>58.5 ± 2.3 (ND)</td>
<td>5.5 ± 2.3 (ND)</td>
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<tr>
<td>4o</td>
<td></td>
<td>66.5 ± 5.1 (ND)</td>
<td>7.3 ± 1.7 (ND)</td>
<td>75.3 ± 2.8 (ND)</td>
<td>16.2 ± 2.8 (ND)</td>
<td></td>
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<tr>
<td>5i</td>
<td></td>
<td>70.6 ± 10.2 (ND)</td>
<td>7.3 ± 1.7 (ND)</td>
<td>53.9 ± 0.7 (ND)</td>
<td>11.7 ± 0.7 (ND)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Percentages of inhibition are reported as the average and standard deviation of at least two independent experiments, each performed in triplicate. IC₅₀ values represent the average and standard deviation of at least two independent experiments. ND = not determined.*
Table 3. Inhibition of rhodesain, cruzain and SmCB1 by 4i analogues without thiosemicarbazones.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>R₁</th>
<th>Rhodesain</th>
<th>Cruzain</th>
<th>SmCB1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% inhibition</td>
<td>IC₅₀ b (µM)</td>
<td>% inhibition</td>
</tr>
<tr>
<td></td>
<td>100 µM a</td>
<td>100 µM a</td>
<td>100 µM a</td>
<td></td>
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<tr>
<td>Galactosyl 1</td>
<td>R₁ = OAc; R₂ = H</td>
<td>33.1 ± 8.6 ND</td>
<td>12.0 ± 2.5 ND</td>
<td>15.2 ± 0.7 ND</td>
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<tr>
<td>Lactosyl 3</td>
<td>R₁ = H; R₂ = tetra-O-acetyl-β-galactosyl</td>
<td>3.7 ± 2.1 ND</td>
<td>6.8 ± 1.8 ND</td>
<td>15.0 ± 2.2 ND</td>
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<tr>
<td>6a</td>
<td>H₂O</td>
<td>20.6 ± 5.9 ND</td>
<td>20.8 ± 1.2 ND</td>
<td>0.0 ± 0.0 ND</td>
</tr>
<tr>
<td>6b</td>
<td>H₂O</td>
<td>10.1 ± 10.8 ND</td>
<td>20.2 ± 3.7 ND</td>
<td>1.6 ± 2.8 ND</td>
</tr>
<tr>
<td>6c</td>
<td>H₂O</td>
<td>0.0 ± 0.0 ND</td>
<td>14.6 ± 0.7 ND</td>
<td>14.4 ± 14.2 ND</td>
</tr>
<tr>
<td>6d</td>
<td>H₂O</td>
<td>18.4 ± 3.7 ND</td>
<td>5.1 ± 3.6 ND</td>
<td>38.9 ± 29.4 ND</td>
</tr>
<tr>
<td>6f</td>
<td></td>
<td>53.8 ± 9.6 ND</td>
<td>39.4 ±1.1 ND</td>
<td>99.1 ± 1.5 5.2 ± 2.8</td>
</tr>
</tbody>
</table>

aPercentages of inhibition are reported as the average and standard deviation of at least two independent experiments, each performed in triplicate. bIC₅₀ values represent the average and standard deviation of at least two independent experiments. ND = not determined.
Table 4. Activity against *Schistosoma mansoni* somules and adult worms.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Severity scores against somules</th>
<th>Severity scores against adult worms</th>
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<tr>
<td></td>
<td>0.1 µM 1 µM 10 µM 5 µM</td>
<td>Day Day Day Day</td>
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<tr>
<td></td>
<td>1 2 3 4</td>
<td>1 2 3 4</td>
</tr>
<tr>
<td>4a</td>
<td>0 0 0 0 0 0 0 0 0</td>
<td>0 0 1 1</td>
</tr>
<tr>
<td>4b</td>
<td>0 0 0 0 0 0 0 0 0</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td>4c</td>
<td>0 0 0 0 0 0 0 0 0</td>
<td>0 0 0 0</td>
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<tr>
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<td>0 0 0 0</td>
</tr>
<tr>
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<td>0 0 0 0 0 0 0 1 0 1 4</td>
<td>0 0 0 0</td>
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<tr>
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<td>5d</td>
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</tbody>
</table>

*Compound evaluated at 50 µM due to solubility limitations. NT = not tested.
\[ \text{R}_1 = \text{R}_3 = \text{OAc}; \text{R}_2 = \text{H} \]
\[ \text{R}_1 = \text{H}; \text{R}_2 = \text{R}_3 = \text{OAc}; \]
\[ \text{R}_1 = \text{H}; \text{R}_2 = \text{tetra-O-acetyl-}\beta-\text{d-galactosyl}; \text{R}_3 = \text{OAc}; \]
R-CHO + H₂N\text{S}N\text{NH}_2 \xrightarrow{\text{AcOH, EtOH, reflux}} \text{H}_2\text{N}\text{SN}N\text{NH}_2 \text{N}N\text{N}\text{N}=\text{R}

51-95 %

\text{O} \text{Br} \xrightarrow{i-PrOH, reflux} \text{N}N\text{N}\text{N}=\text{R}

29-98 %
R1 = Aryl or Heteroaryl

- a R₂ = H; R₃ = H
- b R₂ = CH₃; R₃ = H
- c R₂ = N(CH₃)₂; R₃ = H

- d
- e
- f n = 1
- g n = 2
- h

I R₂ = OH; R₃ = OCH₃
m R₂ = R₃ = OCH₃
n R₂ = OCH₃; R₃ = H
o 3,4,5-trimethoxy

R1 = Cycloalkyl

- i
- j
- k

R1 = Glycosyl