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Graphical Abstract

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Antiprotozoal Glutathione Derivatives with Flagellar Membrane Binding Activity against *T. brucei rhodesiense*

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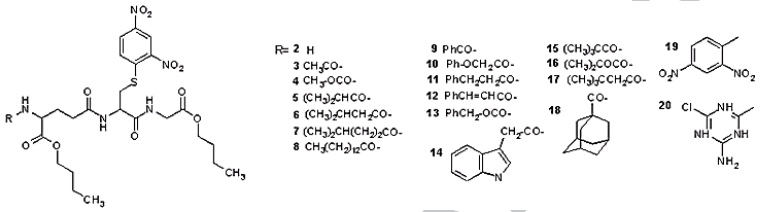

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ABSTRACT

A new series of N-substituted S-(2,4-dinitrophenyl)glutathione dibutyl diesters were synthesized to improve *in vitro* anti-protozoal activity against the pathogenic parasites *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi* and *Leishmania donovani*. The results obtained indicate that N-substituents enhance the inhibitory properties of glutathione diesters whilst showing reduced toxicity against KB cells as in the cases of compounds **5**, **9, 10 , 16**, **18** and **19**. We suggest that the interaction of N-substituted S-(2,4-dinitrophenyl) glutathione dibutyl diesters with *T. b. brucei* occurs mainly by weak hydrophobic interactions such as London and v*an der Waals* forces. A QSAR study indicated that the inhibitory activity of the peptide is associated negatively with the average number of C atoms, N_C and positively to $S_{ZX,}$ the ZX shadow a geometric descriptor related to molecular size and orientation of the compound. HPLC-UV studies in conjunction with optical microscopy indicate that the observed selectivity of inhibition of these compounds against bloodstream form *T. b.brucei* parasites in comparison to *L. donovani* under the same conditions is due to intracellular uptake via endocytosis in the flagellar pocket.

1. Introduction

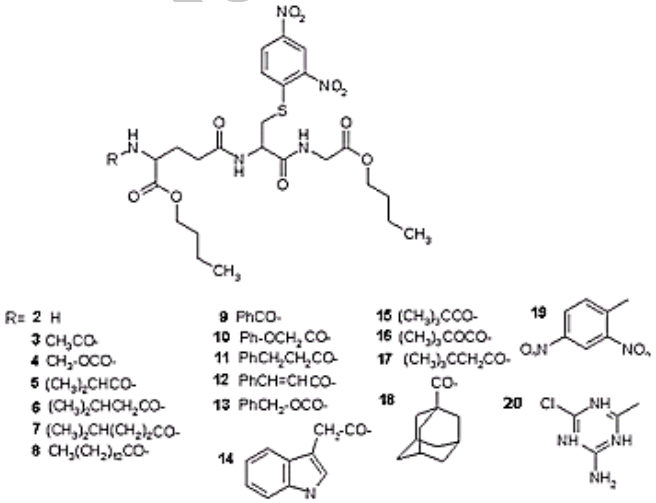
Trypanosomiases and leishmaniases are protozoan parasitic infections of humans and some domestic animals in four continents[1-3]. Human African trypanosomiasis (HAT) is caused by protozoa of the genus *Trypanosoma* transmitted to humans by the bite of infected tsetse fly (genus Glossina), of which two morphologically indistinguishable subspecies exist, that differ in their clinical presentation and epidemiology. *T. brucei gambiense* the slower developing chronic form of HAT, causes 95% of all cases and is prevalent in West and Central Africa. *T. brucei rhodesiense* causes the acute more virulent form of the disease prevalent in East and Central Africa; it has a higher mortality rate due to its rapid progression[4] and poor early diagnosis.[5] Leishmaniases like trypanosomiasis is a disease found in Asia, South America, and East Africa caused by *L.donovani*, transmitted into the host by the bite of infected phlebotomine sandflies[2] and manifests itself in three different forms-visceral (kala-azar) the most serious form of the disease, cutaneous (the most common), and mucocutaneous. American trypanosomiasis (Chagas disease is caused by *T.cruzi*) is a potentially life-threatening disease confined to the region of the Americas primarily Latin America and is transmitted to humans by contact with the faeces or urine of the triatomine bug[3]. Chemotherapy of trypanosomiasis and leishmaniasis is inadequate as many treatments have poor clinical efficacy, with patients suffering side effects or are toxic at the high dosage regimes, used especially in the late chronic stages. This situation has been further aggravated by the emergence of resistance to some of the first line drugs,[6] or the increasing incidence of treatment failure[7]. Genetic approaches have identified some key gene products responsible for resistance[8] in trypanosomes these include the trypanosome P2 adenosine transporter, AT1[9], responsible for the uptake of the front-line drugs melarsoprol and pentamidine used to treat HAT, which was found to be mutated or absent in a number of drug resistant strains[10] . The upregulation of the melarsoprol-trypanothione (Mel-T) transporter, multidrug resistance protein A (MRPA) [11], which caused resistance by the rapid efflux of melarsoprol from the cell

and the loss of the amino acid transporter, AAT6 in resistant strains[12], involved in the uptake of the drug eflornithine used to treat HAT. In *T. cruzi* the down regulation of the trypanosome nitroreductase (NTR) [13], an activator of the front-line pro-drugs nifurtimox and benznidazole used to treat Chagas disease, may lead to resistance. These concerns has highlighted the need for a new generation of drugs.

Focused drug discovery over the past two decades has concentrated on the identification and characterization of different biochemical and molecular targets leading to the identification of some pathways common to all pathogenic trypanosomatids,[14] opening the possibility for the development of clinically useful drugs active against all these parasites. Amongst these is the synthesis of glutathione (GSH), polyamines and trypanothione, essential for the survival of *T. brucei* [14] and related trypanosomatids. The trypanothione cycle maintains the hexapeptide, trypanothione (N^1, N^8-bis (glutathionyl)spermidine) ($T(SH)_2$), an antioxidant replacing glutathione (GSH) via trypanothione reductase (TR), in its reduced form and so decreases the cell's sensitivity to oxidative stress; which is a consequence of rapid growth.[7] A variety of trypanothione reductase inhibitors have been reported to date of both natural and synthetic origin which include peptides, polyamines,[15,16] tricyclics,[17] 2-aminodiphenyl sulfides,[18] 2- and 3-substituted 1,4-naphthoquinone derivatives as subversive substrates[19] and organometallic compounds.[20] Only a few compounds achieve activities in the nanomolar range (100 nM) required to overcome the induced phenotypic changes in TR and a 90% level of inhibition. However, the development of time-dependent irreversible TR inhibitors based on benzofuranyl 3,5-bis-polyamine[21] and spermidine derivatives of the alkaloid lunarine,[22,23] overcome the drawback of competitive inhibitors of TR.

Using a lead directed approach to identify potential antiprotozoal compounds we previously reported N-benzyloxycarbonyl-S-(2,4-dinitrophenyl)glutathione diesters with *in vitro* inhibitory activity against the parasitic growth of *Trypanosoma* and *Leishmania* parasites.[24] Initially these compounds were thought to exert their effect by inhibition of enzymes of the trypanothione cycle due to the similarity of these compounds to trypanothione as they both contained a glutathione peptide backbone. This inference was dismissed, based on an absence of any inhibitory effects of these compounds on enzymes of the trypanothione cycle[25] (trypanothione reductase, tryparedoxin (TXN1 and TXN2) and glutathione peroxidase). A study of the inhibitory properties of lipophilic benzyloxycarbonyl-S-(2,4-dinitrophenyl) glutathione diesters derivatives against *T.b.brucei* and *T.b.rhodesiense*, found a parabolic dependence between $\log(1/ED_{50})$ and log P (lipophilicity) with both species showing an optimium value in the range between 5.0-6.0, which indicated membrane penetration, played a key role in the antiparasitic activity of these compounds[26, 27]. A QSAR analysis of the inhibition data determined a, linear relationship between log P and the Taft's steric parameter (E_s)[26,27] the latter related to lipophilic chain branching. Microscopy studies showed that exposure of the parasite to the glutathione diester derivatives, caused morphological changes to the parasitic cytoskeleton resembling the type of changes seen with antiprotozoal membrane binding peptides responsible for pore formation in trypanosomes,[28,29] suggesting that these peptides might facilitate cell death by a similar process.[25] Studies on glutathione diester peptides, such as **13,** with a range of model membranes in Langmuir trough studies confirmed these peptides form stable monolayers[19] and undergo aggregation and re-orientation of the membrane so altering fluidity, surface relaxation and elasticity. The reduced surface flexibility of the protozoan membrane was suggested to be related to the parasitic killing property of these compounds[30] and membrane integrity.

Apart from disruption of the parasitic membrane integrity, other studies indicate that these peptides at μM levels exerted an inhibitory effect on efflux *via* the trypanosome multidrug resistance protein (MRPA), a putative thiol conjugate transporter,[25,31] indicating an intracellular target for the action of these compounds. The MRPA protein plays an important role in the development of drug resistance [11,32] and is a member of the ATP binding cassette (ABC) transporter superfamily in mammalian systems, proposed to act with the glutathione transferases (GST) to remove endogenous electrophiles and exogenous compounds from the cytosol and reduce intracellular toxicity and oxidative stress.[33] To progress our work, aimed at understanding and improving the antiprotozoal killing power of S-(2,4-dinitrophenyl)glutathione dibutyl diesters *in vitro* whilst minimizing host toxicity we prepared a series of N-substituted derivatives, **2-20**, as shown in Scheme 1.



Scheme 1. Chemical structures of N-blocked-S-2,4-dinitrophenylglutathione di-butyl esters tested for antiparasitic activity.

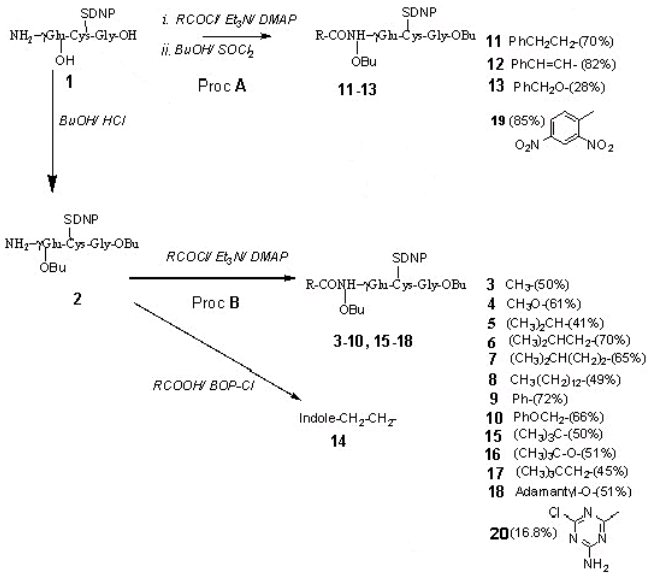
A study of the *in vitro* activity of these compounds against *T.b.rhodesiense* has been used to ascertain the effect changes made at the N-site have on the activity of these peptides. HPLC studies with optical microscopy have been used to further elaborate the mechanism and specificity of action of these compounds with the view to improve their anti-trypanosomal activity.

2. Results & Discussion

2.1. Chemical synthesis

The compounds used in this study were prepared either by procedure A or B using compounds **1** or **2** respectively as starting materials, as shown in Scheme 2 and were obtained in reasonable yield (in parenthesis) with the exception of **20**, that was obtained in low yield <30%. The method used was dependent on the availability of the S-blocked glutathione diacid. In the case of compounds **11 -13** and **19** the availability of the S-blocked glutathione diacid[34] resulted in the use of Method A, N-acylation followed by the $SOCl_2$ esterification of the compound in BuOH to the diester. Compounds **3-10**, **15-18** and **20** were synthesized by the dimethylaminopyridine (DMAP) [34] catalysed

N-acylation or N-arylation of compound **2** as shown in Method B. Compound **14** was prepared by the reaction of N,N-bis[2-oxo-3-oxazolidinyl]phosphinic chloride (BOP-Cl) with indole propionic acid and compound **2** by a variation of Method B. Compound **2** was obtained as an oil, by the esterification of S-2,4-dinitrophenylglutathione **1**, in BuOH/HCl(g) and used without further purification in the above studies. Analytically pure compound **2** was obtained by the N-deprotection of compound **16** using TFA/CH_2Cl_2. The use of either $SOCl_2$/BuOH or BuOH/HCl(g) in esterification was based on the regioselectivity of these reagents with glutathione derivatives.[34]



Scheme 2. General synthetic route to N-blocked -S-2,4-dinitro phenylglutathione di-butyl esters.

2.2. Screening against T. b. rhodesiense, L. donovani & T.cruzi.

The N-substituted glutathione diesters investigated in this study were based on S-(2,4-dinitrophenyl)glutathione (GSDNP; **1**), modified by N-acylation/N-arylation of the amino group with different alkyl and aryl substituents of varying size and volume **3-8**, **15-17**, aryl or aryl/alkyl substituents **9-13**, tricyclo substituent **18**, and heterocyclic substituents **14** and **20**. The N-substituted glutathione derivatives were then converted to the corresponding dibutyl esters by thionyl chloride esterification in butan-1-ol. Compound **8** was prepared because of the importance of N-myristoylation to the survival of *T.b.brucei* [35] in post-transitional modification, membrane targeting and the biological activity of many important proteins. Compound **20** the N-(4-amino-6-chloro-1,3,5-triazin-2-yl)-S-(2,4-dinitrophenyl) glutathione di-n-butyl ester derivative isolated instead of the desired compound, N-(2,4-diamino-1,3,5-triazin-2-yl)-S-(2,4-dinitrophenyl) glutathione di-n-butyl ester derivative, to act as a substrate of the P2 purine transporter.[9] Variation of N-substituents on the S-(2,4-dinitrophenyl)glutathione dibutyl ester scaffold **2-20** (Scheme 1) produced some changes in the inhibitory effects of these compounds against *T.b.rhodesiense* (>1μM). Using the inhibitory activity of compound **2**(1.88 μM), with an unsubstituted nitrogen as a reference, the N-substituents on compounds **4**=**11**>**17**>**3**>**8** were found to weaken the inhibitory properties of these peptides against *T.b.rhodesiense* whilst for **16**<**12**<**14**<**13**<**18**<**9**<**10**=**15**<**5**=**19**<**7**<**6**<**2** they enhanced activity. Of the N-substituents the t-Boc group **16** was found to be the most active glutathione diester derivative(see Table 1) with an ED_{50} value of 0.22 μM; 8.5 fold more active than **2**(see Table 1) with compounds **12-14** being the most active N-aryl/alkyl substituents. As previously observed glutathione compounds with a log P of 4.0-7.0 showed the highest *in vitro* activity against *Trypanosoma*.[26,27] Compound **8** containing the N-myristoyl group(log P: 8.73) proved no exception and showed significantly reduced activity versus **2**. Compound **20**, although similar to the substrates of the P2 receptor showed no enhanced activity in the inhibition of trypanosome growth. An effect of the variation of groups on the N-site was on the cytotoxicity of these compounds, measured using KB cells as a standard cytotoxicity index and cited in many texts,[36,37,38] which were lower in comparison to the unmodified compound **2** especially in the case of compound **3-5**, **8-11** and **17-20**.

Table 1. **The biological properties of glutathione derivatives against *T. b. rhodesiense*[d], *L.donovani*[e] & *T.cruzi*[f].**

Compound ID	ED_{50} (μM)[d] *T. b. rhodesiense*	ED_{50} (μM)[c,e] *L. donovani*	ED_{50} (μM)[c,f] *T. cruzi*	ED_{50} (μM) *KB cells*[b]	*Relative Tox KB/T.b.rhod*	*Log P*[a]	*ΔΔG kJ mol⁻¹*
1	>30	>30	>30				
2	1.88	19.9/t	t/+	107.4	57.1	2.25	0.0
3	6.52	NA	37.8	>300	>46	2.7	-3.1
4	2.15	>30(5.4)	30.4	>300	>139	3.35	-0.3
5	0.73	NA	30.8	>300	>411	3.61	2.3
6	1.16	>30(4.6)	>30(35.6)	59.6	51.4	4.1	1.2
7	1.02	NA	NA	84	82.3	4.59	1.5
8	32.6	NA	>30	>300	>9.2	8.73[a]	-7.1
9	0.55	NA	22.9	>300	>545	4.1	3.05
10	0.6	NA	>30	>300	>500	4.43	2.8
11	2.02	>30(18.4)	>30(29.7)	>300	>148	4.9	-0.2
12	0.28	NA	>30(33.4)	34.4	123	4.68	4.7
13	0.42	>30 (36)	NA	95.2	227	5.06	3.7
14	0.30	NA	35.01	26	87	3.45	4.55
15	0.6	NA	8.4	83.4	139	4.06	2.8
16	0.22	9.9 ±2.9	16.5	70.4	320	4.71	5.3
17	4.38	NA	12.04	250.2	57.1	4.55	-2.1
18	0.5	10.06	4.5	>300	>600	6.74[a]	3.3
19	0.72	NA	NA	>300	>417	4.73	2.4
20	18.8	NA	NA	>300	>15.9	3.27	-5.7

[a] Calculated using *xlogP* [60]

[b] Human oral pharyngeal carcinoma; 72h assay; 3 replication expts.

[c] NA: not active.

[d] Pentamidine isethionate control (ED50, 0.33 nM); 72h assay; 3 replication expts.

[e] Sodium stibogluconate ($NaSb^V$) control. ED50 33ugSb^Vml; 120h assay; 3 replication expts.

[f] Benznidazole control. ED50 4.4uM; 72h assay; 3 replication expts.

[g] $\Delta\Delta G = RTln\Delta Ki$($\Delta$Ki ~$\Delta ED_{50}$ the ratio(*Ki:Cpd ID/ Ki: 2*)

t toxic to macrophages; *t/+* toxic to macrophage + parasite

The relative toxicity expressed as the ratio of the inhibition of KB cells/ *T. b. rhodesiense*, was highest for compounds **5**, **9**, **10, 18** and **19** where the ratio was >400. Of the compounds tested only compound **2** was observed visually by optical microscopy to be toxic (t) to the macrophage host at a concentration of 30μM, as evidenced by lysis of the cell membrane (see Table 1). The toxicity of compound **2** may be due to its amphiphilic nature allowing it to function as a cationic surfactant, disrupting the cell membrane of the macrophage. Compounds **16**, **12-14** showed enhanced parasitic inhibition against *T. b. rhodesiense* that can be related to the interaction of the compound with the parasitic membrane. Assuming that the anti-parasitic activity (ED_{50}) observed is primarily related to binding (K_i) of the compounds with the parasitic membrane then we can calculate an interaction

energy for binding at 25 °C, using $\Delta\Delta G = RT\ln\Delta Ki$ ($\Delta Ki \sim \Delta ED_{50}$ the ratio(*Ki:Cpd ID/ Ki: 2*)) (see Table 1). For compounds **5-7**, **9-10**, **12-16**, **18-19** this agrees with the interaction of N-substituents with the parasitic cell membrane via weak hydrophobic forces i.e. London and v*an der Waals* forces (<1-15.0 kcal mol^{-1}) to enhance binding.

Compounds **16** & **18** in addition to showing good parasitic activity against *T.b.rhodesiense*, also showed appreciable activity against *L.donovani* both at ~10 μM and *T.cruzi* of 16.5 and 4.5 μM respectively. Compound **18,** appears to be an interesting compound with broad spectrum antiparasitic activity against *T.b.rhodesiense*, *L.donovani* and *T.cruzi* and a possible candidate for further development.

2.3 QSAR analysis of data.

QSAR analysis, of the ED_{50} results against *T. b. rhodesiense*, were undertaken using the CODESSA Pro program[39] to determine an inhibition model. Satisfactory multi-linear regression models were obtained using the structural descriptors calculated solely using the program. Of the compounds in Table 1, only the 18 related compounds **2-20** were used in the data set to generate an initial two descriptor multi-linear regression QSAR model with R^2=0.59 and F=11.7. The data set of compounds was then reduced to improve the statistics of this model, by the stepwise removal of outliers **16**, **15**, **3**, **5** and **18** in the order given, to obtain equation 1. Further reduction of the data set did not significantly improve the statistics of the two descriptor QSAR model identified, relating Log ED_{50} negatively to the average number of carbon atoms, N_C and S_{ZX}, the ZX shadow the maximum molecular size of the compound in the ZX plane, equation 1.

$$\text{Log } ED_{50} = -35.5\, N_C + 0.045\, S_{ZX} + 5.85 \qquad (1)$$

N=14, R2 = 0.90, F=49.7, s2= 0.046

The correlation between the predicted and observed log ED50 using equation 1 is shown in Fig. 1**.**

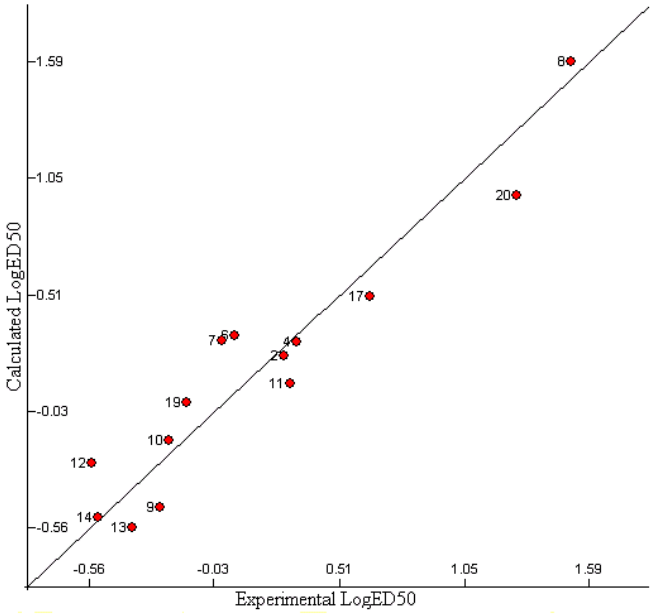
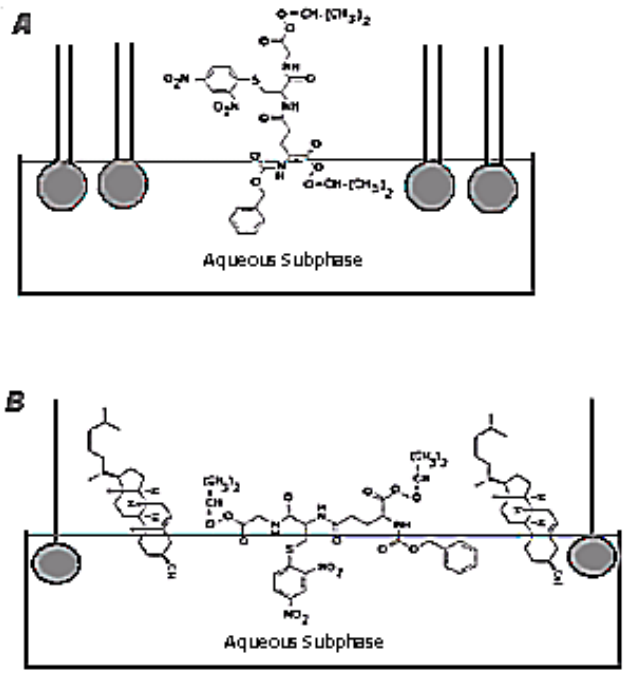


Figure 1. The correlation between calculated and experimental, log ED_{50} values using the two descriptor model (1).

The QSAR model, equation 1, contains a constitutional descriptor, the average number of carbon atoms, N_C that dominates the equation based on its coefficient size and is possibly related to saturation and the hydrophobic character of the compound and its negative association in the equation, a reflection of its solubility in solution/ membrane.[40] The geometrical descriptor, S_{ZX}, the ZX shadow is positive and based on its smaller coefficient, weakly opposes this character and is related to the maximum molecular size and orientation of the compound in the ZX plane.[41] The model was used to predict an ED_{50} value of 7.8 μM for N-(2,4-diamino-1,3,5-triazia-2-yl)-S-(2,4-dinitrophenyl)glutathione di-n-butyl ester that eluded preparation and resulted in the isolation of compound **20**. Outliers **16**, **15**, **5** and **18** occurred in a cluster above the correlation determined by equation 1, indicating that the model underestimated their ED_{50} values to that determined experimentally. Compounds **16**, **15**, **5** and **18** share a common feature that the substituent present contains either a secondary (iso-propyl or t- butyl) substituent suggesting that the N_C descriptor underestimates the hydrophobicity of branched carbon compounds. The membrane binding properties of these type of compounds have been confirmed by LB-trough studies on model membranes that indicated they formed stable monolayers in aqueous solutions with the orientation of the peptide at the air/water interface in the ZX/ZY plane and its orientation/molecular conformation being modulated by the specific composition of the lipids forming the monolayer.[31] In neat membranes composed of phospholipids and dioctadecyl-dimethylammonium bromide (DODAB), N-blocked-S-2,4-dinitrophenylglutathione diesters adopt a confirmation in the ZX/ZY plane with the peptide carbonyl group backbone parallel to the air-water interface[31] and peptide backbone perpendicular to the air –water interface (Scheme 3A). However in monolayers made of sphingomyelin-cholesterol that resemble raft membrane structures, the peptide conformation was found to be preserved to that observed in the aqueous phase, with the C=O group of the peptide backbone being perpendicular to the air-water interface (Scheme 3B). This radical change in conformation adversely affects the proportion of peptide spanning the membrane/aqueous solution interface that relates to binding and helps explain the specificity of these types of compounds.



Scheme 3. A model of the molecular orientation of the peptide in (A) monolayer made with phospholipids with two aliphatic

chains (B) sphingomyelin-cholesterol monolayer . The relative sizes of molecules are only illustrative and out-of-scale.

Raft like structures have been proposed to be specifically enriched in the flagellar membrane of *T.b.brucei* [42] and play an important role in both sequestering and organizing specialized protein complexes, some related to the transport functions of the parasite and its viability to scavenge metabolites.

2.4. HPLC and optical microscopy studies

HPLC and optical microscopy was used to ascertain the location and fate of the *T. b. brucei* inhibitor, N-benzyloxycarbonyl-S-(4-bromobenzyl)glutathione dimethyl ester. N-Benzyloxy carbonyl-S-(4-bromobenzyl)glutathione dimethyl ester, although not the most inhibitory derivative identified against *T. b. brucei*, (40% (30 μM)) was chosen for use in the study as the higher concentrations used aided its detection by absorbance, using HPLC-UV. Exposure of N-benzyloxycarbonyl-S-(4-bromobenzyl) glutathione dimethyl ester (50 μM) with *T.b.brucei* on examination by optical microscopy showed changes in the parasitic cytoskeleton, firstly with immobility of the parasite after 1 min, swelling of the parasite head after 5 min and a circular appearance after 30-40 mins, in which the particles inside the cell showed a high degree of swirling motion similar that seen with H_2O_2 propelled nanomotors ,[43] that slowed down after cell death and lysis, after an hour (see Fig. 2).

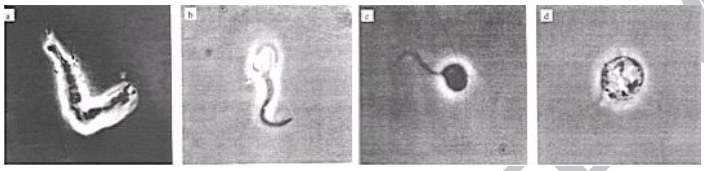


Figure 2. Morphological changes observed for *T. b. brucei* on exposure to N-benzyloxycarbonyl-S-(4-bromobenzyl)glutathione dimethyl ester (50 μM) by microscopy (magnification x 1000) and the recording of images at different times. (a) 1 min (b) 5 min (c) 20 min (d) 30 min.

The time-dependent decrease of the extracellular concentration of N-benzyloxycarbonyl-S-(4-bromobenzyl)glutathione dimethyl ester over 120 mins is not reflected in the observed intracellular change in the concentration of the diester (Fig. 3) which is maximal after 5 mins, indicating that extracellular adsorption is not responsible for the intracellular concentration of the compound but an alternative active mechanism of uptake.

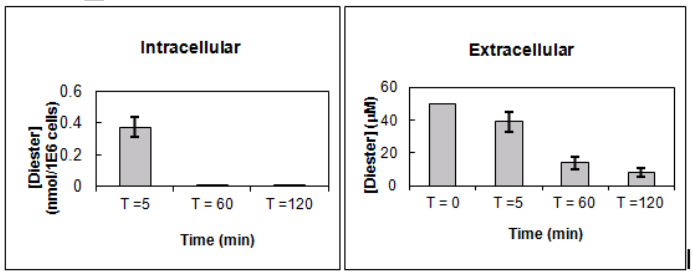


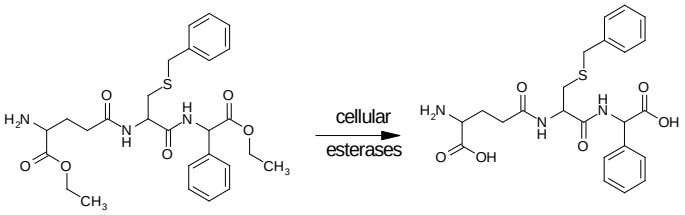
Figure 3. HPLC-UV analysis of extracellular and intracellular concentrations of N-benzyloxycarbonyl-S-(4-bromobenzyl) glutathione dimethyl ester in *T. b. brucei* cells incubated with 50 μM of the drug (HMI-9/10 %) at 37 °C. Error bars represent standard deviations for triplicate determinations.

The fate of the compound in the parasitic membrane was ascertained by treatment of pelleted cells with 1M NaCl or 6M urea and HPLC-UV analysis of the released compound in comparison with standards. The treatment of membranes with 1M NaCl led to the release of N-benzyloxycarbonyl-S-(4-bromobenzyl) glutathione diester and monoester from the membrane in the % ratio 76:24 into solution confirming the diesters susceptibility to hydrolysis. The corresponding 6M urea treated sample led to the release of N-benzyloxycarbonyl-S-(4-bromobenzyl) glutathione diester, monoester and diacid from the membrane into solution in the % ratio 80: 8: 12 indicating the diacid to be tightly associated with the membrane.

Discussion

The results of the present study provide the basis for a QSAR model, with good statistical significance, to describe the inhibitory activity of N-substituted glutathione diester based anti-trypanosomal compounds. Previous QSAR studies on diester derivatives of these peptides proposed a model that bears some similarities to equation 1, where log ($1/ED_{50}$) was related negatively to log P and positively to E_s, the degree of branching and indirectly to molecular size. In that study a model was proposed in which these compounds inserted into the membrane of *T. b. brucei* cells via weak lipophilic forces, compromising structural and membrane fluidity.[26] The studies reported here further support that view, with the N-substituents of glutathione contributing to a small increase in lipophilic binding energy of 1.2-5.3 kJ mol^{-1} (0.29-1.26 kcal mol^{-1}). Model membrane studies indicated that the confirmation of the peptide is preserved to that in the aqueous phase in raft membrane structures (Scheme 3B), which is specifically enriched in the flagellar membrane of *T.b.brucei* [42]. The anti-trypanosomal inhibitory property of glutathione derivatives appear to be related to lipophilicity and molecular size/orientation of the peptide which is related to binding/affinity of the compound and the composition of the membrane. The bloodstream form trypomastigote is covered in a dense protective coat of variant surface glycoproteins, VSGs (variant surface glycoproteins)[44]. The flagellar pocket,[45] located at the base of the flagellum occupies 2-3% of the total plasma membrane and is the only gap in the parasites defenses where nutrient uptake by receptor mediated endocytosis (RME) and secretion by exocytosis occurs and is enriched with raft like membrane structures. Microscopy and HPLC uptake studies on exposure of the compound to bloodstream stage *T. b. brucei* cells suggests that intracellular uptake of the inhibitor occurs quickly via an active transport process that results in the observed immobilization of the parasite within 1 min of exposure to the compound. Nutrient uptake via binding to the flagellar pocket, is the most likely process to explain the rapid intracellular uptake of the compound, after 5 mins (Fig 3). The rate of endocytosis in *Trypanosoma brucei* is equivalent to the internalization of the entire flagellar pocket membrane every 1-2 minutes[46], the same time frame observed for the immobilization of the parasite and the maximal intracellular concentration of compound. Endocytosis has been recently demonstrated as a bona fide drug target,[47] in the African trypanosome, *Trypanosoma brucei*, it is intimately involved in maintaining homeostasis of the cell surface proteome, morphology of the flagellar pocket and *in*

vitro, its shutdown is associated with rapid cell death, as in this study (Fig. 2). The effect of intracellular uptake of glutathione diesters by endocytosis probably results in the inhibition of the MRPA efflux pump and/or TST (trypanothione transferase) protein, the former identified as an inhibitory target of these compounds based on the inhibition of a strain of *T.b.brucei 247* over-expressing the *TbMRPA* protein by 50-100x fold[25]. Both proteins acts in synergy and are responsible for the removal of endogenous electrophiles and exogenous compounds from the cytosol which otherwise would cause toxicity and cell death by creating oxidative stress.[32] Ezatiostat hydrochloride (TLK199; γ-glutamyl-S-(benzyl)cysteinyl -R(-)-phenyl glycine diethyl ester) a glutathione analog diester prodrug is also an inhibitor of the multidrug resistance associated protein-1 (MRP-1)[48] and its de-esterified product γ-glutamyl-S-(benzyl)cysteinyl - R(-)-phenyl glycine (TLK117) both an inhibitor of both the MRP-1 protein and glutathione transferase (GST) enzymes(see Scheme 4).[48,49]



TLK199 TLK117

Scheme 4. Structures of TLK199 and TLK117, inhibitors of the multidrug resistance protein, (MRP-1).

The slow decrease in the extracellular concentration of the compound over 120 mins (see Fig. 3) is a result of its insertion into the membrane bilayer via hydrophobic adsorption compromising cytoskeleton integrity. However as cell death occurs after 30 mins, see Fig 2 this suggests that it is not the major process and endocytosis is the only process consistent with the observed intracellular concentration of the compound, the cell's rapid immobilization and the short time scale for cell death. P-glycoprotein (P-gp/ABCB1), is the model cell membrane ABC transporter [50,51] where high P-gp inhibitory potency has been associated with high lipophilicity and an interaction with the transporter via the membrane bilayer.[52,53]

The direct exposure of the compound to motile *L. donovani*, extracellular promastigote stage parasites, *in vitro,* showed no inhibitory activity over a 2h period indicating that active uptake of the peptide did not occur due to differences in feeding between the extracellular promastigote and amastigote stages of these different parasites. The major difference in the role of the flagellar pocket/membrane of the extracellular form of *T.b.brucei* with *L. donovani* is its importance for parasitic survival due to its restrictive metabolic capability which renders it dependent on salvage pathways and receptor mediated scavenging of metabolites from the host via the flagellar pocket membrane. *T.b. brucei* requires inosine monophosphate (IMP), cholesterol, lipoproteins, serum components and lipids [56] from the host to multiply. The methodology for *L. donovani* and *T. cruzi* assays are similar, using murine peritoneal macrophages as host cells. However, *Leishmania* amastigotes reside in a parasitophorous vacuole within the cell [54] and this is reflected in the 5-day assay as the antimonial control requires a longer period to reach the intracellular, intra-vacuale amastigote whereas *T. cruzi* amastigotes are in the cytoplasm[55] and so only require a three day period for the benznidazole control to be effective. The longer time taken for drugs to reach the intracellular amastigote in the case of *L. donovani* and *T. cruzi* assays may result in the hydrolysis products of the diesters, the monoester and diacid being the inhibitor of the amastigote stage of these parasites.

In conclusion, an understanding of the therapeutic and toxic effects of lipophilic glutathione diesters and their ability to target the flagellar pocket/membrane of *T. b. brucei* will aid selectivity and the further development of new compounds for HAT chemotherapy.

4. Experimental

4.1. Chemistry

4.1.1. General

Precursors and solvents were purchased commercially and purified appropriately. GSH, DMAP, CbzCl, 3-phenylpropionyl chloride, *tert*-butylacetyl chloride and 4-methylvaleric acid were obtained from the Avocado Chemical Co. Ltd (UK). Indolepropionic acid, isobutyryl chloride and phenylacetyl chloride from Lancaster (UK). Di-*tert*-butyl dicarbonate, 1-adamantyl fluoroformate, phenoxyacetyl chloride, trimethylacetyl chloride, 2, 5-dimethoxytetrahydrofuran (DMT) and N,N-bis[2-oxo-3oxazolidinyl] phosphonic chloride (BOP-Cl) were obtained from the Aldrich Chemical Co. Ltd. (UK). Glutathione derivative, N-(benzyloxycarbonyl)-S-(4-bromobenzyl)glutathione dimethyl ester was provided by D'Silva et al.[34] Analytical thin-layer chromatography was performed on Merck silica gel $60F^{254}$ aluminium backed TLC plates and were visualized by fluorescence quenching under UV light or I_2 staining. Preparative thin-layer chromatography (PTLC) was performed on Analtech silica gel GF 2000 microns and were visualized by fluorescence quenching under UV light. Melting points were determined on an Electrothermal apparatus and are reported uncorrected ^{1}H NMR spectra were recorded on a Joel 270 MHz FT-NMR spectrometer, and chemical shifts are reported in parts per million downfield with reference to tetramethysilane. Mass spectra were recorded by B. Stein at the EPSRC Mass Spectrometry service centre, Swansea for EI and CI(NH_3) spectra, on a VG quarto II triple quadropole mass spectrometer and accurate mass Liquid Secondary Ion Mass Spectrometry (LSIMS) measurements on a Finnigan MAT 900 XLT using a Cs+ ions to ionize. Elemental analysis was performed at the Micro Analytical Service, Manchester Univ, UK.

4.1.2. N-Blocked-S-(2,4-dinitrophenyl)glutathione di-n-butyl ester derivatives

The preparation of the compounds of this series is illustrated in **Scheme 1** by the general methods reported below:

Method A : S-(2,4-Dinitrophenyl)glutathione **(1)** was dissolved in MeOH and then Et_3N (3eq), required acyl halide (1.5-2eq) and dimethylaminopyridine (0.05eq). The reaction mixture was stirred overnight at RT, evaporated, acidified to pH 2 with dilute HCl (10%) and the organic layer was then extracted with AcOEt. After drying with $MgSO_4$, filtration and evaporation, the residue was re-suspended in n-butanol and thionyl chloride (2.2eq) was added dropwise. The solution was left stirring overnight at RT and then evaporated. The residue was usually retriturated with

Et_2O and the solid refrigerated, filtered under vacuo and purified by preparative chromatography if required.

Method B : Et_3N (1.1 eq), required acyl halide (1.1eq) and dimethylaminopyridine (0.05eq) were added to a solution of S-(2,4-dinitrophenyl)glutathione di-n-butyl ester **(2)** in MeOH. The reaction mixture was stirred overnight at RT, evaporated and the organic layer was then extracted with AcOEt. After drying with with Et_2O and the solid refrigerated, filtered under vacuo and purified by preparative chromatography if required.

4.1.2.1. S-(2,4-Dinitrophenyl)glutathione (**1**) was prepared according according to Sokolovsky et al [57].

4.1.2.2. S-(2,4-Dinitrophenyl)glutathione di-n-butyl ester (2) was prepared by N-deprotection of **16** with trifluoroacetic acid (2eq) in CH_2Cl_2. The reaction mixture was left stirring for 18hrs at RT. The solvent was then removed under vacuo and the organic layer extracted with AcOEt. The aqueous phase was then neutralised with NaOH (10%) and the organic layer extracted with $CHCl_3$. The latter organic layer was then washed with brine and dried with $MgSO_4$. After filtration and evaporation of the solvent, the residue was triturated with Et_2O. Purification by PTLC ($CHCl_3$/MeOH : 9/1) gave the product as yellow crystals. Yield 50%; mp 98-100°C; ^{1}H NMR (D_6MSO) δ 9.0 (s, 1H), 8.8 (m, 1H), 8.6 (t, J= 9.5 Hz, 2H), 8.1 (d, J= 9.5 Hz, 1H), 4.8 (m, 1H), 4.1-3.9 (m, 5H), 3.95 (m, 2H), 3.6 (m, 2H), 2.3 (t, J= 7.8 Hz, 2H), 2.0 (m, 2H), 1.6 (m, 4H), 1.4 (m, 4H), 1.0 (t, J= 7.0 Hz, 6H). Ir (cm^{-1}) *v* 3289 (NH), 2958 (CH), 1640 (C=O), 1529 (NO_2). Anal. Calcd for $C_{24}H_{35}N_5O_{10}S$, $1.3H_2O$: C, 47.27; H, 6.12; N, 11.5. Found: C, 46.98; H, 5.98; N, 11.77.

4.1.2.3. N-(Acetyl)-S-(2,4-dinitrophenyl)glutathione di-n-butyl ester (3) was prepared according to Method B using acetic anhydride. Purification by PTLC ($CHCl_3$/MeOH : 19/1) gave the product as a yellow crystals. Yield 45%; mp 140-142°C**;** ^{1}H NMR (D_6MSO) δ 8.95 (s, 1H **),** 8.8 (m, 1H), 8.65 (m, 1H), 8.5 (d, J= 9.6 Hz, 1H), 8.3 (m, 1H), 8.05 (m, 1H), 4.7 (m, 1H), 4.25 (m, 1H), 4.1 (m, 4H), 3.9 (m, 2H), 3.6 (m, 2H), 2.3 (m, 2H), 1.9-1.8 (m, 5H), 1.6 (m, 4H), 1.4 (m, 4H), 1.0 (m, 6H). Ir (cm^{-1}) *v* 3279 (NH), 2962 (CH), 1641 (C=O), 1529 (NO_2). LSIMS *m/z* 628.2 ((M + H)$^+$, 43), 650.2 ((M + Na)$^+$, 100); HRLSIMS: Calc for $C_{26}H_{37}N_5O_{11}S$: 628.2289. Found: 628.222**.** Anal. Calcd for $C_{26}H_{37}N_5O_{11}S$: C, 49.74; H, 5.95; N, 11.16. Found: C, 49.56; H, 5.96; N, 10.95.

4.1.2.4. N-(Methoxycarbonyl)-S-(2,4-dinitrophenyl) glutathione di-n-butyl ester (4) was prepared according to Method B using methylchloroformate (1.1 eq). Trituration with Et_2O gave the expected product as yellow crystals. Yield 61%; mp 124-126°C; ^{1}H NMR (D_6MSO**)** δ 8.95 (s, 1H), 8.7 (m, 1H), 8.5 (m, 2H), 8.1 (d, J=9.4 Hz, 1H**),** 7.6 (d, J= 7.0 Hz, 1H**),** 4.75 (m, 1H), 4.1 (m, 5H), 3.9 (d, J= 6.8 Hz, 2H), 3.6 (s, 3H), 3.4 (m, 2H), 2.3 (t, J= 8.1 Hz, 2H), 2.0-1.8 (m, 2H), 1.6 (m, 4H), 1.4 (m, 4H), 1.0 (t, J= 6.8 Hz, 6H). Ir (cm^{-1}) *v* 3294 (NH), 2938 (CH), 1641 (C=O), 1530 (NO_2). LSIMS *m/z* 644.0 ((M + H)$^+$, 100), 666.0 ((M + Na)$^+$, 64); Anal. Calcd for $C_{26}H_{37}N_5O_{12}S$: C, 48.51; H, 5.8; N, 10.89. Found : C, 48.25; H, 5.83; N, 10.89.

4.1.2.5. N-(Isobutyryl)-S-(2,4-dinitrophenyl)glutathione di-n-butyl ester (5) was prepared according to Method B using isobutyryl chloride. Purification by PTLC ($CHCl_3$/MeOH : 19/1) gave the product as yellow crystals. Yield 41%; mp 189-192°C; ^{1}H NMR (D_6MSO) δ 8.85 (s, 1H), 8.6 (t, J= 5.8 Hz, 1H**),** 8.4 (d, J= 8.6 Hz, 2H), 8.1 (d, J=7.5 Hz, 1H), 7.98 (d, J= 8.6 Hz, 1H), 4.65 (m, 1H), 4.2 (m, 1H), 4.0 (m, 4H), 3.75 (d, J= 5.7 Hz, 2H), 3.5 (m, 2H), 2.4 (m, 1H), 2.2 (t, J= 7.9 Hz, 2H), 1.9-1.7 (m, 2H), 1.5 (m, 4H), 1.3 (m, 4H), 1.0 (d, J= 6.6. Hz, 6H), 0.85 (t, J= 7.5 Hz, 6H). Ir (cm^{-1}) *v* 3283 (NH), 2960 (CH), 1642 (C=O), 1531 (NO_2). LSIMS *m/z* 656 ((M + H)$^+$, 17), 678 ((M + Na)$^+$, 15); HRLSIMS: Calc for $C_{28}H_{42}N_5O_{11}S$: 656.2602. Found: 656.2582; Anal. Calcd for $C_{28}H_{41}N_5O_{11}S$: C, 51.28; H, 6.31; N, 10.69. Found:C, 51.04; H, 6.41 ; N, 10.61. .

4.1.2.6. N-(Isovaleryl)-S-(2,4-dinitrophenyl)glutathione di-n-butyl ester (6) was prepared according to Method B using isovaleryl chloride prepared by overnight reflux of isovaleric acid with thionyl chloride. Purification by PTLC ($CHCl_3$/MeOH; 19/1) and trituration with Et_2O gave the product as yellow crystals. Yield 70%; mp 133-135°C; ^{1}H NMR (D_6MSO) δ 9.0 (s, 1H), 8.8 (m, 1H), 8.6 (m, 2H), 8.3 (m, 1H), 8.1 (d, J=9.6 Hz, 1H), 4.7 (m, 1H), 4.3 (m, 1H), 4.1(m, 4H), 4.0 (m, 2H), 3.6 (m, 2H), 2.3 (m, 2H), 2.1-1.8 (m, 3H), 1.6 (m, 4H), 1.4 (m, 4H), 1.0 (m, 12H). Ir (cm^{-1}) *v* 3280 (NH), 2960 (CH), 1638 (C=O), 1525 (NO_2). LSIMS *m/z* 670.3 ((M + H)$^+$, 100), 692.3 ((M + Na)$^+$, 95); Anal. Calcd for $C_{29}H_{43}N_5O_{11}S$, $3H_2O$: C, 48.12; H, 6.82; N, 9.68. Found: C, 47.82 ;H, 6.75 ; N, 9.92.

4.1.2.7. N-(4-Methylvaleryl)-S-(2,4-dinitrophenyl)glutathione di-n-butyl ester (7) was prepared according to Method B using 4-methylvaleryl chloride obtained by overnight reflux of 4-methylvaleric acid with thionyl chloride. Purification by PTLC ($CHCl_3$/MeOH : 19/1) and trituration with Et_2O gave the product as orange crystals. Yield: 65%; mp 148-150°C; ^{1}H NMR (D_6MSO) δ 9.0 (s, 1H), 8.8 (m, 1H), 8.6 (m, 2H), 8.3 (m, 1H), 8.1 (d, J= 9.6 Hz, 1H), 4.7 (m, 1H), 4.3 (m, 4H), 4.1(m, 4H), 4.0 (m, 2H), 3.6 (m, 2H), 2.3 (m, 2H), 2.2 (m, 2H), 2.0-1.8 (m, 2H), 1.6 (m, 5H), 1.4 (m, 6H), 1.0 (m, 12H). Ir (cm^{-1}) *v* 3284 (NH), 2925 (CH), 1637 (C=O), 1532 (NO_2). LSIMS *m/z* 684.5 ((M + H)$^+$,100), 706.5 ((M + Na)$^+$, 90): Calcd for $C_{30}H_{45}N_5O_{11}S.0.5H_2O$: C, 52.04; H, 6.65; N, 10.11. Found: C, 51.83; H, 6.70 ; N, 9.94. .

4.1.2.8. N-(Myristoyl)-S-(2,4-dinitrophenyl)glutathione di-n-butyl ester (8)) was prepared according to Method B using myristoyl chloride. Purification by PTLC ($CHCl_3$/MeOH: 19/1) gave the product as a yellow crystals. Yield: 49 %; mp 172-174 °C; ^{1}H NMR (D_6MSO) δ 8.85 (d, J= 2.3 Hz, 1H), 8.6 (t, J= 5.7 Hz, 1H), 8.4 (dd, J= 8.8, 2.3 Hz, 2H), 8.1 (d, J=7.7 Hz, 1H), 7.98 (d, J= 8.8 Hz, 1H), 4.6 (m, 1H), 4.2 (m, 1H), 4.0 (m, 4H), 3.85 (d, J= 5.6 Hz, 2H), 3.5 (m, 2H), 2.2 (t, J= 7.6 Hz, 2H), 2.1 (t, J=7.5 Hz, 2H, 1.9-1.7 (m, 2H), 1.5 (m, 6H), 1.25-1.22 (m, 24H), 0.8 (t, J= 7.3 Hz, 9H). Ir (cm^{-1}) *v* 3282 (NH), 2960 (CH), 1643 (C=O), 1531 (NO_2). LSIMS *m/z* 796 ((M + H)$^+$, 45), 818 ((M + Na)$^+$, 58): Anal Calcd for $C_{38}H_{61}N_5O_{11}S$. H_2O: C, 56.07; H, 7.80; N, 8.60 . Found: C, 56.18; H, 7.73; N, 8.91.

4.1.2.9. N-(Benzoyl)-S-(2,4-dinitrophenyl)glutathione di-n-butyl ester (9) was prepared according to Method B using benzoyl chloride. Trituration with Et_2O gave the product as yellow crystals. Yield 72%; mp 144-146°C; ^{1}H NMR (D_6MSO**)** δ 8.95 (s, 1H), 8.8 (d, J=8.0 Hz, 1H), 8.7 (m, 1H), 8.5 (m, 2H), 8.1 (d, J=8.0 Hz, 1H**),** 7.95 (d, J=8.0 Hz, 2H), 7.55 (m, 3H**),** 4.75 (m, 1H), 4.5 (m, 1H), 4.1 (t, J=7.1 Hz, 4H), 3.9 (d, J=7.1. Hz, 2H), 3.6 (m, 2H), 2.4 (m, 2H), 2.2-2.0 (m, 2H), 1.6 (m, 4H), 1.4 (m, 4H**),** 0.95 (t, J=8.5 Hz, 6H). Ir (cm^{-1}) *v* 3281 (NH), 2936 (CH), 1644 (C=O), 1533 (NO_2). LSIMS *m/z* 690.2 ((M + H)$^+$, 68), 712.2 ((M + Na)$^+$, 32): Anal. Calcd for $C_{31}H_{39}N_5O_{11}S$: C, 53.97 ; H, 5.70; N, 10.16 . Found: C, 54.56; H, 5.42; N, 10.02.

4.1.2.10. N-(Phenoxyacetyl)-S-(2,4-dinitrophenyl)glutathione di-n-butyl ester (10) was prepared according to Method B using phenoxyacetyl chloride (1.1 eq). Purification by PTLC ($CHCl_3$/MeOH : 19/1) gave the expected product as yellow crystals. Yield 66%; mp 98-100°C; ^{1}H NMR (D_6MSO) δ 8.9 (s, 1H), 8.8 (m, 1H), 8.7 (t , J=7.9 Hz, 2H), 8.5 (d, J=9.5 Hz, 1H),

8.0 (d, J=9.5 Hz, 1H) **,** 7.4 (t, J=7.9 Hz, 2H), 7.0 (d, J=7.9 Hz, 3H), 4.8 (m, 1H), 4.6 (s, 2H), 4.4 (m, 1H), 4.1 (m, 4H), 3.95 (d, J=5.5 Hz, 2H), 3.6 (m, 2H), 2.3 (t, J=7.3 Hz, 2H), 2.1-1.95 (m, 2H), 1.7 (m, 4H), 1.4 (m, 4H), 1.0 (m, 6H). Ir (cm^{-1}) *v* 3284 (NH), 2960 (CH), 1643 (C=O), 1531 (NO_2). LSIMS *m/z* 720.2 ($(M + H)^+$, 43), 742.2 ($(M + Na)^+$, 27); HRLSIMS: Calc for $C_{32}H_{45}N_4O_{12}S$: 737.2816, Found: 737.2811 **;** Anal. Calcd for $C_{32}H_{41}N_5O_{12}S$: C, 53.39 ; H, 5.75; N, 9.73. Found: C, 53.28; H, 5.40; N, 9.63. .

4.1.2.11. N-(3-Phenylpropionyl)-S-(2,4-dinitrophenyl) glutathione di-n-butyl ester (11) was prepared according to Method A using 3-phenylpropionyl chloride. Trituration with Et_2O gave the product as yellow crystals. Yield 70%; mp 143-146°C; ^1H NMR (D_6MSO) δ 9.0 (s, 1H), 8.7 (m, 1H), 8.5 (m, 2H), 8.4 (d, J= 7.0 Hz, 1H), 8.1 (d, J=8.7 Hz, 1H), 7.4-7.2 (m, 5H), 4.8 (m, 1H), 4.3 (m, 1H), 4.1(m, 4H), 4.0 (d, J=7.0 Hz, 2H), 3.6 (m, 2H), 2.9 (t, J= 7.8 Hz, 2H), 2.4 (t, J= 7.0 Hz, 2H), 2.2 (t, J= 7.8 Hz, 2H), 2.0-1.8 (m, 2H), 1.6 (m, 4H), 1.4 (m, 4H), 1.0 (m, 6H). Ir (cm^{-1}) *v* 3294 (NH), 3058 (CH), 1644 (C=O), 1541 (NO_2). HRLISMS calc for $C_{33}H_{43}N_5O_{11}S$ 717.2679, Found 717.2669; Anal. Calcd for $C_{33}H_{43}N_5O_{11}S$: C, 55.21; H, 6.04; N, 9.76. Found: C, 55.20; H, 6.24; N, 9.83. .

4.1.2.12. N-(*trans*-Cinnamoyl)-S-(2,4-dinitrophenyl) glutathione di-n-butyl ester (12) was prepared according to Method A using *trans*-cinnamoyl chloride. Trituration with Et_2O gave the product as yellow crystals. Yield 82%; mp 150-155°C; ^1H NMR (D_6MSO) δ 9.0 (s, 1H), 8.7 (m, 1H), 8.6 (m, 3H), 8.1 (d, J= 9.7 Hz, 1H), 7.6 (d, J= 8.0 Hz, 2H), 7.5 (m, 4H), 6.8 (d, J=14.5 Hz, 1H), 4.8 (m, 1H), 4.4 (m, 1H), 4.2 (m, 4H), 4.0 (d, 2H), 3.6 (m, 2H), 2.3 (t, J=7.0 Hz, 2H), 2.1-1.9 (m, 2H), 1.6 (m, 4H), 1.4 (m, 4H), 1.0 (m, 6H). Ir (cm^{-1}) *v* 3241 (NH), 2943 (CH), 1656 (C=O), 1577 (NO_2). LISMS *m/z* 716.3 ($(M + H)^+$, 25), 738.3 ($(M + Na)^+$, 100); HRLISMS: Calc for $C_{33}H_{42}N_5O_{11}S$: 716.2601, Found: 716.2593 ; Anal. Calcd for $C_{33}H_{41}N_5O_{11}S$: C, 55.37; H, 5.78 ; N, 9.79. Found: C, 55.03; H, 6.04; N, 9.71.

4.1.2.13. N-(Benzyloxycarbonyl)-S-(2,4-dinitrophenyl) glutathione di-n-butyl ester (13) was prepared according to the previously described procedure[24].

4.1.2.14. N-(Indolepropionyl)-S-(2,4-dinitrophenyl) glutathione di-n-butyl ester (14) was prepared according to the method described by Cabre *et al* [58] using indolepropionic acid (1eq), N,N-bis[2-oxo-3-oxazolidinyl]phosphonic chloride (BOP-Cl) (1eq) as activator, Et_3N (1eq) and **2** (1eq) in a dichloromethane solution (~20ml). Purification by PTLC ($CHCl_3$/MeOH : 9 / 1) gave the product as orange crystals. Yield 62%; mp 145-150°C; ^1H NMR (D_6MSO) δ 10.9 (m, 1H), 9.0 (s, 1H), 8.8 (m, 1H), 8.7 (d, J= 9.0 Hz, 1H), 8.5 (d, 9.0 Hz, 1H), 8.4 (d, 9.0 Hz, 1H), 8.1 (d, 9.0 Hz, 1H), 7.6 (d, 9.0 Hz, 1H), 7.4 (d, 9.0 Hz, 1H), 7.0-7.2 (m, 3H), 4.7 (m, 1H), 4.3 (m, 1H), 4.1(m, 4H), 4.0 (m, 2H), 3.6 (m, 2H), 3.0 (m, 2H), 2.3 (m, 2H), 2.0-1.8 (m, 2H), 1.6 (m, 6H), 1.4 (m, 4H), 1.0 (m, 12H). Ir (cm^{-1}) *v* 3285 (NH), 2958 (CH), 1643 (C=O), 1531 (NO_2). ESIMS *m/z* 757 ($(M + H)^+$, 20), 779 ($(M + Na)^+$, 100), 755 ($(M - H)^-$, 80), 791 ($(M + Cl)^-$, 85); Anal. Calcd for $C_{35}H_{44}N_6O_{11}S.4H_2O$: C, 50.72; H, 5.32; N, 10.14. Found: C, 50.69; H, 5.26; N, 10.32. .

4.1.2.15. N-(Trimethylacetyl)-S-(2,4-dinitrophenyl) glutathione di-n-butyl ester (15) was prepared according to Method B using trimethylacetyl chloride (1.1eq). Trituration with Et_2O/ pet ether (40-60) gave the product as yellow crystals. Yield 50%; mp 88-90°C; ^1H NMR (D_6MSO**)** δ 8.9 (s, 1H), 8.6 (t, J=5.6 Hz, 1H), 8.5 (d, J= 7.9 Hz, 2H), 8.0 (d, J= 9.0 Hz, 1H), 7.75 (d, J= 7.9 Hz, 1H), 4.65 (m, 1H), 4.2 (m, 1H), 4.0 (m, 4H), 3.95 (d, J=5.1 Hz, 2H), 3.6 (m, 2H), 2.3 (m, 2H), 2.0-1.8 (m, 2H), 1.5 (m, 4H), 1.3 (m, 4H), 1.1 (m, 9H), 0.85 (t, J= 7.4 Hz, 6H). Ir (cm^{-1}) *v* 3285 (NH), 2918 (CH), 1642 (C=O), 1521 (NO_2). CIMS *m/z* 670.6 ($(M + H)^+$, 100), 687.6 ($(M + NH_4)^+$, 15) ; Anal. Calcd for $C_{29}H_{43}N_5O_{11}S$: C, 52.00; H, 6.48; N, 10.46. Found: C, 51.99; H, 6.52; N, 10.37.

4.1.2.16. N-(*tert*-Butoxycarbonyl)-S-(2,4-dinitrophenyl) glutathione di-n-butyl ester (16) was prepared according to Method B using di-*tert*-butyl dicarbonate (1eq). Purification by PTLC ($CHCl_3$/MeOH : 9/1) gave the expected product as yellow crystals. Yield 51%; mp 108-110°C; ^1H NMR (D_6MSO) δ 9.0 (s, 1H), 8.8 (m, 1H), 8.5 (m, 2H), 8.1 (d, J=8.7 Hz, 1H), 7.4 (d, J=8.7 Hz, 1H), 4.8 (m, 1H), 4.2-3.9 (m, 7H), 3.6 (m, 2H), 2.3 (m, 2H), 2.0-1.8 (m, 2H), 1.6 (t, J=7.8 Hz, 4H), 1.4 (m, 13H), 1.0 (t, J=7.8 Hz, 6H). Ir (cm^{-1}) *v* 3285 (NH), 2918 (CH), 1642 (C=O), 1521 (NO_2). LSIMS *m/z* 686.3 ($(M + H)^+$, 100), 708.3 ($(M + Na)^+$, 85) ; Anal. Calcd for $C_{29}H_{43}N_5O_{12}S$: C, 50.78; H, 6.32 ; N, 10.22. Found: C, 50.40; H, 6.28 ; N, 9.82.

4.1.2.17. N-(*tert*-Butylacetyl)-S-(2,4-dinitrophenyl)glutathione di-n-butyl ester (17) was prepared according to Method B using *tert*-butylacetyl chloride. Purification by PTLC ($CHCl_3$/MeOH : 9/1) gave the product as yellow crystals. Yield 45%; mp 118-120°C; ^1H NMR (D_6MSO) δ 9.0 (s, 1H), 8.8 (m, 1H), 8.5 (m, 2H), 8.2 (d, J=8.7 Hz, 1H), 8.1 (d, J=9.6 Hz, 1H), 4.8 (m, 1H), 4.3 (m, HN-CH-CH_2), 4.1 (m, 4H), 4.0 (d, J=7.0 Hz, 2H), 3.6 (m, 2H), 2.3 (m, 2H), 2.2 (s, 2H), 2.0-1.8 (m, 2H), 1.6 (t, 4H), 1.4 (m, 13H), 1.0 (t, 6H). Ir (cm^{-1}) *v* 3280 (NH), 2959 (CH), 1642 (C=O), 1531 (NO_2). HRLSIMS: Calc for $C_{30}H_{45}N_5O_{11}S$: 683.2836. Found 683.2841; Anal. Calcd for $C_{30}H_{45}N_5O_{11}S$: C, 52.70; H, 6.63; N, 10.24. Found: C, 52.41; H, 7.23, N; 10.34.

4.1.2.18. N-(Adamantyloxycarbonyl)-S-(2,4-dinitrophenyl) glutathione di-n-butyl ester (18) was prepared according to Method B using 1-adamantyl fluoroformate (1eq x 3). Purification by PTLC ($CHCl_3$/AcOH : 25/1) gave the product as yellow crystals. Yield 51%; mp 72-74°C; ^1H NMR (D_6MSO) δ 9.0 (s, 1H), 8.75 (m, 1H), 8.5 (d, J= 8.1 Hz, 2H), 8.05 (d, J=8.7 Hz, 1H), 7.35 (d, J=8.1 Hz, 1H), 4.75 (m, 1H), 4.15 (t, J= . Hz, 4H), 3.9 (d, J= 5.9 Hz, 3H), 3.6 (dd, J= 14.4, 5.9 Hz, 2H), 2.3 (bs, 3H), 2.1 (s, 2H), 2.0 (bs, 8H), 1.65 (bs, 10H), 1.4 (m, 4H), 0.95 (t, J= 8.0 Hz, 6H). Ir (cm^{-1}) *v* 3284 (NH), 2912 (CH), 1641 (C=O), 1514 (NO_2). Anal. Calcd for $C_{35}H_{49}N_5O_{11}S$. 0.5 H_2O: C, 54.43; H, 6.47; N, 9.06. Found: C, 54.39; H, 6.43 ; N, 9.1.

4.1.2.19. N,S-(2,4-Dinitrophenyl)glutathione di-n-butyl ester (19) was prepared by thionyl chloride esterification of N, S-(2,4-dinitrophenyl)glutathione[24] in butanol (Method A). Trituration with Et_2O gave the product as yellow crystals. Yield 85%; mp 130-135°C; ^1H NMR (D_6MSO) δ 9.0 (m, 3H), 8.7 (t, J= 6.7 Hz, 1H), 8.6 (d, J= 8.3 Hz, 1H), 8.5 (m, 1H), 8.3 (m, 1H), 8.0 (d, J= 8.3 Hz, 1H), 7.2 (d, J= 8.3 Hz, 1H), 5.0 (m, 1H), 4.8 (m, 1H), 4.2 (m, 2H), 4.1 (m, 2H), 3.9 (t, J= 6.7 Hz, 2H), 3.6 (m, 2H), 2.3-2.2 (m, 4H), 1.7 (m, 4H), 1.4 (m, 4H), 1.0 (m, 6H). Ir (cm^{-1}) *v* 3283 (NH), 2959 (CH), 1642 (C=O), 1526 (NO_2). LSIMS *m/z* 752.3 ($(M + H)^+$, 67), 774.2 ($(M + Na)^+$, 70); Anal. Calcd for $C_{30}H_{37}N_7O_{14}S.H_2O$: C, 46.80; H, 5.11; N, 12.74. Found: C, 47.24; H, 4.89; N, 12.77.

4.1.2.20. N-(4-amino-6-chloro-1,3,5-triazin-2-yl)-S-(2,4-dinitrophenyl)glutathione di-n-butyl ester (20) was prepared according to Method B using cyanuric chloride and CH_3CN replacing MeOH as the reaction solvent. After reaction for 4hr a saturated solution of ammonia in MeOH (20 ml) was added to the reaction and the solution stirred for a further 1 hr. The solution was then evaporated and the residue dissolved in AcOEt and filtered to remove insoluble material. The organic layer was

washed with HCl, water, dried $MgSO_4$, filtered and evaporated to give a solid. Purification by PTLC ($CHCl_3$/EtOH : 9/1) gave the expected product as pale yellow crystals. Yield 16.8 %; mp 86-90°C; ^{1}H NMR (D_6MSO) δ 8.8(s, 1H), 8.3 (dd, J=8.7, 2.9 Hz, 1H), 7.4 (d, J=7.2 Hz, 1H), 7.3-7.1 (m, 3H), 6.4 (d, J= 8.5 Hz, 1H), 5.8 (bs, 2H), 4.8 (m, 1H), 4.6 (m, 1H), 4.1 (t, J=7.1 Hz, 4H), 4.0 (m, 2H), 3.4 (m, 2H), 2.4 (m, 2H), 2.3-2.0 (m, 2H), 1.6 (m, 4H), 1.4 (m, 4H**),** 0.9 (t, J=7.7 Hz, 6H). Ir (cm^{-1}) *v* 3286 (NH), 2961 (CH), 1642 (C=O), 1530 (NO_2). LSIMS *m/z* 741.1 ($(M + H)^+$, 18), 763.1 ($(M + Na)^+$, 54); HRLSIMS: calc for $C_{30}H_{41}N_8O_{10}$SCl: 741.2433. Found 741.2431; Anal. Calcd for $C_{30}H_{40}N_8O_{10}$SCl: C, 48.63; H, 5.58; N, 15.13. Found: C, 48.61; H, 6.10; N, 14.89.

4.2. Biological Procedures

4.2.1. ***Quantitative Structure Activity Regression (QSAR).*** Initial studies were undertaken using the SciQSAR module of Alchemy 2000 (Tripos) running under Win 98 for data analysis as previously reported.[26] However in the absence of a simple graphical relationship between the experimental and calculated parameters and the large size of the data set used this led us to use the CODESSA Pro program for statistical analysis. This program uses diverse statistical structure property/activity correlation techniques for the analysis of experimental data in combination with independently calculated molecular descriptors. The program is an advanced fully featured QSAR program for analysis of experimental data sets, commercially available (SemiChem, Inc), which was provided to us in 2004 by Professor Katritzky, of the Univ of Florida during its evaluation and to whom we are indebted. Structures inputted into the program were constructed using Alchemy 2000, optimized and saved to file in the mdl.mol format. A text input file was created, excluding tabs, containing compound names, mol file path, one property (log ED_{50}) and one extra descriptor(log P) for each of the 19 di-ester compounds **2-19**. The data file was loaded into the Codessa Pro program and used to calculate constitutional, topological, geometrical and electrostatic descriptors totaling 166 for each compound. The large number of descriptors was filtered using the program's heuristic method which was used to determine a multi-linear regression QSAR relationship assuming zero for missing descriptors and the constraint of three or less descriptors. The heuristic method (HM)[39] was implemented in Codessa Pro to select the 'best' regression models. It quickly gives an estimation as to the quality of correlation to expect from the data, and derives several best regression models excluding descriptors that have bad or missing values, descriptors that are insignificant, and descriptors which are highly inter-correlated. This information is then used to reduce the number of descriptors involved in identifying the best QSAR model. The goodness of the fit of each correlation was tested by the regression coefficient(R2), where a value close to 1.0 represents the better fit to the model. The *F*-test the Fisher ratio of the variance explained by the model and the variance due to the error in the model (i.e., not explained by the model), where high values of F indicate that the model is statistically significant. The standard error shown by the squared cross-validated coefficient (s^2), expresses the variation of the residuals or the variation about the regression line and measures the model error. If the model is correct, it is an estimate of the error of the data variance. The t-test measures the statistical significance of the regression coefficients, high t-test values correspond to the relatively more significant regression coefficients. The confidence limits of the regression coefficient (DX), are also reported. The model, which passed the above statistical diagnosis with as few descriptors as possible was chosen. The regression analysis was further improved by diagnosis during the stepwise removal of outliers from the data set in whilst examining its effect not to significantly enhance the statistics of the model. When the optimum QSAR set was achieved for the identified descriptors the QSAR model was constructed on the basis of the reduced dataset.

The molecular property descriptor, log P (log K_{OW}) , was included in the descriptor set and was calculated for each of the individual molecules using the desktop version of this program EPE suite program obtained from the Environmental Service Centre, Syracuse Research Corporation[59] running under WinXP. The program uses the atom/fragment contribution method to estimate the log octanol-water partition coefficient, log K_{OW} of compounds. This method uses a dataset from full compounds, or fragments, that are experimentally determined, and then modelled using QSPR or other regression techniques into small fragments than per atom. Fragment contributions are then added up, with correction factors. Log K_{OW} values are calculated by the program by importing MDL mol structures of molecules constructed using Alchemy 2000 with the exception of compound **18** where due to large number of atoms, the program, xlog P [60] was used.

4.2.2. General Methods

Parasites: *Trypanosoma brucei rhodesiense* (STIB900) bloodstream form trypomastigotes were maintained in HMI-18 medium [61,62] with 20% heat inactivated foetal calf serum (HIFCS) (Harlan Sera-Lab., Crawley, UK) at 37°C in 5% CO_2-air mixture. *Leishmania donovani* (strain MHOM/ET/67/HU3) was maintained routinely in special pathogen free (SPF) female Golden hamsters by serial passage every 6 to 8 weeks. *Trypanosoma cruzi* (strain MHOM/BR/OO/Y) trypomastigotes were derived from MDCK fibroblasts in Dulbecco's Modified Eagle medium (Life Technologies Ltd., Paisley, Scotland) with 10% HICFS at 37°C in a 5% CO_2-air mixture.

4.2.3. Evaluation of the parasitic activity of glutathione derivatives *in vitro*

4.2.3.1. *T. brucei rhodesiense***:** All compounds were tested in triplicate in a three-fold dilution series from a top concentration of 30 μM. Parasites were diluted to $2x10^5$/mL and added in equal volumes to the test compounds in 96-well, flat bottom Microtest III™ tissue culture plates (Becton Dickinson and Company, NJ, USA). Appropriate controls with pentamidine isethionate (Sanofi, UK) as the positive, were set up in parallel. Plates were maintained for 3 days at 37°C in a 5% CO_2-air mixture as pentamidine is known to be efficacious in this time frame and the ultimate aim is to match or improve upon its potency. Compound activity was determined on day three by the use of the Alamar Blue assay.[63]

4.2.3.2. *L. donovani* and *T. cruzi.* Peritoneal macrophages were harvested from female CD1 Mice (Charles Rivers Ltd., Margate, UK) by peritoneal lavage 24 h after starch (Merck Ltd., Leics, UK) induced recruitment. After washing cells were dispensed into 16-well Lab-tek™ tissue culture slides (Nunc Inc., IL, USA) at $4x10^4$/well in a volume of 100 mL of RPMI-1640 medium (Sigma-Aldrich Company Ltd., Dorset, UK) and 10% HIFCS. After 24h, macrophages were infected at a ratio of 10:1 ($4x10^5$/well) with *L. donovani* amastigotes or 5:1 ($2x10^5$/well) with *T. cruzi* trypomastigotes. Infected macrophages were then maintained in the presence of drug in a three-fold dilution series in quadruplicate for 5 days in the case of *L. donovani* and 3 days with *T. cruzi*, the standard time frame taken for the respective control drugs to work. Drug activity was evaluated from the percentages of macrophages cleared of amastigotes in treated

cultures. Sodium stibogluconate ($NaSb^{v}$) (GSK, UK) and benznidazole (Bayer, UK) were used as the respective controls .[64]

4.2.4. Evaluation of the cytotoxicity of glutathione derivatives *in-vitro.*

Cytotoxicity testing on KB cells (human oral pharyngeal carcinoma) was evaluated using a one step fluorometric assay.[65] Cell cultures were grown in RPMI medium with 10% calf foetal serum (CFS) at 37°C in a 5% CO_2-air mixture in a humidified incubator. Plates were incubated with compound for 3 days at 37°C in a 5% CO_2-humidified air mixture prior to determination of activity.

4.2.5. Membrane location studies by HPLC-UV.

T. b. brucei S247 cells (1 x 10^6 cells/ml; bloodstream form) grown in HMI-9 medium were incubated in the same media with 10% calf foetal serum (CFS) at 37°C for 3h with N-benzyloxycarbonyl-S-(4-bromobenzyl)glutathione dimethyl ester (50 μM) in 1% DMSO (ED_{50} ~30 μM [14]). Aliquots (1 ml) were taken at the start of the reaction and time intervals 5, 60 and 120 mins and sedimented by centrifugation (2 mins; 7000 rpm) and the supernatant removed. Trichloroacetic acid (TCA)(20μl) was then added to the supernatant (~1ml) and the sample analysed by HPLC-UV (extracellular). The sedimented cell pellet was washed with PBS (1ml) and centrifuged (2 mins; 7000 rpm). The cell pellet was re-suspended in HMI-9 media (1ml), TCA (20μl), lysed and then centrifuged (13,000 rpm, 10mins) and the supernatant removed and stored at -20°C for HPLC analysis (intracellular). The metabolites of N-benzyloxycarbonyl-S-(4-bromobenzyl)glutathione dimethyl ester, N-benzyloxycarbonyl-S-(4-bromobenzyl)glutathione monomethyl ester and N-benzyloxycarbonyl-S-(4-bromobenzyl)glutathione, formed in the extracellular or intracellular medium of *T.b.brucei* cells in culture were analysed in triplicate by reversed-phase HPLC-UV. A control was run without inhibitor to ascertain that 1 % DMSO had no effects on the activity of parasites. Analysis was undertaken on a Hewlett Packard series 1100 HPLC system using a Techsphere 5μ ODS (25cm x 4.6mm) column eluted with a mobile phase of CH_3CN/H_2O (1:1), TFA (50mM) at a flow rate of 2ml/min, OD 235nm. The retention times of N-benzyloxycarbonyl-S-(4-bromobenzyl)glutathione dimethyl ester, N-benzyloxycarbonyl-S-(4-bromobenzyl)glutathione monomethyl ester and N-benzyloxycarbonyl-S-(4-bromobenzyl) glutathione (mean (± SD), n = 16) were 6.3 (± 0.2), 3.9 (± 0.5) and 2.3 (± 0.2) mins respectively. Calibration curves were produced for analysis of N-benzyloxycarbonyl-S-(4-bromobenzyl) glutathione dimethyl ester and N-benzyloxycarbonyl-S-(4-bromobenzyl)glutathione[24] over a range of concentrations of 0.5-500μM and 0.5-100μM respectively. The concentration ranges were limited due to solubility problems and the detection limit of these compounds by HPLC-UV of <0.5μM. Analysis of diester breakdown products were undertaken as above for the analysis of the intracellular contents of parasitic cells where the HMI-9 media (1ml) was replaced either with 1M NaCl or 6M urea.

4.2.6. Optical Microscopy

Morphological changes induced by S-bromobenzylglutathione dimethyl ester ($SBrBzGSMe_2$) [24] were investigated by incubation of *T. b. brucei* cells (1 x 10^6/mL; 1 x 10^6) for 1hr in the presence of 50 μM ($SBrBzGSHMe_2$). A sample of the treated solution was monitored by optical microscopy using a Leitz Aristoplan microscope, X40 PL fluorotar objective with Nomarski interference illumination. Photographs of the parasites were taken over a 1hr period on Fuji sensia 100 film using a Nikon 801 and Nikon SB25 flash on a TTL cord to freeze the motion.

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Supplementary data

Supplementary data (Detailed NMR characterizations of all compounds) associated with this article may be found, in the online version, at doi:

References and notes

1. Control and Surveillance of Human African Trypanosomiasis.; World Health Organization: Geneva, 2013; http://apps.who.int/iris/bitstream/10665/95732/1/_9789241209847_eng.pdf (accessed 21 Oct 2014) http:/www.who; int/mediacentre/factsheets/fs259/en/ (30 Jul 2012).
2. Leishmaniasis.; World Health Organization: http://www.who.int/mediacentre/factsheets/fs375/en/(30 Mar 2016).
3. Chagas disease (American trypanosomiasis).; World Health Organization:Geneva:http://www.who.int/mediacentre/factsheets/fs340/en/(30 Mar 2016).
4. Franco, J.R.: Simarro, P.P.: Diarra, A.; Jannin, J.G. Epidemiology of human African trypanosomiasis, *Clin Epidemiol*. **2014**, 6, 257–275.
5. Ettari, R.; Tamborini, L.; Angelo, I.C.; Micale, N.; Pinto, A.; Micheli, C.De.; Conti, P. Inhibition of Rhodesain as a Novel therapeutically modality for Human African Trypanosomiasis, *J. Med.Chem.* **2013**, 56, 5637-5658.
6. Chakravarty, J.; Sundar, S. Drug resistance in leishmaniasis, *J Glob Infect Dis*. **2010**, 2, 167-176.
7. Legros, D.; Evans, S.; Maiso, F.; Enyaru, J.C.; Mbulamberi, D. Risk factors for treatment failure after melarsoprol for *Trypanosoma brucei gambiense* trypanosomiasis in Uganda. *Trans R Soc Trop Med Hyg.* **1999**, 93, 439-442.
8. Alsford, S.; Kelly, J.M.; Baker, N.: Horn, D. Genetic dissection of drug resistance in trypanosomes. *Parasitology*. **2013**, 140, 1478–1491.
9. Carter, N. S.; Fairlamb, A. H. Arsenical-resistant trypanosomes lack an unusual adenosine transporter. *Nature*. **1993**, 361, 173–176.
10. Maser, P.; Sutterlin, C.; Kralli, A.; Kaminsky, R. A nucleoside transporter from *Trypanosoma brucei* involved in drug resistance. *Science*. **1999**, 285, 242–244.
11. Shahi, S. K.; Krauth-Siegel, R. L.; Clayton, C. E. Overexpression of the putative thiol conjugate transporter TbMRPA causes melarsoprol resistance in *Trypanosoma brucei*. *Molecular Microbiology*. **2002**, 43, 1129–1138.
12. Vincent, I. M.; Creek, D.; Watson, D. G.; Kamleh, M. A.; Woods, D. J.; Wong, P. E.; Burchmore, R. J.; Barrett, M. P. A molecular mechanism for eflornithine resistance in African trypanosomes. *PLoS Pathogens*. **2010**, 6, e1001204.
13. Wilkinson, S. R.; Taylor, M. C.; Horn, D.; Kelly, J. M.; Cheeseman I. A mechanism for cross-resistance to nifurtimox and benznidazole in trypanosomes. *Proceedings of the National Academy of Sciences*, USA. **2008**, 105, 5022–5027.

14. Berriman, M.; Ghedin, E.; Hertz-Fowler, C.; Blandin, G.; Renauld, H.; Bartholomeu, D.C.; Lennard, N.J.; Caler, E.; Hamlin, N.E.; Haas, B.; Böhme, U.; Hannick, L.; Aslett, M.A.; Shallom, J.; Marcello, L.; Hou, L.; Wickstead, B.; Alsmark, U.C.; Arrowsmith, C.; Atkin, R.J.; Barron, A.J.; Bringaud F, Brooks K, Carrington M, Cherevach I, Chillingworth TJ, Churcher C, Clark LN, Corton CH, Cronin A, Davies RM, Doggett J, Djikeng A, Feldblyum T, Field MC, Fraser A, Goodhead I, Hance Z, Harper D, Harris BR, Hauser H, Hostetler J, Ivens A, Jagels K, Johnson D, Johnson J, Jones K, Kerhornou AX, Koo H, Larke N, Landfear S, Larkin C, Leech V, Line A, Lord A, Macleod A, Mooney PJ, Moule S, Martin DM, Morgan GW, Mungall K, Norbertczak H, Ormond D, Pai G, Peacock CS, Peterson J, Quail MA, Rabbinowitsch E, Rajandream MA, Reitter C, Salzberg SL, Sanders M, Schobel S, Sharp S, Simmonds M, Simpson AJ, Tallon L, Turner CM, Tait A, Tivey AR, Van Aken S, Walker D, Wanless D, Wang S, White B, White O, Whitehead S, Woodward J, Wortman J, Adams MD, Embley TM, Gull K, Ullu E, Barry JD, Fairlamb AH, Opperdoes F, Barrell BG, Donelson JE, Hall N, Fraser CM, Melville SE, El-Sayed, NM. The Genome of the African Trypanosome. *Trypanosoma brucei*, *Science.* **2005**, 309, 416-422.
15. O'Sullivan, M.C.; Zhou, Q. Novel Polyamine derivatives as potent competitive inhibitors of *Trypanosoma cruzi* trypanothione reductase. *Bioorg. Med. Chem. Lett.* 5, (1995), 1957-1960**.**
16. Li, Z.; Fennie, M.W.; Ganem, B.; Hancock, M.T.; Kobaslija, M,; Rattendi, D.; Bacchi, C.J.; O'Sullivan, M.C. Polyamines with N-(3-phenylpropyl) substituents are effective competitive inhibitors of trypanothione reductase and trypanocidal agents. *Bioorg. Med. Chem. Lett.* **2001**, 11, 251-254.
17. Khan, O.F.; Austin, S.E.; Chan, C.; Yin, H.; Marks, D.; Vaghjiani, S.N.; Kendrick, H.;, Yardley, V.; Croft, S.L.; Douglas, K.T. Use of an Additional Hydrophobic Binding Site, the Z Site , in the Rational Drug Design of a New Class of Stronger Trypanothione Reductase Inhibitor, Quaternary Alkylammonium Phenothiazines**.** *J. Med. Chem.* **2000**, 43**,** 5148-3156.
18. Girault, S.; Davioud-Charvet, E.; Maes, L.; Dubremetz, J-F.; Debreu, M-A.; Landry, V.; Sergheraet, C. Potent and specific inhibitors of Trypanothione reductase from *Trypanosoma cruzi:* Bis(2-aminodiphenylsulfides) for fluorescent labeling studies. *Bioorg. Med. Chem.* **2001**, 9, 837-846.
19. Salmon-Chemin, L.; Buisine, E.; Yardley, V.; Kohler, S.; Debreu, M-A.; Landry, V.; Sergheraet, C.; Croft, S.L.; Krauth-Siegel, R.L.; Davioud-Charvet, E. 2- and 3-Substituted 1,4-Naphthoquinone derivatives as subversive substrates of trypanothione reductase and lipoamide dehydrogenase from *Trypanosoma cruzi:* Synthesis and correlation between redox cycling activities and in vitro cytotoxicity. *J. Med. Chem.* **2001**, 44, 548-565.
20. Bonse, S.; Richards, J.M.; Ross, S.A.; Lowe, G.; Krauth-Siegel, R.L. (2,2':6', 2''-Teryridine)platinum(II) Complexes are Irreversible Inhibitors of *Trypanosoma cruzi* Trypanothione Reductase but not of Human Glutathione Reductase. *J. Med. Chem.* **2000**, 43, 4812-4821.
21. Bond, C.S.; Zhang, Y.; Berriman, M.; Cunningham, M.L.; Fairlamb, A.H.; Hunter, W.N. Crystal structure of *Trypanosoma cruzi* trypanothione reductase in complex with trypanothione, and the structure-based discovery of new natural product inhibitors. *Structure.* **1999**, 7, 81-89.
22. Hamilton, C.J.; Saravanamuthu, A.; Fairlamb, A.H.; Eggleston, I.M. Benzofuranyl 3,5-bis-Polyamine as Time-Dependent Inhibitors of Trypanathione Reductase. *Bioorg. Med. Chem*. **2003**, 11, 3683-3693.
23. Hamilton, C.J.; Saravanamuthu, A.; Poupat, C.; Fairlamb, A.H.; Eggleston, I.M. Time-dependent inhibitors of trypanothione reductase: Analogues of the spermidine alkaloid lunarine and related natural products. *Bioorg. Med. Chem*. **2005**, 11, 3683-3693.
24. D'Silva, C.; Daunes, S.; Rock, P.; Yardley, V.; Croft. S. L. Structure-Activity Study on the In Vitro Antiprotozoal Activity of Glutathione Derivatives. *J. Med. Chem*. **2000**, 43, 2072-2078
25. Alibu, V.; Daunes, S.; D'Silva, C. The *T. b. brucei* MRPA protein is an intracellular target for the antiparasitic activity of N-benzyloxy-S-(2,4-dinitrophenyl)glutathione diesters. *Bioorg.Biomed.Lett.* **2013**, 23, 4351–4353.
26. Daunes, S.; D'Silva, C.; Kendrick, H.; Yardley, V.; Croft, S. L. A QSAR Study on the Contribution of Log P and E_s to the in vitro Antiprotozoal Activity of Glutathione Derivatives. *J. Med. Chem*. **2001**, 44, 2976-2983.
27. Daunes, S.; D'Silva, C., Glutathione Derivatives Active against *T.b.Rhodensiense* & *T.b.Brucei in vitro*. Antimicrob. Agents Chemother. **2002**, 46, 434-437.
28. McGwire, B. S.; Olson, C. l.; Tack, B.F.; Engman, D.M. Killing of African Trypanosomes by Antimicrobial Peptides. *JID*.**2003**, 188, 146-152.
29. Roch, P.; Beschin, A.; Bernard, E. Antiprotozoan and Antiviral Activities of Non-cytoxic Truncated and Variant Analogues of Mussel Defensin. *Evid Based Complement Alternat Med*. **2004**, 1, 167-174.
30. Pascholati, C. P.; Lopera, E. P.; Pavinatto, F. J.; Caseli, L.; Zucolotto, V.; Nobre, T.M.; Maria, E.D.; Zaniquelli, M.E.D.; Viitala, T.; D'Silva, C.; Oliveira Jr, O.N. The Interaction of an Antiparasitic peptide Active against African Sleeping Sickness with Cell Membrane Models. Colloids and Surfaces B: *Biointerfaces*, **2009**, 74(2) 504-510.
31. Herculano, R.D.; Pavinatto, F.J.; Caseli, L.; D'Silva, C.; Oliveira Jr. O.N. The Lipid Composition of a Cell Membrane Model Modulates the Action of an Antiparasitic Peptide at the Air-Water Interface, *Biochimica et Biophysica Acta,* **2011**, 1808(7), 1907-1912.
32. Fairlamb, A.H.; Chemotherapy of human African trypanosomiasis: current and future prospects. *Trends in Parasitol*, **2003**, 19(11), 488-494.
33. Li, W-S.; Lam, W.S.; Liu, K-C.; Wang, C-H.; Chang, H.C.; Jen, Y.C.; Hsu, Y-T.; Shivatare, S.S.; Jao, S-C.; Overcoming the Drug Resistance in Breast Cancer Cells by Rational Design of Efficient Glutathione *S*-Transferase. *Org. Letts.* **2010**, 12, 20-23.
34. Natesan, S.K.A.; Black, A,; Matthews, K.R.; Mottram, J.C.; Field. M.C.; *Trypanosoma brucei brucei*: Endocytic recycling is important for mouse infectivity. *Exp. Parasitol.* **2011**, 127, 777-783.
34. D'Silva, C.; Synthesis of carboxyl modified coenzyme derivatives as probes to the mechanism of glutathione dependent enzymes. *Biochem. J*. **1990**, 271, 167-169.
35. Frearson, J.A.; Brand, S.; McElroy, S.P.; Cleghorn, L.A.T.; Smid, O.; Stojanovski, L.; Price, H.P.; Lucia, M.; Guther, S.; Torrie, L.S.; Robinson, D.A.; Hallyburton, I.; Mpamhanga, C.P.; Brannigan, J.A.; Wilkinson, A.J.; Hodgkinson, M.; Hui, R.; Qiu, W.; Raimi, G.O.; van Aalten, Daan M. F.; Brenk, R.; Gilbert, I.H.; Kevin, D.; Read, K.D.; Fairlamb, A.H.; Ferguson, M.A.J.; Smith, D.F.; Wyatt, P.G. N-myristoyl-transferase inhibitors as new leads to treat sleeping sickness. *Nature*. **2010**, 464, 728-734.
36. Chuprajob, T.; Chokchaisiri, R.; Chunglok, W.; Sornkaew, N.; Suksamrarn, A. Synthesis, cytotoxicity against human oral cancer KB cells and structure-activity relationship studies of trienone analogues of curcuminoids. *Bioorg.Med.Chem.Lett.* **2014**, 24, 2839-2844.
37. Tai, K.W.; Chang, Y.C.; Chou, L.S.; Chou, M.Y. Cytotoxic effect of pingyangmycin on cultured KB cells. *Oral Oncol.* **1998**, 34, 219-223.
38. T.Okimoto, T.Seguchi, M.Ono, Y.Nakayama, G.Funatsu, T.Fujiwara, Y.Ikehara, M.Kuwano.*Cell.Struct.Funct.* **1993**, 18, 241-51.
39. Katritzky, A. R.; Kulshyn, O.V.; Slavova, I. S.; Dobchev, D. A.; Kuanar, M.; Faraa, D.C.; and Karelson, M. Antimalarial activity: A QSAR modeling using CODESSA PRO software. *Bioorg. Med. Chem*, **2006**, 14, 2333–2357.
40. Fitch, W.L.; McGregor, M.; Katritzky, A. R.; Lomaka, A.; Petrukhin, R.; Karleson, M. Prediction of Ultraviolet Spectral Absorbance Using Quantitative Structure-Property Relationships. *J. Chem. Inf. Comput. Sci.*, **2002**, 42 (4), 830–840.
41. Rouhrbaugh, R.; and Jurs. P.C. Descriptions of molecular shape applied in studies of structure/activity and structure/property relationships. *Analytica Chimica Acta,* **1987**, 199, 99-109.
42. Tyler, K.M.; Fridberg, A.; Toriello, K.M.; Olson, C.L.; Cieslak, J.A.; Hazlett, T.L.; Engman. D.M. Flagellar membrane localization via association with lipid rafts, *J. Cell. Sci*. **2009,** 122, 859-865.
43. http://en.wikipedia.org/wiki/Nanomotor
44. Aitcheson, N.; Talbot, S.; Shapiro, J.: Hughes, K; Adkin, C.; Butt, T.; Sheader, K.; and Rudenko, G. VSG switching in *Trypanosoma brucei:* antigenic variation analysed using RNAi in the absence of immune selection, *Mol Microbiol.* **2005**, 57, 1608–1622.
45. Field, M.C.; Carrington. M. The trypanosome flagellar pocket, *Nat Rev Microbiol*. **2009**, 7, 775-786.
46. Morgan, G.W.; Allen, C.L.; Jeffries, T.R.; Hollinshead, M.; Field. M.C. Developmental and morphological regulation of clathrin-mediated endocytosis in *Trypanosoma brucei. J.Cell. Sci.* **2001**, 114, 2605-2615.
47. Natesan, S.K.A.; Black, A,; Matthews, K.R.; Mottram, J.C.; Field. M.C. *Trypanosoma brucei brucei*: Endocytic recycling is important for mouse infectivity. *Exp. Parasitol.* **2011**, 127, 777-783.
48. O'Brien, M.L.; Vulevic, B.; Freer, S.; Boyd, J.; Shen, H.; Tew, K.D. Glutathione peptidomimetic drug modulator of multidrug resistance-associated protein. *J. Pharmacol. Exp. Ther*. **1999**, 291, 1348–1355.
49. Townsend, D.M.; Tew, K.D. The role of glutathione-S-transferase in anti-cancer drug resistance. *Oncogene*. **2003**, 22, 7369–7375.
50. Juliano, R.; Ling, V.; Graves. J. Drug-resistant mutants of Chinese hamster ovary cells possess an altered cell surface carbohydrate component. *J. Supramol. Struct*. **1976**, 4, 521−526.
51. Ford, R.C.; Kamis, A.B.; Kerr, I.D.; Callaghan. R. The ABC Transporters: Structural Insights into Drug Transport. *In Transporters*

as Drug Carriers; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, Germany, **2010**; pp 1−48.
52. Sharom, F.J. The P-glycoprotein multidrug transporter. *Essays Biochem*. **2011**, 50, 161−178.
53. Aller, S.G.; Yu, J.; Ward, A.; Weng, Y.; Chittaboina, S.; Zhuo, R.; Harrell, P.M.; Trinh, Y.T.; Zhang, Q.; Urbatsch, I.L.; Chang, G. Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. *Science.* **2009**, 323, 1718−1722.
54. Antoine, J.C.; Jouanne, C.; Lang, T.E.; Prina, E.; De Chastellier, C.; Frehel, C. The biogenesis and properties of the parasitophorous vacuoles that harbour *Leishmania* in murine macrophages. *Trends Microbiol*. **1998**, 6, 392-401.
55. Andrade, L.O.; Andrews, N.W. The *Trypanosoma cruzi*-host-cell interplay: location, invasion, retention. *Nat Rev Microbiol*. **2005**, 3, 819-23.
56. Green, H.P.; Del Pilar Molina Portela, M.; St Jean, E.N.; Raper, J. Evidence for a *Trypanosoma brucei* lipoprotein scavenger receptor. *J Biol Chem*. **2003**, 278, 422-427.
57. Sokolovsky,M.; Sadeh, T.; Patchornik, A. Nonenzymatic cleavages of peptide chains at the cysteine and serine residues through their conversion to Dehydroalanine (DHAL). II. The specific chemical cleavage of cysteinyl peptides. *J. Am. Chem. Soc*. **1964**, 86, 1212-1217.
58. Cabre, J.; Palomo, A.L. New Experimental Strategies in Amide Synthesis using *N,N*-Bis[2-oxo-3-oxazolidinyl] phosphorodiamidic Chloride. *Synth. Commun*. **1984**, 5, 413-417.
59. Environmental Service Centre. Syracuse Research Corporation (SRC). http://esc-plaza.syrres.com/interkow/kowdemo.htm.
60. http://www.ics.uci.edu/~dock/manuals/xlogp2.1/usage.html
61. Ellis, J.A.; Fish, W.R.; Sileghem, M.; McOdimba, F. A colorimetric assay for trypanosome viability and metabolic function. *Vet Parasitology*. **1993**, 50, 143-149.
62. Hirumi, H.; Hirumi, K. Continuous cultivation of *Trypanosoma brucei* bloodstream forms in a medium containing a low concentration of serum proteins without feeder cell layers. *J. Parasitol*. **1989**, 75, 985–989.
63. Riaz, B.; Iten, M.; Grether-Buhler, Y.; Kaminsky, R.; Brun, R. The Alamar Blue assay to determine drug sensitivity of African Trypanosomes (*T. b. rhodesiense* and *T. b. gambiense*) in vitro. *Acta Tropica*. **1997**, 68, 139-147.
64. Neal, R.A.; Croft, S.L. An *in vitro* system for determining the activity of compounds against the intracellular amastigote form of *Leishmania donovani*. *J. Antimic. Chemother*. **1984**, 14, 463-475.
65. Nociari, M.M.; Shalev, A.; Benias, P.; Russo, C. A novel one-step, highly sensitive Fluorometric assay to evaluate cell-mediated cytotoxicity. *J. Immunol Methods*. **1998**, 213(2), 157-167.

Compound ID	ED_{50} (μM)[d] *T. b. rhodesiense*	ED_{50} (μM)[c,e] *L. donovani*	ED_{50} (μM)[c,f] *T. cruzi*	ED_{50} (μM) *KB cells*[b]	*Relative Tox KB/T.b.rhod*	*Log P*[a]	*ΔΔG kJ mol*$^{-1}$
1	>30	>30	>30				
2	1.88	19.9/t	t/+	107.4	57.1	2.25	0.0
3	6.52	NA	37.8	>300	>46	2.7	-3.1
4	2.15	>30(5.4)	30.4	>300	>139	3.35	-0.3
5	0.73	NA	30.8	>300	>411	3.61	2.3
6	1.16	>30(4.6)	>30(35.6)	59.6	51.4	4.1	1.2
7	1.02	NA	NA	84	82.3	4.59	1.5
8	32.6	NA	>30	>300	>9.2	8.73[a]	-7.1
9	0.55	NA	22.9	>300	>545	4.1	3.05
10	0.6	NA	>30	>300	>500	4.43	2.8
11	2.02	>30(18.4)	>30(29.7)	>300	>148	4.9	-0.2
12	0.28	NA	>30(33.4)	34.4	123	4.68	4.7
13	0.42	>30 (36)	NA	95.2	227	5.06	3.7
14	0.30	NA	35.01	26	87	3.45	4.55
15	0.6	NA	8.4	83.4	139	4.06	2.8
16	0.22	9.9 ±2.9	16.5	70.4	320	4.71	5.3
17	4.38	NA	12.04	250.2	57.1	4.55	-2.1
18	0.5	10.06	4.5	>300	>600	6.74[a]	3.3
19	0.72	NA	NA	>300	>417	4.73	2.4
20	18.8	NA	NA	>300	>15.9	3.27	-5.7

Highlights

- **Antiparasitic glutathione derivatives**
- **Inhibitors of *Trypanosomiasis rhodesiense***
- **Membrane binding hydrophobic compounds**
- **Inhibitors targeting the flagellar membrane pocket**