Glutathione Derivatives Active against Trypanosoma brucei rhodesiense and T. brucei brucei In Vitro

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Trypanosomiasis and leishmaniasis are parasitic diseases that cause severe infections in humans and domestic animals in the tropics. These infections pose a serious health problem to the countries in tropical regions, in terms of the suffering they inflict and the effects on their economies (22). Chemotherapy for treatment of these diseases is inadequate, because many treatments have poor clinical efficacy, produce side effects, or are toxic, especially in the late chronic stages, which inevitably lead to death. Parasites display a variety of unique metabolic reactions not present in other microorganisms and eukaryotes. One general approach to the development of novel antiparasitic drugs is to identify key differences in metabolism between the host and pathogen and use them in the design of selective toxic agents. Thiol metabolism in the trypanosomatids (15) is characterized by dependence on the hexapeptide, trypanothione [N^1, N^6-bis-(glutathionyl) spermidine] [T(SH)2] (Fig. 1), an antioxidant that replaces glutathione (GSH), which is the major antioxidant of eukaryotic cells. The importance of trypanothione as an antiprotozoal drug target is highlighted by the fact that existing trypanocidal drugs, notably the arsencals (e.g., melarsoprol) (3) and difluoromethylornithine (23), may work by interfering with the metabolism or synthesis of this hexapeptide. The central roles of trypanothione in trypanosomiasis and leishmaniasis make thiol-dependent enzymes potential targets for the development of chemotherapeutic drugs (15). Enzymes investigated to date include glutathionylperoxidase synthetase, by using substrate analogues (6, 9), and trypanothione reductase, by using substrate analogues (12), substractive substrates (19), irreversible inhibitors (5), and pe- nothiazine derivatives (1). However, many of these compounds have proven to be inactive against trypanosomes in vitro (6, 9). By using a lead-directed approach to identify potential anti- protozoal compounds, we recently reported the identification of several GSH derivatives active in vitro against trypanosomiasis and leishmaniasis (10). A structure-function study of S-bromo- benzylglutathione derivatives identified the antiparasitic activity to be exclusively associated with N, S-blocked GSH diester derivatives (10), with the nature of the N and S groups contributing to the compound’s activity and ability to differentiate between the parasite and host (10). GSH diesters, due to their ease of membrane penetration and hydrolysis by nonspecific esterases to free acids, have been proposed as chemical delivery systems for transport of GSH into cells (16, 20). The nature of the ester group in the case of Trypanosoma brucei brucei control membrane penetration and susceptibility to hydrolysis (8) and the hydrophobicity parameter (log P) and Taft steric parameter (E_s) are an index of these two factors. To identify differences in the antiprotozoal activity of GSH diesters against Trypanosoma brucei rhodesiense and T. brucei brucei, we have undertaken a comparative study of the dependence of hydrophobicity (log P) and E_s on inhibitory activity.

**MATERIALS AND METHODS**

GSH diester derivatives. Diester derivatives 3 to 25 (Fig. 2) were prepared as previously described (8, 10) with either S-(2,4-dinitrophenyl) GSH (GSDNP) or N-benzyloxycarbonyl-S-(2,4-dinitrophenyl) GSH (Cbz-GSDNP) as the starting material suspended in the appropriate alcohol, to which was added 2 to 3 mol eq of thiomethyl chloride.

QSAR. For quantitative structure activity regression (QSAR), SciQSAR, a module of Alchemy 2000 (Tripos), was used for data analysis. This program searched Alchemy 2000 for low-energy molecular conformations and used the information to calculate several molecular descriptors (MW, W, D, etc). The Wienn (W) descriptor is a topological parameter (21) whose value is larger for extended molecules than compact ones. The log P (log K_ow) descriptor was calculated for each of the molecules (8) by using the interactive demo program established at the Environmental Service Centre, Syracuce Research Corpora- tion, in which structures were entered in SMILES notation (limited to 100 characters). The program estimated the octagonal/water partition coefficient (log P) by using an atom/fragment contribution method. The Taft steric parameter (E_s) is based on values determined experimentally for a series of menthol esters, of which values were reported for 13 of the 17 esters used, while the remainder were calculated by regression analysis or extrapolation (8).

The creation of a regression equation by using these descriptors and their statistical regression analysis was undertaken by using the LINES program provided with Excel (Microsoft Corporation). The program calculated the coefficients of the equation: the multiple correlation coefficient (R^2) and Fisher statistic value (F).
Evaluation of the parasitic activity of GSH derivatives in vitro: parasites. T. brucei brucei (S427) and T. brucei rhodesiense (STIB900) bloodstream-form trypanomastigotes were maintained in HM1-18 medium (11, 13) with 20% heat-inactivated fetal calf serum (HIFCS) (Harlan Sera-Lab., Crawley, United Kingdom) at 37°C in a 5% CO₂-air mixture.

All compounds were tested in triplicate in a threefold dilution series from a top concentration of 30 μM. Parasites were diluted to 2 × 10⁶/ml and added in equal volumes to the test compounds in 96-well, flat-bottom Microtest III tissue culture plates (Becton Dickinson and Company, Paramus, N.J.). Appropriate controls with pentamidine isethionate (Aventis, Sussex, United Kingdom) as the culture plates (Becton Dickinson and Company, Paramus, N.J.). Appropriate equal volumes to the test compounds in 96-well, fl

rhodesiense and a tetrazolium salt colorimetric assay (11) for T. brucei activity was determined on day 3 with the Alamar blue assay (18) for determination of activity by the use of a tetrazolium salt colorimetric assay (11) on day 5.

RESULTS AND DISCUSSION

The diesters investigated in this study were based on N-benzoxycarbonyl-S-2,4-dinitrophenylglutathione (CbzGSDNP) and were prepared with either linear alcohols 3 to 9, branched alcohols 10 to 20, or heteroatom (-F, -Cl, -OMe)-containing linear alcohols 21 to 25.

Diester compounds 5 to 7, 10 to 19, 23, and 24 (Table 1) showed significant inhibitory activity against T. brucei rhodesiense (≥1 μM). Of the linear GSH diester derivatives (Table 1), compound 6, the butyl diester (log P = 5.06), proved to be compound 7, the pentyl diester (log P = 6.04), with an ED₅₀ of 0.38 μM, while for the heteroatom-substituted diesters 21 to 25, the most active linear diester against T. brucei rhodesiense (ED₅₀ ∼0.47 μM) was the chlorobutyl diester 24, as well as the chloroethyl diester 22 for T. brucei brucei. However, the toxicity of these electrophilic substituted diesters to KB cells was high, as seen by the small relative toxicity values that showed less than unity (Table 1) observed for compounds 23 to 25. In the branched series, compounds 10 to 19 displayed the highest activity against T. brucei rhodesiense—between 0.18 and 0.65 μM. The antiparasitic activity of diester compounds 3 to 25 was, in most cases, better for T. brucei rhodesiense than for T. brucei brucei. The exceptions were compound 22, which displayed a 16-fold-higher activity against T. brucei rhodesiense, and compound 10, which displayed a 55-fold-higher activity against T. brucei rhodesiense than T. brucei brucei (Table 1).

A plot of log (1/ED₅₀) versus log P with the data in Table 1 indicated a parabolic dependence between these parameters for T. brucei rhodesiense based on regression analysis, with an

![FIG. 1. Structure of trypanothione [T(SH)₂].](image1)

![FIG. 2. Structures of GSH derivatives.](image2)
optimum value of log $P$ in the range 4.5 to 5.5 compared to 5.0 to 6.0 for $T. brucei brucei$ (8). This value is in agreement with those for compounds possessing good membrane penetration, with log $P$ values in the range ~4 to 7 (2, 4). The optimum value for log $P$ (log $P^{*}$) of 5.03 determined from the parabolic curve was lower than the value of 5.8 determined for $T. brucei brucei$. Similarly, a plot of log (1/ED$_{50}$) versus $E_{s}$ showed optimum values for $E_{s}$ of −0.84 for $T. brucei rhodesiense$ and −0.70 for $T. brucei brucei$. This result indicates that some degree of branching on the diester is beneficial to membrane penetration, but beyond the optimum value ($E_{s} = −0.7$ or −0.84), the benefits decrease. Examination of the $E_{s}$ values reported in Table 1 indicates that branched diesters with values in the optimum range (compounds 10, 17, and 18), are branched on the carbon directly attached to the alcohol.

The parabolic dependence of log (1/ED$_{50}$) versus log $P$ and $E_{s}$ for $T. brucei rhodesiense$ as found for $T. brucei brucei$ indicates that, in both cases, the activity of these GSH diester derivatives is primarily controlled by their ability to enter parasitic cells, as previously suggested (8). The best QSAR equation derived, using a set of 17 compounds, excluding the outliers 11, 14, and 15, was equation 1 with the multiple correlation coefficient $R^{2} = 0.86$ and Fisher statistic value $F = 14.0$:

$$\log (1/ED_{50}) = -1.02 \times \log P + 0.21 \times E_{s} + 0.1 \times MW - 0.0014 \times W - 50.6$$

Equation 1 indicates that the therapeutic activity of these compounds decreases with increasing values of log $P$ and $E_{s}$, the latter a result of the overall negative character of these parameters. Log $P$ is positive in character (Table 1), but its coefficient is negative (see equation 1), while $E_{s}$ values are predominantly negative (Table 1). These two parameters render the overall equation negative in character and so decrease the positive value of log (1/ED$_{50}$). For high therapeutic activity, log $P$, $E_{s}$, and their corresponding coefficients have to be small. Comparison of equation 1 with that identified for $T. brucei brucei$ (equation 2) (8) shows that log $P^{*}$, $E_{s}$, and their associated coefficients make an overall smaller negative contribution to the equation, thus resulting in a higher degree of therapeutic activity for these compounds against $T. brucei rhodesiense$ than against $T. brucei brucei$, as observed (Table 1):

$$\log (1/ED_{50}) = -2.57 \times \log P + 1.87 \times E_{s} + 0.20 \times MW - 0.002 \times W - 102$$

Equation 2 shows that the dip in log $P$ is lower than in equation 1: $-2.57$ compared to $-1.02$. This suggests that the log $P$ region is a factor impacting the therapeutic activity of these compounds against $T. brucei rhodesiense$ more than against $T. brucei brucei$. Therefore, the therapeutic activity of the heteroatom diester against $T. brucei rhodesiense$ would be affected by the log $P$ and $E_{s}$, as shown in equation 2, which has a lower coefficient value than in equation 1.
teins among species rather than biochemistry. In view of these results, we have altered our testing regime and now use *T. brucei rhodesiense* instead of *T. brucei brucei* to test these compounds.

The pharmacological values of the compounds identified in this study, 6, 7, 10 to 18, and 24, are dependent on their specificity. The relative toxicity values given in Table 1, expressed as the ratio of the activity of KB cells to that of *T. brucei rhodesiense* cells, are a suitable indicator of this: 7 < 16 < 10 < 12 < 14 < 11. The significant activity of several of these compounds combined with their relatively low toxicity to KB cells indicates the potential of GSH derivatives for use in the treatment of trypanosomiasis infections.

GSH diesters have been previously proposed as therapeutic agents for use in cancer chemotherapy targeted against the glyoxalase system, with branched diesters showing the highest activity against HL60 cells (median growth-inhibitory concentration [GC50] ≈ 4.2 μM) (20). The toxicity data presented in Table 1 indicate that linear diesters 23 to 25 are the most active compounds against KB cells (ED50 ≈ 0.38 to 0.70 μM), while both linear diesters 7 and 24 and branched diesters 12, 16, 18, and 19 are the most active against A2780 cells (ED50 ≈ 1.5 to 5.0 μM), and only the branched diester 13 is significantly active against K562 cells (ED50 ≈ 4.8 μM). In the case of compounds 23 and 24, the nature of the diester groups appears to contribute directly to the high toxicity of these compounds against KB cells. The electrophilic nature of the ester groups suggests that these GSH diesters either function as alkylating agents or act as carriers (prodrugs) of these toxic reagents into cells, prior to cleavage and release by nonspecific esterases. The latter explanation seems the most plausible, based on a systematic study of some 90 GSH derivatives (7), of which only 4 were found with comparable activity against KB cells, and their toxicity could be associated with the nature of the groups attached to the S-site of GSH. This result indicates an alternative strategy for the design of anticancer compounds based on GSH with high in vitro activities.

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**REFERENCES**


