Chagas Disease Drug Discovery: Multiparametric Lead Optimization against *Trypanosoma cruzi* in Acylaminobenzothiazole Series

Charlotte Fleau, Angel Padilla, Juan Miguel-Siles, Maria T. Quesada-Campos, Isabel Saiz-Nicolás, Ignacio Cotillo, Juan Cantizani Perez, Rick L. Tarleton, Maria Marco, and Gilles Courtemanche

Bioaster, Microbiology Technology Institute, 28 rue du Docteur Roux, 75015 Paris, Ile-de-France, France

Global Health R&D, GlaxoSmithKline, Calle Severo Ochoa 2, 28760 Tres Cantos, Madrid, Spain

Center for Tropical and Emerging Global Infectious Diseases and Department of Cellular Biology, University of Georgia, 30602 Athens, Georgia, United States

Supporting Information

**ABSTRACT:** Acylaminobenzothiazole hits were identified as potential inhibitors of *Trypanosoma cruzi* replication, a parasite responsible for Chagas disease. We selected compound 1 for lead optimization, aiming to improve in parallel its anti-*T. cruzi* activity (IC$_{50}$ = 0.63 μM) and its human metabolic stability (human clearance = 9.57 mL/min/g). A total of 39 analogues of 1 were synthesized and tested in vitro. We established a multiparametric structure--activity relationship, allowing optimization of antiparasite activity, physicochemical parameters, and ADME properties. We identified compound 50 as an advanced lead with an improved anti-*T. cruzi* activity in vitro (IC$_{50}$ = 0.079 μM) and an enhanced metabolic stability (human clearance = 0.41 mL/min/g) and opportunity for the oral route of administration. After tolerability assessment, 50 demonstrated a promising in vivo efficacy.

**INTRODUCTION**

Chagas disease is a neglected tropical disease caused by a protozoan kinetoplastid parasite, *Trypanosoma cruzi*. A total of 70 million people live in areas at risk of contracting the disease, and approximately 6–7 million are actually infected. Chagas disease kills more than 10,000 people a year, but is also responsible for substantial disabilities with significant social and economic impacts (unemployment, loss of resources, and a vicious circle of poverty). T. cruzi is transmitted to humans by an insect of the family Reduviidae, trivially known as the “kissing bug,” during its blood meal. More precisely, T. cruzi is frequently transmitted when the vector insect bites the host and defecates near its bite. The trypomastigote, a non-replicative but mobile and infectious form can then infect host cells. Inside the cells, the parasite transforms to an amastigote form and replicates by binary fission. Once the host cell is saturated with amastigotes (usually 4–5 days postinfection), the parasites convert back into trypomastigotes and leave the cell, spreading into the bloodstream and other host tissues.

Blood transfusion, organ transplants, congenital transmission, and ingestion of contaminated food are other means of infection. The acute phase of *T. cruzi* infection, during which parasites can often be detected in the blood, is often asymptomatic and resolved by immune recognition and control of the parasite load within a few months. The majority of the infected subjects will continue their life unaffected. However, some, estimated at approximately 30%, will progress to chronic disease some 10–25 years after the initial infection. Such individuals might experience severe cardiac and/or digestive changes that, if left unmanaged, can end in death. Chagas disease is today the leading cause of cardiomyopathy in the world.

There is no vaccine, and the currently available drugs, benznidazole and nifurtimox, were discovered more than 40 years ago. Their effectiveness has been demonstrated consistently in acute infections but efficacy in chronically infected subjects is less dependable. Treatment duration is frequently long (60–90 days), and treated subjects may suffer from side effects which could be quite serious. In the 2010s, a new class of molecules, CYP51 inhibitors (such as posaconazole and E1224, the prodrug of ravuconazole) reached clinical development. Unfortunately, they proved to be ineffective in humans with treatment failure in patients reaching at least 70%, as opposed to maximum 30% failure for benznidazole-treated patients, and thus, they were all abandoned, leaving the R&D pipeline nearly completely empty today.

Endemic in 21 Latin American countries, Chagas disease is now spreading beyond the American continent to Europe, Japan, Australia, and North America because of the globalization of movements of people carrying the parasite without restriction.
knowing. At the same time, global warming widens the potential housing area of insect vectors.

That is why, it is now urgent to revise research to fight Chagas disease, with the ambitious goal of discovering an effective and safe molecule, active in both phases, and compatible with the field.

## RESULTS AND DISCUSSION

### From Hit Identification to Lead Selection

We selected the acylaminobenzothiazole (AAB) series from an HTS campaign performed in 2009 by the Broad Institute on the NIH compound collection. 303 286 compounds were tested in a luminescence cell-based/microorganism bioassay to identify 4065 inhibitors of *T. cruzi* replication (AID 1885). Our selection criteria was *T. cruzi* activity < 1.2 μM, selectivity index (SI) versus host cell > 100,17 following the Lipinski rule of 5,18 novel chemotypes (i.e., not already described in the NIH compound collection. 303 286 compounds were tested in a campaign performed in 2009 by the Broad Institute on the activity (IVA) assessment and seven of these demonstrated an efficacy similar to benznidazole. Three of them were from the same AAB series (CID 673490, CID 16194665 and CID 12006028) (Figure 1).

![Figure 1. Structure of the AAB compounds selected from HTS to in vivo screening.](image)

Because of its in vitro and in vivo antiparasitic activities (Table 1, Figure 2), compound 1 was selected to be further profiled. Compound 1 is readily synthesized in one step from cheap raw materials (Scheme 1).

### Table 1. In Vitro Parasitology Determined by GSK for Compound 1

<table>
<thead>
<tr>
<th>Test</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. cruzi</em> growth inhibition IC$_{50}$ (μM)</td>
<td>0.63</td>
</tr>
<tr>
<td>Cytotoxicity (μM)</td>
<td>&gt;50 (H9C2)</td>
</tr>
<tr>
<td>SI = TC$<em>{50}$/IC$</em>{50}$</td>
<td>&gt;80</td>
</tr>
</tbody>
</table>

In vitro metabolite identification demonstrated a different behavior of compound 1 when subjected to human and mice hepatocytes. While mouse hepatocytes induced a hydrolysis of the amide bond, human hepatocytes induced a cascade of hydroxylation, epoxidation, and dichlorination reactions on the benzo ring (Figure 3).

![Figure 3. In vitro metabolite identification.](image)

With these data in hand, began the optimization of the AAB series, with the objective of improving their anti-*T. cruzi* potency and metabolic stability.

### General Strategy

SAR exploration was carried out, varying substituents at R1, R2, and R3 positions. Initially, varying substitution at R1 with different electron withdrawing and electron donating groups and displacing chlorine atoms were investigated. Second, substitution of the cyclopropene by diverse groups R2 was evaluated. Third, the amide bond between the thiazole and the R2 group was replaced by different linkers, and the importance of the hydrogen was evaluated by its substitution by alkyl groups (Figure 4). Finally, after determining the best substituents, the disubstitution of the aromatic core was explored in order to increase metabolic stability by blocking position 5.

### Chemistry

SAR exploration was focused on finding the best position for the chlorine atom in the benzo group in terms of the antiparasite activities of the AAB series. Hence, AAB analogues substituted at positions 4, 5, and 7 were synthesized in one to four steps (Scheme 2). Precursor 8 was prepared in three steps using 3-chloro-2-iodophenylamine 5 as the starting material. Compound 5 was converted into 1-benzoyl 3-(3′-chloro-2′-iodophenyl) thiourea 6 which was treated by K$_2$CO$_3$ to obtain 1-(3-chloro-2-iodophenyl) thiourea 7 followed by a copper-catalyzed cyclization reaction to furnish the desired aminobenzothiazole 8. 20,21 Aminobenzothiazole 10 was obtained from 3-chloroaniline 9 and potassium thiocyanate using a process commonly described in the literature.22,23 Then, AAB 12, 13, and 14 were obtained by reacting precursor 8, 10, and 11 with cyclopropanecarbonyl chloride, respectively.

The electronic effect of the aromatic substituent on potency was studied by synthesizing several AABs with different electron withdrawing and donating groups at position 6. This synthesis was accomplished from the corresponding aniline following a well-known literature procedure which yielded compounds 17 to 21 in only two steps (Scheme 3). 24,25 Compounds 22 to 26 were obtained in one step from the corresponding aminobenzothiazole.

Exploration of the cyclopropyl group replacement was also considered in order to improve the potency of 1. Several R2 groups were envisaged as heterocycle, linear, ramified, and alicyclic carbon chains. Two procedures were used depending on the availability of starting materials (Scheme 4). Aminobenzothiazole 4 was converted into acylaminobenzo-thiazole 27 to 30 in the presence of the corresponding acyl chloride reagent, as described previously. N acylation of 4 was accomplished by activation of the corresponding carboxylic acid 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU) in the presence of Hunig’s base to furnish final compounds 31 to 34.

To determine the importance of the hydrogen of the amide bond, it was replaced by different alkyl groups as methyl, ethyl, and butyl (Scheme 5). Alkylaminobenzothiazole 37–c were obtained in four steps starting from the commercially available 6-chloroaminobenzothiazole 4 which was first protected using di-tert-butyl dicarbonate (Boc anhydride) to get 35. Protected intermediate 35 was then subjected to N-alkylation to get compounds 36a to 36c which were then deprotected with trifluoroacetic acid (TFA) to get N-alkylated aminobenzothiazoles 37–c. Additionally, the introduction of a more polar ether alkyl chain was considered. As such, 37d was obtained in one step by reacting 4 with 2-bromoethanol. Then, N-alkylated aminobenzothiazole was engaged in the amide bond formation reaction using conditions described in Scheme 2.

The effect of the amide bond replacement on the anti-*T. cruzi* activity of AAB derivatives was investigated by preparing compounds 42, 44, and 45 (Scheme 6). Reduction of the amide bond of 1 using lithium aluminium hydride yielded secondary amine 42.27 Compound 44 was synthesized in two
steps by conversion of 16j to benzothiazol-2-hydrazine 43 followed by hydrazone formation in the presence of cyclopropanecarboxaldehyde. Sulfonamide 45 was obtained in one step by reacting 16j with cyclopropanesulfonyl chloride.

Another issue for this series was the observed low metabolic stability of 1. In order to improve it, synthesis of disubstituted acylaminobenzothiazole was accomplished (Scheme 7). Using the classical route with potassium thiocyanate and bromine, only traces of the disubstituted aminobenzothiazole were obtained in the case of 50 because these conditions are in favor of bromination at position 6 of the starting aniline 46a. Hence, a new synthetic approach for disubstituted aminobenzothiazole was necessary. We based our strategy on the synthesis of 8. Aniline precursors 46a–c were commercially available and
anilines 46d–h were prepared using the previously described procedures (see Experimental Section). First, anilines were submitted to an iodination reaction catalyzed by p-toluene sulfonic acid or by boron trifluoride etherate to control the regioselectivity at position 6. Intermediates 47a–h were converted to the corresponding 1-benzoyl 3-(3′-R1-4′-R1′-6′-iodophenyl) derivatives using benzoyl isothiocyanate under microwave irradiation and were treated by K2CO3 to yield disubstituted aminobenzothiazole 49a–h (Scheme 7). Final compounds 50−57 were obtained by reacting the corresponding aminobenzothiazole with cyclopropanecarbonyl chloride. Finally, after improving anti-T. cruzi activity and metabolic stability by changing R1, R2, and R3 compounds, 58, 59, and 60 were designed using a mix and match approach. Compounds 58, 59, and 60 were synthesized using conditions, as described in Scheme 4 (Scheme 8).

**Biology.** Compounds were tested against T. cruzi using an intracellular assay with cardiomyocytes H9c2 as the host cell, permitting, in the same time, the determination of the cytotoxicity. Initial frontrunner 1 was used as the reference to evaluate the effect of different substituents at R1, R2, and R3 positions.

**In Vitro Anti-T. cruzi Activity and Aromatic Chlorine Position.** The first SAR exploration was focused on the investigation of the best position for the aromatic substituent around the final group. When chlorine was moved from position 6 to position 5, higher anti-T. cruzi activity and SI (13 IC50 = 0.32 μM vs 1 IC50 = 0.63 μM) was observed while substitution at positions 7 and 4 led to the loss of antiparasite activity (12 IC50 = 24 μM and 14 IC50 > 50 μM) suggesting the importance for R1 substitution to be placed at positions 5 or 6 (Table 2).

**In Vitro Anti-T. cruzi Activity and 6-Substitution.** After determining the importance of the position in the phenyl ring for antiparasite activity, investigation around the effect of varying the nature of the substituent was carried out (Table 3). Replacing the chlorine by alkyl carbon chains such as isopropyl 18, tertbutyl 19, or methyl 23 decreased the activity; however, substitution by an ethyl group 17 increased the anti-T. cruzi activity. Mesomere-donating groups such as a fluoride 22, ethoxy 21, methoxy substituents 24 led to lower anti-T. cruzi activity. Replacement of the chlorine by a trifluoromethoxy substituent 20 yielded a higher potency (20 IC50 = 0.19 μM), however, with some cytotoxicity against the host cell observed (20 SI = 28). These results suggest that mesomere-donating groups do not improve antiparasite activity of the series. Derivatives 25 and 26, that hold nitro and trifluoromethyl groups, respectively, presented higher activity and good SIs pointing toward a beneficial effect of electron withdrawing groups the anti-T. cruzi activity of AAB derivatives (25 IC50 = 0.04 μM, 26 IC50 = 0.1 μM).

**In Vitro Anti-T. cruzi Activity and Cyclopropyl Replacement.** In parallel of the determination of the best substituents on the aromatic core, the acyl group R2 was explored (Table 4). The cyclopropyl moiety was first replaced by an alkyl carbon chain (propyl) leading to a significant decrease of activity (27 IC50 = 4.73 μM), whereas a ramified such tertbutyl slightly decreased potency (29 IC50 = 0.95 μM) and isopropyl marginally increased antiparasite potency (28 IC50 = 0.41 μM). Substitution of the cyclopropyl moiety by cyclobutyl...
decreased the activity by twofold (IC$_{50}$ = 0.63 μM and IC$_{50}$ = 1.32 μM). Increasing the flexibility of the R2 group by introducing methylene linkers between the amide bond and cyclopropyl led to lower antiparasite potency (IC$_{50}$ = 5.4 μM). Introducing polarity at R2 through heterocyclic group substitutions such as tetrahydrofuran (THF) 32, oxetane 34, and an ethanol chain 33 had a detrimental effect on anti-T. cruzi activity suggesting that ramified alkyls such as cyclopropyl or isopropyl are better tolerated in terms of anti-T. cruzi activity of AAB compounds.

**In Vitro Anti-T. cruzi Activity and Hydrogen Amide Bond Replacement.** Substitution of the amide hydrogen by different R3 groups represented another site of SAR investigation. Hydrogen replacement by the alkyl group such as methyl 38, ethyl 39, and butyl 40 or an ethanol chain 41 turned compounds completely inactive, suggesting this hydrogen is required to maintain activity (Table 5).

<table>
<thead>
<tr>
<th>compound</th>
<th>R1</th>
<th>IC$_{50}$ (μM) vs T. cruzi</th>
<th>IC$_{50}$ (μM) vs host cell (H9C2)</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6-Cl</td>
<td>0.63</td>
<td>&gt;50</td>
<td>&gt;80</td>
</tr>
<tr>
<td>12</td>
<td>7-Cl</td>
<td>24</td>
<td>&gt;50</td>
<td>&gt;2</td>
</tr>
<tr>
<td>13</td>
<td>5-Cl</td>
<td>0.32</td>
<td>&gt;50</td>
<td>&gt;158</td>
</tr>
<tr>
<td>14</td>
<td>4-Cl</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
</tbody>
</table>

Table 3. Effect of 6-Substituent on Anti-T. cruzi Activity and on Cytotoxicity

substitutions such as tetrahydrofuran (THF) 32, oxetane 34, and an ethanol chain 33 had a detrimental effect on anti-T. cruzi activity suggesting that ramified alkyls such as cyclopropyl or isopropyl are better tolerated in terms of anti-T. cruzi activity of AAB compounds.
antiparasite activity. Summary, the amide bond seems to be important for tri

In Vitro Anti-T. cruzi Activity and Amide Bond Replacement. Having evaluated the effect of substituents at position 6 on the anti-T. cruzi activity and on microsomal stability, we decided to explore disubstitution of the aromatic core at positions 5 and 6 looking for a further improvement in metabolic stability of these derivatives. Position 5 was chosen because it was suggested that the first hydroxylation of 1 occurred at this position during the metabolic degradation process. A trifluoromethyl group was selected in the first place because it is an electron withdrawing group with the potential for development (as opposed to a nitro substituent, 25, which can induce formation of free radical species during its reduction and lead to toxicity). This risk together with an unsatisfactory improvement of human metabolic stability and a very low solubility pushed us to discard compound 25 despite its very good in vitro activity. Then, the effect of several other substituents at 6-position was explored. Introduction of a fluorine atom as in 50 slightly increased the activity of 26 (IC50 = 0.1 μM, 50 IC50 = 0.079 μM), but a contrary effect was observed when methyl 53, nitrile 54, or methoxy 55 substituents were introduced, resulting compounds being less potent or inactive. Interestingly, reversing the substituent position of 50 completely inhibited the anti-T. cruzi activity (51 IC50 = 31 μM). Then, it was suggested that an electron withdrawing group at position 6 with a fluorine at position 5 was the best option in term of potency. Hence, other electron withdrawing groups to replace trifluoromethyl were investigated such as nitrile 52, sulfonamide 56, and sulfone 57, but they yielded a loss in antiparasite activity (Table 8). Intrinsic clearance of 50 was then determined in mouse and in human microsomes, being higher for mouse (5.26 mL/min/g) than for human (0.41 mL/min/g), however, representing a marked improvement in human microsomal stability. Using this improved scaffold, additional exploration of the R2 position was carried out (Table 9). Following the previous observations, an isopropyl group was introduced; however, in this case a slight decrease in antiparasite activity was observed instead (50 IC50 = 0.079 μM and 58 IC50 = 0.25 μM), an effect that

SAR Conclusion. It has been demonstrated that an electron withdrawing group as trifluoromethyl at position 6 of the aromatic core led to a potent antiparasite compound. Furthermore, ramified alkyls as cyclopropyl or isopropyl as the R2 group maintained anti-T. cruzi activity. Finally, hydrogen of the amide bond is needed to observe antiparasite activity. Using those preliminary results, additional compounds were synthesized and tested to improve the anti-T. cruzi activity and the metabolic stability.

In Vitro Anti-T. cruzi Activity and Amide Bond Replacement. The importance of the amide bond for anti-T. cruzi activity was also investigated (Table 6). Reduction of the amide bond to get the corresponding secondary alkylidine amine resulted in the loss of antiparasite activity (42 IC50 = 27 μM). When the amide was transformed into sulfonamide 45, the compound was completely inactive. Substitution of the amide bond by hydrazine maintained an activity but increased drastically cytotoxicity (44 IC50 = 2.5 μM and SI = 5). In summary, the amide bond seems to be important for antiparasite activity.

Metabolic Stability. Because compound 1 had already been identified as metabolically unstable in vitro versus human microsomes, the most promising compounds in terms of their antiparasite activity were assessed against mouse and human liver microsomes (Table 7). Replacement of chlorine by ethyl 17 drastically increases in vitro mouse clearance and had no effect on human microsomal stability, but when it was replaced by an electron withdrawing group as nitro 25 or trifluoromethyl 26, improved metabolic stability was observed although compounds were still not stable (Table 7).
was more pronounced when a 2-fluoroisopropyl substituent was introduced, (50 IC_{50} = 0.079 μM and 60 IC_{50} = 1 μM). In conclusion, the cyclopropyl moiety gave the best results in terms of antiparasite activity and metabolic stability, compound 50 having the best overall profile and was selected for further profiling.

In Vitro ADME Properties of 50. The physicochemical, and in vitro and in vivo DMPK profiles for compound 50 were evaluated in order to evaluate its progression. Compound 50 displayed low-moderate kinetic solubility [charged aerosol detection (CAD)] and low-moderate aqueous solubility in fasted simulated intestinal fluid (FaSSIF) media. Permeability values measured with MDCKII-MDR1 were high in both directions with no evidence for significant efflux. Plasma protein binding measured in both human and mouse species was high, and blood to plasma partitioning in mouse resulted was moderate (Table 10).

Table 10. 50 in Vitro ADME Profile

<table>
<thead>
<tr>
<th>compound</th>
<th>R1'</th>
<th>R2</th>
<th>IC_{50} (μM) vs T. cruzi</th>
<th>IC_{50} (μM) vs host cell (H9C2)</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>58</td>
<td>F</td>
<td>iPr</td>
<td>0.25</td>
<td>&gt;50</td>
<td>&gt;200</td>
</tr>
<tr>
<td>59</td>
<td>H</td>
<td>iPrF</td>
<td>0.13</td>
<td>&gt;50</td>
<td>&gt;384</td>
</tr>
<tr>
<td>60</td>
<td>F</td>
<td>iPrF</td>
<td>1</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
</tbody>
</table>

In Vivo Pharmacokinetics of 50. Compound 50 in vivo pharmacokinetics was investigated after intravenous and oral administration in mice. The compound exhibited moderate blood clearance, which correlates with the observed clearance in mouse microsomes (less than two-fold difference when comparing in vitro and in vivo results), moderate half-life, and high volume of distribution. Metabolite 49a was detected (percentage of metabolite = 32%), and its C_{max} (634 ng/mL) and AUC (10,065.32 ng h/mL) were estimated. The blood concentration versus time data profile shows that metabolism is saturated at 50 mg/kg after oral gavage administration, and compound is well absorbed. T_{max}, C_{max} and AUC parameters were estimated. Bioavailability was not estimated as different mouse strains were used for intravenous and oral administration.

In Vivo Anti-T. cruzi Activity of 50. The IVA of compound 50 was assessed in an acute model of T. cruzi infection in mice (Figure 5). A single oral dose of 50 mg/kg showed efficacy in controlling the parasite load at the site of infection compared to untreated animals. The specific IVA for compound 50 (78.5) approached that for the positive control benznidazole (101.6).

CONCLUSIONS

Synthesis and evaluation of anti-T. cruzi activity of several AAB derivatives was accomplished starting from 1. Modifications at R1 introducing electron withdrawing groups such as trifluoromethyl or nitro substituents at position 6 improved antiparasite activity of these derivatives, whereas blocking position 5 of the benzothiazole scaffold with a fluoroine had a positive effect on lowering human microsomal clearance. Only small ramified or small cyclic carbon groups are tolerated at position R2, but the initial cyclopropyl remained the best substituent. Finally, the amide bond with an unsubstituted hydrogen is required for anti-T. cruzi activity. As a result of this optimization, a new lead compound 50 was identified. Compound 50 showed a favorable ADME and pharmacokinetic profile. Furthermore, 50 showed an interesting in vivo antiparasite activity confirming the potential of AAB series to furnish progressable compounds. The target of this series, which is not CYP51 (result not presented here), is still to be identified.

ETHICAL STATEMENT

“All studies were conducted in accordance with the GSK Policy on the Care, Welfare, and Treatment of Laboratory Animals and were reviewed the Institutional Animal Care and Use Committee either at GSK or by the ethical review process at the institution, where the work was performed.”

EXPERIMENTAL SECTION

Chemistry. General. All starting materials were purchased from commercial suppliers (Sigma-Aldrich, Fluorochem, Enamine and combi-blocks). 1H NMR spectra were recorded on a Bruker AVANCE 400 MHz. The following abbreviations were used: s (singlet), d (doublet), dd (doublet of doublet), t (triplet), q (quartet),
**Characterization of N-(Benzothiazol-2-yl)cyclopropanecarboxamide 12 to 14.** It was obtained in 41% yield. Purity (LC–MS) > 95%. NMR: 1H (DMSO, 400 MHz): J = 12.85 (NH, 1H, br), 7.73 (H4, 1H, dd, J = 6.8 Hz and J = 7.8 Hz), 7.48 (H5, 1H, t, J = 8.1 Hz), 7.40 (H6, 1H, dd, J = 8.1 Hz and J = 0.8 Hz), 3.02 (CH2Pr, 1H, m) and 0.98 (CH2Pr, 4H, m). HRMS: mass calculated for C12H12Cl2N2OS, 219.0175 and mass found, 230.0202.

**N-(5-Chlorobenzothiazol-2-yl)cyclopropanecarboxamide (13).** It was obtained in 32% yield. Purity (LC–MS) > 95%. NMR: 1H (DMSO, 400 MHz): δ = 10.66 (NH, 1H, br), 7.08 (H4, 1H, dd, J = 6.8 Hz and J = 7.8 Hz), 7.83 (H5, 1H, t, J = 8.1 Hz) and 7.64 (H6, 1H, dd, J = 8.1 Hz and J = 0.8 Hz), 3.14 (CH2Pr, 1H, m) and 0.96 (CH2Pr, 4H, m). HRMS: mass calculated for C12H12Cl2N2OS, 233.0197 and mass found, 235.0188.

**N-(4-Chlorobenzothiazol-2-yl)cyclopropanecarboxamide (16).** It was obtained in 45% yield. Purity (LC–MS) > 95%. NMR: 1H (DMSO, 400 MHz): δ = 7.41 (H7, 1H, d, J = 5.8 Hz), 7.31 (NH2, 1H, d, J = 8.1 Hz), 7.04 (H5, 1H, d, J = 8.1 Hz and J = 1.8 Hz), 2.89 (CH, 1H, dd, J = 8.1 Hz and J = 1.8 Hz). MS: M + H+, 193 (exact mass of C9H9N3S: 192.07)

**6-Isopropylbenzothiazol-2-amine (16b).** It was obtained in 16% yield. Purity (LC–MS) > 95%. NMR: 1H (DMSO, 400 MHz): δ = 7.36 (H7, 1H, d, J = 5.8 Hz), 7.31 (NH2, 2H, br), 7.23 (H4, 1H, d, J = 8.1 Hz), 7.07 (H5, 1H, dd, J = 8.1 Hz and J = 1.8 Hz), 2.89 (CH, 1H, dd, J = 8.1 Hz and J = 1.8 Hz). MS: M + H+, 193 (exact mass of C9H9N3S: 192.07)

**6-(Trifluoromethoxy)benzothiazol-2-amine (16d).** It was obtained in 61% yield. NMR: 1H (DMSO, 400 MHz): δ = 7.76 (H7, 1H, d, J = 5.8 Hz), 7.31 (NH2, 2H, br), 7.23 (H4, 1H, d, J = 8.1 Hz), 7.07 (H5, 1H, dd, J = 8.1 Hz and J = 1.8 Hz). MS: M + H+, 233.00 (exact mass of C10H8FN3OS: 234.01)

**6-Ethoxybenzothiazol-2-amine (16e).** It was obtained in 45% yield. Purity (LC–MS) > 95%. NMR: 1H (DMSO, 400 MHz): δ = 7.26 (H7, 1H, d, J = 5.8 Hz), 7.21 (H4, 1H, d, J = 8.8 Hz), 7.19 (NH2, 1H, br), 6.79 (H5, 1H, dd, J = 8.8 Hz and J = 2.5 Hz), 3.98 (CH2H, 2H, q, J = 7.1 Hz) and 1.31 (CH2H, 3H, t, J = 7.1 Hz). MS: M + H+, 195 (exact mass of C10H13N3O: 194.05)

**Characterization of N-(Benzothiazol-2-yl)cyclopropanecarboxamide 17 to 26.** N-(6-Ethylbenzothiazol-2-yl)cyclopropanecarboxamide (17). It was obtained in 52% yield. Purity (LC–MS) > 95%. NMR: 1H (DMSO, 400 MHz): δ = 12.54 (NH, 1H, br), 7.77 (H7, 1H, d, J = 6.8 Hz), 7.63 (H4, 1H, d, J = 8.3 Hz), 7.27 (H5, 1H, dd, J = 8.3 Hz and J = 1.6 Hz), 2.69 (CH2H, 2H, q, J = 7.6 Hz), 1.99 (CH2Ph, 1H, m). MS: M + H+, 193.00 (exact mass of C12H12N3O2S: 194.05)

**N-(6-Iso-propylbenzothiazol-2-yl)cyclopropanecarboxamide (18).** It was obtained in 61% yield. Purity (LC–MS) > 95%. NMR: 1H (DMSO, 400 MHz): δ = 12.54 (NH, 1H, br), 7.81 (H7, 1H, d, J = 5.8 Hz), 7.64 (H4, 1H, d, J = 8.3 Hz), 7.31 (H5, 1H, dd, J = 8.3 Hz and J = 1.8 Hz). MS: M + H+, 193.00 (exact mass of C12H12N3O2S: 194.05)
(CH₃)₂CO, 1H, t, J = 7.4 Hz). HRMS: mass calculated for C₁₃H₁₅N₂O₂S, 267.0353 and mass found, 267.0346. 

**Conclusion**

In summary, we have developed a novel protocol for the synthesis of N-Substituted(benzothiazol-2-yl)cyclopropanecarboxamide derivatives. The method is facile, scalable, and yields high purity products. The synthesized compounds exhibit promising biological activities, which open up new avenues for the development of potential drug candidates. Further studies on their pharmacological properties are currently underway.

**Acknowledgment**

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**References**


**Author Contributions**

J. Doe and S. Smith contributed equally to this work. J. Doe designed the experiments and analyzed the data. S. Smith performed the experiments and wrote the manuscript. All authors contributed to the discussion and reviewed the manuscript.
The synthesis of N-Alkylated Aminobenzothiazole produced a beige solid (0.014 g, 46%).

NMR: 8'H (DMSO, 400 MHz): δ 8.42 (NH, 1H, t), J_{NH} = 7.85 Hz). HRMS: mass calculated for C_{12}H_{12}ClN_{2}OS, 267.0353 and mass found, 267.0358.

Synthesis of 6-Chloro-N-(cyclopropylmethyl)benzothiazole-2-amine (42). To an ice-cold solution of 1 (0.030 g, 0.18 mmol) in THF (0.2 mL) was added a solution of LiAlH_{4} in THF (0.47 mL, 0.47 mmol). The solution was warmed up to room temperature and stirred for 17 h. The mixture was diluted in water (4 mL). The product was extracted with EtOAc (3 × 10 mL), and the organic layers were combined, dried over Na_{2}SO_{4} and filtered and reduced under vacuum. Crudes were purified on preparative TCL eluting with cyclohexane/2-propanol/DCM (7:2:1) to furnish 37a as a beige solid (0.014 g, 46%).

Purity (LC–MS) > 95%, NMR: 8'H (DMSO, 400 MHz): δ 9.32 (NH, 1H, t), J_{NH} = 2.3 Hz). HRMS: mass calculated for C_{12}H_{12}ClN_{2}OS, 267.0353 and mass found, 267.0358.

Synthesis of 2-Hydrazinyl-6-(trifluoromethyl)benzothiazole (43). To an ice-cold solution of hydrazine monohydrate (0.066 mL, 1.37 mmol) were successively added hydrochloric acid (0.062 mL, 0.756 mmol), ethylene glycol (0.5 mmol), and 6-trifluoromethylbenzothiazol (0.050 g, 0.229 mmol). The solution was warmed up to reflux (160 °C) and stirred for 3 h. Then, the mixture was cooled down to room temperature and dissolved in 10 mL of EtOAc. The organic phase was washed with brine, dried over Na_{2}SO_{4}, and filtered and concentrated under vacuum. The crude was purified by chromatography eluting with cyclohexane/EtOAc (95/5 to 85/15) to furnish 37d as a white solid (11 mg, 18% yield).

Purity (LC–MS) > 95%, NMR: 8'H (DMSO, 400 MHz): δ 8.09 (H7, 1H, d, J_{7-8} = 2.3 Hz), 7.79 (H4, 1H, d, J_{4-5} = 8.6 Hz and J_{4-5} = 2.3 Hz), 3.92 (CH_{2}Pr, 1H, m) and 1.05 (CH_{2}Pr, 4H, m). HRMS: mass calculated for C_{9}H_{14}ClN_{2}OS, 267.0353 and mass found, 267.0358.

N-(6-Chlorobenzothiazol-2-yl)-N-ethylcyclopropanecarboxamide (38). It was obtained in 30% yield. Purity (LC–MS) > 90%, NMR: 8'H (DMSO, 400 MHz): δ 8.10 (H7, 1H, d, J_{7-8} = 2.3 Hz), 7.8 (H4, 1H, d, J_{4-5} = 8.8 Hz), 7.45 (H5, 1H, d, J_{4-5} = 2.3 Hz) and 2.45 (CH_{2}Pr, q, J_{CH_{2}-CH_{2}} = 7.8 Hz). 2.36 (CH_{2}Pr, 4H, m) and 0.97 (CH_{2}Pr, 3H, t). HRMS: mass calculated for C_{15}H_{18}ClN_{2}OS, 280.0510 and mass found, 280.0514.

N-(6-Chlorobenzothiazol-2-yl)-N-butyrylcyclopropanecarboxamide (40). It was obtained in 30% yield. Purity (LC–MS) > 95%, NMR: 8'H (DMSO, 400 MHz): δ 8.09 (H7, 1H, d, J_{7-8} = 2.3 Hz), 7.79 (H4, 1H, d, J_{4-5} = 8.6 Hz), 7.45 (H5, 1H, d, J_{4-5} = 8.6 Hz and J_{4-5} = 2.3 Hz), 4.48 (CH_{2}Pr, 2H, m), 2.33 (CH_{2}Pr, 1H, m) and 1.80 (CH_{2}, 2H, m), 1.41 (CH_{2}, 2H, m), 1.06 (CH_{2}Pr, 4H, m), 0.92 (CH_{2}Pr, 4H, m) and 0.97 (CH_{2}Pr, 3H, t). HRMS: mass calculated for C_{19}H_{22}ClN_{2}OS, 297.0495 and mass found, 297.0496.

Characterization of N-Alkylated 6-chlorobenzothiazol-2-yl-cyclopropanecarboxamide 38 to 41. N-(6-Chlorobenzothiazol-2-yl)-N-methylcyclopropanecarboxamide (38). It was obtained in 75% yield.
ratory eluting with cyclohexane/EtOAc (95/5 to 70/30) to furnish 44 as a yellow solid (0.003 g, 17%).

Purity (LC-MS) > 95%. NMR: 1H (DMSO, 400 MHz): δ 8.11 (NH, 1H, br), 7.64 (H7, 1H, d, J = 1.5 Hz), 7.31 (H5, 1H, d, J = 9.3 Hz), 6.81 (CPCr, 1H, m) and 1.87 (CH2cPr, 6H, m). MS: M + H+ 237.0 (exact mass of C12H9FN3OS: 236.0447).

Characterization of N-(5-6-Disubstituted-benzothiazol-2-yl)cyclopropanecarboxamide (51). It was obtained in 33% yield. Purity (LC-MS) > 95%. NMR: 1H (DMSO, 400 MHz): δ 13.16 (NH, 1H, br), 7.30 (H4, 1H, d, J = 9.3 Hz), 7.38 (H7, 1H, d, J = 1.5 Hz), 7.31 (H5, 1H, d, J = 9.3 Hz), 7.38 (H7, 1H, d, J = 1.5 Hz). MS: M + H+ 261.1 (exact mass of C13H12F3N3S2O3: 260.0612).
Characterization of 58 to 60. N-(5-Fluoro-6-(trifluoromethyl)benzothiazol-2-yl)cyclopropanecarboxamide (58). It was obtained in 46% yield. Purity (LC–MS) > 95%. NMR: δ 1.04 (H7, 1H, s), 7.95 (H4, 1H, d, JF = 8.3 Hz), 7.77 (H5, 1H, d, JF = 8.3 Hz and JCH-F = 1.8 Hz) and 1.64 (CH2PrF, 6H, d, JCH-F = 22 Hz). HRMS: mass calculated for C12H10F5N2OS, 329.0424 and mass found, 329.0431.

Characterization of 59. 2-Fluoro-2-methyl-N-(6-(trifluoromethyl)benzothiazol-2-yl)propenamide (59). It was obtained in 58% yield. Purity (LC–MS) > 95%. NMR: δ 1.04 (H7, 1H, br), 8.54 (H7, 1H, s), 7.95 (H4, 1H, d, JF = 8.3 Hz), 7.84 (H5, 1H, d, JF = 8.3 Hz and JCH-F = 1.8 Hz) and 1.64 (CH2PrF, 6H, d, JCH-F = 22 Hz). HRMS: mass calculated for C12H11F4N2O3S, 325.0429 and mass found, 325.0431.

Characterization of 60. 2-Fluoro-N-(5-fluoro-6-(trifluoromethyl)benzothiazol-2-yl)-2-methylpropenamido (60). It was obtained in 50% yield. Purity (LC–MS) > 95%. NMR: δ 1.28 (NH, 1H, br), 8.59 (H7, 1H, s), 7.89 (H4, 1H, d, JF = 6.6 Hz), 7.89 (H5, 1H, d, JF = 6.6 Hz) and 1.16 (CH3, 6H, d, JCH-F = 1.8 Hz). HRMS: mass calculated for C10H13F3N2OS, 233.0428 and mass found, 233.0431.

Plasma Protein Binding. In vitro plasma protein binding of 50 was measured in mouse and human plasma using equilibrium dialysis at a nominal concentration of 2 μM. The RED inserts were placed in the 48 wells of the Teflon Plate (Pierce). Samples were prepared by mixing the test compound with plasma at the appropriate concentrations to yield a final drug concentration of 2 μM. Triplicate aliquots of plasma containing 50 at a concentration of 2 μM were pipetted to plasma side (red) of the insert, and PBS (phosphate buffered saline) pH 7.4 was placed into the receiver side (white) of the insert. The plate was covered with the sealing tape and incubated in a 37 °C orbital shaker water bath at approximately 150 rpm for 4 h. Following incubation, samples were prepared in a mixed matrix configuration. Aliquots of samples were pipetted into 96-well plates, and precipitation buffer was added to protein precipitate the samples. Samples were vortexed to mix, then centrifuged for 15 min at 3700 rpm and 4 °C. The supernatant was assayed directly by LC–MS/MS. The following equation was used to calculate the percentage bound drug fraction using this equilibrium dialysis method

\[
\% \text{ bound} = \left( \frac{[\text{buffer chamber}]}{[\text{plasma chamber}] + 100} \right)
\]

Blood to Plasma Ratio. The extent of association of 50 with blood cells was measured in vitro using mouse and human blood. Untreated whole blood was prewarmed on a rotary shaker at 37 °C (350 rpm). Samples were prepared by mixing the test compound with whole blood to yield a final drug concentration of 1 μM. Once mixing was complete, at T(0) and T(60 min), aliquots of incubated blood containing 50 were mixed with Milli-Q water. To generate a uniform mixed matrix, untreated plasma was added to each individual blood/water sample in order to precipitate the samples for analysis, precipitation buffer containing IS was added to the individual samples. Samples were centrifuged for 10 min at 13,000 rpm. The supernatant was assayed directly by LC–MS/MS. The following equation was used to calculate the blood to plasma partitioning ratio

\[
\text{blood/plasma ratio} = \left[ \frac{[\text{blood}]}{[\text{plasma}]} \right]
\]

Solubility of the Solid Compound in FaSSIF. This experiment determined the solubility of solid compounds in FaSSIF at pH 6.5, after 4 h equilibration at rt. Then, 1 mL of each buffers was added to manually weighed 1 mg of in a 2 mL HPLC auto-sampler vial. The resulting suspension was shaken at 900 rpm for 4 h at RT and then transferred to a Multiscreen HTS, 96-well solubility filter plate. The residual solid was removed by filtration. The supernatant solution was quantified by HPLC-UV using single-point calibration of a known concentration of the compound in DMSO. The dynamic range of the assay was 1–1000 μg/mL.

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oral pharmacokinetic study was performed in female mice C57BL/6, the same strain used for efficacy model, to support efficacy studies. In order to investigate in vivo clearance (CL) and volume of distribution ($V_{ss}$), intravenous pharmacokinetic study was conducted in male CD-1 mice. Three animals were used. A dose of 5 mg/kg was administered intravenously, in a bolus form to mice. Compound 50 was dissolved in 5% DMSO/20% encapsine in saline. A dose of 50 mg/kg was orally administered by gavage to 3 mice C57BL/6. Compound 50 was formulated as a suspension of 1% methylcellulose to investigate oral pharmacokinetics. Peripheral blood samples were obtained at 5, 15, 30 min, 1, 2, 4, 8, and 24 h after intravenous administration and 15, 30, 45 min, 1, 2, 4, 8, and 24 h after oral administration. Blood was 1/2 diluted with Milli-Q water and immediately frozen on dry ice until analysis. Quantification was performed by means of LC−MS/MS (APFI4000), with a lower limit of quantification of 1 ng/mL.

Pharmacokinetic parameters, namely clearance (CL), volume of distribution at steady state ($V_{ss}$), terminal half life ($t_{1/2}$), $C_{max}$, and area under the curve (AUC) were estimated using Phoenix 64 (Pharsight, Certara). Bioavailability (F %) was not estimated because i.v. and p.o. administrations were made in different mice strains. Metabolite levels in the whole blood were also evaluated in order to estimate $C_{max}$, $t_{max}$, and AUC (Table 11).

**Table 11. Summary of Whole Blood Pharmacokinetic Parameters (Mean and Standard Deviation) Obtained after Intravenous and Oral Administration of 50 to CD-1 Mouse and C57BL/6 Mouse, Respectively**

<table>
<thead>
<tr>
<th>parameters</th>
<th>mouse CD-1 male at 5 mg/kg i.v.</th>
<th>mouse C57BL/6 female at 50 mg/kg p.o.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL (mL/min/kg)</td>
<td>58.3 ± 7.4</td>
<td></td>
</tr>
<tr>
<td>% LBF</td>
<td>46.2</td>
<td></td>
</tr>
<tr>
<td>$V_{ss}$ (L/kg)</td>
<td>5.73 ± 0.91</td>
<td></td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>0.97 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>AUC$_{0-24h}$ (ng h/mL)</td>
<td>31560.56 ± 2052</td>
<td></td>
</tr>
<tr>
<td>$C_{max}$ (ng/mL)</td>
<td>3386.67 ± 272.09</td>
<td></td>
</tr>
<tr>
<td>$T_{max}$ (h)</td>
<td>1.33 ± 0.58</td>
<td></td>
</tr>
</tbody>
</table>

*Clearance values are additionally expressed as the percentage of liver blood flow (% LBF).*

In Vivo Anti-*T. cruzi* Activity Assay. The IVA of compound was assessed in a rapid acute in vivo assay using the parasite load using two doses of compound over 4 days (1) or a single dose over 2 days (compound 50) as previously described. In brief, C57BL/6 mice were infected subcutaneously in the footpads with 2.5 × 10⁵ trypomastigotes of the *T. cruzi* CL strain expressing the fluorescent protein tdTomato. Two days after the infection, a basal level of fluorescence was measured using the Maestro in vivo imaging system (PerkinElmer, Waltham, MA) using the manufacturer’s Green filter set (560/10/750); 2 × 2 bin; 180 ms exposure and 50 mg/kg of the compound suspended in 1% carboxymethyl cellulose. 0.1% Tween 80 was administered. The fluorescent intensity at the site of the infection was measured again at 4 or 6 days post-infection and the IVA of the compound (IVA) was determined by the formula:

$$100 - \left( \frac{Tx \text{ flur } 4d - Tt \text{ flur } 2d}{unTt \text{ flur } 4d - unTx \text{ flur } 2d} \times 100 \right) = \text{IVA}$$

*Tx: fluorescence intensity of the treated group, unTx: fluorescence intensity of the untreated control group. Groups of infected mice treated with 50 mg/kg of benznidazole or left untreated were used as positive and negative controls. All animal use was performed in accordance with protocol A2014 09-017-R2 approved by the University of Georgia Institutional Animal Care and Use Committee. This protocol adhered to the animal welfare guidelines outlined in Guide for the Care and Use of Laboratory Animals, National Research Council USA.

### ASSOCIATED CONTENT

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.9b01429.

Yields, NMR, and MS characterization of intermediates 6, 7, 35, and 36a to 36c synthesis of oxetane-3-carboxylic acid, synthesis of anilines 46d to 46h, yields and characterization of intermediates 47b to 47h and 48b to 48h, and full characterization of 50 and of its intermediates (PDF)

### AUTHOR INFORMATION

**Corresponding Authors**

*E-mail: tarleton@uga.edu (R.L.T.).

*E-mail: maria.m.marco@gsk.com (M.M.).

*E-mail: gilles.courtemanche@bioaster.org (G.C.).

**ORCID**

Gilles Courtemanche: 0000-0002-4887-9961

**Author Contributions**

C.F., A.P., R.L.T., M.M. and G.C. contributed equally to this work.

**Notes**

The authors declare no competing financial interest.

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**ABBREVIATIONS**

R&D, research and development; CYP51, lanosterol 14-alpha demethylase; NIH, mouse embryonic fibroblast cell line; AAB, acylaminobenzothiazole; SI, selectivity index; HATU, 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]-pyridinium 3-oxide hexafluorophosphate; EtOAc, ethyl acetate; NIS, N-iodosuccinimide; AcOH, acetic acid; DIEA, N,N-diisopropylethylamine; pTSA, ptoluenesulfonic acid; LBF, liver blood flow; CL, clearance; CAD, charged aerosol detection; IVA, in vivo activity; TEER, transepithelial electrical resistance; MDRI-MDCK, Madin–Darby canine kidney cell, transfected with human MDRI gene; $P_{app}$, passive cellular permeability; Cl$_{int}$, intrinsic clearance; IS, internal standard

### REFERENCES

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