TRYPANOCIDAL BISBENZYLISOQUINOLINE ALKALOIDS ARE INHIBITORS OF TRYPANOTHIONE REDUCTASE

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Eleven bisbenzylisoquinoline (BBIQ) alkaloids were studied for \textit{in vitro} trypanocidal activity against trypanostigotic forms of the \textit{Y} strain of \textit{Trypanosoma cruzi}. The inhibitory activity of these compounds against trypanothione reductase (TR), a target enzyme for chemotherapy against Chagas disease, was also studied. Six BBIQ alkaloids (antioquine, cepharanthine, daphnoline, irminine, cycleanine and \((-\)curine) displayed a 90\% lethal concentration (LC\textsubscript{90}) against \textit{T. cruzi} of less than 100\,\mu M. Daphnoline and curine, with LC\textsubscript{90} values of 10\,\mu M, are attractive for further investigation as potential anti-Chagasic drugs. Kinetic analyses suggested the BBIQ alkaloids are mixed inhibitors of TR. These compounds are reasonably potent inhibitors of TR; the best TR inhibitor, cepharanthine, had an IC\textsubscript{50} of 15\,\mu M, which is in the same order of magnitude as its LC\textsubscript{90} against \textit{T. cruzi}. The similar magnitudes of the IC\textsubscript{50} and LC\textsubscript{90} values suggest that inhibition of TR could contribute to the trypanocidal activity exhibited by the BBIQ alkaloids.

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INTRODUCTION

Trypanosomatid parasites are pathogens that cause a wide range of tropical illnesses including Chagas disease, a condition which affects approximately 20 million people worldwide.\textsuperscript{1} Compounds that belong to the bisbenzylisoquinoline (BBIQ) alkaloid family have been shown to inhibit parasite growth in cultures of the trypanosomatid \textit{Trypanosoma cruzi}, the causative agent of Chagas disease.\textsuperscript{2,3} Toxicology studies conducted on some BBIQ alkaloids in mice showed that these compounds cause no short-term toxic effects at bioactive doses.\textsuperscript{4} Six of the eleven BBIQ alkaloids studied here have previously been shown to decrease growth of bloodstream forms in a variety of strains (Tulahuen, C8C11, 1979 CL1, and Y) of \textit{T. cruzi} in vitro.\textsuperscript{2,3} Because the potency of these compounds has been shown to vary significantly among strains of \textit{T. cruzi},\textsuperscript{3} this study focuses on obtaining trypanocidal activity data for all compounds against only one strain. Also, previous studies have not conformed to a uniform method for measuring trypanocidal activity.\textsuperscript{2,3} In this study, the concentration of all eleven BBIQ at which 50% lysis (LC$_{50}$) of trypomastigote forms of the Y strain of \textit{T. cruzi} occurs was determined in a uniform manner.

Trypanothione reductase (TR, EC 1.6.4.8) is an enzyme found in \textit{T. cruzi} which catalyzes the reduction of trypanothione disulfide (T(S)$_2$), a compound which then acts as a buffer against reactive oxygen species within the parasites. TR plays a role analogous to that of human glutathione reductase (GR, EC 1.6.4.2).\textsuperscript{5} TR is an attractive target for rational drug design for several reasons: trypanosomatid parasites are more sensitive to oxidative stress than their hosts,\textsuperscript{1} the structure of TR has been solved to high resolution,\textsuperscript{6} and TR exhibits at its active site structural differences from its human equivalent, GR.\textsuperscript{6} Thus, obtaining potent inhibitors of TR has become a focus of many structure-based drug design studies.\textsuperscript{1,5–7}

Because the BBIQ alkaloids are similar to previously studied TR inhibitors which are positively charged molecules with a large hydrophobic group,\textsuperscript{5} steady-state kinetic experiments were conducted to determine whether or not the BBIQ alkaloids inhibition of TR could be the mechanism...
for their trypanocidal activity. *Crithidia fasciculata* trypanothione reductase (cfTR) was used because it is readily available from an expression system and because previous studies have shown that compounds which inhibit cfTR also inhibit *T. cruzi* TR to the same degree. The concentration of each BBIQ at which 50% of normal cfTR activity was observed (IC50) was determined, and a full kinetic analysis of two BBIQ alkaloids was conducted. While a clear, direct correlation of the data was not observed, the LC50 and IC50 values for each compound generally fell within the same order of magnitude, suggesting that the inhibition of TR is a possible contributor to BBIQ trypanocidal activity.

**MATERIALS AND METHODS**

**A Compounds**

Eleven BBIQ alkaloids (of five different types) were used in this study, each differing in the position of the linkages between monomeric BBIQ components (see Figure 1 for chemical structures). The eleven BBIQ alkaloids used in this study were obtained from the following sources: Lindoldhamine was extracted from *Abuta pahni* (Menispermaceae), daphnoline and limacine from *Albertisia papuana* (Menispermaceae), limacusine from *Curare condicans* (Menispermaceae), gyrocarpine and pheanthine from *Gyrocarpus americanus* (Hernandiaceae), isotetrandrine from *Limaciodis loangensis* (Menispermaceae), clyceanine and curine from *Isoloma hexaloba* (Annonaceae) and antioquine from *Pseudoxandra sclerocarpa* (Annonaceae). Cepharanthine was purchased by Pr. H. Guinaudeau (Angers, France) from tuberous roots of *Stephania cepharanthia* (Menispermaceae). All BBIQ alkaloids were identified for chemical structure by physical and spectral data (1H NMR and mass spectrometry) against reference samples and literature values.

**B Effect of BBIQ Alkaloids on Trypanosoma cruzi Trypanastigote Forms in vitro**

To obtain the parasites, albino mice were infected with *T. cruzi* Y strain. Seven days after infection, blood was obtained by cardiac puncture using 3.8% sodium citrate as anticoagulant in a 7:3 blood/anticoagulant ratio. The parasitemia in infected mice ranged between $1 \times 10^5$ to $5 \times 10^5$. 
The eleven compounds have been divided into six categories: type I, BBIQ with one diaryl ether bridge (11 to 12'), lindoldhamine; type II, BBIQ with one diaryl ether bridge (8 to 7') and one biphenyl bridge (11 to 8'), antioquine; type III, BBIQ with two diaryl ether bridges (7 to 8' and 11 to 12'), daphnoline, gyrocarpine and limacusine; type IV, BBIQ with two diaryl ether bridges (8 to 7' and 11 to 12'), cepharantine, daphnoline, gyrocarpine and limacusine; type V, BBIQ with two diaryl bridges (8 to 12' and 12 to 8') cycleanine; type VI, BBIQ with two diaryl bridges (8 to 12' and 11 to 7') curine.

**FIGURE 1 Structure and stereochemistry of BBIQ alkaloids.**
parasites per milliliter. BBIQ alkaloids were dissolved in phosphate buffered saline (PBS) when they were in salt form (HCl salts) or in cold DMSO (final concentration 1%) when they were in basic form, to a final concentration of 250 μg/mL. Aliquots of 10 μL of each extract of different concentrations (4, 20, 40, 100 and 250 μg/mL) were mixed in microtiter plates with 100 μL of infected blood containing different parasite concentrations (1 × 10^5 and 10^6 parasites per mL). Infected blood and infected blood containing gentian violet at 250 μg/mL were used as controls. The plates were shaken for 10 min at room temperature and kept at 4° for 24 h. Each solution was microscopically observed at 400×, placing a 5 μL-sample on a slide and covering it with a 22 × 22 mm coverglass for parasite counting. The LC50 of each compound (μM) was determined from graphical plots of BBIQ alkaloid concentration vs. percentage lysis using the probit method of analysis (software Toxicologie, G. Febvay, INSA 406, F-69621 Villeurbanne, France).

C Effect of BBIQ Alkaloids on Trypanothione Reductase

*Crithidia fasciculata* trypanothione reductase (cfTR) was purified from *E. coli*, using the expression system described by Strickland. Steady-state kinetic constants of the cfTR used were K_m = 54 μM T(S)_2 and V_max = 10600 min⁻¹. T(S)_2 was purchased from Bachem Bioscience Inc. The activity of the cfTR was observed from the rate of oxidation of the co-substrate NADPH to NADP⁺ as measured by a decrease in absorbance at 340 nm. The assay mixture contained 0.014 μM cfTR, 0.1 M Hepes pH 7.8, 50 μM EDTA, 250 μM NADPH, 50 μM T(S)_2, and varying concentrations of inhibitor in a total volume of 1 mL. The reactions were initiated by the addition of NADPH. All kinetics experiments were conducted at 25°. A 10 mM stock solution of each BBIQ alkaloids was made in distilled water or DMSO as specified in Table I. Full kinetic analyses were conducted at inhibitor concentrations of 0.5, 1.0 and 2.0 times the estimated K_m from initial experiments. At each of these inhibitor concentrations, assays were performed at 12, 24, 50, 100, and 200 μM T(S)_2. The kinetic data were graphically analyzed for mechanism with Lineweaver–Burk plots and fitted to the general rate equation using the computer programs of Cleland. The values of K_i and K_ii for mixed inhibitors were estimated by fitting data to the equation:

\[
ν = \frac{V_{max}[S]}{K_m(1 + [I]/K_{i})} + [S](1 + [I]/K_{ii})
\]
TABLE I LC50 and IC50 values of bisbenzylisoquinoline alkaloids

<table>
<thead>
<tr>
<th>BBIQ</th>
<th>Type</th>
<th>MW</th>
<th>Solvent</th>
<th>Mechanism</th>
<th>T. cruzi LC50 (µM)</th>
<th>TR IC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daphnoline</td>
<td>III</td>
<td>653</td>
<td>H2O</td>
<td>N/D</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>(-) Curine</td>
<td>VI</td>
<td>667</td>
<td>H2O</td>
<td>N/D</td>
<td>10</td>
<td>60</td>
</tr>
<tr>
<td>Cepharanthine</td>
<td>III</td>
<td>679</td>
<td>H2O</td>
<td>Mixed; ( K_a = 7.6, K_d = 51.6 )</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>Antioquine</td>
<td>III</td>
<td>680</td>
<td>DMSO</td>
<td>N/D</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>Limacine</td>
<td>IV</td>
<td>681</td>
<td>H2O</td>
<td>N/D</td>
<td>40</td>
<td>90</td>
</tr>
<tr>
<td>Cyclamine</td>
<td>V</td>
<td>695</td>
<td>H2O</td>
<td>Mixed; ( K_a = 194.2, K_d = 260.8 )</td>
<td>60</td>
<td>303</td>
</tr>
<tr>
<td>Pheanthine</td>
<td>IV</td>
<td>695</td>
<td>H2O</td>
<td>N/D</td>
<td>100</td>
<td>122</td>
</tr>
<tr>
<td>Isotetrandrine</td>
<td>IV</td>
<td>695</td>
<td>H2O</td>
<td>N/D</td>
<td>100</td>
<td>235</td>
</tr>
<tr>
<td>Gyrocarpine</td>
<td>III</td>
<td>608</td>
<td>DMSO</td>
<td>N/D</td>
<td>110</td>
<td>58</td>
</tr>
<tr>
<td>Limacine</td>
<td>III</td>
<td>608</td>
<td>DMSO</td>
<td>N/D</td>
<td>420</td>
<td>55</td>
</tr>
<tr>
<td>Lindoldhamine</td>
<td>I</td>
<td>568</td>
<td>DMSO</td>
<td>N/D</td>
<td>470</td>
<td>27</td>
</tr>
</tbody>
</table>

RESULTS

The eleven BBIQ alkaloids examined have been divided into six categories (Figure 1). The structures of these compounds suggest that their amine groups are likely to be protônated at a neutral pH, so they are likely to be positively charged.

The in vitro LC50 of the BBIQ alkaloids against trypomastigote forms of T. cruzi were all lower than 500 µM and six BBIQ alkaloids had LC50 values less than 100 µM (Table I). Two of the most active compounds, daphnoline and cepharanthine, were type III BBIQ alkaloids.

BBIQ alkaloid IC50 values for the inhibition of TR are shown in Table I. The most active BBIQ alkaloids were cepharanthine and lindoldhamine with IC50 values of 15 µM and 27 µM, respectively. Eight BBIQ alkaloids had IC50 values lower than 100 µM and three were less active. Two of the least potent inhibitors, isotetrandrine and pheanthine, were both type IV BBIQ alkaloids.

Full kinetic analyses were performed on cepharanthine and cyclamine, the most and the least potent inhibitors, respectively (Figure 2). In both cases the kinetics showed a mixed mechanism of inhibition with respect to trypanothione. It is unlikely that these compounds would bind to the NADPH binding site on TR, because the BBIQ alkaloids lack the negative charge characteristic of NADPH. For this reason, kinetic analyses with respect to NADPH were not conducted. Because these two compounds are mixed inhibitors with respect to trypanothione, it is possible that the other nine BBIQ alkaloids are mixed inhibitors as well, although more experiments would be necessary to confirm this.
INHIBITING TR WITH TRYPIACIDAL ALKALOIDS


discussion

The LC_{50} values for the BBIQ alkaloids confirm that these compounds exhibit trypanocidal activity in the micromolar range. The most potent compounds, daphnoline and curine, both with LC_{50} = 10 \mu M, are potent enough to justify further testing for use in the treatment of Chagas disease and to suggest that structure–function studies of other members of the BBIQ alkaloid family might lead to even more potent compounds. The fact that BBIQ alkaloids have exhibited antiprotozoal activity against Plasmodium and Leishmania parasites also encourages further studies of these compounds.

The BBIQ alkaloids were also TR inhibitors in the micromolar range. For example, the best TR inhibitor, cepharanthine, had an IC_{50} of 15 \mu M. This value falls within the same order of magnitude as its LC_{50} (30 \mu M), suggesting that at trypanocidal doses, there could be considerable TR inhibition. Such a similarity between LC_{50} and IC_{50} is also observed for most of the other BBIQ alkaloids. While a direct relationship between LC_{50} and IC_{50} is not observed, it is likely that the bioavailability of each compound to the parasites varies. Despite the lack of a linear relationship in the data, TR inhibition cannot be ruled out as a possibility for the mechanism of trypanocidal activity. However, the activity of these compounds could also result from another mechanism or a combination of mechanisms. Studies of BBIQ alkaloids have shown their capacity to block calcium channels, and more recently it has been demonstrated that changes in Ca^{2+} concentration in tissue culture cells or parasite cultures are an important
mechanism for the penetration of the parasite into the host cell. Further studies are needed before the mechanism of these compounds can be established. Also, a comprehensive study of structure–activity relationships should be conducted to improve the potency of these compounds against T. cruzi. The uniform method for testing trypanocidal activity used in this study can be applied to develop a robust structure–activity relationship.

Though their mechanism of inhibition is not strictly competitive, the BBIQ alkaloids share many of the characteristics of previously studied competitive inhibitors of TR; that is they combine a positive charge attached to a hydrophobic group. The positively charged BBIQ alkaloids could be attracted to TR's negatively charged active site. Although the BBIQ alkaloids are mixed inhibitors of TR, their IC₅₀ and Kᵢ values obtained compare favorably with those of the best known TR inhibitors: kukoamine (Kᵢ = 1.5 μM, Kᵢ = 13 μM), chlorpromazine (Kᵢ = 14 μM), and mepacrine (Kᵢ = 21 μM). The Kᵢ of 15 μM for cepharanthine highlights it as a potential lead compound for drug design.

Acknowledgments

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References

INHIBITING TR WITH TRYPANOCIDAL ALKALOIDS
