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

Nayara Cristina Fonseca,[a] Luana Faria da Cruz,[b] Filipe da Silva Villela,[b] Glaécia Aparecida do Nascimento Pereira,[b,c] Jair Lage de Siqueira-Neto,[d] Danielle Kellar,[e] Brian M. Suzuki,[e] Debalina Ray,[e] Thiago Belarmino de Souza,[a] Ricardo José Alves,[a] Policarpo Ademar Sales Júnior,[f] Alvaro José Romanha,[f,g] Silvane Maria Fonseca Murta,[f] James H. McKerrow,[d] Conor R. Caffrey,[e] Renata Barbosa de Oliveira,[a] Rafaela Salgado Ferreira [b,#]

Departamento de Produtos Farmacêuticos, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Belo Horizonte, MG 31270-901, Brazil[a]; Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG 31270-901, Brazil[b]; CAPES Foundation, Ministry of Education of Brazil, Brasília, DF 70040-020, Brazil[c]; Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, San Diego, CA 92093, USA[d]; Center for Discovery and Innovation in Parasitic Diseases and the Department of Pathology, University of California San Francisco, San Francisco, CA 94158, USA[e]; Centro de Pesquisas René Rachou, FIOCRUZ, Belo Horizonte, MG 30190-002, Brazil[f]; Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de Santa Catarina, Florianópolis, SC 88040-900, Brazil[g]

Running Head: Thiosemicarbazones as cysteine protease inhibitors

#Address **correspondence to** Rafaela S. Ferreira, rafaelasf@icb.ufmg.br

N.C.F. and L.F.C. contributed equally to this work

ABSTRACT

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 ± 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 δ (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 ºC (123-124 ºC(37)); $[\alpha]_D$ -8.1 (*c* 0,49, CH_2Cl_2); IR ($\bar{\upsilon}/cm^{-1}$): 2988, 2901 (C-H sp^3), 1752, 1740 (C=O), 1693 (C=O), 1590, 1514 (C=C), 1370 (C-H sp^3); ^1H NMR (400 MHz; $CDCl_3$) 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, OCH_3); 2.17-2.02 (4s, 12H,$COCH_3$); ^{13}C NMR (100 MHz; $CDCl_3$) δ 190.89 (CHO); 170.33-169.35 (4C O<u>C</u>OCH_3); 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 (OCH_3); 20.69-20.58 (4C, CO<u>C</u>H_3).

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 ºC (135-137 ºC(38)); $[\alpha]_D$ -39.2 (*c* 0,51, CH_2Cl_2); IR ($\bar{\upsilon}/cm^{-1}$): 2988, 2901 (C-H sp^3), 1753, 1737 (C=O), 1694 (C=O), 1591, 1510 (C=C), 1378 (C-H sp^3); ^1H NMR (400 MHz; $CDCl_3$) 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, OCH_3); 3.85-3.70 (m, 1H); 2.07-2.04 (4s, 12H, $COCH_3$); ^{13}C NMR (100 MHz; $CDCl_3$) δ 190.89 (CHO); 170.52-169.25 (4C O<u>C</u>OCH_3); 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 (OCH_3); 20.67-20.59 (4C, CO<u>C</u>H_3).

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 ºC (121-124 ºC(39)); $[\alpha]_D$ -12.5 (*c* 0.48, CH_2Cl_2); IR ($\bar{\upsilon}$/cm^{-1}): 2942 (C-H sp^3), 1741 (C=O), 1687 (C=O), 1592, 1507 (C=C), 1424, 1370 (C-H sp^3); ^{1}H NMR (400 MHz; $CDCl_3$) 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); ^{13}C NMR (100 MHz; $CDCl_3$) δ 190.89 (CHO); 170.38-169.10 (7C $\underline{C}OCH_3$); 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 (OCH_3); 20.79-20.50 (7C, $CO\underline{C}H_3$).

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 ºC (lit. 157-159 ºC(40)). IR, ($\bar{\upsilon}$/cm^{-1}): 3418 (NH), 1589 (C=N), 1539, 1447 (C=C aromatic). ^{1}H NMR (200 MHz, DMSO-d_6), δ/ppm: 11.43 (1 H, s, N$\underline{H}$); 8.20 (1 H, s, N$\underline{H}_2$); 8.05 (1 H, s, C$\underline{H}$=N); 7.99 (1 H, s, N$\underline{H}_2$); 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 ºC (lit. 162-163 °C(40)). IR, ($\bar{\upsilon}$/cm^{-1}): 3398 (NH), 1596

(C=N), 1509, 1462 (C=C aromatic).

2-(4-pyridinylmethylene)hydrazinecarbothioamide (**4d**). Obtained from the general procedure B, as a pale yellow solid (80% yield). Mp 235-236 ºC (lit. 240 ºC(41)). IR, ($\bar{\upsilon}$/cm^{-1}): 3420 (NH), 1591 (C=N), 1536 (C=C aromatic). ^{1}H NMR (200 MHz, DMSO-d_6), δ/ppm: 11.69 (1 H, s, NH); 8.58 (2 H, d, H-2 pyridine); 8.04 (1 H, s, NH$_2$); 8.21 (1 H, s, NH$_2$); 8.00 (1 H, s, CH=N); 7.76 (2 H, d, H-3 pyridine).

2-(1*H*-pyrrol-2-ylmethylene)hydrazinecarbothioamide (**4e**). Obtained from the general procedure B, as a solid (66 % yield). Mp 191.9-193.6 ºC (lit. 195-197 ºC(42)). IR, ($\bar{\upsilon}$/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 ºC (lit. 152-154 ºC(43)). IR, ($\bar{\upsilon}$/cm^{-1}): 3375 (NH), 1586 (C=N), 1508, 1448 (C=C aromatic). ^{1}H NMR (200 MHz, $CDCl_3$), δ/ppm: 8.61 (1 H, s, NH); 7.33 (1 H, s, NH$_2$); 6.67 (1 H, s, NH$_2$); 2.36 (4 H, m, CH$_2$); 1.85 (4 H, m, CH$_2$).

1-Cyclohexylidenethiosemicarbazide (**4g**). Obtained from the general procedure B, as a solid (65 % yield). Mp 160.2-161.1 ºC (lit. 154-155 ºC(43)). IR, ($\bar{\upsilon}$/cm^{-1}): 3375 (NH), 1583 (C=N), 1505, 1461 (C=C). ^{1}H NMR (200 MHz, $CDCl_3$), δ/ppm: 8.93 (1 H, s, NH); 7.30 (1 H, s, NH$_2$); 6.60 (1 H, s, NH$_2$); 2.32 (4 H, m, CH$_2$); 1.67 (6 H, m, CH$_2$).

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 ºC; $[\alpha]_D$ -18.9 (*c* 0,53, EtOH); IR ($\bar{\upsilon}$/cm^{-1}): 3454 (NH), 1743 (C=O), 1597 (C=N), 1504, 1450 (C=C), 1068 (C-O); ^{1}H NMR (200 MHz; $CDCl_3$) δ/ppm: 10.27 (1H, s, NH); 7.90 (1H, s, CH=N); 7.27-

7.12 (4H, m, ArH); 6.64 (1H, s, NH_2); 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, OCH_3); 2.17-2.02 (12H, 4s, $COCH_3$); ^{13}C NMR (50 MHz; $CDCl_3$) δ/ppm: 177.55 (C=S); 170.02-169.09 (4C, $OCOCH_3$); 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 (OCH_3); 20.34-20.25 (4C, $COCH_3$); HRMS (m/z) 556.1590 $[M+H]^+$, calcd 556.1596 $C_{23}H_{30}N_3O_{11}S^+$.

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; $[\alpha]_D$ -28.6 (c 0,28, MeOH); IR ($\bar{\upsilon}$/cm^{-1}): 3278 (NH), 1739 (C=O), 1597 (C=N), 1504, 1450 (C=C), 1030 (C-O); ^{1}H NMR (200 MHz; $CDCl_3$) δ/ppm: 10.37 (1H, s, NH); 7.91 (1H, s, CH=N); 7.21-7.10 (4H, m, ArH); 6.72 (1H, s, NH_2); 5.31-5.00 (m, 4H); 4.32-4.15 (2H, m); 3.84 (4H, m, OCH_3 + H-5); 2.07-2.04 (12H, 4s, $COCH_3$); HRMS (m/z) 556.1591 $[M+H]^+$, calcd 556.1596 $C_{23}H_{30}N_3O_{11}S^+$.

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 220.1-121.8 ºC; $[\alpha]_D$ -12.0 (c 0.5, MeOH); IR ($\bar{\upsilon}$/cm^{-1}): 3469 (NH), 1739 (C=O), 1594 (C=N), 1528, 1505 (C=C), 1046 (C-O); ^{1}H NMR (200 MHz; $CDCl_3$) δ/ppm: 10.0 (1H, s, NH); 7.86 (1H, s, CH=N); 7.27-7.08 (4H, m, ArH); 6.60 (1H, s, NH_2); 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); ^{13}C NMR (50 MHz; $CDCl_3$) δ 178 (C=S); 170.33-169.05 (7C $COCH_3$); 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 (OCH_3); 20.74-20.45 (7C, $COCH_3$); HRMS (m/z) 844.2423 $[M+H]^+$, calcd 844.2441 $C_{35}H_{46}N_3O_{19}S^+$.

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 $NaHCO_3$ 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 ºC (lit. 186-187 ºC(44)). IR, ($\bar{\upsilon}$/cm^{-1}): 3306 (NH), 1557 (C=N), 1482, 1428 (C=C). ^{1}H NMR (200 MHz, DMSO-d_6), 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 ºC (lit. 195-196 ºC(44)). IR, ($\bar{\upsilon}$/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 ºC (lit. 207-208 ºC(45)). IR, ($\bar{\upsilon}$ /cm^{-1}): 3288 (NH), 1603 (C=N), 1520, 1480 (C=C). ^{1}H NMR (200 MHz, DMSO-d_6), 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 ºC (lit. 250-252 ºC (46)). IR, ($\bar{\upsilon}$/cm^{-1}): 3450 (NH), 1571 (C=N), 1482, 1441 (C=C). ^{1}H NMR (200 MHz, DMSO-d_6), 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 ºC (lit. 125 ºC(47)). IR, ($\bar{\upsilon}$/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 ºC (lit. 156-157 ºC(48)). IR, ($\bar{\upsilon}$/cm^{-1}): 3438 (NH), 1626 (C=N), 1560, 1481 (C=C). ^{1}H NMR (200 MHz, $CDCl_3$), 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, CH_2); 1.99-1.79 (4H, m, CH_2).

2-[(2-Cyclohexylmethylene)hydrazino]-4-phenyl-thiazole (**5g**). Obtained from the general procedure C as a solid, yield 51%, mp 149-151 ºC (lit. 148-149 ºC(48)). IR, ($\bar{\upsilon}$/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 ºC. IR, ($\bar{\upsilon}$/cm^{-1}): 3363 (NH), 1615 (C=N), 1495, 1470 (C=C). ^{1}H NMR (200 MHz, DMSO-d_6), 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, CH_2, CH); 2.14 (1H, m, CH_2); 1.33 (3H, s, CH_3); 1.13-1.03 (1H, m, CH_2); 0.78 (3H, s, CH_3). HRMS (m/z) 324.1530 $[M+H]^+$, calcd 324.1529 $C_{19}H_{22}N_3S^+$.

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 ºC; $[\alpha]_D$ -36 (c 0,5, MeOH); IR ($\bar{\upsilon}$/cm^{-1}): 2952 (NH), 1748 (C=O), 1602 (C=N), 1567, 1510 (C=C), 1071 (C-O); ^{1}H NMR (200 MHz; $CDCl_3$) δ/ppm: 7.80 (2H, d, J = 7.0 Hz, ArH); 7.44-7.29 (4H, m, ArH and

N=CH); 7.05-6.97 (3H, m, ArH); 6.81-6.75 (2H, m, H-thiazole and NH); 5.31-4.96 (4H, m); 4.33-4.13 (2H, m); 3.82 (4H, m, OCH_3 and H-5); 2.08-2.04 (12H, 4s, $COCH_3$); HRMS (*m*/*z*) 656.1905 $[M+H]^+$, calcd 656.1909 $C_{31}H_{34}N_3O_{11}S^+$.

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.

263

264 ***T. brucei brucei* 221 maintenance.** The parasites were cultured in HMI-9 medium supplemented with
265 20% of heat-inactivated Fetal Bovine Serum (FBS Gibco) starting at a density of 2 x 10^4 parasites/ml
266 and sub-culturing every other day.

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

272

273 ***T. brucei brucei* screening assay.** Five thousand (5 x 10^3) parasites were seeded in a volume of 25 µl
274 in the 384 wells plate already containing the compounds in serial dilution, being the highest
275 concentration tested 10 µM. After 72 hours incubation at 37C and 5% CO_2, each well received 12.5 µl
276 of Sybr Green in lysis solution (30 mM Tris pH 7.5, 7.5 mM EDTA, 0.012% saponin, 0.12% Triton X-
277 100 and 0.3µl/ml of Sybr Green). After addition of lysis solution the plates were sealed with plastic
278 film and the mixture was vortexed for 45 seconds at 1700 rpm (MixMate). The mixture was incubated
279 for 1 hour at room temperature and the plate was read in Flexstation (Molecular Devices) to detect the
280 fluorescence signal corresponding to parasite viability (Ex 485nm/Em 530nm). Raw viability data
281 consists of values of relative fluorescence unit (R.F.U.) obtained from the reading of Sybr Green that
282 binds to the viable parasite's DNA, and includes max and min controls and measured values.
283 Thymerasol (2 µM) was used as the reference drug EC_{100}. The activity normalization was done based
284 on the non-treated (negative control, 0% activity) and the reference drug at the EC_{100} concentration
285 (positive control, 100% activity), with at least 16 wells for each control per plate.

286

287 ***T. cruzi* screening assay.** The assay was performed using *T. cruzi* (Tulahuen strain) expressing the

288 *Escherichia coli* β-galactosidase as reporter gene (52, 53). Infective trypomastigote forms were
289 obtained through culture in monolayers of mouse L929 fibroblasts in RPMI-1640 medium (pH 7.2-7.4)
290 without phenol red (Gibco BRL) plus 10% fetal bovine serum and 2 mM glutamine. For the bioassay,
291 4,000 L929 cells in 80 µl of supplemented medium were added to each well of a 96-well microtiter
292 plate. After an overnight incubation, 40,000 trypomastigotes in 20 µl were added to the cells and
293 incubated for 2 h. The medium containing extracelullar parasites was replaced with 200 µl of fresh
294 medium and the plate was incubated for an additional 48 h to establish the infection. For IC_{50}
295 determination, the cells were exposed to active samples at serial decreasing dilutions starting at 1000
296 µM in DMSO (less than 1% in RPMI-1640 medium), and the plate was incubated for 96 h. After this
297 period, 50 µl of 500 µM chlorophenol red β-D-galactopyranoside (CPRG) in 0.5% Nonidet P40 was
298 added to each well, and the plate was incubated for 16 to 20 h, after which the absorbance at 570 nm
299 was measured. Controls with uninfected cells, untreated infected cells, infected cells treated with
300 benznidazole at 1 µg/ml = 3.8 µM (positive control) or DMSO 1% was used (Faria J et al, manuscript
301 *in press*). The results were expressed as the percentage of *T. cruzi* growth inhibition in compound-tested
302 cells as compared to the infected cells and untreated cells. IC_{50} values were calculated by linear
303 interpolation. Tetraplicates were run in the same plate, and the experiments were repeated at least once.
304
305 ***In vitro* assay for analysis of cell viability.** The active compounds were tested *in vitro* for cytotoxicity
306 versus L-929 cells using the alamarBlue® dye. Were used the same cell number, time of the cell
307 development and time of compound exposure used for the beta-galactosidase assay. The cells were
308 exposed to compounds at increasing concentrations starting at the IC_{50} value of *T. cruzi*. The
309 compounds were tested in tetraplicate. After 96 hours of compound exposure, alamarBlue® was added
310 and the absorbance at 570 and 600 nm measured after 4-6 h. The cell viability was expressed as the
311 percentage of difference in the reduction between treated and untreated cells (53). IC_{50} values were
312 calculated by linear interpolation and the Selectivity Index (SI) was determined by the ratio between

313 the cytotoxic concentration (CC_{50}) and the IC_{50} value against the parasite for each compound.

314

315 ***S. mansoni* screening assay.** The acquisition, preparation and *in vitro* maintenance of newly
316 transformed *S. mansoni* schistosomula (derived from infective stage cercariae) and adult parasites have
317 been described by us (54, 55). We employ a Puerto Rican isolate of *S. mansoni* that is cycled between
318 *Biomphalaria glabrata* snails and female Golden Syrian hamsters (infected at 4-6 weeks of age) as
319 intermediate and definitive hosts, respectively. Maintenance and handling of small mammals are
320 carried out in accordance with a protocol approved by the Institutional Animal Care and Use
321 Committee (IACUC) of the University of California San Francisco. For schistosomula, 200-300
322 parasites are incubated in 96w flat-bottomed plates (Corning Costar 3599) containing 200 µL in Basch
323 medium 169 (56) supplemented with 2.5% FBS and 1x penicillin-streptomycin solution in a 5% CO_2
324 atmosphere at 37 ˚C. For adult parasites, 5 male worms are incubated in 2 ml of the above medium in
325 24w flat-bottomed plates (Corning Costar 3526) under the same conditions. Compound, 100% DMSO,
326 is added at the final concentrations indicated in Table 4. Controls employ the equivalent volume of
327 DMSO at a final concentration never exceeding 0.5%. Parasite responses to chemical insult are
328 adjudicated visually each day using a constrained descriptive nomenclature (54, 57). The types and
329 number of phenotypic responses recorded are then converted into a 'severity score' ranging from 0 (no
330 effect) to 4 (severely compromised). Thus, for schistosomula and adults, alterations in shape (e.g.,
331 'rounding'), motility ('slow' or 'overactive') and density ('darkening') are each awarded a score of 1
332 up to the maximum of 4. In addition, for adults, the inability to adhere to the bottom of the well is
333 awarded a score of 1: damage to the integrity of the outer surface (tegument) is considered lethal to the
334 parasite and is awarded the maximum score of 4.

335

336 **RESULTS AND DISCUSSION**

337 **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; IC_{50} 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**, IC_{50} = 3.0 ± 0.8 μM), as does the replacement of the phenyl by a pyridine (**4d**, IC_{50} = 3.3 ± 0.9 μM) or by an imidazol (**4e**, IC_{50} = 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 IC_{50} 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**, IC_{50} = 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**, IC_{50}= 6.6 ± 3.2 μM, and **4e**, IC_{50} =9.7 ± 5.2 μM) and SmCB1 (**4c**, IC_{50}= 1.5 ± 0.4 μM, and **4e**, IC_{50} = 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_{50}= 22.4 ± 3.1 μM vs **4b**, IC_{50}= 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_{50} = 1.2 ± 1.0 μM), it was approximately 35-fold less potent against cruzain (IC_{50} = 37.7 ± 9.8 μM) and essentially inactive against SmCB1 (IC_{50} 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**) where 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_{50} = 7.3 ± 4.0 μM; **4m,** IC_{50} = 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_{50} 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 ≥ 10 might be considered prototypes for new trypanocidal drugs, they are not recommended for *in vivo* tests as the SI values do not cross a decision gate threshold of 50 (53).

For *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 μM) and modest potency against cruzain (IC_{50} = 37.7 ± 9.8 μM). 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 $IC_{50} \leq 10$ μM. 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 *T. cruzi*, *T. brucei* and *S. mansoni* showed some anti-parasitic activity but also suggested that further SAR modifications will be needed to produce lead compounds.

ACKNOWLEGDMENTS

RSF acknowledges CNPq (Grant 477435/2012-2), Capes (Grants A118/2013 and Edital

462 Biocomputacional AUXPE 3379/2013) and FAPEMIG (Grant PPM-00357-14) and RBO acknowledges
463 CNPq (Grant 4041130/2012-0) for financial support. GANP acknowledges a postdoctoral fellowship
464 from CAPES Grant A118/2013. JHM and JLdeSN received funding from the European Community's
465 7th Framework Programme (602777) Project Kindred. Research by CRC is supported in part by NIH-
466 NIAD R21AI107390 and R01AI089896 awards. The authors also thank the Center of Flow Cytometry
467 and Fluorimetry at the Biochemistry and Immunology Department (UFMG) and the Program for
468 Technological Development of Tools for Health-PDTIS-FIOCRUZ for use of its facilities and
469 Plataforma de Bioprospecção RPT10A-PDTIS-CPqRR-Fiocruz for HRMS measurements.

REFERENCES

1. **Caffrey CR, Secor WE**. 2011. Schistosomiasis: from drug deployment to drug development. Curr. Opin. Infect. Dis. **24**:410–417.
2. **Coura JR, Vinas PA**. 2010. Chagas disease: a new worldwide challenge **465**:S6–S7.
3. **McKerrow JH**. 1999. Development of cysteine protease inhibitors as chemotherapy for parasitic diseases: insights on safety, target validation, and mechanism of action. Int J Parasitol **29**:833–837.
4. **Sajid M, McKerrow JH**. 2002. Cysteine proteases of parasitic organisms. Mol Biochem Parasit **120**:1–21.
5. **Renslo AR, McKerrow JH**. 2006. Drug discovery and development for neglected parasitic diseases. Nat Chem Biol **2**:701–710.
6. **McKerrow JH, Doyle PS, Engel JC, Podust LM, Robertson SA, Ferreira R, Saxton T, Arkin M, Kerr ID, Brinen LS, Craik CS**. 2009. Two approaches to discovering and developing new drugs for Chagas disease. Mem. Inst. Oswaldo Cruz **104 Suppl 1**:263–269.
7. **Brak K, Kerr ID, Barrett KT, Fuchi N, Debnath M, Ang K, Engel JC, McKerrow JH, Doyle PS, Brinen LS, Ellman JA**. 2010. Nonpeptidic tetrafluorophenoxymethyl ketone cruzain inhibitors as promising new leads for Chagas disease chemotherapy. J Med Chem **53**:1763–1773.
8. **Bryant C, Kerr ID, Debnath M, Ang KKH, Ratnam J, Ferreira RS, Jaishankar P, Zhao D, Arkin MR, McKerrow JH, Brinen LS, Renslo AR**. 2009. Novel non-peptidic vinylsulfones targeting the S2 and S3 subsites of parasite cysteine proteases. Bioorg. Med. Chem. Lett. **19**:6218–6221.
9. **Choe Y, LS B, MS P, JC E, M L, C G, SG W, PV P, H C, LW H, DS H, M M, RF T, CM B, CS. C**. 2005. Development of alpha-keto-based inhibitors of cruzain, a cysteine protease implicated in Chagas disease. Bioorg Med Chem Lett **13**:2141–2156.
10. **Ferreira RS, Bryant C, Ang KKH, McKerrow JH, Shoichet BK, Renslo AR**. 2009. Divergent

Modes of Enzyme Inhibition in a Homologous Structure−Activity Series. J. Med. Chem. **52**:5005–5008.

11. **Ferreira RS, Simeonov A, Jadhav A, Eidam O, Mott BT, Keiser MJ, McKerrow JH, Maloney DJ, Irwin JJ, Shoichet BK**. 2010. Complementarity Between a Docking and a High-Throughput Screen in Discovering New Cruzain Inhibitors. J. Med. Chem. **53**:4891–4905.
12. **Ferreira RS, Dessoy MA, Pauli I, Souza ML, Krogh R, Sales AIL, Oliva G, Dias LC, Andricopulo AD**. 2014. Synthesis, Biological Evaluation, and Structure–Activity Relationships of Potent Noncovalent and Nonpeptidic Cruzain Inhibitors as Anti- *Trypanosoma cruzi* Agents. J. Med. Chem. **57**:2380–2392.
13. **Greenbaum DC, Mackey Z, Hansell E, Doyle P, Gut J, Caffrey CR, Lehrman J, Rosenthal PJ, McKerrow JH, Chibale K**. 2004. Synthesis and structure-activity relationships of parasiticidal thiosemicarbazone cysteine protease inhibitors against *Plasmodium falciparum*, *Trypanosoma brucei*, and *Trypanosoma cruzi*. J Med Chem **47**:3212–3219.
14. **Magalhaes Moreira DR, de Oliveira ADT, Teixeira de Moraes Gomes PA, de Simone CA, Villela FS, Ferreira RS, da Silva AC, dos Santos TAR, Brelaz de Castro MCA, Pereira VRA, Leite ACL**. 2014. Conformational restriction of aryl thiosemicarbazones produces potent and selective anti-*Trypanosoma cruzi* compounds which induce apoptotic parasite death. Eur. J. Med. Chem. **75**:467–478.
15. **Mallari JP, Shelat A, Kosinski A, Caffrey CR, Connelly M, Zhu F, McKerrow JH, Guy RK**. 2008. Discovery of trypanocidal thiosemicarbazone inhibitors of rhodesain and TbcatB. Bioorg Med Chem Lett **18**:2883–2885.
16. **Mott BT, Ferreira RS, Simeonov A, Jadhav A, Ang KK-H, Leister W, Shen M, Silveira JT, Doyle PS, Arkin MR, McKerrow JH, Inglese J, Austin CP, Thomas CJ, Shoichet BK, Maloney DJ**. 2010. Identification and Optimization of Inhibitors of Trypanosomal Cysteine Proteases: Cruzain, Rhodesain, and TbCatB. J. Med. Chem. **53**:52–60.

17. **Dos Santos Filho JM, Moreira DRM, de Simone CA, Ferreira RS, McKerrow JH, Meira CS, Guimarães ET, Soares MBP**. 2012. Optimization of anti-*Trypanosoma cruzi* oxadiazoles leads to identification of compounds with efficacy in infected mice. Bioorg. Med. Chem. **20**:6423–6433.

18. **Abdulla MH, O'Brien T, Mackey ZB, Sajid M, Grab DJ, McKerrow JH**. 2008. RNA Interference of *Trypanosoma brucei* Cathepsin B and L Affects Disease Progression in a Mouse Model. PLoS Negl Trop Dis **2**:e298.

19. **Abdulla M-H, Lim K-C, Sajid M, McKerrow JH, Caffrey CR**. 2007. Schistosomiasis mansoni: Novel chemotherapy using a cysteine protease inhibitor. Plos Med. **4**:130–138.

20. **Doyle PS, Zhou YM, Engel JC, McKerrow JH**. 2007. A cysteine protease inhibitor cures Chagas disease in an immunodeficient-mouse model of infection. Antimicrob Agents Chemother **51**:3932.

21. **Engel JC, Doyle PS, Hsieh I, McKerrow JH**. 1998. Cysteine protease inhibitors cure an experimental *Trypanosoma cruzi* infection. J Exp Med **188**:725–734.

22. **Palmer JT, Rasnick D, Klaus JL, Bromme D**. 1995. Vinyl sulfones as mechanism-based cysteine protease inhibitors. J Med Chem **38**:3193–3196.

23. **Roush WR, Gwaltney SLI, Cheng J, Scheidt KA, McKerrow JH, Hansell E**. 1998. Vinyl sulfonate esters and vinyl sulfonamides: potent, irreversible inhibitors of cysteine proteases. J Am Chem Soc **120**:10994–10995.

24. **Roush WR, Cheng J, Knapp-Reed B, Alvarez-Hernandez A, McKerrow JH, Hansell E, Engel JC**. 2001. Potent second generation vinyl sulfonamide inhibitors of the trypanosomal cysteine protease cruzain. Bioorg Med Chem Lett **11**:2759–2762.

25. **Kerr ID, Lee JH, Farady CJ, Marion R, Rickert M, Sajid M, Pandey KC, Caffrey CR, Legac J, Hansell E, McKerrow JH, Craik CS, Rosenthal PJ, Brinen LS**. 2009. Vinyl sulfones as antiparasitic agents and a structural basis for drug design. J Biol Chem **284**:25697–25703.

26. **Brak K, Doyle PS, McKerrow JH, Ellman JA**. 2008. Identification of a new class of

nonpeptidic inhibitors of cruzain. J Am Chem Soc **130**:6404–6410.

27. **Du X, Guo C, Hansell E, Doyle PS, Caffrey CR, Holler TP, McKerrow JH, Cohen FE**. 2002. Synthesis and Structure−Activity Relationship Study of Potent Trypanocidal Thio Semicarbazone Inhibitors of the Trypanosomal Cysteine Protease Cruzain. J. Med. Chem. **45**:2695–2707.

28. **Caputto ME, Fabian LE, Benítez D, Merlino A, Ríos N, Cerecetto H, Moltrasio GY, Moglioni AG, González M, Finkielsztein LM**. 2011. Thiosemicarbazones derived from 1-indanones as new anti-*Trypanosoma cruzi* agents. Bioorg. Med. Chem. **19**:6818–6826.

29. **Fujii N, Mallari JP, Hansell EJ, Mackey Z, Doyle P, Zhou YM, Gut J, Rosenthal PJ, McKerrow JH, Guy RK**. 2005. Discovery of potent thiosemicarbazone inhibitors of rhodesain and cruzain. Bioorg. Med. Chem. Lett. **15**:121–123.

30. **Siddiqui N, Ahsan W**. 2011. Synthesis, anticonvulsant and toxicity screening of thiazolyl–thiadiazole derivatives. Med. Chem. Res. **20**:261–268.

31. **Bharti SK, Nath G, Tilak R, Singh SK**. 2010. Synthesis, anti-bacterial and anti-fungal activities of some novel Schiff bases containing 2,4-disubstituted thiazole ring. Eur. J. Med. Chem. **45**:651–660.

32. **Singh N, Bhati SK, Kumar A**. 2008. Thiazolyl/oxazolyl formazanyl indoles as potent anti-inflammatory agents. Eur. J. Med. Chem. **43**:2597–2609.

33. **Havrylyuk D, Mosula L, Zimenkovsky B, Vasylenko O, Gzella A, Lesyk R**. 2010. Synthesis and anticancer activity evaluation of 4-thiazolidinones containing benzothiazole moiety. Eur. J. Med. Chem. **45**:5012–5021.

34. **Rawal RK, Tripathi R, Katti SB, Pannecouque C, De Clercq E**. 2008. Design and synthesis of 2-(2,6-dibromophenyl)-3-heteroaryl-1,3-thiazolidin-4-ones as anti-HIV agents. Eur. J. Med. Chem. **43**:2800–2806.

35. **Wisastra R, Ghizzoni M, Maarsingh H, Minnaard AJ, Haisma HJ, Dekker FJ**. 2011. Isothiazolones; thiol-reactive inhibitors of cysteine protease cathepsin B and histone

acetyltransferase PCAF. Org. Biomol. Chem. **9**:1817.

36. **Goud PM, Sheri A, Desai PV, Watkins EB, Tekwani B, Sabnis Y, Gut J, Rosenthal PJ, Avery MA**. 2005. Design, Synthesis and Evaluation of Trisubstituted Thiazoles Targeting *Plasmodium falciparum* Cysteine Proteases. Med. Chem. Res. **14**:74–105.

37. **Yuen C-T, Price RG, Chattagoon L, Richardson AC, Praill PFG**. 1982. Colorimetric assays for N Aacetyl-β-D-glucosaminidase and β-D-galactosidase in human urine using newly-developed ω-nitrostyryl substrates. Clin. Chim. Acta **124**:195–204.

38. **Mohri K, Watanabe Y, Yoshida Y, Satoh M, Isobe K, Sugimoto N, Tsuda Y**. 2003. Synthesis of Glycosylcurcuminoids. Chem. Pharm. Bull. (Tokyo) **51**:1268–1272.

39. **Chen C-Y, Sun J-G, Liu F-Y, Fung K-P, Wu P, Huang Z-Z**. 2012. Synthesis and biological evaluation of glycosylated psoralen derivatives. Tetrahedron **68**:2598–2606.

40. **Rajak H, Agarawal A, Parmar P, Thakur BS, Veerasamy R, Sharma PC, Kharya MD**. 2011. 2,5-Disubstituted-1,3,4-oxadiazoles/thiadiazole as surface recognition moiety: Design and synthesis of novel hydroxamic acid based histone deacetylase inhibitors. Bioorg. Med. Chem. Lett. **21**:5735–5738.

41. **Konig HB, Seifken W, Hans O**. 1954. Sulfur-containing derivatives of pyridinecarboxylic acids and compounds derived therefrom. Chem. Ber. **87**:825–834.

42. **Anderson FE, Duca CJ, Scudi JV**. 1951. Some Heterocyclic Thiosemicarbazones. J. Am. Chem. Soc. **73**:4967–4968.

43. **Liu J, Cao R, Yi W, Ma C, Wan Y, Zhou B, Ma L, Song H**. 2009. A class of potent tyrosinase inhibitors: Alkylidenethiosemicarbazide compounds. Eur. J. Med. Chem. **44**:1773–1778.

44. **Shih M-H, Su Y-S, Wu C-L**. 2007. Syntheses of Aromatic Substituted Hydrazino-thiazole Derivatives to Clarify Structural Characterization and Antioxidant Activity between 3-Arylsydnonyl and Aryl Substituted Hydrazino-thiazoles. Chem. Pharm. Bull. (Tokyo) **55**:1126–1135.

595 45. **Alam MS, Liu L, Lee Y-E, Lee D-U**. 2011. Synthesis, Antibacterial Activity and Quantum-
596 Chemical Studies of Novel 2-Arylidenehydrazinyl-4-arylthiazole Analogues. Chem. Pharm. Bull.
597 (Tokyo) **59**:568–573.
598 46. **Bilinski S, Tyburczyk W, Urban T**. 1961. Condensation of thiosemicarbazones of nicotinic and
599 isonicotinic aldehydes with halo ketones. Ann Univ Mariae Curie-Sklodowska **15**:123–128.
600 47. **Yurttaş L, Özkay Y, Kaplancıklı ZA, Tunalı Y, Karaca H**. 2013. Synthesis and antimicrobial
601 activity of some new hydrazone-bridged thiazole-pyrrole derivatives. J. Enzyme Inhib. Med.
602 Chem. **28**:830–835.
603 48. **Zhang D-N, Li J-T, Song Y-L, Liu H-M, Li H-Y**. 2012. Efficient one-pot three-component
604 synthesis of N-(4-arylthiazol-2-yl) hydrazones in water under ultrasound irradiation. Ultrason.
605 Sonochem. **19**:475–478.
606 49. **Caffrey CR, Hansell E, Lucas KD, Brinen LS, Alvarez Hernandez A, Cheng J, Gwaltney SL,**
607 **Roush WR, Stierhof Y-D, Bogyo M, Steverding D, McKerrow JH**. 2001. Active site mapping,
608 biochemical properties and subcellular localization of rhodesain, the major cysteine protease of
609 *Trypanosoma brucei rhodesiense*. Mol. Biochem. Parasitol. **118**:61–73.
610 50. **Jilkova A, Rezacova P, Lepsik M, Horn M, Vachova J, Fanfrlik J, Brynda J, McKerrow JH,**
611 **Caffrey CR, Mares M**. 2011. Structural Basis for Inhibition of Cathepsin B Drug Target from the
612 Human Blood Fluke, *Schistosoma mansoni*. J. Biol. Chem. **286**:35770–35781.
613 51. **Sajid M, McKerrow JH, Hansell E, Mathieu MA, Lucas KD, Hsieh I, Greenbaum D, Bogyo**
614 **M, Salter JP, Lim KC, Franklin C, Kim J-H, Caffrey CR**. 2003. Functional expression and
615 characterization of *Schistosoma mansoni* cathepsin B and its trans-activation by an endogenous
616 asparaginyl endopeptidase. Mol. Biochem. Parasitol. **131**:65–75.
617 52. **Buckner FS, Verlinde CL, La Flamme AC, Van Voorhis WC**. 1996. Efficient technique for
618 screening drugs for activity against *Trypanosoma cruzi* using parasites expressing beta-
619 galactosidase. Antimicrob. Agents Chemother. **40**:2592–2597.

620 53. **Romanha AJ, Castro SL de, Soeiro M de NC, Lannes-Vieira J, Ribeiro I, Talvani A, Bourdin**
621 **B, Blum B, Olivieri B, Zani C, Spadafora C, Chiari E, Chatelain E, Chaves G, Calzada JE,**
622 **Bustamante JM, Freitas-Junior LH, Romero LI, Bahia MT, Lotrowska M, Soares M,**
623 **Andrade SG, Armstrong T, Degrave W, Andrade Z de A**. 2010. *In vitro* and *in vivo*
624 experimental models for drug screening and development for Chagas disease. Mem. Inst. Oswaldo
625 Cruz **105**:233–238.
626 54. **Abdulla M-H, Ruelas DS, Wolff B, Snedecor J, Lim K-C, Xu F, Renslo AR, Williams J,**
627 **McKerrow JH, Caffrey CR**. 2009. Drug Discovery for Schistosomiasis: Hit and Lead
628 Compounds Identified in a Library of Known Drugs by Medium-Throughput Phenotypic
629 Screening. PLoS Negl. Trop. Dis. **3**.
630 55. **Štefanić S, Dvořák J, Horn M, Braschi S, Sojka D, Ruelas DS, Suzuki B, Lim K-C, Hopkins**
631 **SD, McKerrow JH, Caffrey CR**. 2010. RNA Interference in *Schistosoma mansoni*
632 Schistosomula: Selectivity, Sensitivity and Operation for Larger-Scale Screening. PLoS Negl.
633 Trop. Dis. **4**:e850.
634 56. **Basch PF**. 1981. Cultivation of *Schistosoma mansoni in vitro*. I. Establishment of cultures from
635 cercariae and development until pairing. J. Parasitol. **67**:179–185.
636 57. **Rojo-Arreola L, Long T, Asarnow D, Suzuki BM, Singh R, Caffrey CR**. 2014. Chemical and
637 genetic validation of the statin drug target to treat the helminth disease, schistosomiasis. PloS One
638 **9**:e87594.
639 58. **Conchie J, Levvy GA, Marsh CA**. 1957. Methyl and phenyl glycosides of the common sugars.
640 Adv. Carbohydr. Chem. **12**:157–187.
641 59. **Tenchiu (Deleanu) A-C, Kostas ID, Kovala-Demertzi D, Terzis A**. 2009. Synthesis and
642 characterization of new aromatic aldehyde/ketone 4-(β-d-glucopyranosyl)thiosemicarbazones.
643 Carbohydr. Res. **344**:1352–1364.
644 60. **De Oliveira RB, de Souza-Fagundes EM, Soares RPP, Andrade AA, Krettli AU, Zani CL**.

645 2008. Synthesis and antimalarial activity of semicarbazone and thiosemicarbazone derivatives.

646 Eur. J. Med. Chem. **43**:1983–1988.

647

648

649 **FIGURE LEGENDS**

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

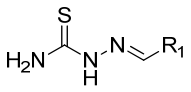
651 **Figure 2 –** General synthetic route for preparation of thiosemicarbazones and their corresponding

652 thiazole heterocycles.

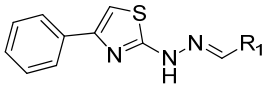
653 **Figure 3 –** Chemical structure of the thiosemicarbazones **4a-o** and cyclic analogues **5a-i** synthesized.

654 **Table 1**. Inhibition of rhodesain, cruzain and SmCB1 by a series of thiosemicarbazones and cyclic

655 analogues.

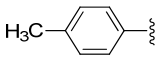
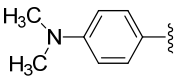
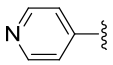
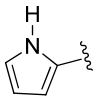


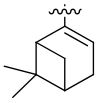
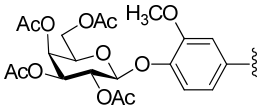
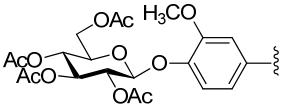
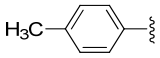
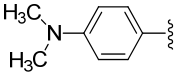
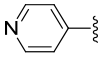
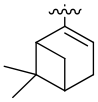
4a-k



5a-h

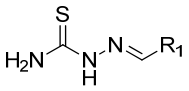
656

Compound	R_1	Rhodesain		Cruzain		SmCB1	
		% inhibition at 100 µM[a]	IC_{50}[b] (µM)	% inhibition at 100 µM[a]	IC_{50}[b] (µM)	% inhibition at 100 µM[a]	IC_{50}[b] (µM)
4a	Ph	72.5 ± 12.7	ND	53.7 ± 1.2	ND	84.4 ± 8.5	22.4 ± 3.1
4b		77.6 ± 2.7	ND	41.9 ± 1.1	ND	87.2 ± 11.9	2.5 ± 1.9
4c		100.0 ± 0.0	3.0 ± 0.8	91.9 ± 0.2	6.6 ± 3.2	100.0 ± 0.0	1.5 ± 0.4
4d		81.7 ± 2.1	3.3 ± 0.9	60.8 ± 4.4	ND	73.1 ± 23.4	42.4 ± 2.6
4e		100 ± 0.0	4.0 ± 1.8	97.2 ± 0.1	9.7 ± 5.2	86.1 ± 4.4	6.8 ± 2.1
4f		1.8 ± 9.3	ND	0.0 ± 0.0	ND	0.0 ± 0.0	ND
4g		0.0 ± 0.0	ND	0.0 ± 0.0	ND	0.0 ± 0.0	ND

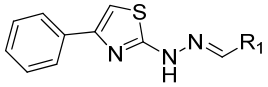
4h		69.6 ± 7.0	ND	32.7 ± 2.8	ND	56.1 ± 3.0	ND
4i		96.9 ± 0.6	1.2 ± 1.0	71.4 ± 1.7	37.7 ± 9.8	59.9 ± 10.6	ND
4j		95.4 ± 1.2	26.2 ± 1.5	70.3 ± 4.9	ND	52.6 ± 5.3	ND
5a**	Ph	72.0 ±13.3	ND	74.6 ± 2.6	ND	9.3 ± 3.8	ND
5b*		64.6 ± 3.6	ND	39.4 ± 6.1	ND	53.6 ± 16.0	ND
5c**		65.3 ± 2.0	ND	80.9 ± 0.2	5.1 ± 1.6	0.0 ± 0.0	ND
5d		13.6 ± 5.4	ND	37.3 ± 5.7	ND	0.0 ± 0.0	ND
5e		30.2 ± 12.4	ND	27.5 ± 5.4	ND	41.0 ± 2.7	ND
5f		0.0 ± 0.0	ND	16.9 ± 4.8	ND	0.0 ± 0.0	ND
5g		5.1 ± 8.7	ND	23.7 ± 1.0	ND	38.0 ± 9.8	ND
5h		34.7 ± 29.1	ND	47.8 ± 3.4	ND	53.7 ± 11.5	ND

[a]Percentages of inhibition are reported as the average and standard deviation of at least two independent experiments, each performed in triplicate. [b] IC_{50} 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.

661 **Table 2.** Inhibition of rhodesain, cruzain and SmCB1 by **4i** analogues modified in the sugar moiety.

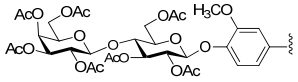
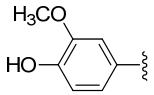
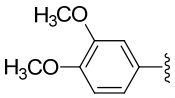
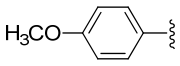
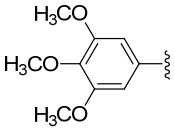
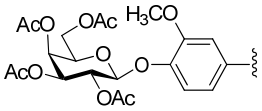


4l-q



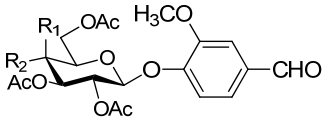
5i-j

662

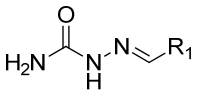
Compound	R_1	Rhodesain % inhibition at 100 μM[a]	Rhodesain IC_{50}[b] (μM)	Cruzain % inhibition at 100 μM[a]	Cruzain IC_{50}[b] (μM)	SmCB1 % inhibition at 100 μM[a]	SmCB1 IC_{50}[b] (μM)
4k		37.6 ± 8.7	ND	30.9 ±2.1	ND	87.6 ± 14.1	7.7 ± 1.7
4l		85.6 ± 1.5	7.3 ± 4.0	80.4 ± 0.8	11.0 ± 1.8	60.7 ± 6.9	ND
4m		89.8 ± 0.7	6.2 ± 2.7	89.9 ± 1.5	9.0 ± 2.8	98.4 ± 2.7	14.6 ± 0.7
4n		60.6 ± 6.4	ND	58.5 ± 2.3	ND	1.5 ± 2.7	ND
4o		66.5 ± 5.1	ND	75.3 ± 2.8	ND	18.7 ± 16.2	ND
5i		70.6 ± 10.2	ND	53.9 ± 0.7	ND	71.0 ± 11.7	ND

663 [a] Percentages of inhibition are reported as the average and standard deviation of at least two
664 independent experiments, each performed in triplicate. [b] IC_{50} values represent the average and standard
665 deviation of at least two independent experiments. ND = not determined.

666 **Table 3.** Inhibition of rhodesain, cruzain and SmCB1 by **4i** analogues without thiosemicarbazones.

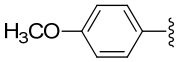
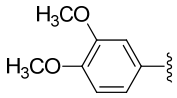
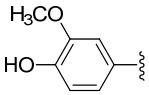
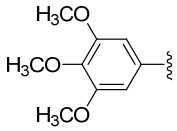
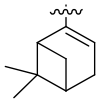


1 R_1 = OAc; R_2 = H
3 R_1 = H; R_2 = tetra-*O*-acetyl-β-galactosyl



6a-f

667

Compounds	R_1	Rhodesain % inhibition 100 µM[a]	Rhodesain IC_{50}[b] (µM)	Cruzain % inhibition 100 µM[a]	Cruzain IC_{50}[b] (µM)	SmCB1 % inhibition 100 µM[a]	SmCB1 IC_{50}[b] (µM)
Galactosyl **1**		33.1 ± 8.6	ND	12.0 ± 2.5	ND	15.2 ± 0.7	ND
Lactosyl **3**		3.7 ± 2.1	ND	6.8 ± 1.8	ND	15.0 ± 2.2	ND
6a		20.6 ± 5.9	ND	20.8 ± 1.2	ND	0.0 ± 0.0	ND
6b		10.1 ± 10.8	ND	20.2 ± 3.7	ND	1.6 ± 2.8	ND
6c		0.0 ± 0.0	ND	14.6 ± 0.7	ND	14.4 ± 14.2	ND
6d		18.4 ± 3.7	ND	5.1 ± 3.6	ND	38.9 ± 29.4	ND
6f		53.8 ± 9.6	ND	39.4 ±1.1	ND	99.1 ± 1.5	5.2 ± 2.8

668 [a]Percentages of inhibition are reported as the average and standard deviation of at least two
669 independent experiments, each performed in triplicate. [b]IC_{50} values represent the average and standard
670 deviation of at least two independent experiments. ND = not determined.

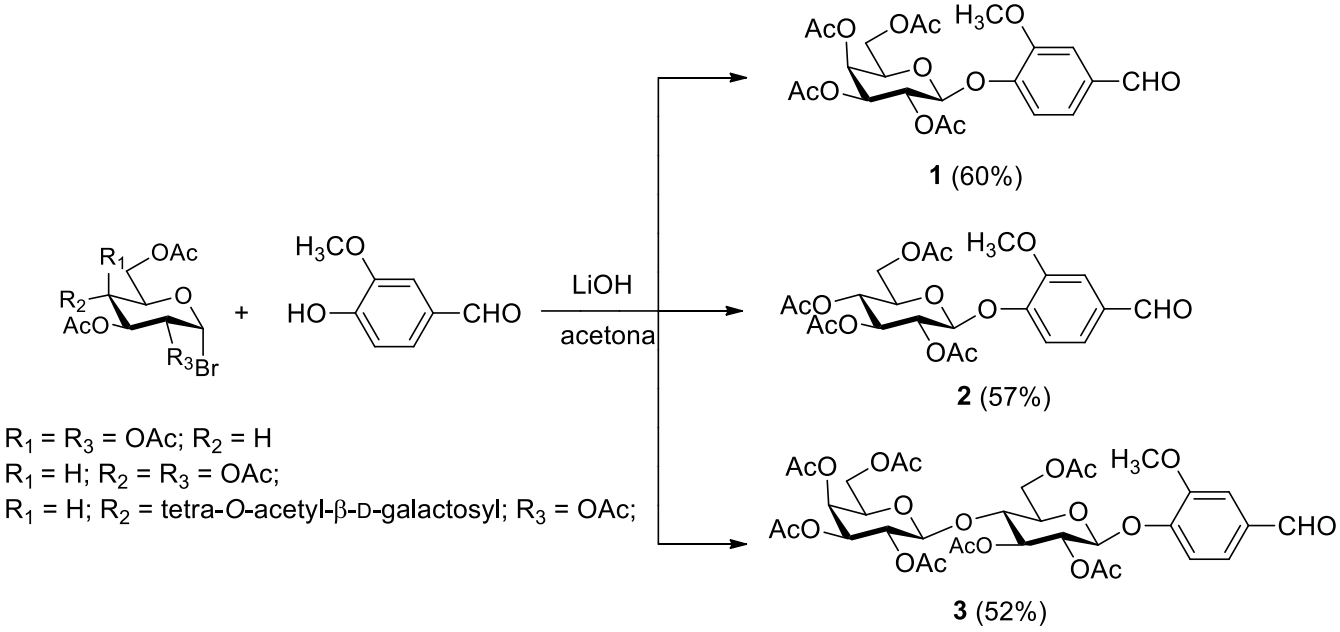
671 **Table 4.** Activity against *Schistosoma mansoni* somules and adult worms.

Compound	Severity scores against somules												Severity scores against adult worms		
	0.1 µM				1 µM				10 µM				5 µM		
	Day				Day				Day				Day		
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	5
4a	0	0	0	0	0	0	0	0	0	0	**1**	**1**	NT	NT	NT
4b	0	0	0	0	0	0	0	0	0	0	0	0	NT	NT	NT
4c	0	0	0	0	0	0	0	0	0	0	0	0	NT	NT	NT
4d	0	0	0	0	0	0	0	0	0	0	0	0	NT	NT	NT
4e	0	0	0	0	0	0	0	0	0	0	0	0	NT	NT	NT
4f	0	0	0	0	0	0	0	0	0	0	0	0	NT	NT	NT
4g	0	0	0	0	0	0	0	0	0	0	0	0	NT	NT	NT
4h	0	0	0	0	0	0	**3**	**2**	0	0	**2**	**2**	0	0	0
4i	0	0	0	0	0	0	0	0	**1**	0	0	0	NT	NT	NT
4j	0	0	0	0	0	0	0	0	0	0	0	0	NT	NT	NT
4k	0	0	0	0	0	0	0	0	0	0	0	0	NT	NT	NT
4l	0	0	0	0	0	0	0	0	0	0	0	0	NT	NT	NT
4n	0	0	0	0	0	0	0	0	0	0	0	0	NT	NT	NT
4o	0	0	0	0	0	0	0	0	0	0	0	0	NT	NT	NT
4q	0	0	0	0	0	0	0	0	0	0	0	0	NT	NT	NT
5a*	0	0	0	0	0	**1**	0	**1**	**2**	0	**4**	**4**	0	0	0
5b	0	0	0	0	0	0	0	**1**	0	0	**1**	**4**	0	0	0

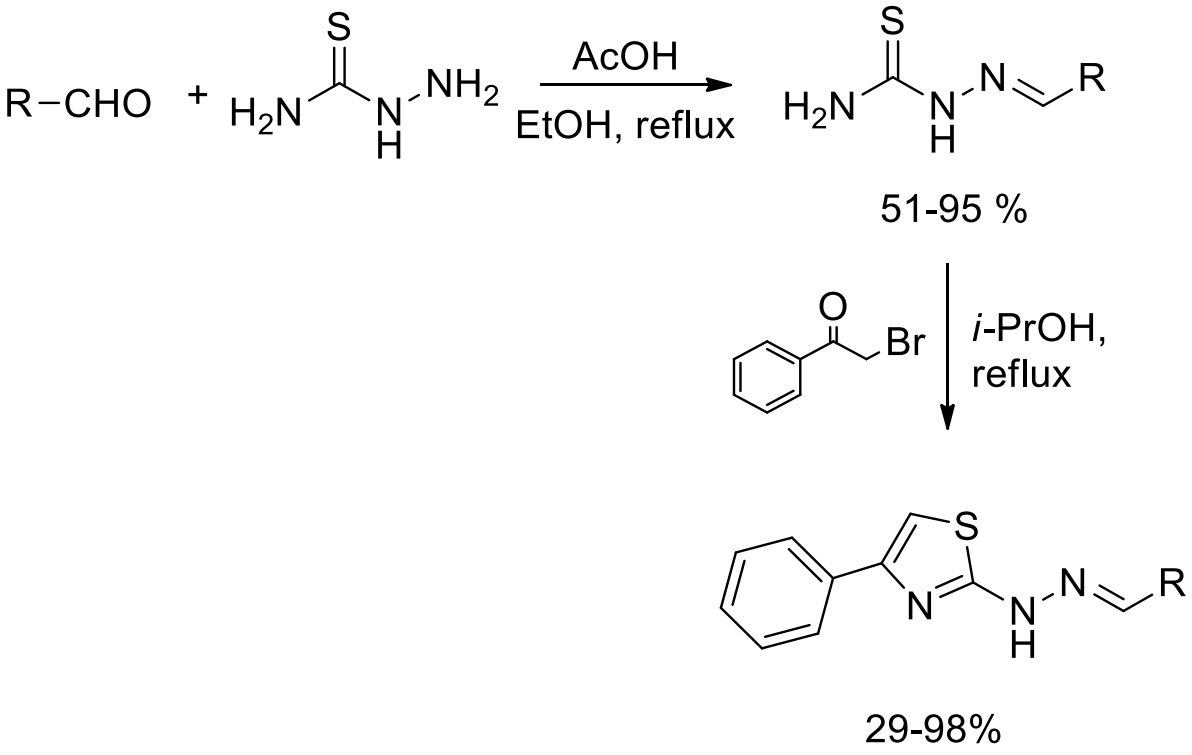
5c	0	0	0	0	0	0	**1**	**4**	0	0	**4**	**4**	0	0	0
5d	**1**	0	**1**	**4**	**2**	**2**	**3**	**4**	**1**	**4**	**4**	**4**	0	**1**	**2**
5e	0	0	0	0	0	0	0	0	**1**	0	**1**	**1**	NT	NT	NT
5f	0	0	0	0	0	0	0	0	0	0	0	0	NT	NT	NT
5g	0	0	0	0	0	0	0	0	0	0	0	0	NT	NT	NT
5h	0	0	0	0	0	0	0	0	0	0	0	0	NT	NT	NT
6a	0	0	0	0	0	0	0	0	0	0	0	0	NT	NT	NT
6b	0	0	0	0	0	0	0	0	0	0	0	0	NT	NT	NT
6c	0	0	0	0	0	0	0	0	0	0	0	0	NT	NT	NT
6d	0	0	0	0	0	0	0	0	0	0	0	0	NT	NT	NT
6f	0	0	0	0	0	0	0	0	0	0	0	0	NT	NT	NT
Galactosyl 1	0	0	0	0	0	0	0	0	0	0	0	**2**	NT	NT	NT
Lactosyl 3	0	0	0	0	0	0	0	0	0	0	0	0	NT	NT	NT

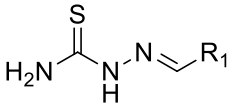
672 *Compound evaluated at 50 μM due to solubility limitations. NT = not tested

673

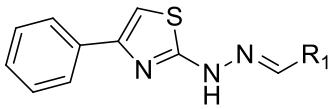


$R_1 = R_3 = OAc$; $R_2 = H$
$R_1 = H$; $R_2 = R_3 = OAc$;
$R_1 = H$; R_2 = tetra-*O*-acetyl-β-D-galactosyl; $R_3 = OAc$;



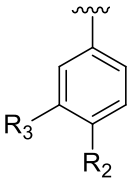


4a-o

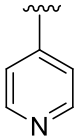


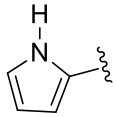
5a-i

R1 = Aryl or Heteroaryl



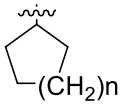
a R_2 = H; R_3 = H
b R_2 = CH_3; R_3 = H
c R_2 = $N(CH_3)_2$; R_3 = H

d = 

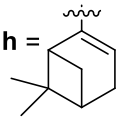
e = 

l R_2 = OH; R_3 = OCH_3
m R_2 = R_3 = OCH_3
n R_2 = OCH_3; R_3 = H
o 3,4,5-trimethoxy

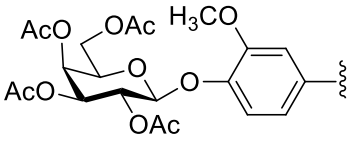
R1 = cycloalkyl



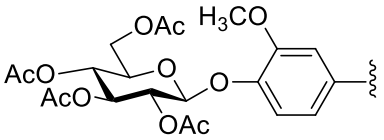
f n = 1
g n = 2

h = 

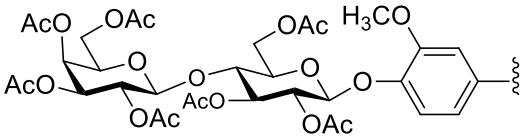
R1 = glycosyl



i



j



k