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Carbonic anhydrases from Trypanosoma cruzi and Leishmania donovani chagasi are inhibited by benzoxaboroles

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ABSTRACT

A series of 6-substituted ureido- and thioureido-benzoxaboroles were investigated as inhibitors of carbonic anhydrases from Trypanosoma cruzi (TcCA), and Leishmania donovani chagasi (LdCA). Both enzymes were inhibited by benzoxaboroles in the micromolar range. Preferential inhibitory potency against the \( \beta \)-CA LdCA versus the \( \alpha \)-CA TcCA was observed with submicromolar inhibitory activities. Some derivatives displayed excellent inhibitor and selectivity profile over the ubiquitous and physiological relevant human off-target hCA II. This study provides a convincing opportunity to study benzoxaborole scaffold for the design of antiprotozoan potential drugs targeting the pathogen’s carbonic anhydrases.

Introduction

Chagas disease (American trypanosomiasis) and leishmaniasis belong to the list of neglected tropical diseases developed by the World Health Organization (WHO). Both of these diseases are caused by parasites belonging to the kinetoplastidae family and belong to the vector-borne diseases which are responsible for more than 17% of infectious diseases, affecting 20 million people and killing more than 50,000 every year\textsuperscript{1}. Trypanosoma is transmitted by a variety of bedbugs, appeared in Latin America before spreading to other continents. In 30% of cases, it manifests itself in cardiac disorders, and, in 10% of cases, in digestive or neurological disorders. Leishmania, transmitted by the bite of an infected phlebotomia, causes skin or visceral ailments that are very debilitating or even fatal if left untreated. Current treatment used today has many limitations in terms of cost and toxicity, as well as the emergence of resistance phenomena throughout the world. Finding new therapeutic targets to develop new drugs is therefore urgent for these parasites, which WHO now classifies as priority infections (category 1: re-emerging or uncontrolled infections)\textsuperscript{2,3}.

The availability of the complete genome sequence of both protozoans has given the possibility of large-scale analysis, which lead to consequent identification of novel drug targets. Protozoan carbonic anhydrases (CAs, EC 4.2.1.1) were thus recently identified as novel promising targets for chemotherapeutic interventions\textsuperscript{4-6}. Because of the universal reaction they catalyze, i.e. reversible hydration of CO\textsubscript{2} to bicarbonate with a proton release, the prokaryotic metalloproteins are considered as important components in the growth and virulence of pathogenic microorganisms. In 2013, carbonic anhydrases from the two unicellular protozoans Trypanosoma cruzi (TcCA) and Leishmania donovani chagasi (LdCA) were cloned and characterized\textsuperscript{7,8}, paving the way for new opportunities in the design of novel inhibitors exploitable as anti-protozoal agents and acting by a totally new mechanism of action, lacking of cross-resistance to existing drugs.

Important classes of carbonic anhydrase inhibitors (CAIs) have been investigated in detail for their inhibitory profile against these two protozoans carbonic anhydrases.

The \( \alpha \)-CA TcCA, which is characterized by very high catalytic activity for the CO\textsubscript{2} hydration reaction (\( k_{\text{cat}} \) of 1.21 × 10\textsuperscript{8} s\textsuperscript{-1} and \( k_{\text{cat}}/K_{M} \) of 1.49 × 10\textsuperscript{6} M\textsuperscript{-1} s\textsuperscript{-1}), was shown to be inhibited in the nanomolar range by many types of aromatic/heterocyclic sulfonamides\textsuperscript{7,9,10}, sulfamates\textsuperscript{7}, thiol\textsuperscript{7} and hydroxamates\textsuperscript{11}; the two last families of inhibitors demonstrated anti-trypanosomal activity, inhibiting \textit{in vivo} the three phases of the pathogen’s life cycle\textsuperscript{5,7,11}. Inorganic anions and other small molecules were also reported to inhibit TcCA, with a simple dithiocarbamate showing low micro-molar activity \textit{in vitro}\textsuperscript{5,12}.

The \( \beta \)-CA LdCA which also possess an effective catalytic activity for the CO\textsubscript{2} hydration reaction (\( k_{\text{cat}} \) of 9.35 × 10\textsuperscript{5} s\textsuperscript{-1} and \( k_{\text{cat}}/K_{M} \) of 5.9 × 10\textsuperscript{2} M\textsuperscript{-1} s\textsuperscript{-1}), has been reported to be efficiently inhibited by sulfonamides and heterocyclic thiol with nanomolar inhibition constants\textsuperscript{8,13}. Preliminary \textit{in vivo} assays demonstrated that selected inhibitors in thiol series possessed anti-leishmania activity, being able to reduce parasites growth and causing their death\textsuperscript{8}.

Considering the druggability of protozoans CAs, the design of new inhibitors with potent anti-trypanosomal or anti-leishmania activities is worth of investigation.

Previously, we reported a new generation of CAIs based on benzoxaborole scaffold. This new family of inhibitors was shown to act \textit{via} a new binding mode, and to be effective against human \( \alpha \)-CA as well as \( \beta \)-CA from pathogenic fungi\textsuperscript{14,15}.

Encouraged by these results but also by the interesting profile of the trypanocidal orally active benzoxaborole compound.
SCYX-7158\textsuperscript{16,17} which enter Phase IIb/III trials in 2016 for the treatment of African trypanosomiasis, we report in this paper, the inhibitory activities of a series of 6-substituted urea/thiourea benzoxaboroles against C\textalpha s from the two pathogenic protozoans TcCA and LdcCA in order to detect possible candidates for anti-protozoans studies.

\textbf{Material and methods}

\textbf{Chemistry}

Compounds 1–23 were previously reported by this group\textsuperscript{14}.

\textbf{CA inhibition assay}

An applied photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO\textsubscript{2} hydration activity\textsuperscript{18}. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as buffer for testing the \textbeta-CA isoforms and with 20 mM TRIS buffer (pH 8.3) for testing the \textbeta-class enzyme. Na\textsubscript{2}SO\textsubscript{4} of 20 mM were also added to the assay system for maintaining constant the ionic strength. The initial rates of the CA-catalyzed CO\textsubscript{2} hydration reaction were followed for a period of 10–100 s. The CO\textsubscript{2} concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10\% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng–Prusoff equation, as reported earlier\textsuperscript{19–23} and represent the mean from at least three different determinations. All CA isoforms used in these experiments were recombinant ones obtained in-house as reported earlier\textsuperscript{7,8}.

\textbf{Results and discussion}

Inhibition data of benzoxaborole 1–23 against TcCA and LdcCA were measured by a stopped flow CO\textsubscript{2} hydrase assay and are shown in Table 1. Acetazolamide, a clinically used sulfonamide inhibitor, was used as standard. SCYX-7158 being not commercially available, Tavaborole, a commercially benzoxaborole used as topical antifungal medication was also used as standard in our inhibition assays. Inhibitory activities were displayed in comparison with the ones against the two physiologically relevant and off-target human isoforms hCA I and hCA II (Table 1).

Regarding the data in Table 1, the following structure activity relationship for compounds 1–23 may be noted:

- Ureido and thioureido benzoxaborole 1–23 exhibited micromolar to low micromolar inhibitory activities against protozoans TcCA and LdcCA, showing a pronounced selectivity against the \textbeta-CA LdcCA with inhibition constant ranging from 3 to 0.65 \textmu M, and selectivity ratio ($K_{i}(\text{TcCA})/K_{i}(\text{LdcCA})$) ranging from

| Compounds | \(K_{i}\) (\mu M)\textsuperscript{a} | Selectivity ratio | \(K_{i}(\text{TcCA})/K_{i}(\text{LdcCA})\) |
|-----------|-------------------------------|------------------|-----------------------------------------------|
| 1 H       | >100                          | 3.79             | >26                                           |
| 2 NO\textsubscript{2}       | >100                          | 4.01             | >25                                           |
| 3 NH\textsubscript{2}       | >100                          | 2.37             | >42                                           |
| 4 NHCONH-CH\textsubscript{2}Ph | 75.1                     | 2.04             | 36.8                                          |
| 5 NHCONH-CH\textsubscript{2}(3-Cl,5-CH\textsubscript{3}-Ph) | 87.6                     | 4.30             | 20.4                                          |
| 6 NHCONH-Ph | 32.7                      | 0.74             | 43.6                                          |
| 7 NHCONH-(4-CI-Ph) | 37.8                     | 0.62             | 61                                            |
| 8 NHCONH-CH\textsubscript{2}(3,4,5-Cl\textsubscript{3}-Ph) | 67.6                     | 3.54             | 19.1                                          |
| 9 NHCONH-(4-F-Ph) | 24.0                     | 0.78             | 30.8                                          |
| 10 NHCONH-(4-CF\textsubscript{3}-Ph) | 60.7                     | 2.59             | 23.4                                          |
| 11 NHCONH-(2,4,6-Cl\textsubscript{3}-Ph) | 69.5                     | 3.85             | 18.1                                          |
| 12 NHCONH-(2-Ome,5-CH\textsubscript{3}-Ph) | 38.6                     | 0.67             | 57.6                                          |
| 13 NHCONH-(4-COCH\textsubscript{3}-Ph) | 33.6                     | 0.48             | 70                                            |
| 14 NHCSNH-(2,4,5-OMe-Ph) | 72.8                     | 3.20             | 22.7                                          |
| 15 NHCSNH-(4,5-Cl-Ph) | 32.5                     | 0.67             | 48.5                                          |
| 16 NHCSNH-(4-CF\textsubscript{3}-Ph) | 58.8                     | 3.06             | 19.2                                          |
| 17 NHCSNH-(4-OCH\textsubscript{3}-Ph) | 12.6                     | 0.59             | 21.3                                          |
| 18 NHCSNH-(4-NO\textsubscript{2}-Ph) | 46.8                     | 0.91             | 51.4                                          |
| 19 NHCSNH-CH\textsubscript{2}Ph | 59.6                     | 4.37             | 13.6                                          |
| 20 NHCSNH-(4-F-Ph) | 19.7                     | 0.66             | 29.8                                          |
| 21 NHCSNH-CH\textsubscript{2}(3,4-Cl\textsubscript{2}-Ph) | 42.1                     | 4.36             | 9.6                                           |
| 22 NHCSNH-(4-CF\textsubscript{3}-Ph) | 16.6                     | 0.85             | 19.5                                          |
| 23 NHCSNH-Ph | 23.4                     | 0.65             | 36                                            |

\textsuperscript{a}Mean from three different assays, by a stopped flow technique (errors were in the range of ±5–10\% of the reported values).
The most performing inhibitor was found to be the para-methoxy derivative 17.

- It is worth noting that simple benzoxaborole 1, 6-nitro-2 and 6-amino benzoxaboroles 3 exhibited low micromolar activities against LdcCA, as the same level of magnitude as against hCA I, with inhibition constants ranging from 2.37 to 4.01 μM. No activity was noticed for these three compounds against TcCA (K_i>100 μM). Tavaborole, a benzoxaborole used as standard in this study, showed inhibition potency similar to compound 2, and 3, against LdcCA, and a slight selectivity against human isoforms hCA II with a 5.5 magnitude selectivity ratio (K_i(LdcCA)/K_i(hCA II)).

- From a general point of view, change of the ureido by a thio-ureido group did not affect significantly inhibitory potency. Inhibitory activity against LdcCA was comparable with the ones observed for the off-targets hCA I and hCA II. A slight change of selectivity may be noticed for some compounds when we compare LdcCA versus hCA II. For example, if we compare inhibition data for the phenylureido benzoxaborole 6 and phenylthio ureido benzoxaborole 23, a selectivity of 2.5 magnitude for LdcCA was observed with the thioureido derivative 23 and no selectivity with the ureido compound 6. Substitution in the para position of the phenyl ring (e.g. 4-F, 20 and 4-CF_3, 22) also showed a 2.2 magnitude of selectivity against LdcCA over CA II in thioureido series. An inversion of selectivity can be noticed for these two compounds in ureido series where compounds 9 (4-F derivative) and 10 (4-CF_3) were, respectively, more selective against hCA II over LdcCA with selectivity ratio of 1.6 and 5.6 (K_i(LdcCA)/K_i(hCA II)).

- The most performing inhibitor was found to be the para-nitrophenyl thioureido derivative 18 with a high-selectivity ratio against the pathogenic isofrom LdcCA over hCA II (around 110). This compound could represent a slightly better solution compared with the standard clinically used sulfonamide acetazolamide, as acetazolamide showed selectivity for hCA II over LdcCA and TcCA with selectivity ratio, respectively, of 6 and 9.

**Conclusion**

We report for the first time the activity of benzoxaborole derivatives against protozoans CAs. 6-Substituted ureido and thioureido benzoxaborole derivatives 4–23 investigated here showed a preferential inhibitory activity against the β-CA from *Leishmania donovani chagasi* (LdcCA) versus the α-CA from TcCA. Some derivatives such as 18 displayed excellent inhibitory and selectivity profile for LdcCA over the human off-target hCA II.

The present study demonstrates that benzoxaborole chemotype offers interesting opportunities for the inhibition of CA from pathogenic protozoans and for the development of anti-trypanosomal or anti-leishmanial compounds with a new mechanism of action, warranting further development.

**Disclosure statement**

The authors declare no conflict of interest.

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