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Potent trypanocidal curcumin analogs bearing a mono-enone linker motif act on *Trypanosoma brucei* by forming an adduct with trypanothione

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Abbreviations:

COSY, Correlation Spectroscopy; EC₅₀, 50% effective concentration; FBS, fetal bovine serum; γ -GCS, γ -glutamylcysteine synthetase; GS, glutathione synthetase; HILIC, hydrophilic interaction liquid chromatography; HEK, human embryonic kidney cells; HMBC, Heteronuclear Multiple Bond Correlation Spectroscopy; HMQC, Heteronuclear multiple-quantum correlation spectroscopy; LC, liquid chromatography; mBBr, monobromobimane; Mel-T, melarsoprol-trypanothione adduct; S-AdoMet, S-adenosyl methionine; TA014, trypanosomes adapted to AS-HK014;

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ABSTRACT

We have previously reported that curcumin analogs with a C₇ linker bearing a C₄-C₅ olefinic linker with a single keto group at C3 (enone linker) display mid-nanomolar activity against the bloodstream form of *Trypanosoma brucei*. However, no clear indication of their mechanism of action, or of their superior antiparasitic activity relative to analogs with the original di-ketone curcumin linker, was apparent. In order to further investigate their utility as antiparasitic agents, we here compare the cellular effects of curcumin and the enone linker lead compound, AS-HK014. An AS-HK014-resistant line, TA014, was developed by *in vitro* exposure to the drug. Metabolomic analysis revealed that exposure to AS-HK014, but not curcumin, rapidly depleted glutathione and trypanothione in the wild-type line, although almost all other metabolites were unchanged relative to control. In TA014 cells thiol levels were similar to untreated wild-type cells, and were not significantly depleted by AS-HK014. Adducts of AS-HK014 with both glutathione and trypanothione were identified in AS-HK014-exposed wild-type cells, and reproduced by chemical reaction. However, adduct accumulation in sensitive cells was much lower than in resistant cells. TA014 cells did not exhibit any changes in sequence or protein levels of glutathione synthetase and γ -glutamylcysteine synthetase relative to wild-type cells. We conclude that mono-enone curcuminoids have a different mode of action than curcumin, rapidly and specifically depleting thiol levels in trypanosomes by forming an adduct. This adduct may ultimately be responsible for the highly potent trypanocidal activity antiparasitic activity of the mono-enone curcuminoids.

Introduction

Curcumin is the active ingredient of turmeric, which is obtained from the roots and rhizomes of the plant *Curcuma longa* L. Turmeric has been used as a food flavoring, dye, and for many centuries in traditional Indian, Nepali and Chinese medicine, for instance to treat abdominal pain, sprains, fever and swellings (Eigner and Scholz, 1999; Goel et al., 2008). Curcumin has now been widely accepted to have many genuinely medicinal qualities and clinical applications are being investigated for a multitude of applications, including in cancer, and anti-inflammatory and neuroprotective treatments (Kuttan et al., 2007). Multiple clinical trials are assessing the clinical uses of curcumin and its derivatives (Goel et al., 2008; Gupta et al., 2013a,b).

In addition, curcuminoids have been proposed as anti-infective agents. For instance, curcuminoids displayed activity to the HIV-1 protease and integrase enzymes (Vajragupta et al., 2005; Itokawa et al., 2008), displayed better antifungal activity than fluconazole against *Paracoccidioides braziliensis* and inhibited adhesion of *Candida* species to human epithelial cells (Martins et al., 2009). Isoxazole curcuminoids were highly active against *Mycobacterium tuberculosis* (Changtam et al., 2010b). In particular, there is a growing literature on antiprotozoal activities of curcumin and its analogs.

Although curcumin itself displays only modest antimalarial activity, it was suggested that curcumin potentiates the action of the antimalarial α,β -arteether (Nandakumar et al., 2006). This was later shown to be due to immunomodulation by curcumin rather than a direct effect of the compound on the *Plasmodium berghei* parasites (Vathsala et al., 2012). Curcumin similarly protected mice against infection with *Trypanosoma evansi* through immunomodulation (Wolkmer et al., 2013), and protected fibroblasts against invasion by *T. cruzi* (Nagajyothi et al., 2012). Indeed, despite modest in vitro leishmanicidal activity (Koide et al., 2002; Salaheen et al., 2002), long-term use of curcumin reportedly exacerbates visceral

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leishmaniasis (Adapala and Chan, 2008) and certain bacterial infections (Marathe et al., 2012), in part by suppressing type I immunity (Adapala and Chan, 2008; Kang et al., 1999).

While the available evidence thus suggests predominantly indirect effects of curcumin against parasitic infections, curcuminoids with much improved activity against the human malaria parasite *P. falciparum* (Mishra et al., 2008; Manohar et al., 2013), *Leishmania mexicana amastigotes* and *L. major* promastigotes (Changtam et al., 2010a), experimental *L. amazonensis* infections (Alves et al., 2003), and *Trypanosoma brucei* (Changtam et al., 2010a) have recently been reported. In particular, a series of monoketo enone curcuminoids displayed highly promising in vitro activity against *T. brucei* bloodstream forms (Changtam et al., 2010a). In the current manuscript we investigate the mode of action of these compounds, and whether they act through a different mechanism than the parent compound curcumin.

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Materials and Methods

Trypanosome Strains and Cultures. *T. b. brucei* bloodstream forms of strain Lister 427 were cultured at 37 °C and under a 5% CO₂ atmosphere, exactly as described previously (Gudin et al., 2006), in HMI9 media (Invitrogen) supplemented with 10% fetal bovine serum (FBS; BioSera, Ringmer, UK). Strains adapted to curcumin or its analog AS-HK014 analogs were derived from s427 through in vitro exposure to increasing levels of the agent over several months, as described for diminazene (Teka et al., 2011) and pentamidine (Bridges et al., 2007). Clonal populations were obtained by limiting dilution.

Drug sensitivity assays. Sensitivity of trypanosome cultures to curcumin and its analogs was determined using the redox-sensitive indicator dye Alamar blue (resazurin sodium salt, Sigma) as described (Rodenko et al., 2007). The assay was performed in white 96-well plates, each of which contained 2×10^5 cells and the appropriate drug (at doubling dilution) in 200 μ l HMI-9/FBS. After 48 h of incubation (37 °C/5% CO₂) 20 μ l of sterile resazurin solution (125 μ g/ml in phosphate-buffered saline (PBS)) was added to each well, followed by a further incubation of 24 h. Fluorescence was read in a FLUOstar Optima (BMG Labtech; λ_{exc} = 530 nm, λ_{em} =590 nm). The diamidine trypanocides pentamidine and diminazene (both from Sigma-Aldrich) were used as controls. Drug sensitivity assays with human embryonic kidney (HEK) cells were performed similar using a resazurin-based assay, exactly as described previously (Changtam et al., 2010a; Rodenko et al., 2007).

Measurements of intracellular calcium and cAMP, and of plasma membrane potential.

Intracellular Calcium level was measured using the Screen Quest™ Fluo-8 Calcium Kit produced by ABD Bioquest (Ibrahim et al., 2011). Briefly, cultures of bloodstream form *T. b. brucei* Lister 427 were harvested and washed by centrifugation at $2500 \times g$ for 10 min at 4 °C, and re-suspended to a final density of 4×10^6 cells/ml in the Fluo-8 dye-loading solution and further incubated at 37 °C for 30 min. The cells were then washed twice with assay buffer to remove the dye-loading solution; 90 μ l of cells were added to each well in a black-

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bottomed 96-well plate. The positive control was 10 μ M calcium ionophore A23187 (Sigma) and the negative control was the assay buffer. The plate was then incubated in a FLUOstar OPTIMA fluorimeter at 37°C, and the fluorescence was observed at 485 nm excitation and 520 nm emission for 2 minutes before the addition of the test compounds and a further recording of 15 minutes (250 cycles – 4 sec/cycle).

Plasma membrane potential was recorded using the fluorescent probe bisoxonol as described (De Koning and Jarvis 1997). Briefly, 100 μ M of a cell suspension of 10^7 cells/mL was added to 2.9 mL of 0.1 μ M bisoxonol in a quartz cuvette loaded in a PerkinElmer LS55 Luminescence Spectrometer. Fluorescence was monitored at an excitation wavelength of 540 nm and emission at 580 nm. Test compounds were added 140 s after the start of recording in order to establish a stable baseline. Control traces without cells and without added compound were recorded as well.

The cellular cAMP content of trypanosomes was determined using the Direct Cyclic AMP Enzyme Immunonassay kit (Assay Designs) as described (De Koning et al., 2012; Gould et al 2013), following the manufacturer's instructions. The cAMP phosphodiesterase inhibitor CpdA (De Koning et al., 2012) was used as positive control.

Metabolomics sample preparation and analysis. Metabolomic analysis of AS-HK014-exposed trypanosome cultures was performed in triplicate, essentially as described for exposure to 5-fluoruracil (Ali et al., 2013). Briefly, log phase trypanosomes (Lister 427 or AS-HK014-adapted cells (TA014)) were resuspended at 2×10^6 cells/ml in 50 ml HMI-9/FBS and incubated for 30 min with or without 0.75 μ M of AS-HK014 (37 °C/5% CO₂) after which cells were rapidly cooled to 4 °C using a dry ice/ethanol bath. This culture was centrifuged (4 °C, 1250 \times g, 10 min) and the supernatant was removed. For MS analysis of thiols and other metabolites, we used the modified method for thiol derivatisation with the thiol specific reagent, monobromobimane (mBBr) (Fahey et al., 1983; Fairlamb et al., 1987).

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50 μ l of 40 mM *N*-(2-hydroxyethyl)piperazine-*N'*-3-propanesulfonic acid (EPPS), 2 mM EDTA, pH 8.0 and 50 μ l of 2 mM mBBR in absolute ethanol were added to the pellet, and vigorously mixed. Each solution was incubated at 70 °C for 5 min and cooled on ice for a min. Then, 100 μ l of 100 % acetonitrile precooled at 4 °C was added and incubated on ice for 10 min. Precipitated proteins and cellular debris were removed by centrifugation (13000 \times g, 3 min) and metabolite extracts were stored at -80 °C. Control samples included untreated cells grown in parallel; unused growth medium; test compound dissolved in HMI9/10% FBS; and extraction solvent blanks. Analysis was by hydrophilic interaction liquid chromatography (HILIC) coupled to high resolution mass spectrometry (MS). LC separation utilized a zwitterionic ZIC-HILIC column (Merck Sequant) with formic acid gradient as previously described (Zhang et al., 2012). The method was performed on a Dionex U3000 RSLC (Thermo Fisher) LC system coupled to an Exactive Orbitrap (Thermo Fisher) operating at 50,000 resolution in positive and negative mode ESI (rapid switching) with MS parameters as described (Creek et al., 2011). Metabolomics data was analyzed using the IDEOM application (<http://mzmatch.sourceforge.net/ideom.php>) with default parameters (Creek et al., 2012). Metabolites were identified by accurate mass and retention time (or predicted retention time where standards were unavailable) according to the IDEOM metabolite database (Creek et al 2011), with the addition of possible mBBR adducts for common cellular thiols. Molecular formulae for unidentified metabolites were determined based on accurate mass using the IDEOM application.

Cell survival assays. The effect of curcumin and its analogs on trypanosome growth was determined using microscopic cell counts, using an improved Neubauer hemocytometer; all determinations were in triplicate. Real-time cell survival was monitored over 500 min using the fluorescent probe propidium iodide, which fluoresces upon binding to nucleic acids. As this fluorophore does not cross the intact *T. b. brucei* plasma membrane, monitoring its fluorescence (λ_{exc} = 544 nm, λ_{em} = 620 nm) generates an output proportionate to the number of

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cells with compromised cell membrane integrity (Gould et al., 2008). The assay was performed in 96-well plates with doubling dilutions of the test compounds (as well as no-drug controls), 9 μM propidium iodide and 5×10^6 cells/ml in a FLUOstar Optima fluorimeter fitted with environmental controls (37 °C, 5% CO_2) (Ibrahim et al., 2011).

Western blotting. Log-phase bloodstream forms of *T. b. brucei* were resuspended in PBS at 10^6 ml^{-1} and lysed in 300 μl lysis buffer (20 mM MOPS pH 8.0, 4 mM MgCl_2 plus Roche Complete Protease Inhibitor Cocktail (Roche Diagnostics, Germany)), vigorously mixed, and flash frozen in liquid nitrogen; freeze/thaw cycles were repeated three times before cell debris was removed by centrifugation (13,000 $\times g$, 3 min). Protein content in the resulting lysates was determined using the Bradford method and 40 μg of protein from each sample was heated at 98 °C for 5 min in 4 \times sample buffer for SDS polyacrylamide gel electrophoresis. A 15 μl sample was separated on a 4-12% NuPAGE Novex Bis-Tris gradient gel using MES/SDS running buffer (both Invitrogen) at 180 V. Protein transfer used a buffer containing 25 mM Tris-base, 192 mM glycine and 20% ethanol and a nitrocellulose membrane (Hybond-ECL, Amersham) blocked with 5% milk and TBS/T (20 mM Tris-base, 137 mM NaCl, pH 7.4, 0.1% v/v Tween 20). After three washes in TBS/T the membrane was incubated for 2 h with primary antiserum (rabbit polyclonal anti- γ -glutamylcysteine synthetase (γ -GCS) or anti-glutathione synthetase (GS), both generous donations of Professor Meg Phillips, University of Texas Southwestern Medical Center, Houston, TX) at 1:2000 dilution in TBS/T/5% milk, followed by three washes with TBS/T and incubation with a secondary antirabbit horseradish peroxidase antibody (Amersham Biosciences; 1:1000 in TBS/T/5% milk, 1 h). Detection was by X-ray film after addition of chemoluminescence substrate.

Chemical reaction between glutathione and AS-HK014. In order to verify whether curcumin analog AS-HK014 reacts with glutathione to form an adduct, the two chemicals were mixed in a cell-free environment. Glutathione (130 mg; 0.42 mmol) was dissolved in 1.5 ml phosphate buffer pH 7.4, and 130 mg (0.37 mmol) of AS-HK014 in 3 ml ethanol was

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added. The reaction was stirred at ambient temperature (30 °C – 32 °C), and the reaction was observed by thin layer chromatography. The plate was developed using an anisaldehyde reagent, followed by heating on a hot plate in order to visualize spots. After 15 min stirring, an apparent reaction product was detected by TLC and stirring continued for 7 h. The reaction mixture was concentrated and the aqueous solution was washed with dichloromethane (3 × 10 ml). Acetone (2 ml) was added and the solution was left to stand. The colorless amorphous solid which separated out was filtered, washed with water-acetone and dried to yield the adduct (75 mg, 31%).

The reaction product of glutathione and AS-HK014 yielded a white solid with a melting point of 170 °C (foam) that decomposed at 210 °C. It had the following spectral properties: IR ν_{\max} : 3391, 3067, 2937, 2860, 1705, 1651, 1602, 1519, 1398, 1274, 1154, 1033, 817, 544 cm^{-1} . $^1\text{H-NMR}$ (400 MHz, D_2O): δ 1.53 (m, 2H, H-6), 1.98 (m, 2H, H-8'''), 2.27–2.37 (m, 4H, H-7 and H-7'''), 2.56–2.61 (m, 7H, H-1, H-2 and H-4), 2.64 (dd, $J = 13.3, 8.3$ Hz, 1H, H-1'''a), 2.77 (obscured signal, 1H, H-1'''b), 2.78 (obscured signal, 1H, H-5), 3.60 (obscured signal, 3H, H-4''' and H-9'''), 3.63 (br s, 2 × 3H, $\text{CH}_3\text{O-3}'$ and $\text{CH}_3\text{O-3}''$), 4.32 (br dd, $J = 8.3, 5.3$ Hz, 1H, H-2'''), 6.40 and 6.45 (each d, $J = 7.3$ Hz, 2 × 1H, H-6' and H-6''), 6.65–6.68 (m, 4H, H-2', H-2'', H-5' and H-5''); $^{13}\text{C-NMR}$ (100 MHz, D_2O): δ 26.1 (C-8'''), 28.9 (C-1), 31.4 (C-1''' and 7'''), 31.6 (C-7), 35.8 (C-6), 39.7 (C-5), 43.2 (C-4'''), 43.8 (C-2), 47.6 (C-4), 53.3 (C-2'''), 54.1 (C-9'''), 55.8 ($\text{OCH}_3 \times 2$), 112.6 (C-2', C-2''), 115.3 (C-5', C-5''), 120.8 and 120.9 (C-6', C-6''), 133.2 (C-1'), 133.9 (C-1''), 142.9 (C-4'), 143.1 (C-4''), 147.22 and 147.29 (C-3', C-3''), 171.5 (C-3'''), 173.8 and 174.5 (C-6''', C-10'''), 175.8 (C-5'''), 213.3 (C-3); ESMS (–ve): m/z (% rel. abund.) 662 $[\text{M} - \text{H}]^-$ (100).

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Results

Production and characterization of a *Trypanosoma brucei* cell line resistant to AS-HK014. Two adapted cell lines were established, by exposure to curcumin and to AS-HK014, with initial drug concentrations of 5 μM and 75 nM for curcumin and AS-HK014, respectively (structures and EC_{50} values, see Table 1). The concentration was systematically increased over many passages until the maximum tolerated concentration was reached; resumption of normal growth signaled adaptation and a further increase in the drug concentration. After 11 months, the concentration of curcumin reached only 8 μM , indicating an inability of *T. brucei* to meaningfully adapt to curcumin. For AS-HK014, the maximum tolerated concentration in culture eventually reached 4.5 μM (supplementary Fig 1A). Figure 1 shows that, whereas the long-term exposure to curcumin did not significantly change the curcumin EC_{50} value, the value for AS-HK014 was 28-fold ($P < 0.001$) increased in the adapted line, which was named TA014. In vitro growth rates of WT 427 and TA014 trypanosomes were identical (supplementary Fig 1B).

Interestingly, the TA014 line was not cross-resistant to curcumin ($P > 0.05$; Table 1) and indeed only showed cross-resistance to other analogues bearing the exact same mono-enone linker between the two aryl groups, with resistance factors of 4.7 - 5.2. Although this level of tolerance is modest, it was highly significant ($P < 0.01$) in all cases regardless of substituents on the aromatic rings, and not significant in analogs with any other linker, even with those having a higher number of conjugated olefinic groups such as AS-HK015, AS-HK017 and AS-HK118 (Table 1). This clearly establishes that the mono-enone analogs have a mode of action that is distinct from all the other curcumin analogs.

Effects of ASHK014 on *T. b. brucei* growth and survival. In order to investigate the mechanism of action of the mono-enone curcumin analogs, it is necessary to establish how

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quickly they act on parasite growth and survival at what concentration, so that the correct incubation conditions can be established for measuring early events rather than advanced cellular toxicity.

Cultures of s427WT and TA014 were incubated with either 150 nM or 500 nM AS-HK014, or without drug (control), and samples were taken over a 24-h period for cell counting. WT cells were highly sensitive to the compound, with 150 nM resulting in growth arrest, and 500 nM causing complete lysis of the cell population at the first (4 h) time point (Fig. 2A); neither concentration had much effect on TA014 growth (Fig. 2B) and we thus assessed the effects of a larger range of concentrations in real time over a period of 8 h, using an assay based on the cellular penetration of the fluorophore propidium iodide (Gould et al., 2008). This allowed us to establish that s427WT cells succumb to AS-HK014 at concentrations $\geq 500 \mu\text{M}$ in approximately 40 min and to as little as $250 \mu\text{M}$ in 3 h, whereas 100 nM had no measurable effect on cellular integrity over the course of the 500 min experiment (Fig. 2C). TA014 cells were vulnerable to $4 \mu\text{M}$, with lysis completed in approximately 3 h, and partly sensitive to $1.5 \mu\text{M}$ AS-HK014, with effects becoming apparent after 4 h (Fig. 2D). Curcumin only started to affect plasma membrane integrity after 4h and only at concentrations $\geq 25 \mu\text{M}$ (Fig. 2E).

Assessment of the effects of curcumin and AS-HK014 on plasma membrane conductance and signaling in wild-type *T. brucei*.

It has previously been reported that curcumin, and several other common phytochemicals such as EGCG from green tea, exerts a non-specific effect on biological membranes and generally interact with many membrane proteins rather than a specific target (e.g. Ingolfsson et al., 2014). There is certainly evidence to support this, yet this does not necessarily negate the many reports of beneficial medical and physiological effects of curcumin and green tea or imply that these compounds impact on membrane integrity at therapeutic levels. In order to

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assess the effects of curcumin and the mono-enone analogs AS-HK014 and AS-HK096 on plasma membrane potential and calcium conductance as well as on intracellular levels of cAMP as a further indicator signaling events that could be triggered by non-specific activation of membrane proteins. None of the test compounds had any effect on calcium conductance (Fig. 3), or plasma membrane potential (Supplementary Figure S2) although the concentrations used were much higher than the EC₅₀ values (Curcumin 50 μM; AS-HK, 10 μM; AS-HK096, 20 μM) and the controls (calcium ionophore A23187 and gramicidin, respectively) had immediate and pronounced effects on the parameters measured. Furthermore, none of the three curcuminoids had a significant effect on cAMP, although the phosphodiesterase inhibitor CpdA (De Koning et al., 2012) stimulated cAMP levels to almost 300% of the no-drug control (P<0.01) under the same conditions (Supplementary Figure S3). Several ionophores with well-described effects on the plasma membrane potential or proton-motive force of *T. brucei* bloodstream forms (De Koning and Jarvis, 1997; De Koning et al 1998) were included in the cAMP experiment to assess whether such perturbations directly affected cAMP levels over the 15 minutes of the experiments; only the calcium ionophores A23187 and ionomycin caused an apparent reduction in cellular cAMP content, but this did not reach statistical significance (Supplementary Figure S3).

Metabolomic analysis of the action of AS-HK014 on *T. b. brucei*. In order to measure early effects of AS-HK014 on s427WT trypanosomes, we incubated a culture of these cells with and without 200 nM AS-HK014 for 30 minutes. This is a sufficient concentration to impact on the cells, but the incubation was terminated, and the metabolites extracted, well before the test compound had a measureable effect on cell survival or plasma membrane integrity (Fig 2A,C). The specificity of the metabolic changes observed was evaluated by comparison with an identical analysis of TA014 cells incubated with the same concentration of AS-HK014 in parallel.

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1. Wild-type s427 cells. As we used untargeted metabolomics there was no a priori assumption on a mechanism of action and the entire metabolome was analyzed to the limit of the technology. A global overview of most-changed metabolites shows that AS-HK014 impacted strongly on intracellular thiol levels (Fig. 4), with trypanothione content reduced by >99%, and glutathione and S-glutathionyl-L-cysteine levels reduced by >90% in AS-HK014-treated WT cells, relative to non-treated controls. In the subsequent metabolomic analyses described below the cellular levels of the relevant thiols (cysteine, glutathione and trypanothione) were determined by a modification of the monobromobimane method (Fahey & Newton, 1983; Fairlamb *et al.*, 1987). This method can derivatise intracellular sulfhydryls, to prevent their oxidation and allow detection of both oxidised and reduced forms of intracellular thiols.

The relevant pathway (supplementary Fig S4) starts with the biosynthesis of glutathione from three amino acids: cysteine, glutamate and glycine. Two molecules of glutathione are then coupled consecutively to spermidine to form trypanothione, the main trypanosome thiol. In WT trypanosomes, treatment with 200 nM of AS-HK014 for 30 min led to very clear reductions in thiol levels (Fig. 5). Trypanothione levels, both in reduced form (detected as di-monobromobimane adduct (di-mBBr)) and as disulfide, were dramatically reduced under these conditions (to $12 \pm 3\%$ ($P < 0.01$) and $6 \pm 2\%$ ($P < 0.001$), respectively). Similar reductions in glutathione mBBr ($P < 0.01$), S-glutathionyl-L-cysteine ($P < 0.02$) and glutathionylspermidine mBBr ($P < 0.01$) were also observed, opening the strong possibility that biosynthesis of trypanothione was inhibited due to a depletion of glutathione. Further upstream, there were small but significant reductions in the levels of L-cysteine mBBr and of glycine (both $P < 0.05$), but there was no change in glutamate levels (Fig. 5). All three of these amino acids are present at high levels in the HMI-9 culture medium so any changes might be difficult to unearth with the conditions used. In addition, the pathway leading up to spermidine (supplementary Fig S5) was analyzed. There was no change in spermidine levels

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upon treatment with AS-HK014, nor in any of the metabolites of its biosynthesis pathway: ornithine, *S*-adenosyl methionine (*S*-AdoMet) and putrescine (Fig. 5). Furthermore, there was no significant change in the level of 5'-methylthioadenosine (data not shown), the reaction product after the aminopropyl donation by *S*-AdoMet in the synthesis of spermidine.

The above shows that the only metabolomic perturbations upon incubation with AS-HK014 were the dramatically reduced levels of thiols, whereas the constituent metabolites in their biosynthetic pathways displayed at most very minor changes. This contrasts with treatment of the same trypanosome strain with the inhibitor of ornithyl decarboxylase, eflornithine, which resulted in rapid changes in intracellular levels of putrescine, ornithine (and both of their acetylated forms) and 5'-thioadenosine metabolites (Vincent *et al.*, 2012).

One explanation for the rapid depletion of thiols would be the reaction with AS-HK014 to form a stable adduct. This type of mechanism has been proposed for melarsoprol, which forms the trypanothione adduct Mel-T (Fairlamb *et al.*, 1989), which in turn inhibits trypanothione reductase (Cunningham *et al.*, 1994). Mel-T can be exported from the cells by ABC transporters (Shahi *et al.*, 2002). Unlike ASHK-014, however, melarsoprol treatment does not significantly decrease the cellular trypanothione concentration (Fairlamb *et al.*, 1992), presumably because it is used at a much lower concentration than AS-HK014. Stable adducts were indeed found in AS-HK014-exposed trypanosomes, both with trypanothione and glutathione (Fig. 6). However, the adducts were observed not only in cells exposed to AS-HK014 for the 'standard' 30 min at 37 °C, but also when AS-HK014 was added after 30 min drug-free incubation and the cells had been rapidly cooled to 4 °C in a dry-ice/ethanol bath; these cells were then immediately processed for metabolite extraction (quenched cells). Indeed, the levels of adduct were similar in the incubated and in the quenched cells, showing clearly that the AS-HK014 reaction with thiols is a rapid chemical reaction with free thiols, rather than an enzymatic reaction.

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2. TA014 cells. The entire metabolomic analysis was performed in parallel with the AS-HK014-resistant line TA014 as well. The same trend on thiol levels as in WT *T. b. brucei* was apparent (Fig. 5), but none of the reductions in thiol concentrations reached significance under the assay conditions of 200 nM for 30 minutes - consistent with the lack of impact that this concentration has on the TA014 cells. More interesting was that the levels of thiols, polyamines, relevant amino acids and 5'-thioadenosine metabolites were all statistically identical in unexposed WT, unexposed TA014 and AS-HK014 exposed TA014 cells (Fig. 5). This shows that the resistance adaptation was not the result of significantly higher cellular levels of trypanothione and/or glutathione. Nor was AS-HK014 accumulation prevented (loss of import transporter or expression of export transporter), as AS-HK014 levels (detected intact or as a C₁₀H₁₀O₂ MS-generated fragment) were statistically identical in wild-type and resistant cells (Supplementary Fig. S6). Furthermore, much higher levels of glutathione (P<0.05) and trypanothione (P<0.02) adducts were found in AS-HK014 exposed TA014 cells than in WT trypanosomes (Fig. 5), possibly reflecting the higher thiol levels present in the exposed TA014 cells. This observation provides strong confirmation that it is not the adduct that is deleterious to the cells and is consistent with the diffusion model of AS-HK014 uptake, driven by adduct formation. Yet, in the resistant cells, the thiol depletion is minimal, indicating an increased capacity of cells to maintain their glutathione and trypanothione levels in the presence of the drug.

3. Metabolomics of curcumin.

A highly similar metabolomics analysis was subsequently performed using 3 μM curcumin on wild-type or TA014 cells and a 30 minute or 5 h incubation time; AS-HK014 was used as a positive control. Whereas AS-HK014 again had profound effects on thiol levels, particularly glutathione and trypanothione, there was no effect of curcumin on thiol levels.

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Indeed, no clear pattern on the composition of the metabolome was apparent (data not shown).

Adduct 2 from chemical reaction between glutathione and AS-HK014. AS-HK014

reacted with glutathione to form adduct **2** in 31% yield (Fig. 7). It should be noted that, without the buffer, only a very small quantity of the adduct **2** was detected after 7 h stirring at the same temperature. The rate of addition reaction increased with increased temperature. The structure of the adduct **2** was elucidated by spectroscopic methods. Negative ion electrospray mass spectrum (ESMS) showed the $[M - H]^-$ peak at m/z 662, which was compatible with the molecular formula of $C_{31}H_{41}N_3O_{11}S$ of the adduct **2**. The 1H NMR spectra revealed the presence of a glutathione and AS-HK014 moieties, except for the absence of two olefinic protons at the 4- and 5-positions of the latter. The 1H and ^{13}C NMR data were assigned based on the COSY, HMQC and HMBC experiments. Apart from four carbonyl carbon resonances of the glutathione part at δ 171.5, 173.8, 174.5 and 175.8, the carbon resonance of the non-conjugated keto function of the curcuminoid part appeared at δ 213.3, indicating the addition of glutathione to the 5-position of AS-HK014 (**1**) has taken place. Linkage between the two moieties was evident from the key HMBC correlation between H-5 (δ 2.78) and C-1''' (δ 31.4); H-1'''a (δ 2.64) and H-1'''b (δ 2.77) and C-5 (δ 39.7).

Extracellular L-glutathione affects the trypanocidal activity of AS-HK014. The standard Alamar blue assay for trypanosome viability was used to determine the EC_{50} of curcumin compounds with different concentrations of L-Glutathione in the medium (0, 10, 100, 1000 μ M). Whereas extracellular glutathione up to 1 mM had no effect on the activity of curcumin on WT trypanosomes (Fig. 8A), co-incubation of AS-HK014 with different concentrations of L-glutathione led to a progressive decrease in the activity of the drug (Fig. 8B). As little as 10 μ M glutathione significantly increased the AS-HK014 EC_{50} value against WT cells ($P < 0.05$);

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however, the EC₅₀ values were not significantly different with different concentrations of L-glutathione for the TA014 line (Fig. 8C), indicating that the residual effect of AS-HK014 in the resistant line might not be due to the same glutathione-sensitive mechanism.

Sequence analysis of *T. b. brucei* glutathione synthetase and γ -glutamylcysteine synthetase. In *T. b. brucei*, the two key enzymes of glutathione biosynthesis are γ -glutamylcysteine synthetase (γ -GCS; Tb427.10.12370) and glutathione synthetase (GS; Tb427.07.4000) (see Fig. S4). As the adaptation in TA014 giving protection to AS-HK014 was connected with thiol levels, we used polymerase chain reaction (PCR) to verify the presence of both genes in trypanosome wild-type and resistance lines. Bands of the expected size were amplified from both strains (Fig. 9A). The PCR products were cloned, and amplified in *E. coli*. For each reaction, 9 colonies were picked and the inserts were sequenced. Both genes were heterozygous in the wild-type and in the TA014 strains, but no allelic differences were observed between the two strains. Coding polymorphisms in γ -GCS were G63S, G237S, L477V and T670K (allele-1/allele-2). For GS, the only coding difference between the two alleles was N523T.

Analysis of protein levels of glutathione synthetase and γ -glutamylcysteine synthetase.

As the sequence analysis detected no mutations in the GS and γ -GCS genes in s427WT and TA014 strains, we next examined whether there was any difference in protein levels, using Western blotting with specific antibodies. Primary antibody (rabbit polyclonal anti- γ -GCS antibody and rabbit polyclonal anti-GS (both generously provided by Professor Margaret Phillips, University of Texas Southwestern Medical Center) was used to measure the protein expression level. s427WT and TA014 (10^6 cells/mL) were treated with 200 nM of AS-HK014 for 30 min, with untreated cells incubated in parallel serving as controls; protein extracts were separated by polyacrylamide gel electrophoresis (PAGE) and probed by

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Western blot. There were no detectable differences in the protein levels of the two strains and the protein levels were not changed by the treatment with AS-HK014 (Fig. 9B).

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Discussion

It has been long known that curcumin has beneficial effects for some conditions and ailments, due to now well-documented anti-inflammatory effects (Shehzad et al., 2013). Anticarcinogenic, antiangiogenesis and antimetastatic effects of this natural polyphenol, by a plethora of proposed mechanisms, have also been described (Kuttan et al., 2007; Norris et al., 2013; Fan et al., 2013). Although anti-infective properties of curcumin have also been described, particularly with reference to parasitic disease (Haddad et al., 2011), the potential here is mostly in synthetic curcuminoids, curcumin itself having insufficient antiparasitic activity. Efforts have been made to address the structure-activity relationships of these antiparasitic curcuminoids through the synthesis of small chemical libraries but it is not clear whether the new curcumin analogs do in fact represent optimized curcumins, or constitute separate chemical entities with an entirely new mechanism of action.

We recently described the synthesis and antitrypanosomal evaluation of a series of 57 curcumin analogs, all with a C7 linker between phenolic rings, of which the most active compound, 1,7-bis(4-hydroxy-3-methoxyphenyl)hept-4-en-3-one (AS-HK014) displayed an EC₅₀ value of 0.053 ± 0.007 μ M against *T. b. brucei* bloodstream forms (Changtam et al., 2010a), approximately 50-fold better than curcumin and 2-fold better than diminazene aceturate. AS-HK014 displayed an in vitro selectivity index of 221 versus HEK cells, compared with only 13.7 for curcumin itself. Other curcuminoids with the same mono-enone linker displayed similar activity. However, a realistic optimization strategy would require knowledge of the mechanism of action, both for curcumin and for the enone analogues.

In the current paper, we establish that curcumin and AS-HK014 do not have the same mechanism of action. First of all, we were unable to induce resistance to curcumin, although we have previously had much success with this strategy (Bridges et al., 2007; Gould et al., 2013; Ali et al., 2013; Eze and De Koning, unpublished), and we were able to induce resistance to AS-HK014 without problems. More importantly, the resultant AS-HK014-

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resistant strain was not cross-resistant with curcumin, a clear indication of a different mode of action. Furthermore, incubation of wild-type trypanosomes with AS-HK014 rapidly and specifically depleted cellular thiol levels, including glutathione, S-glutathionyl-L-cysteine, glutathionylspermidine, and trypanothione, whereas curcumin had no effect on these metabolites. Thus, the much higher trypanocidal activity of AS-HK014, relative to curcumin, is not the result of an improved interaction with the same target, but rather the result of interaction with a different or additional target, i.e. trypanosomal thiol metabolism.

In contrast, the observations that (1) resistance to curcumin could not be induced in vitro, and (2) that the compound had no specific effect on the metabolome, seem to suggest that its trypanocidal action was multifactorial and not very specific. As curcumin displays a very similar low micromolar activity against a number of protozoa in vitro, including *Leishmania* spp (Salaheen et al., 2002; Changtam et al., 2010a), *Giardia lamblia* (Pérez-Arriaga et al., 2006), *Plasmodium falciparum* (Mishra et al., 2008), and *Trypanosoma brucei* (Nose et al., 1998; Changtam et al., 2010a), the argument of limited specificity is compelling. In this respect it is of interest to note that curcumin is known to alter membrane fluidity in an apoptosis-independent way (Jaruga et al., 1998; Atsumi et al., 2005; Hung et al., 2008; Ingolfsson et al., 2014).

We thus propose the possibility that the main antiprotozoal activity of curcumin may be through its effects on membrane fluidity and function, whereas the principal activity of many synthetic curcuminoids is unrelated to this mechanism. However, curcumin did not affect plasma membrane integrity very rapidly: neither curcumin nor AS-HK014 affected plasma membrane potential over 15 minutes, or intracellular calcium levels over 20 minutes, at $>10\times EC_{50}$ concentrations. Although curcumin did affect plasma membrane permeability in the propidium iodide fluorescence assay ($\geq 5\times EC_{50}$, >4 h), we cannot exclude the possibility that this reflects cell death by a different mechanism, unrelated to membrane fluidity. We also find no evidence that mono-enone curcuminoids have a deleterious effect on plasma

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membrane stability or permeability, especially at the mid-nanomolar concentrations at which they exert their trypanocidal effects. Indeed, a non-specific action on the cells would not explain the in vitro selectivity of several hundred fold over human cell lines, or the >100-fold lower activity against *Leishmania major* promastigotes and *L. mexicana* amastigotes (Changtam et al 2010a).

The untargeted metabolomics analysis, however, gave a very clear indication of the AS-HK014 mechanism of action. The cellular levels of all major thiols including cysteine were significantly reduced after treatment with this compound, whereas this did not happen in the AS-HK014-resistant line TA014. No other significant changes were observed in the metabolomic analysis. A possible explanation for these observations is that AS-HK014 directly oxidizes thiols. This was tested by including glutathione in the trypanosome culture medium, which dose-dependently inhibited the activity of AS-HK014, but not of curcumin. The chemical reaction was confirmed by mixing glutathione and AS-HK014 together at ambient temperature, and the product was identified.

We thus conclude that AS-HK014 undergoes a rapid chemical reaction with reactive intracellular thiols, particularly glutathione and trypanothione, effectively depleting these essential thiols and leading to, or strongly contributing to, cell death. It is possible that the compound furthermore reacts with additional thiols, perhaps in proteins, leading to irreversible inhibition of the function of these proteins. This mechanism of action is highly reminiscent of the mechanism of action of the melaminophenyl arsenical class of trypanocides, which also form a stable adduct with trypanothione; again this adduct is the only metabolic change detected in treated trypanosomes (Fairlamb et al., 1989). The authors of that study concluded that the adduct itself could well be the toxic agent, responsible for the selective activity of these organo-arsenic compounds, as the adduct was an inhibitor of the essential enzyme trypanothione reductase (Fairlamb et al., 1989). We conclude that although mono-enone curcuminoids like AS-HK014 may react non-selectively with glutathione, the

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evidence shows that they could still induce rapid and selective lysis of *T. brucei* spp, most likely through adduct formation with trypanothione.

As curcuminoids may be able to efficiently diffuse into the cells (AS-HK014 has a cLogP of 3.84; ChemDraw Pro 10.0, CambridgeSoft), it is highly likely that uptake is driven by the reaction with trypanothione, and continues until thiol content is effectively depleted. However, observed adduct levels in the treated cells were not greater than those obtained from unexposed, quenched cells, which did not exhibit significant thiol depletion. This suggests that the adduct may undergo metabolic degradation, or possibly complexes/reacts further with an intracellular target. The metabolomics data does not provide any direct evidence for either pathway, as no novel metabolite signals were specifically enriched in cells exposed to drug for 30 minutes.

As an alternative to thiol depletion directly by chemical reaction with AS-HK014, the depletion could be explained by inhibition of glutathione synthesis, but it is unlikely that this mechanism alone would cause such a dramatic effect on the total thiol pool within 30 minutes. Moreover, inhibition of the enzymes of glutathione synthesis would likely have lead to accumulation of the enzyme substrates (See Supplementary Figure S4), However, L- γ -glutamylcysteine, the substrate of glutathione synthetase, was not detected in the treated cells, and levels of L-cysteine and L-glutamate, the substrates of γ -glutamylcysteine synthetase, were significantly reduced and unchanged, respectively, which is not compatible with inhibition of glutathione and trypanothione biosynthesis.

In the AS-HK014-resistant line TA014, AS-HK014 did not significantly deplete thiols under the assay conditions chosen (200 nM, 30 min), although glutathione, glutathione-cysteine, glutathionylspermidine, and trypanothione all trended somewhat lower in the AS-HK014-treated TA014 cells. Clearly, TA014 cells have adapted to become more resistant to the oxidative effects of enone curcuminoids, but by which mechanism they have done so is unclear. Thiol levels were not different in unchallenged wild-type and TA014 cells, nor could

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we find evidence for mutations in the open reading frames of the main enzymes involved in glutathione synthesis, glutathione synthetase and γ -glutamylcysteine synthetase, nor evidence of differential expression in TA014. Yet, the amount of AS-HK014 adduct with trypanothione and glutathione was significantly higher in TA014 cells, and the resistance mechanism might be at the level of the adduct's toxicity by a change in sequestration of this product, or a mutation in the cellular target. The absence of a reaction (or strong complex) of the adduct with a cellular target would explain its apparently higher accumulation observed in the metabolomic analysis of the resistant cells. Alternatively, if the resistant cells have a mechanism (enzymatic or otherwise) of regenerating free thiols from the increased levels of adduct, this would help maintain total free GSH and TSH pools within the cell.

In summary, we have established that mono-enone curcuminoids act through a different and much more specific mechanism than curcumin on trypanosomes, and identify this mechanism as a chemical reaction with thiols, depleting the cells of glutathione and trypanothione, likely followed by a toxic action of the reaction product. Whereas chemicals based on the curcumin scaffold have undoubted potential against protozoan pathogens, these should not be considered as improved curcumins in the sense of improved action on a common target.

Authorship contributions

Participated in research design: DJC, MPB, HPdK

Conducted experiments: AAMA, DJC, HI, D-HK, KB, CC, NBQ

Contributed new reagents or analytic tools: AS

Performed data analysis: DJC

Wrote or contributed to the writing of the manuscript: DJC, MPB, AS, HPdK

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Footnotes

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Figure Legends

Fig. 1. Average EC₅₀ values of the clonal lines that were long-term exposed to curcumin (A) or AS-HK014 (B) compared with wild-type *T. b. brucei* bloodstream forms. The experiment was performed on three separate occasions, and the data shown are the average \pm SEM of these three experiments.

Fig 2. Effect of different concentrations of AS-HK014 on the growth and integrity of bloodstream form *T. brucei brucei* s427WT and TA014 lines. Frames A and B show growth of WT (A) and resistant (B) cell populations in the presence or absence of AS-HK014. The results shown are the average of three independent determinations; error bars depict standard errors. In frames C (s427WT) and D (TA014), fluorescence of 9 μ M propidium iodide was monitored in real time, with cells in HMI-9/FBS (37 C, 5% CO₂) incubated in the presence or absence of various concentrations of AS-HK014. Frame E, propidium iodide assay with curcumin on WT s427 trypanosomes. Fluorescence was also recorded in parallel without cells, for each drug concentration, and the fluorescence recorded was subtracted as background from the traces obtained in the presence of cells. a, 8 μ M; b, 4 μ M; c, 1.50 μ M; d, 500 nM; e, 250 nM; f, 100 nM; g, control, no drug; h, 50 μ M; i, 25 μ M; j, 12.5 μ M; k, 3.1 μ M; l, 0.78 μ M.

Fig. 3. Effect of curcumin compounds on the intracellular calcium level in bloodstream-form *T. b. brucei*. Parasites (4×10^6 /mL) were loaded with Fluo-8 dye, and the fluorescence intensity was measured. The fluorescence was read for 120 s before addition of the curcumin compound or calcium ionophore was added to the cells (arrow), and the fluorescence was recorded for an additional 18 min. Separate traces were recorded in the absence of cells and were subtracted from those presented in the figure, in order to compensate for any innate fluorescence of the compounds or buffer components.

Fig. 4. Relative abundance of 915 putative metabolites from cells treated with ASHK-014 relative to untreated cells. Untargeted metabolomics was conducted with LC-MS, metabolite

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abundance is measured by raw peak height and putative identification was based on accurate mass. Metabolites are grouped by metabolic pathways where red = amino acid metabolism, blue = carbohydrate metabolism, green = lipid metabolism, orange = metabolism of cofactors and vitamins, purple = nucleotide metabolism, black = peptides and brown = unknown pathways.

Fig. 5. Metabolomic analysis of *T. b. brucei*-WT and TA014 resistance line cell extracts treated with the AS-HK014 compound. For each cell sample, 25 mL of cell culture (2×10^6 cells/mL) was incubated for 30 min at 37 °C with and without AS-HK014 (200 nM) for both strains. Relative quantification was measured according to peak intensity from the LC-MS metabolomic platform and is expressed relative to untreated WT (mean and SEM). mBBr adducts indicate reduced thiol species derivitised by monobromobimane. *P<0.05, **P<0.02, ***P<0.01, ****P<0.001

Fig. 6. Adduct levels of trypanothione and glutathione in extracts produced under different conditions. Time indicated is incubation time of live cells with 200 nM AS-HK014 before metabolomics sample processing. Zero time denotes addition of AS-HK014 to quenched cells. *P<0.05, **P<0.02

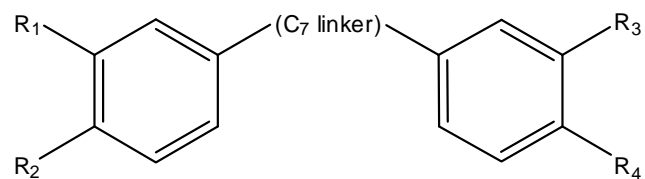
Fig.7. Chemical reaction between curcumin AS-HK014 and glutathione . 1: AS-HK014 and 2: adduct.

Fig. 8. EC₅₀ values for curcumin (A) and AS-HK014 (B,C) with various concentrations of glutathione, as determined by Alamar Blue assay for the s427 WT *T. b. brucei* (A,B) and TA014 resistance lines (C). The experiment was performed on three separate occasions, and the data shown are the average \pm SEM of these three experiments. P-values indicate significant differences from control (0 μ M of L-glutathione), * P<0.05, ** P<0.01, *** P<0.001.

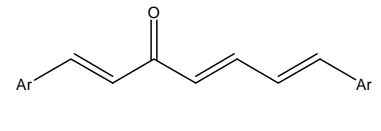
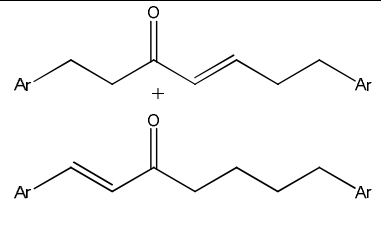
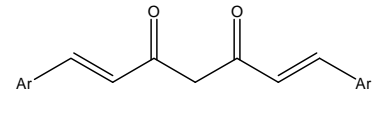
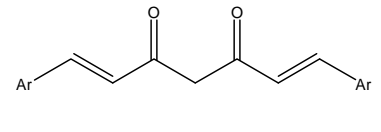
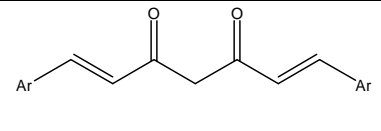
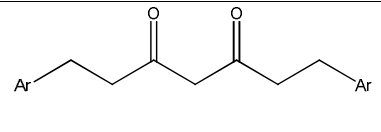
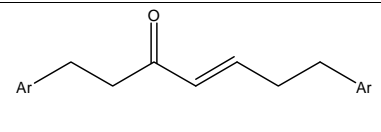
Fig. 9. A. PCR amplification of G1 (γ -GCS) and G2 (GS) for both strains of bloodstream-form *T. b. b.* WT and TA014 resistance line, NG: negative control which consists of all PCR

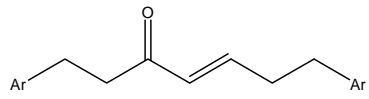
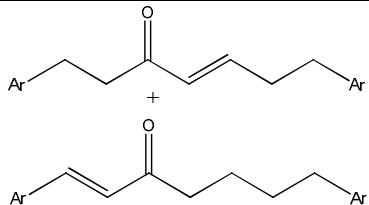
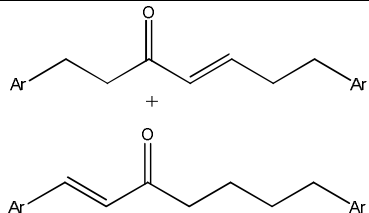
MOL #96016

components except genomic DNA. B. Western blot of the *T. b. brucei*-WT and TA014 resistance lines at 1×10^6 cells/mL. The cells were incubated for 30 min in the presence or absence of 200 nM of AS-HK014 and. Two antibodies were used for both strains: γ -GCS and GS. (1) *T. b. b.* WT, drug free, (2) *T. b. b.* WT + AS-HK014, (3) TA014, drug free, (4) TA014 + AS-HK014.



Compound	s427 EC ₅₀ (μ M)	TA014 EC ₅₀ (μ M)	RF	t-test	R1	R2	R3	R4	HEK EC ₅₀ (μ M)	SI	Linker structure
curcumin	2.7 \pm 0.4	2.9 \pm 0.13	1.06	NS	OCH ₃	OH	OCH ₃	OH	37.1 \pm 6.3	13.7	
AS-HK014	0.11 \pm 0.01	3.1 \pm 0.1	28.2	P < 0.001	OCH ₃	OH	OCH ₃	OH	24.3 \pm 2.4	221	
AS-HK015	3.8 \pm 0.12	4.1 \pm 0.19	1.08	NS	OCH ₃	OH	OCH ₃	OH	75.6 \pm 3.0	19.9	
AS-HK016	0.21 \pm 0.007	0.17 \pm 0.013	0.81	NS	OCH ₃	OH	OCH ₃	OH	8.1 \pm 1.0	38.8	

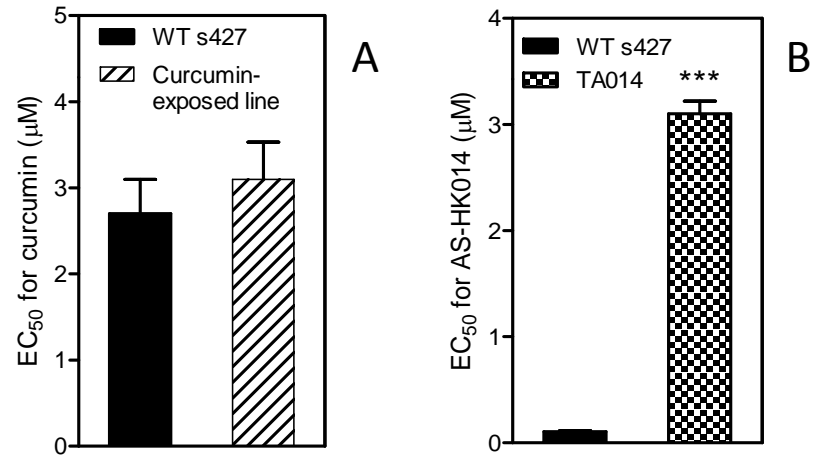
AS-HK017	1.07 ± 0.04	1.04 ± 0.02	0.97	NS	OCH ₃	OH	OCH ₃	OH	22.0 ± 0.14	20.6	
AS-HK018	0.18 ± 0.008	0.84 ± 0.08	4.7	P < 0.01	OCH ₃	OH	OCH ₃	OCH ₃	19.7 ± 1.6	109	
AS-HK028	1.15 ± 0.07	1.08 ± 0.02	0.94	NS	OCH ₃	OH	OCH ₃	OBz	93.7 ± 11.2	81.5	
AS-HK033	0.80 ± 0.005	0.73 ± 0.06	0.92	NS	OCH ₃	OH	OH	OH	36.3 ± 3.6	45.4	
AS-HK073	0.41 ± 0.03	0.45 ± 0.03	1.1	NS	OCH ₃	OCH ₃	OCH ₃	OCH ₃	202 ± 54	493	
AS-HK085	0.52 ± 0.03	0.47 ± 0.04	0.9	NS	OCH ₃	OH	OH	OH	132 ± 19	255	
AS-HK093	0.079 ± 0.006	0.40 ± 0.01	5.2	P < 0.001	OCH ₃	OCH ₂ CN	OCH ₃	OCH ₂ CN	21 ± 2.3	270	

AS-HK096	0.047 ± 0.002	0.24 ± 0.04	5.1	P < 0.001	OCH ₃	OAc	OCH ₃	OAc	29.9 ± 1.8	637	
AS-HK097	0.090 ± 0.001	0.47 ± 0.02	5.2	P < 0.001	OCH ₃	OH	OCH ₃	OAc	24.8 ± 0.8	275	
AS-HK118	0.44 ± 0.019	0.43 ± 0.005	1	NS	OCH ₃	OH	OCH ₃	OC ₅ H ₁₀ Br	17.1 ± 0.6	38.8	
Diminazene	0.13 ± 0.03	0.15 ± 0.04	1.15	NS							
Pentamidine	0.005 ± 0.001	0.003 ± 0.001	0.6	NS							
Eflornithine	39.5 ±	43.17 ±	1.1	NS							

	3.76	3.64									
Cymelarsan	0.0059 ± 0.0015	0.0078 ± 0.0021	1.33	NS							
Nifurtimox	9.27 ± 1.09	7.21 ± 1.38	0.78	NS							
Suramin	0.025 ± 0.005	0.036 ± 0.006	1.44	NS							

TABLE 1. EC₅₀ values of curcumin and its analogs against wild type trypanosomes (Lister s427) and the strain adapted to AS-HK014 (TA014). All values are average and SEM of at least three independent determinations, with EC₅₀'s against the two strains determined in parallel. Also shown is the resistance factor (RF) which is the ratio EC₅₀ TA014 / EC₅₀ s427. When more than 1 linker structure is given, it represents a mixture of both isomers. Significance was determined using a paired Student's t-test. OBz, benzoyl. HEK, Human Embryonic Kidney cells. SI, selectivity index, being the ratio of the EC₅₀ values against HEK cells and wild-type trypanosomes. Some of the HEK EC₅₀ values listed here were previously reported by Changtam et al. (2010a).

Fig. 1



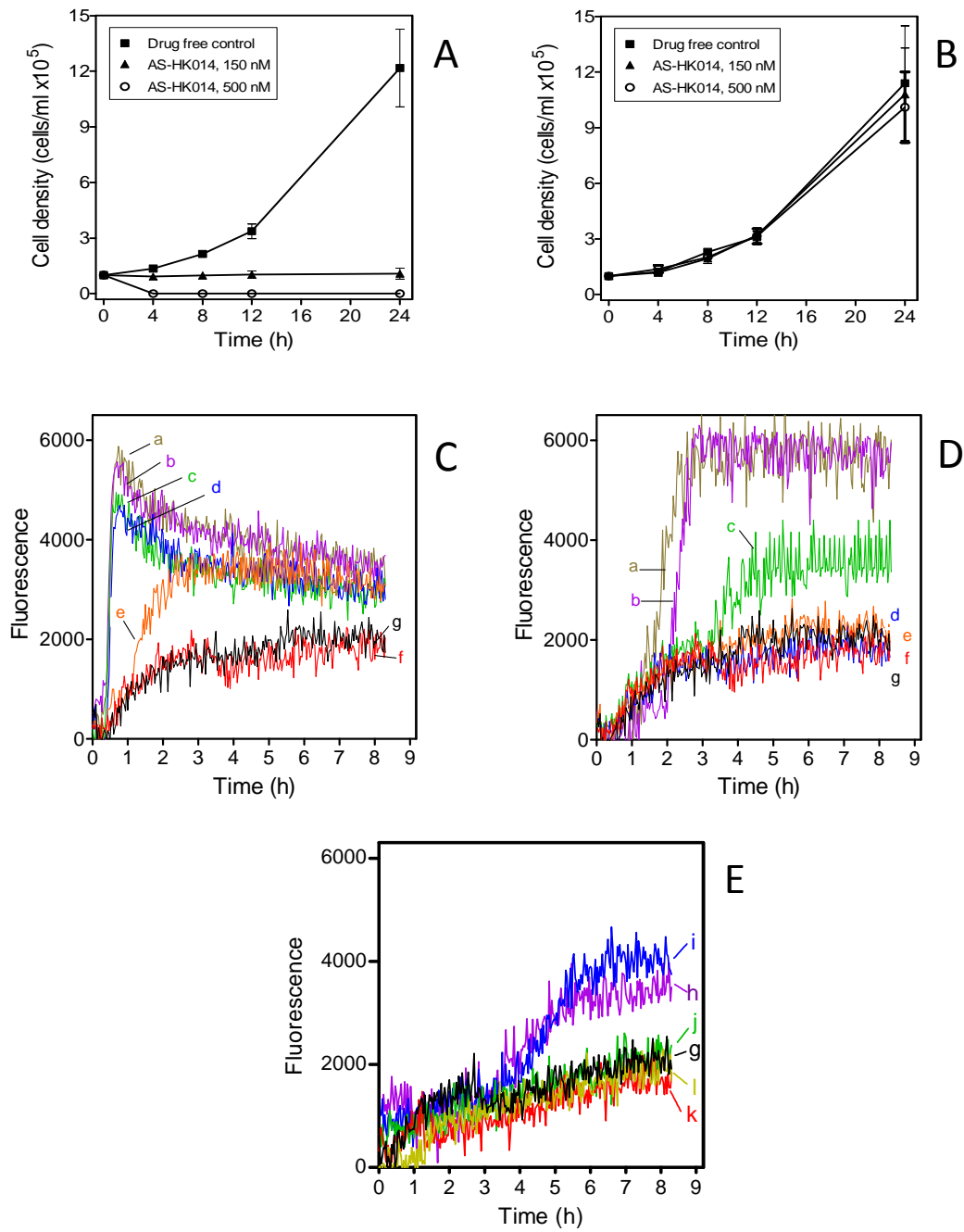


Fig. 2

Fig. 3

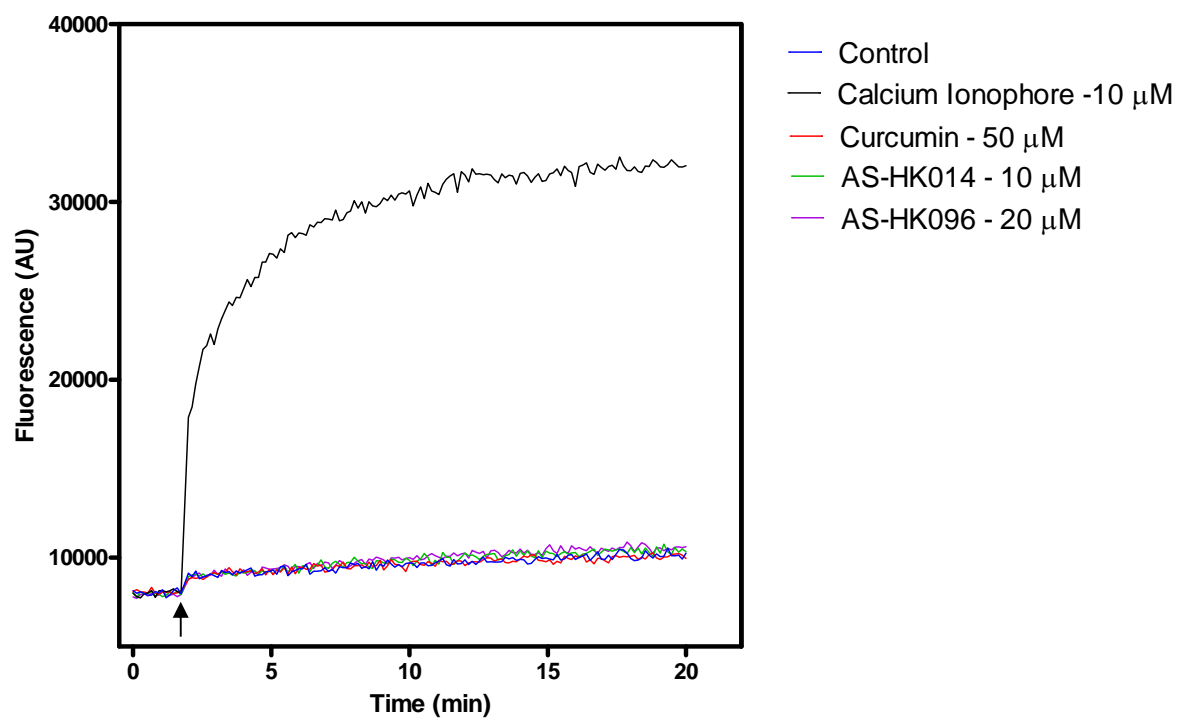


Fig. 4

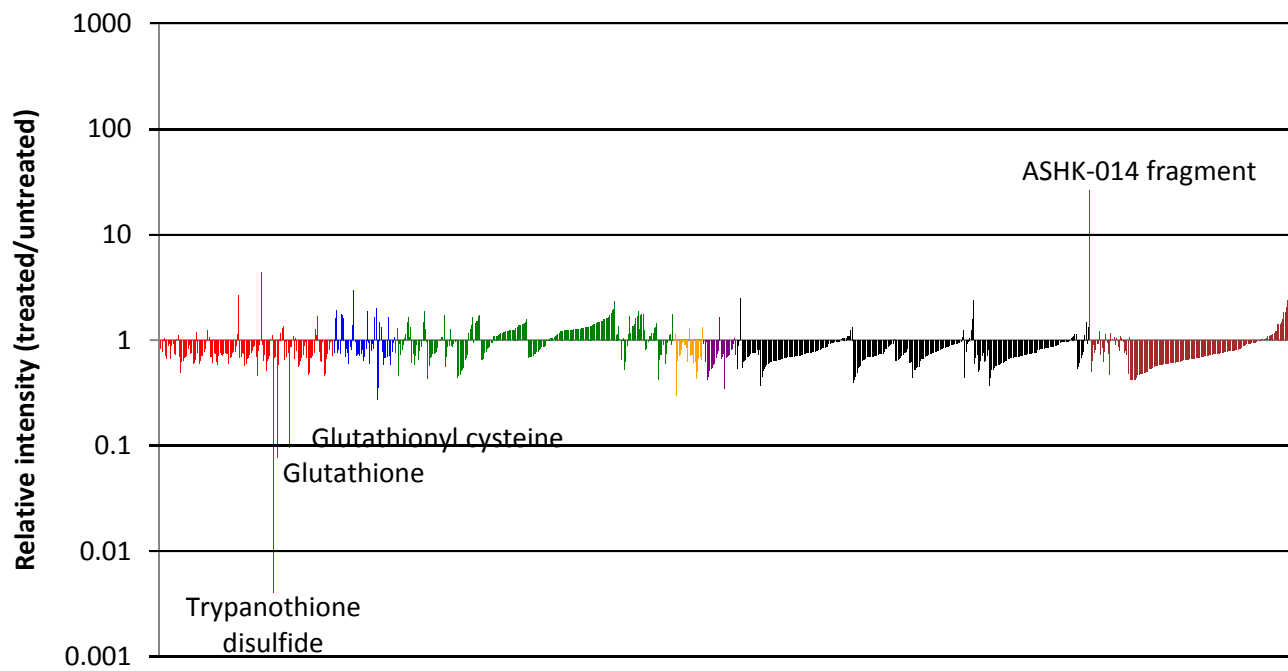


Fig. 5

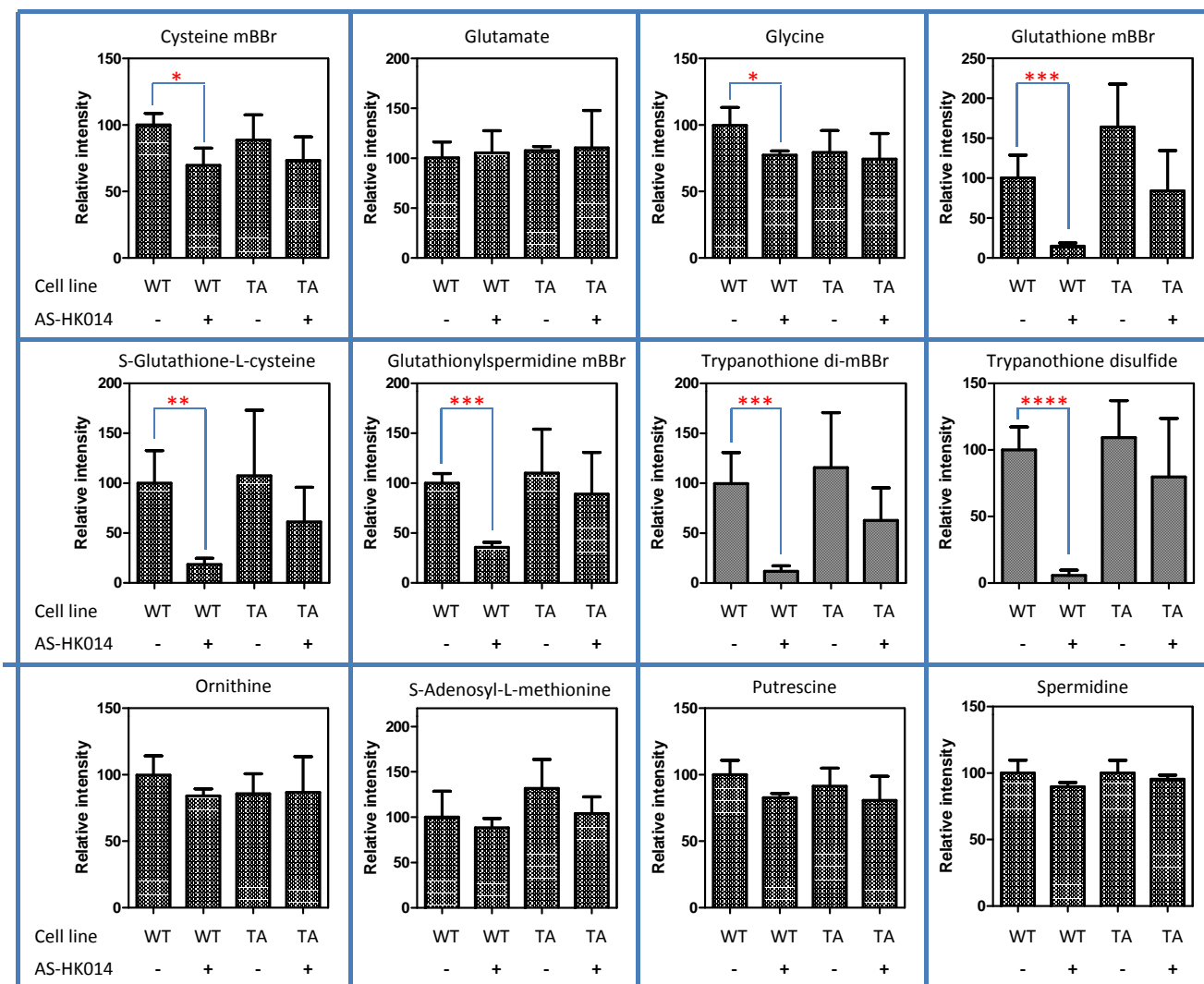


Fig. 6

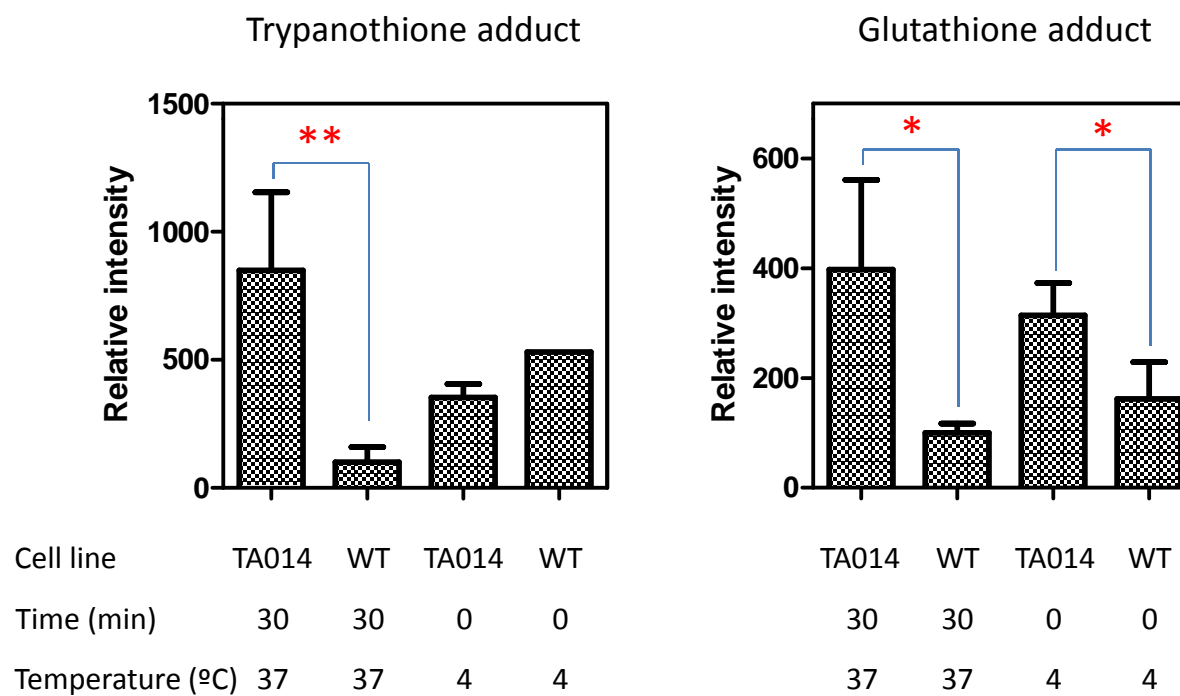


Fig. 7

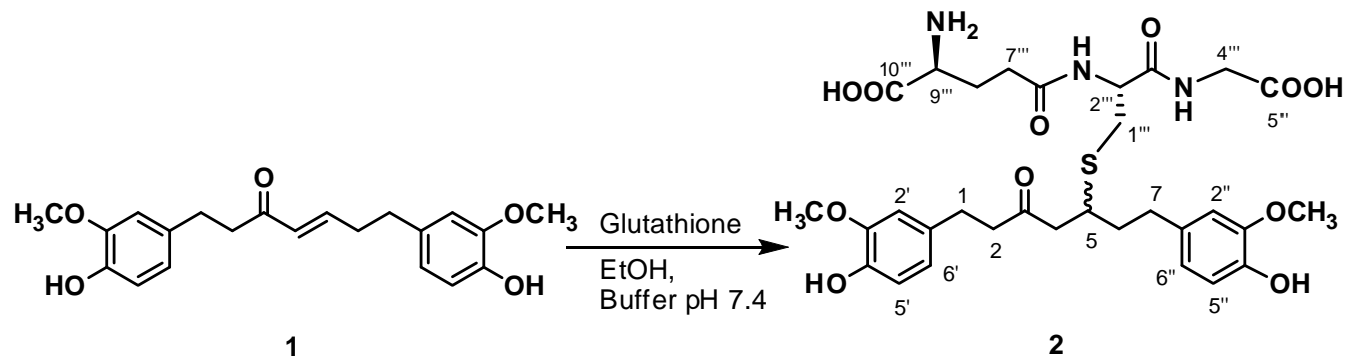


Fig. 8

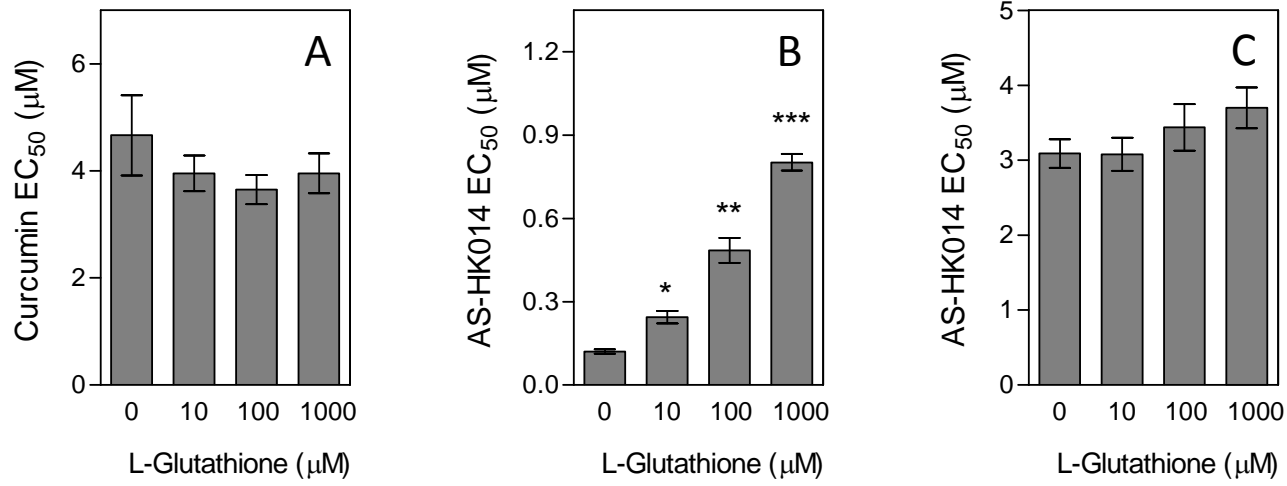
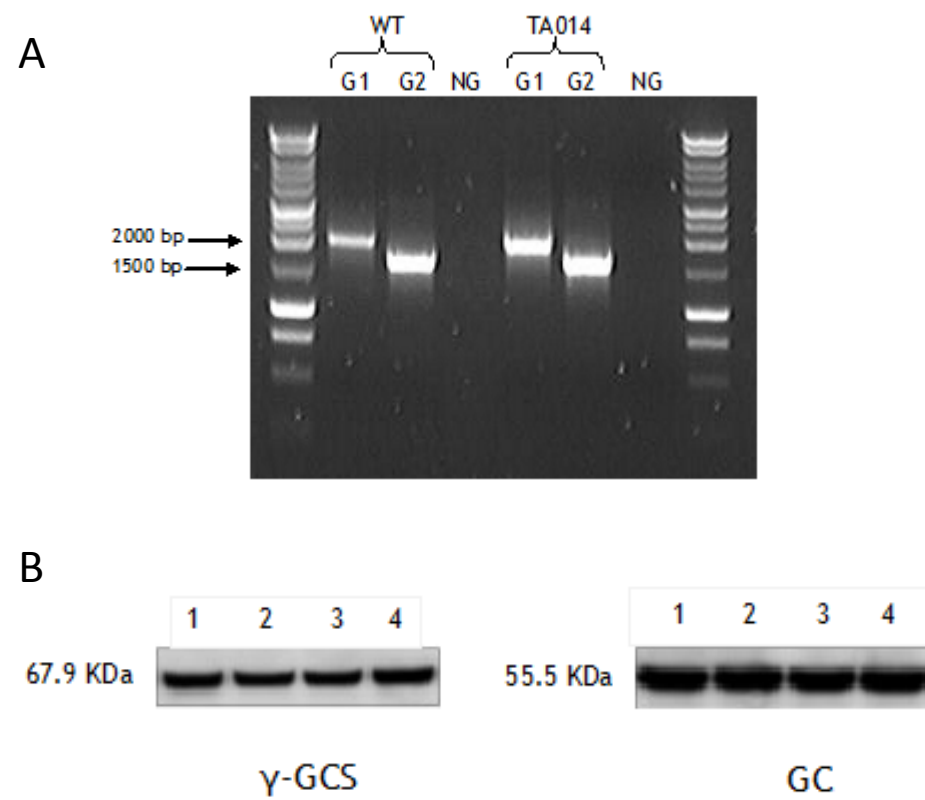


Fig. 9

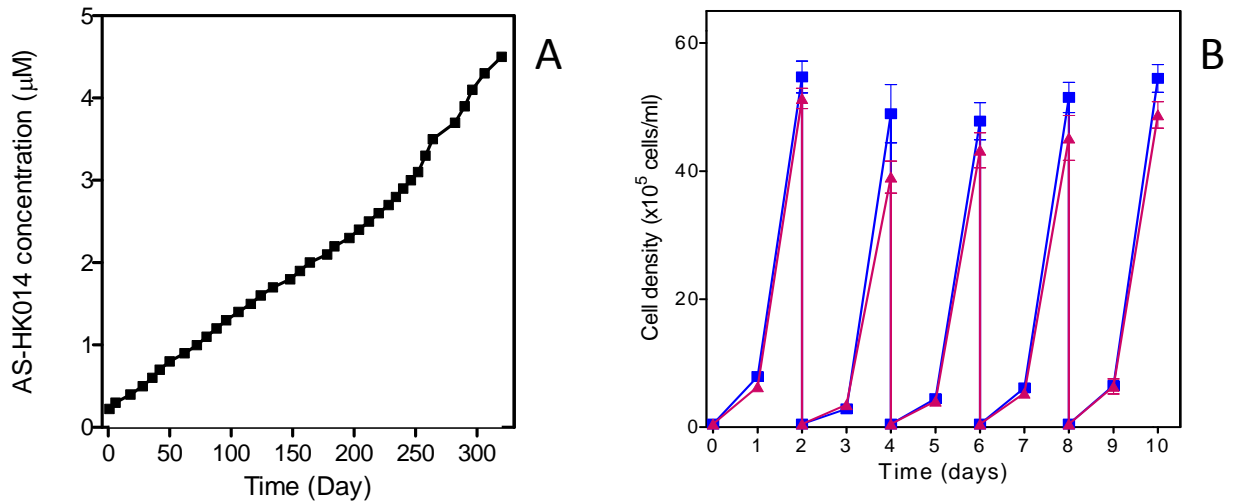


Molecular Pharmacology

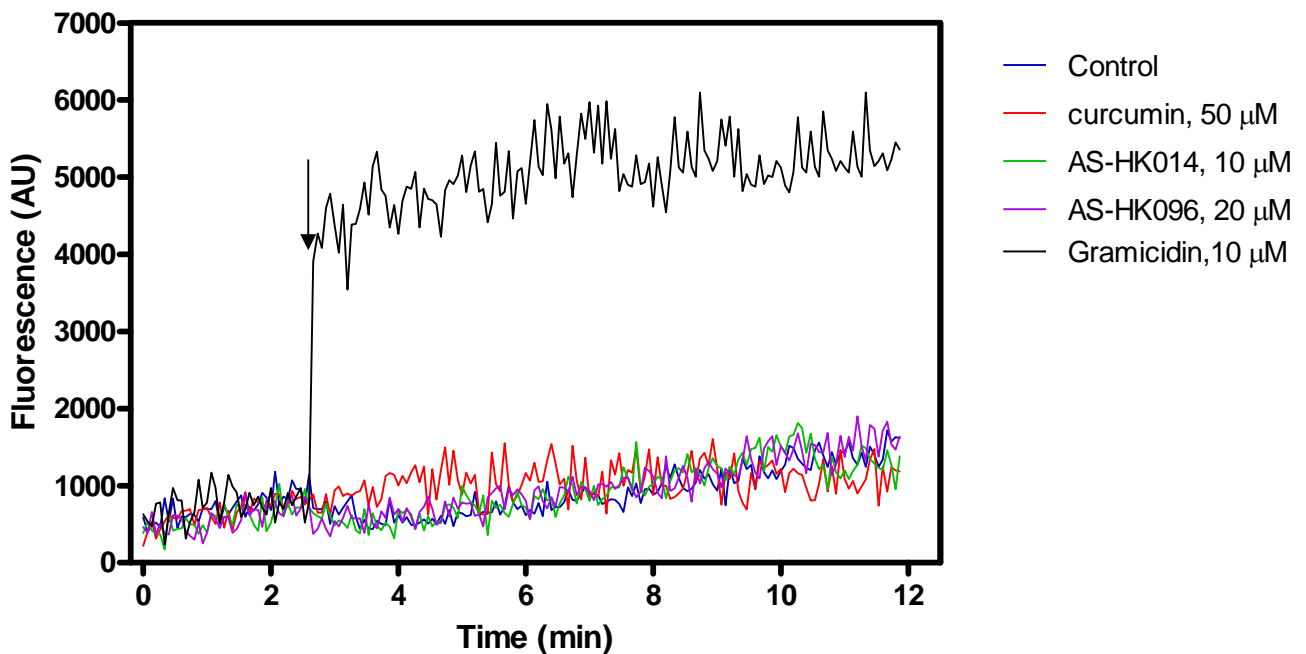
supplementary figures and figure legends

Potent trypanocidal curcumin analogs bearing a mono-enone linker motif act on *Trypanosoma brucei* by forming an adduct with trypanothione

Abdulsalam A. M. Alkhalidi, Darren J. Creek, Hasan Ibrahim, Dong-Hyun Kim, Neils B. Quashie, Karl E. Burgess, Chatchawan Changtam, Michael P. Barrett, Apichart Suksamrarn and Harry P. de Koning

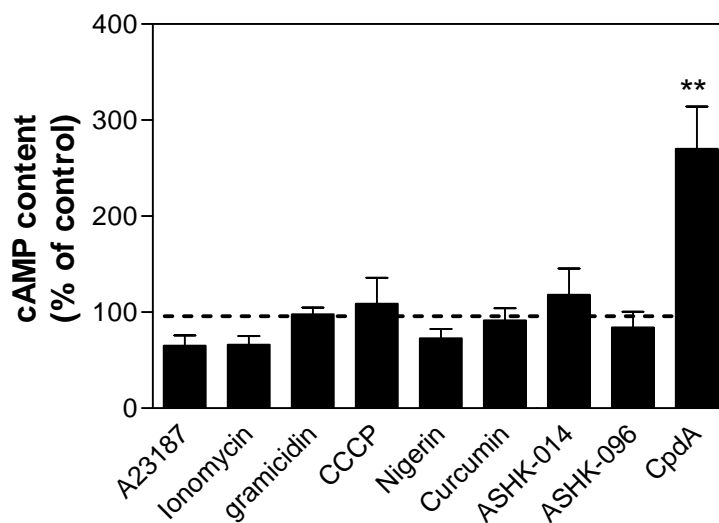
**Supplementary Figure 1.**

- A. Creation of the TA014 cell line. The tolerated drug concentration in the medium steadily increased for a period of about 11 months. Starting concentration of AS-HK014 was 0.25 μM
- B. Growth of TA014 (red triangles) and WT (blue squares) in HMI9/FBS. Seeding density was 5×10^4 cells/ml and cells were passaged to the same density every second day. Data represents averages and SEM of three independent experiments.



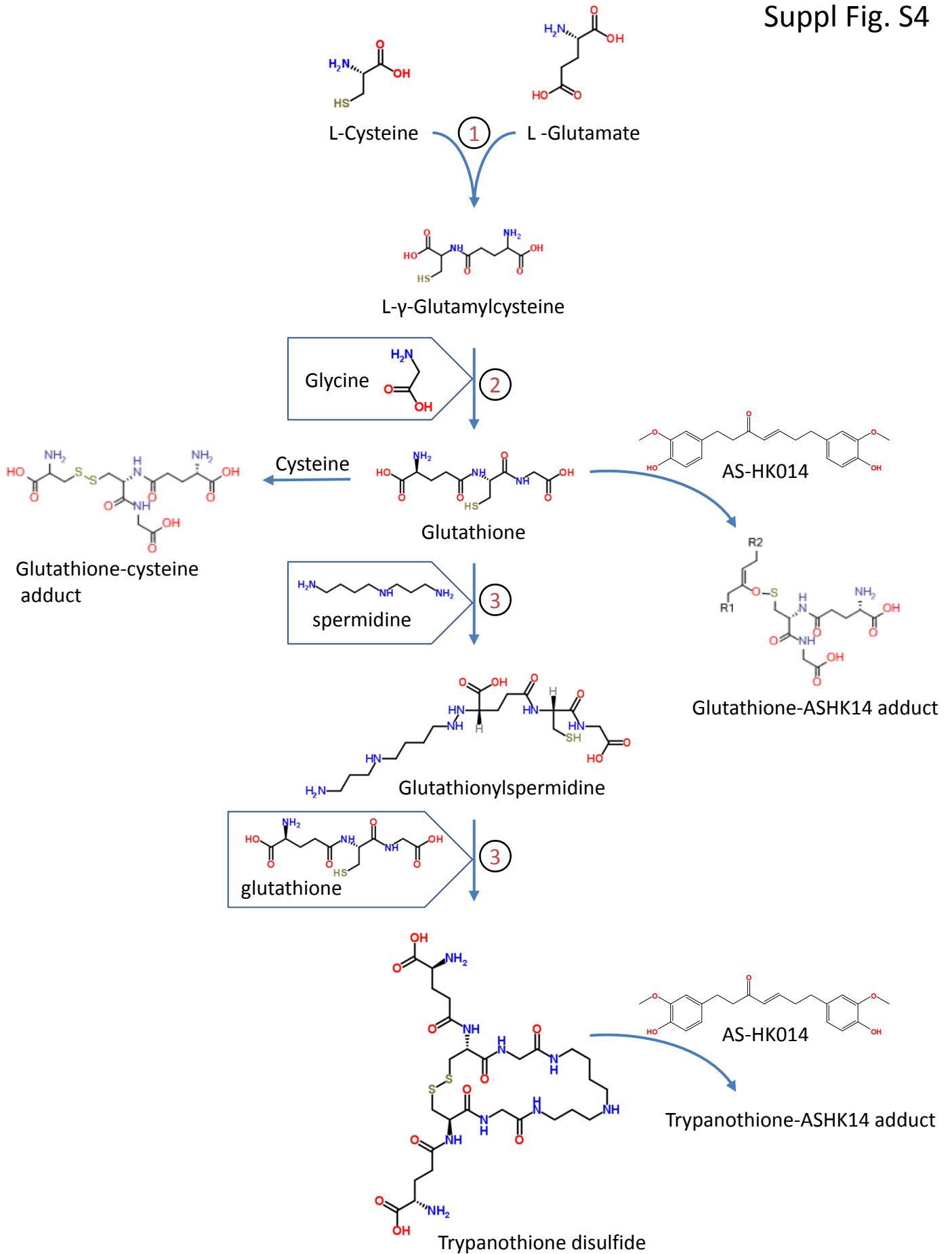
Effect of curcumin compounds on the fluorescence of the plasma membrane potential indicator bisoxonol in bloodstream-form *T. b. brucei*. Cells (1×10^7 /mL) were pre-incubated in an assay buffer containing $0.1 \mu\text{M}$ of bisoxonol at room temperature. The fluorescence was read for 160 sec before different compounds (at the final concentration presented in the figure) were added to the cells (arrow), and the fluorescence was recorded for an additional 10 min. Separate traces were recorded in the absence of cells and were subtracted from those presented in the figure.

Effect of ionophores on basal levels of cAMP over 15 minutes.



Average intracellular cAMP in bloodstream-form *T. b. brucei* incubated with different test compounds for 15 min. cAMP was measured with the use of an ELISA kit and the density of the cells was 5×10^6 cell/mL. The experiment was performed on three separate occasions, and the data shown are the average \pm SEM of these three experiments. The data are expressed as % of cAMP content of the control cells, incubated for the same time in the absence of drug free. Statistical differences from the control group were calculated using the unpaired Student's t-test ($P < 0.01$). Concentrations: A23187, 10 μ M; Ionomycin, 10 μ M; gramicidin, 1 μ M; CCCP, 10 μ M; Nigericin, 20 μ M; Curcumin, 1.5 μ M; AS-HK014 0.5 μ M; AS-HK-098 0.5 μ M; CpdA 1 μ M.**

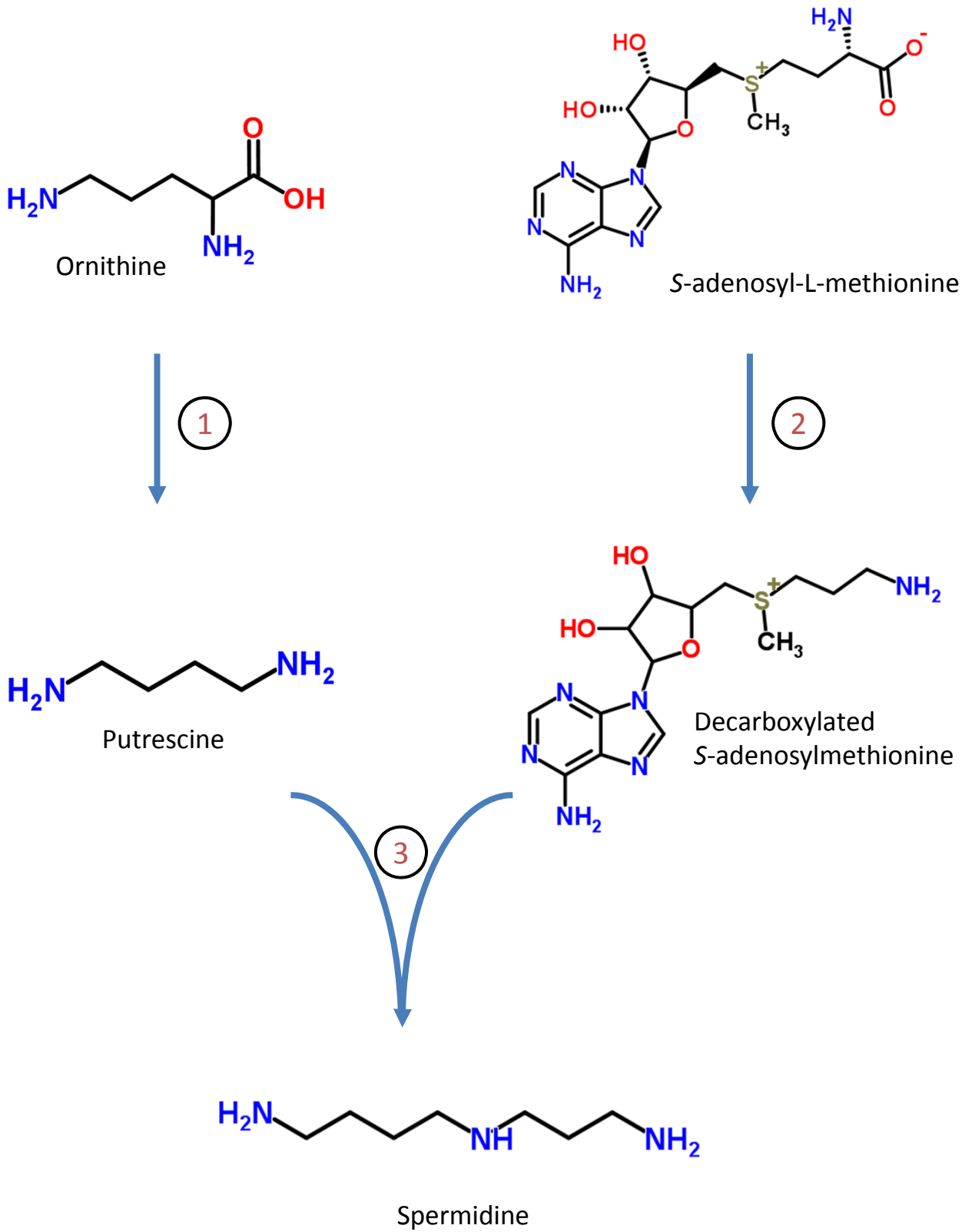
Suppl Fig. S4

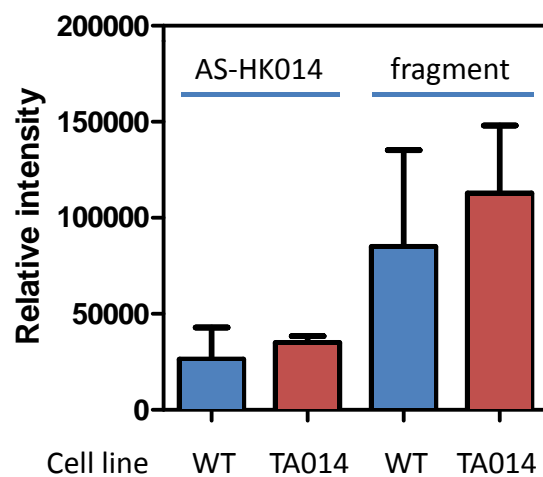


Supplementary Fig 4. Trypanothione pathway. 1: Gamma glutamylcysteine synthetase. 2: Glutathione synthetase. 3: Trypanothione synthetase

Supplementary Fig 5. Spermidine synthesis pathway. 1: Ornithine decarboxylase. 2: S-adenosylmethionine decarboxylase. 3: Spermidine synthetase

Supplementary Fig. S5





Supplementary Fig. 4. Levels of AS-HK014 in WT s427 and TA014 trypanosomes as determined in a metabolomic experiment performed without mBBR treatment. Incubation was with 0.75 μ M AS-HK014 for 30 minutes at 37 $^{\circ}$ C. Data shown are the average of triplicate determinations within a single experiment. Error bars are SEM. There were no significant differences between the cell lines ($P > 0.05$). Experiments analysed after extraction in the presence of mBBR showed essentially similar results but with much lower relative intensities (not shown).