Minor Groove Binders for DNA as Antitrypanosomal Agents: the Veterinary Context

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1

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Graphical Abstract

Minor groove binders designed and synthesised at the University of Strathclyde (S-MGBs) are trypanocidal and curative in mouse models of disease caused by the animal parasites, *Trypanosoma congolense* and *Trypanosoma vivax*.
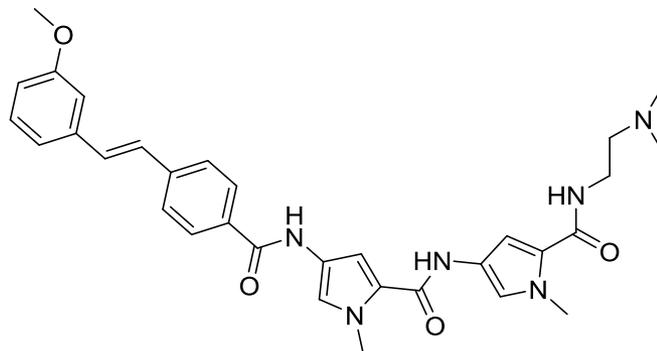
Abstract: Animal African trypanosomiasis (or nagana) is a wasting livestock disease found in sub-Saharan Africa and caused by protozoan parasites *Trypanosoma congolense*, *T. vivax* and *T. brucei*. Chemotherapy (diamidine diminazene aceturate) and chemoprophylaxis (phenanthridine isometamidium) are essential for disease control. Current treatments have reduced efficacy due to increased drug resistance, the need for new veterinary trypanocides becomes a high-priority. All three of the above African species are susceptible to minor groove binding drugs from Strathclyde (S-MGBs) which is important because compounds useful in the field must have activity across the range of infectious species so that characterisation of the infection is not required at diagnosis. Over 100 S-MGBs have been evaluated in Glasgow and Switzerland, and compounds with development potential have been identified. As an example, S-MGB 234 has been shown to be curative in *in vivo* models of trypanosome infection in mice. Importantly S-MGB 234 does not show cross resistance with other antitrypanosomal drugs such as diminazine, isometamidium, or ethidium bromide, which is consistent with a different route into the parasite's cell. S-MGBs that contain alkene links, such as in S-MGB 234, are the most active sub-class of S-MGB and point the way towards structural optimisation.

Keywords: antitrypanosomal compounds; DNA minor groove binders; cross resistance.

Introduction

From the early stages of our MGB programme we have had evidence for activity against kinetoplastid parasites, especially *Trypanosoma brucei*. Over the last five years the study has been developed in collaboration with the University of Glasgow and the Swiss Tropical Health Institute, Basel, to reach the stage at which advanced leads and potential development candidates have emerged. In reaching this point, some generalities concerning structure-activity relationships have been observed. This paper summarises the salient features by selecting specific small groups of closely related compounds.

An effective anti-trypanosomal compound for use against African Animal Trypanosomiasis (AAT) must be active against all of the species causing disease so that identification of a species is not a necessary part of diagnosis. Moreover such a compound must also be effective against strains of trypanosomes resistant to currently used drugs such as isometamidium and diminazine. Evidence is presented to show that S-MGBs do not show cross resistance.

Lastly, a development candidate must be active in an *in vivo* model of disease. Data showing that S-MGBs are curative in a mouse model of trypanosomiasis are presented.

Results and discussion

Part of the S-MGB library (about 100 compounds) was screened *in vitro* against *T. congolense* (IL3000 BF) and *ex-vivo* against *T. vivax* (STIB 719/ILRAD 560 BF) for trypanocidal activity. To evaluate selectivity against mammalian cells, the toxicity of each compound to the L6 murine cell line was determined.

The S-MGB library consists of compounds of general structure illustrated by S-MGB **234**, shown on the next slide. Three different N-terminal (head groups) are included, amide, amidine, and alkene, of which the alkene in general shows the best anti-infective activity. Examples of differences cause by these structural changes are shown in the following slides.

Five S-MGBs had IC_{50} less than 100 nM against *T. congolense* and 7 had IC_{50} less than 100 nM against *T. vivax.* There was a broad but not exact correlation between the two activities (see following slides). On the basis of their determined activities and selectivity indices, 7 S-MGBs were chosen for further evaluation of which 3, S-MGBs **234**, **235**, and **248** were progressed to the *in vivo* disease model stage.

The data are summarised on the following chart, which shows the activity against *T. congolense* in comparison with that against *T. vivax.* The bubble size represents the effect on L6 cells; the larger the bubble, the lower the toxicity. Perhaps unsurprisingly some of the most active compounds (lower left quadrant) are also the most toxic. However, as the data presented in the following slides show, several S-MGBs have sufficiently large selectivity indices to warrant further investigation.

Structural features of S-MGBs
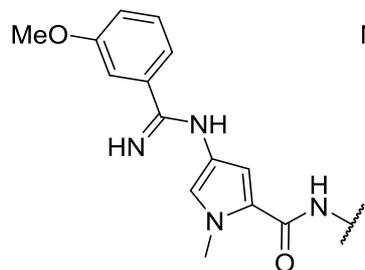
Amidine linked head group   Amide linked head group   Alkene linked head group
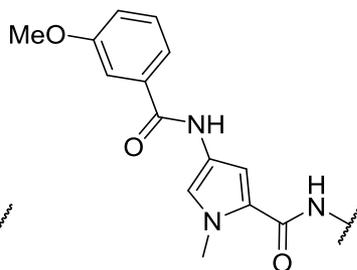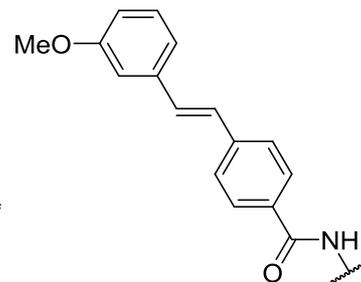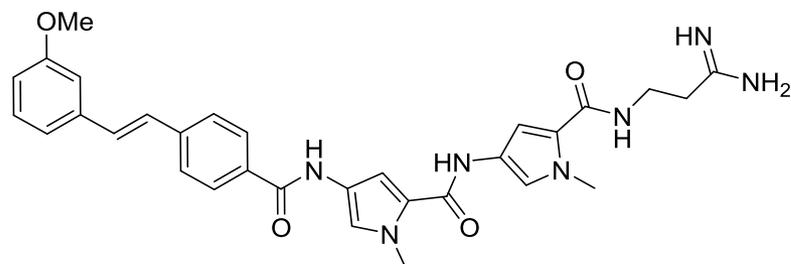
S-MGB **234**. An example of a full length trypanocidal S-MGB

Strathclyde MGBs (S-MGBs) of all three structural types have been investigated. Tertiary amine and amidine tail groups have been included. The following chart shows the activity for the S-MGBs against *Trypanosoma congolense* and *T. vivax;* the bubbles represent the toxicity against the L6 cell line, the larger the bubble, the lower the toxicity. Significant compounds in this study have been numbered.

Log *T. vivax* EC$_{50}$ (µM)

Log *T. congolense* EC$_{50}$ (µM)

Highest activity

235
234
248

100.000
10.000
1.000
0.100
0.010
0.001

0.010
0.100
1.000
10.000
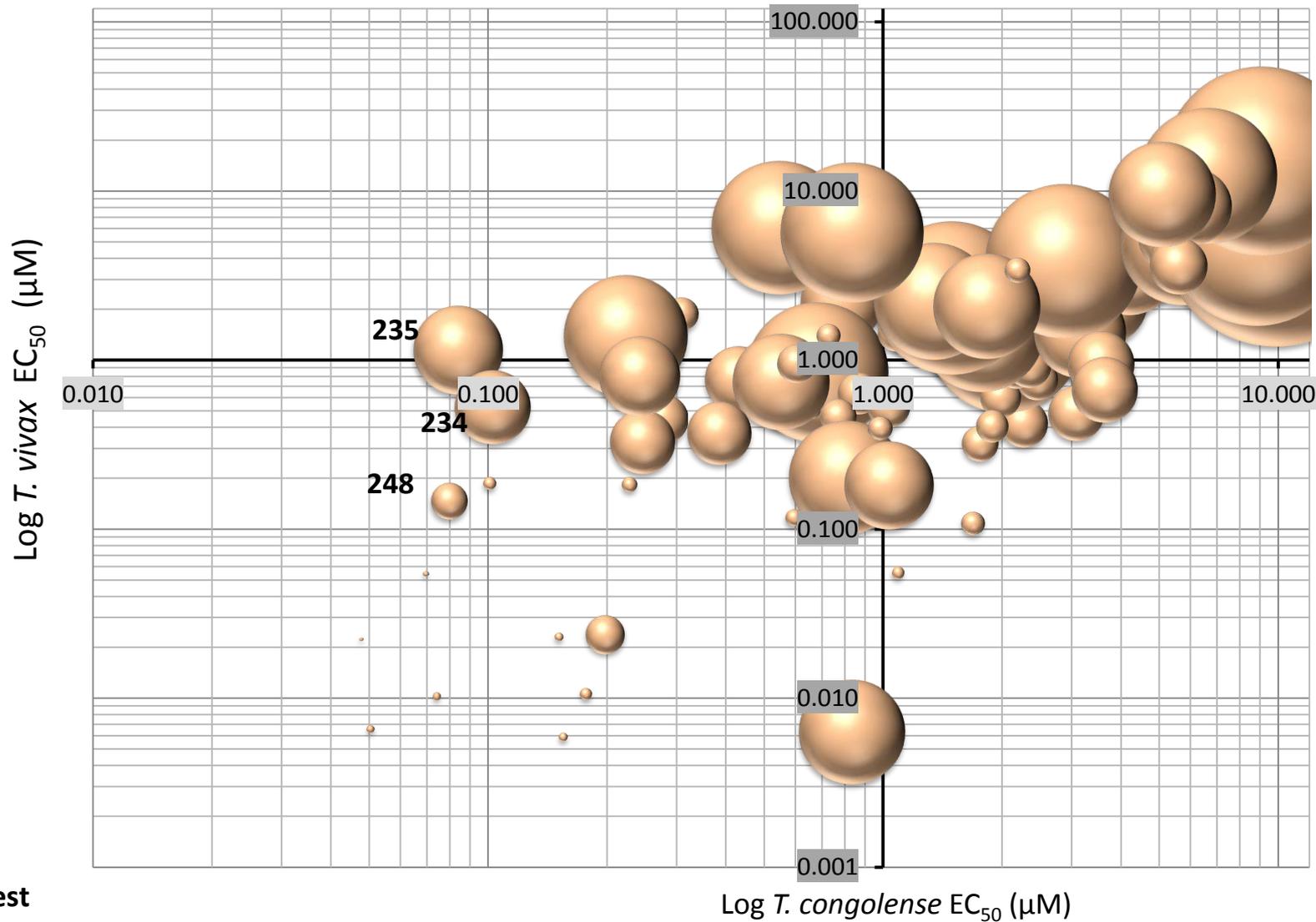
Essential trends of SAR – 1
Amidine tail group compounds have low toxicity

1
Tc 0.94 μM SI 1.8

235
Tc 0.084 μM SI 120
Tv 0.9 μM SI 11

2
Tc 0.89 μM SI 3.85
Tv 0.18 μM SI 19.1

234
Tc 0.1 μM SI > 320
Tv 0.33 μM SI > 97
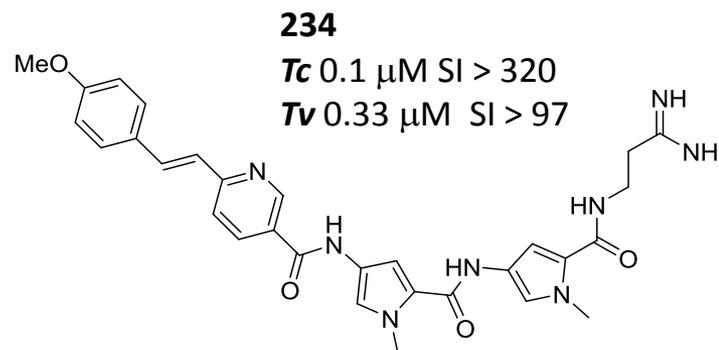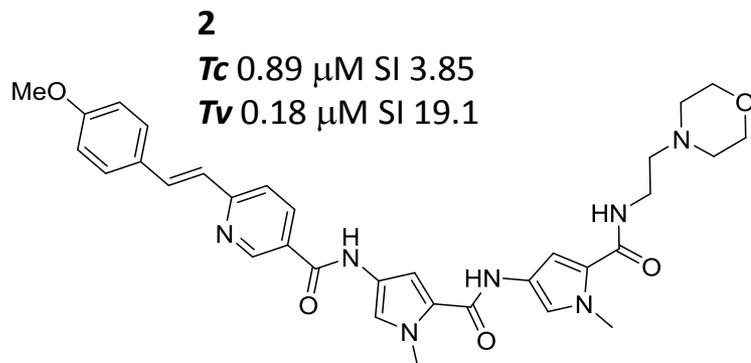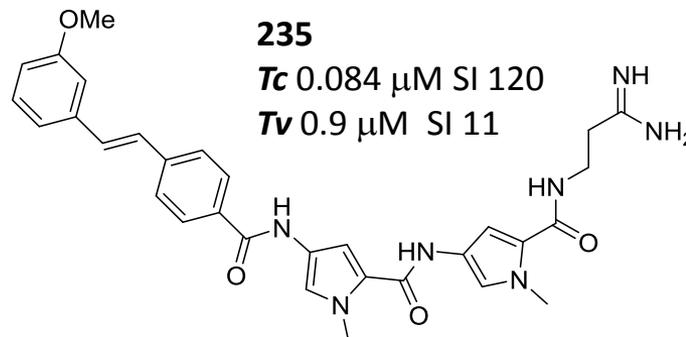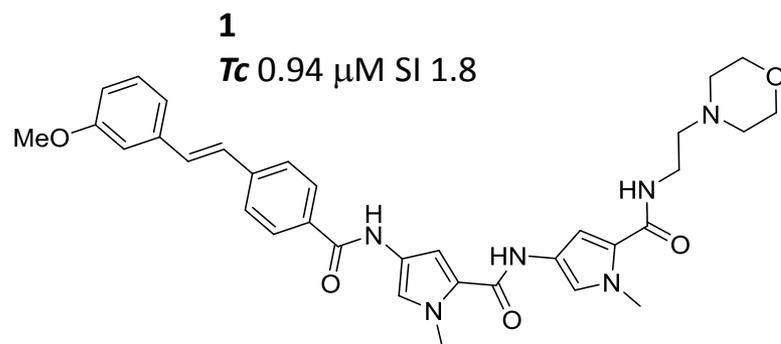
Whilst some activities in the pairs above are very similar, the selectivity indices (SIs) differ greatly. Two factors may contribute strongly to this. 1. The morpholino tail groups are weakly basic because of self association of the MGB and largely unprotonated at physiological pH leading to highly lipophilic compounds. 2. In contrast, the amidine tail groups are essentially permanently protonated giving more soluble compounds that could be substrates for specific carrier proteins.

Essential trends of SAR – 2
Head groups have a great influence on activity

248
T. congo 0.018 µM SI 283
T. vivax 0.147 µM SI 35

246
T. congo 0.94 µM SI 2.6

247
T. congo 1.03 µM SI 6.9
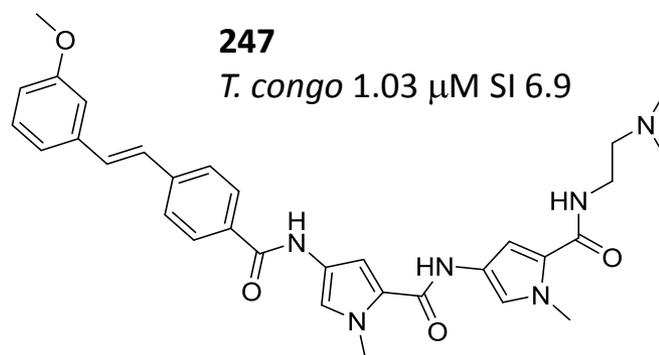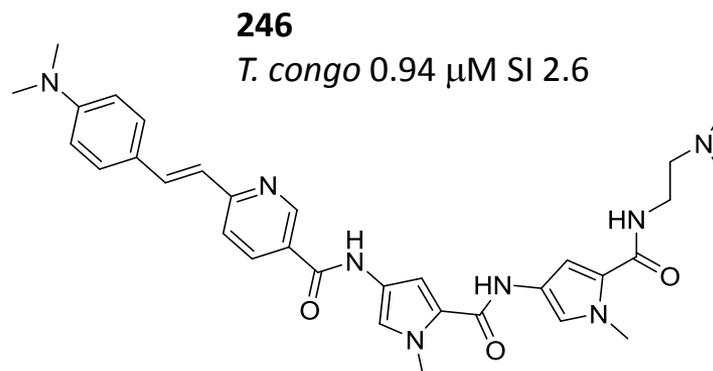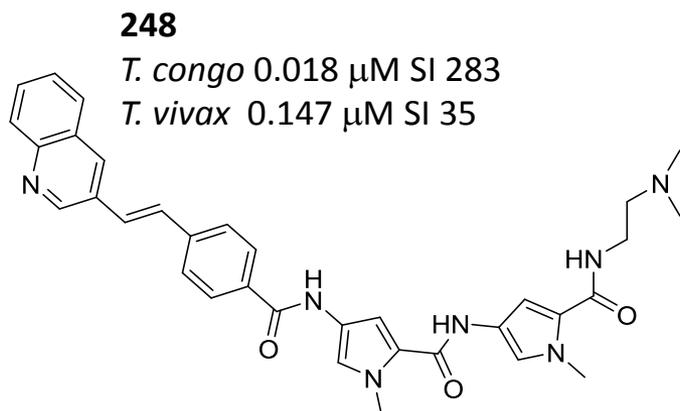
Bicyclic heteroaryl head groups, such as 3-quinolyl in **248**, in general have higher activity than substituted monocyclic head groups such as 4-dimethylaminophenyl (**246**) and 3-methoxyphenyl **(247)**.

Essential trends of SAR – 3
Alkenes are the best head group links for activity

200 inactive

224 inactive

388 *T. congo* 2.40 μM SI 36.4

390 *T. congo* 0.09 μM SI 634
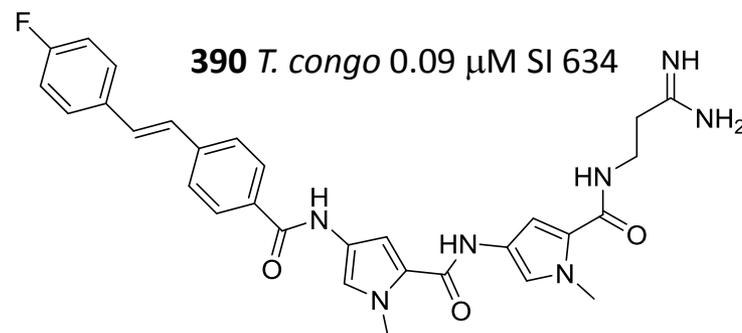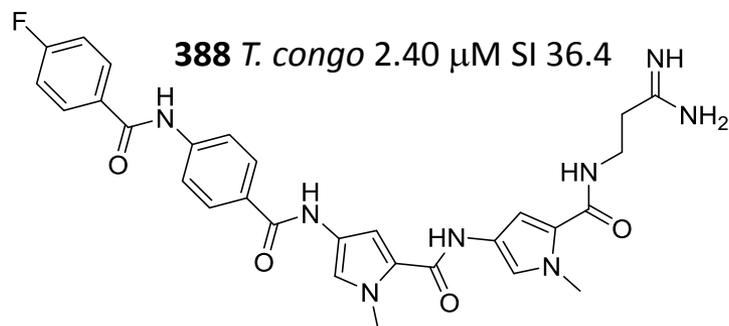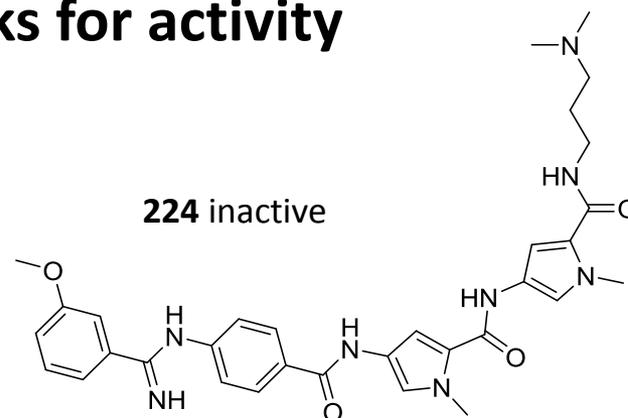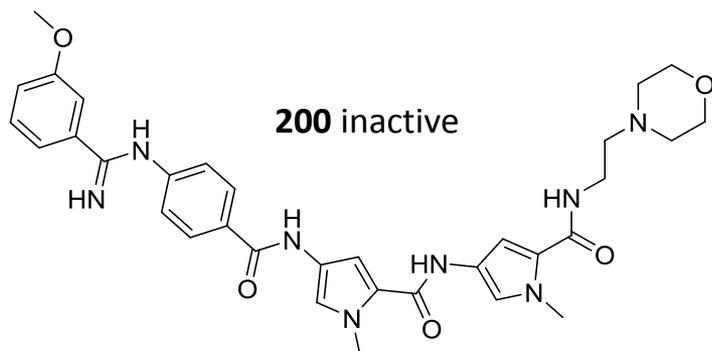
For this antitrypanosomal activity, but not others, amidine head groups are inactive. Amide head groups give activity as good as some alkenes but the one-to-one comparisons, as here, always substantially favour the alkene. The importance of the amidine is also evident from the large SI of **390**.

Comments on SAR

The trends observed in the previous 3 slides are representative of observations from the full collection of S-MGBs studied. Different structural features turn out to contribute to the best profiles discovered so far for several different applications of S-MGBs (antibacterial, antifungal and antiparasitic). The currently available generalisations are described in the accompanying paper 'Selectivity in anti-infective minor groove binders'.

With respect to this representative set of compounds and the antitrypanosomal activity, there is no evident correlation between the activity and typical medicinal chemical parameters such as clogP and tPSA. Indeed S-MGBs are characterised by large polar surface areas (110 – 150 for the most active alkene-containing compounds) and S-MGBs with clogP values in the range 2.5 to 5.0 have all been found to be active. An added complication is the known tendency of S-MGBs to aggregate in solution which makes an SAR basis in physicochemical properties difficult to define (see Parkinson et al*., Med. Chem. Commun.,* **2013**, *4*, 1105-1108).

The range of activities observed might be understood through different DNA-binding properties and by different uptake / efflux into and from target cells. Because of the multiple local targets expected on trypanosomal DNA there are no data with which to discriminate between compounds for DNA binding. The following biological evaluation, however, implicates uptake as important in the activity of S-MGBs.

Evidence for lack of cross resistance to diminazene

S-MGB	*T. congolense* WT EC$_{50}$ (μM)	*T. congolense* DimR EC$_{50}$ (μM)	RF	*T. congolense* EMS MUT DimR EC$_{50}$ (μM)	RF
248	0.27 ± 0.02	0.18 ± 0.01	0.7	0.22 ± 0.03	0.8
234	0.51 ± 0.06	0.64 ± 0.03	1.3	0.70 ± 0.005	1.4
235	1.40 ± 0.24	0.66 ± 0.08	0.5	1.19 ± 0.12	0.9
246	2.13 ± 0.17	1.82 ± 0.06	0.9	2.03 ± 0.07	1.0
247	2.36 ± 0.10	1.66 ± 0.10	0.7	1.78 ± 0.10	0.8
2	4.50 ± 0.22	4.41 ± 0.25	1.0	4.52 ± 0.18	1.0
1	7.99 ± 0.85	5.26 ± 0.15	0.7	6.68 ± 1.06	0.8
Diminazene	0.20 ± 0.01	2.06 ± 0.10	10.4	2.36 ± 0.10	12.0

In vitro trypanocidal activity of selected S-MGBs against two diminazene-resistant *T. congolense* lines (DimR and EMS MUT DimR) as compared to wild type (WT) (mean ± SEM, n≥3) were determined at the University of Glasgow. Ratios (RF) close to unity indicate that the two strains compared are of essentially equal sensitivity to the S-MGB. The data suggest that the S-MGBs do not act by the same mechanism as diminazene; both differences in uptake and differences in intracellular activity may contribute.

(Note: The EC$_{50}$ data here differ from those reported in the SAR comparisons because the latter were obtained at the Swiss Tropical Health Institute under slightly different conditions.)

In vivo activity against *T. congolense*

S-MGB	Dose (mg/kg × number of treatment days)	Cured/ infected	MRD (days)	MSD (days)
234	50 × 2	4/4	n/a	>60
	10 × 4	1/4	19.3	34.8
235	10 × 4	4/4	n/a	>60
	10 × 2	4/4	n/a	>60
Untreated	n/a	0/4	n/a	11

MRD = mean day of relapse. MSD = mean days of survival

S-MGB **234** and **235** have significant *in vivo* activity against *T. congolense* STIB 736/IL1180 mouse model of infection. The positive curative effects and the significant length of survival (at least to end of experiment) are notable. In view of this and the lack of cross resistance, understanding the mechanism of action of S-MGBs becomes important. Some relevant information is presented in the following slides.

Evidence for significance of kinetoplast in S-MGB activity

Conventional diamidine minor groove binding trypanocidal drugs cause destruction of the kinetoplast, which is an essential component of their mechanism of action. S-MGBs might be expected to show similar effects. This has been tested using ISMR1, which is a *T. b. brucei* in vitro-generated isometamidium resistant line with reduced mitochondrial membrane potential and loss of kinetoplast.

Compound	*T. b. brucei* WT EC_{50} (μM ± SD)	*T. b. brucei* ISMR1[#] EC_{50} (μM ± SD)	Resistance Factor
S-MGB 234	0.46 ± 0.33	0.39 ± 0.22	0.8
Diminazene	0.06 ± 0.05	0.61 ± 0.34	9.6
Isometamidium	0.06 ± 0.05*	1.10 ± 0.36	19.8
EtBr	0.49 ± 0.41*	1.19 ± 0.16	2.4

S-MGB **234** is as active against akinetoplastic *T. b. brucei*, ISMR1, as it is against wild type. This strain is resistant to diminazene and to the phenanthridines, isometamidium and ethidium bromide.

The presence of the kinetoplast therefore is not necessary for the trypanocidal activity of the S-MGBs. This is a distinctive feature of their mechanism of action.

# The effect of S-MGB 234 on kinetoplast and nucleus
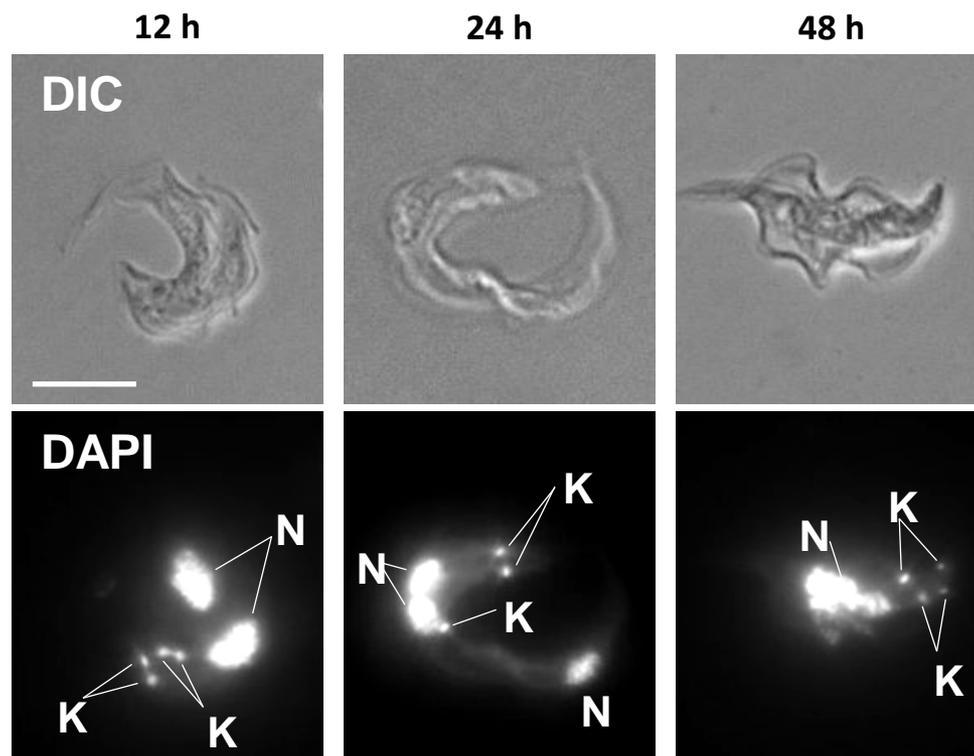
Although S-MGB **234** does not cause destruction of the kinetoplast it has major effects on the DNA-containing organelles. Treatment of *T. b. brucei* with S-MGB **234** causes aberrations in the DNA-containing organelles, their segregation and their morphology as well as an apparent block of cytokinesis. An accumulation of cells with multiple kinetoplasts and nuclei (MKMN) is observed over time in the treated parasite population.

Exposure to S-MGB **234** ($5 \times EC_{50}$) for 10 h has irreversible effects on *T. congolense* survival showing that this S-MGB, and presumably others, is cytocidal.

Time to kill and cytocidal activity

T. b. brucei Lister 427 BF

T. congolense IL3000 BF
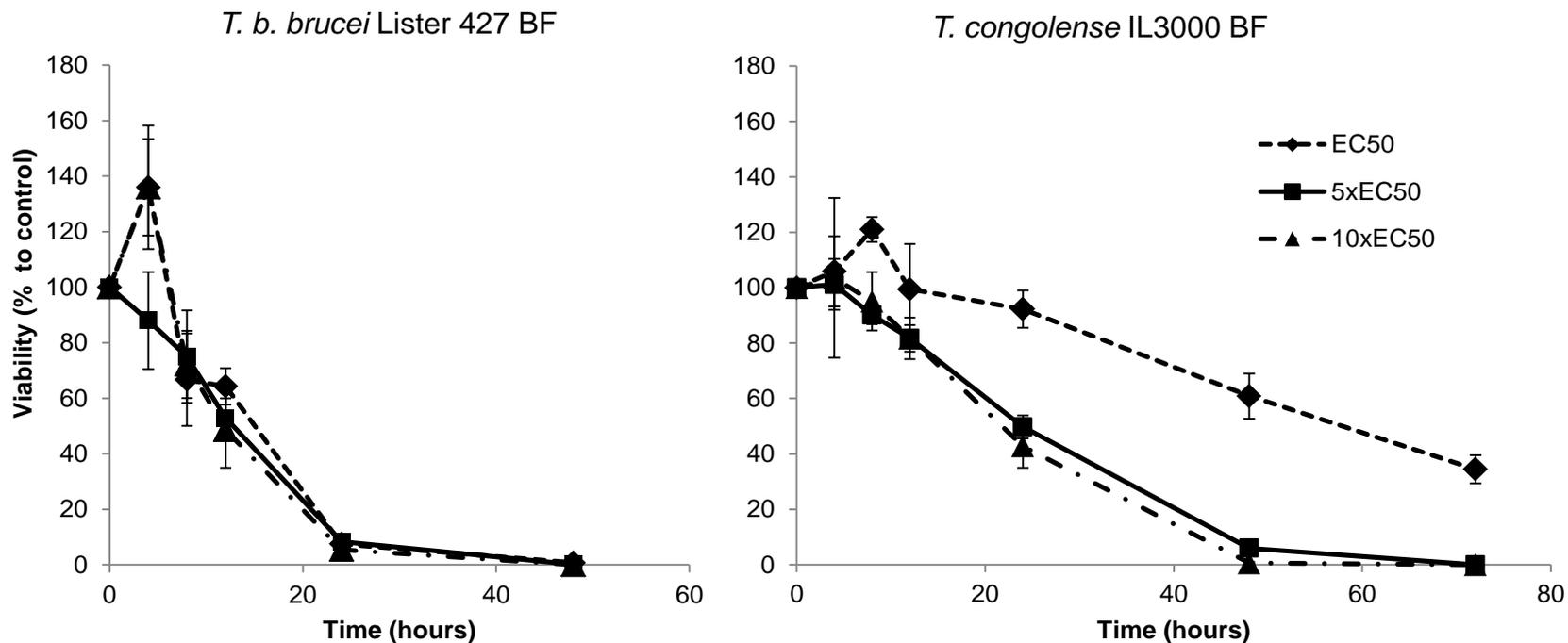
S-MGBs do not act fast. Exposure to $5 \times EC_{50}$ S-MGB **234** kills most *T. b. brucei* within 48 h and sterilises *T. congolense* cultures within 72 h. Abnormal, sick cells are visible before death occurs. Treated cells do not proliferate, which is further evidence for cytocidal activity.

Antitrypanosomal activity compared with antibacterial

Evidence has been obtained from metabolomics studies which show that many metabolite levels are altered on treatment of *T. b. brucei* with S-MGB **234** ($5 \times EC_{50}$, 8 h). The major changes in identified metabolites were in the nucleoside / nucleotide pool together with some derived cofactors (pyridoxine and pyridoxamine). 2-Oxoglutarate was also found to have a significant change. These changes relate to two of those observed in the bacterial experiments using RNA-seq methodology.

The metabolomics observations can be compared with the results from RNA-seq experiments using another S-MGB (**3**) attacking *Staphylococcus aureus* (see paper 'Why antibacterial minor groove binders are a good thing.). In the latter case, challenge with S-MGB-**3** caused hundreds of changes in gene expression, up and down regulation, that could be mapped on to several areas of metabolism. One of the most prominent of these was nucleotide metabolism. Energy production and lipid metabolism were also affected. There are thus some similarities between the effects of the S-MGBs on the two very different species.

It can be suggested, therefore, that S-MGB **234** by binding to trypanosomal DNA causes multiple biological effects in a manner related to that by which S-MGB **3** affects *S. aureus*. Since the selectivity of S-MGB **234** appears to be due to an uptake mechanism different from that of current drugs, a change in transporter availability or effectiveness may lead to resistance.

Conclusions

S-MGBs as a class have significant antitrypanosomal activity that shows clear structure-activity patterns.

- Amidine tail groups provide the best balance between activity and *in vitro* toxicity.
- Weakly basic tail groups are associated with compounds with higher toxicity.
- The most active compounds have arylalkenyl head groups and amidine tail groups.

S-MGBs are promising leads for the treatment of animal African trypanosomiasis.

- Good *in vitro* activity combines with proved *in vivo* efficacy against relevant *Trypanosoma* species;
- They are active *in vitro* against parasites resistant to currently used veterinary trypanocides.

Despite being both minor groove binders, S-MGBs and diamidines are unlikely to be cross-resistant.

- S-MGBs do not share the same transporters involved in diamidine uptake and resistance in *T. b. brucei*.
- The kinetoplast, which is disintegrated by diamidine exposure, is not the main target of the S-MGBs.

S-MGBs are cytocidal and affect cell division.

- Treated parasites do not proliferate and are unable to undergo correct cytokinesis.
- Some nucleotides accumulate in treated parasites, possibly due to decrease in DNA synthesis or to DNA disintegration.

Acknowledgments

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