Potent Trypanocidal Curcumin Analogs Bearing a Monoenone Linker Motif Act on Trypanosoma brucei by Forming an Adduct with Trypanothione

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ABSTRACT

We have previously reported that curcumin analogs with a C7 linker bearing a C2-C9 olefinic linker with a single keto group at C3 (enone linker) display midnanomolar activity against the bloodstream form of Trypanosoma brucei. However, no clear indication of their mechanism of action or superior antiparasitic activity relative to analogs with the original di-ketone curcumin linker was apparent. To further investigate their utility as antiparasitic agents, we compare the cellular effects of curcumin and the enone linker lead compound 1,7-bis(4-hydroxy-3-methoxyphenyl)hept-4-en-3-one (AS-HK014) here. An AS-HK014–resistant line, trypanosomes adapted to AS-HK014 (TA014), was developed by in vitro exposure to the drug. Metabolomic analysis revealed that exposure to AS-HK014, 1,7-bis(4-hydroxy-3-methoxyphenyl)hept-4-en-3-one; FBS, fetal bovine serum; ABBREVIATIONS: AS-HK014, 1,7-bis(4-hydroxy-3-methoxyphenyl)hept-4-en-3-one; FBS, fetal bovine serum; γ-GCS, γ-glutamylcysteine synthetase; GS, glutathione synthetase; mBBr, monobromobimane; MS, mass spectrometry; TA014, trypanosomes adapted to AS-HK014; WT, wild-type.
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 Trypanosoma cruzi (Nagajyothi et al., 2012). Indeed, despite modest in vitro leishmanicidal activity (Koide et al., 2002; Saleheen et al., 2002), long-term use of curcumin reportedly exacerbates visceral leishmaniasis (Adapala and Chan, 2008) and certain bacterial infections (Marathe et al., 2012), in part by suppressing type I immunity (Kang et al., 1999; Adapala and Chan, 2008).

While the available evidence thus suggests predominantly indirect effects of curcumin against parasitic infections, curcuminoids with much improved activity against the human malaria parasite Plasmodium falciparum (Mishra et al., 2008; Manohar et al., 2013), Leishmania mexicana amastigotes, and Leishmania major promastigotes (Changtam et al., 2010a), experimental Leishmania amazonensis infections (Alves et al., 2003), and Trypanosoma brucei (Changtam et al., 2010a) have recently been reported. In particular, a series of monoketone eno 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.

### Materials and Methods

**Trypanosome Strains and Cultures.** T. brucei brucei blood-stream 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 HMI-9 media (Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum (FBS) (BioSera, Ringmer, UK). Strains adapted to curcumin or its analog 1,7-bis(4-hydroxy-3-methoxyphenyl)hept-4-en-3-one (AS-HK014) 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 (Bridge 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-Aldrich, St. Louis, MO), as described by Rodenko et al. (2007). The assay was performed in white 96-well plates, each of which contained 2 × 105 cells and the appropriate drug (at doubling dilution) in 200 μl HMI-9/FBS. After 48 hours of incubation (37°C/5% CO2), 20 μl of sterile resazurin solution (125 μg/ml in phosphate-buffered saline) was added to each well, followed by a further incubation of 24 hours. Fluorescence was read in FLUOstar OPTIMA (BMG Labtech, Aylesbury, UK; 530 nm; λex = 530 nm; λem = 590 nm). The diamidine trypanocides pentamidine and diminazene (both from Sigma-Aldrich) were used as controls. Drug sensitivity assays with human embryonic kidney cells were performed similarly using a resazurin-based assay, exactly as described previously (Rodenko et al., 2007; Changtam et al., 2010a).

**Measurements of Intracellular Calcium and cAMP and Plasma Membrane Potential.** The intracellular calcium level was measured using the Screen Quest Fluor-8 Calcium Kit produced by ABD Bioquest (Sunnyvale, CA) (Ibrahim et al., 2011). Briefly, cultures of bloodstream-form T. b. brucei Lister 427 were harvested and washed by centrifugation at 2500g for 10 minutes at 4°C, resuspended to a final density of 4 × 106 cells/ml in the Fluo-8 dye-loading solution, and further incubated at 37°C for 30 minutes. The cells were then washed twice with an assay buffer to remove the dye-loading solution. Ninety microliters of cells were added to each well in a black-bottomed 96-well plate. The positive control was 10 μM calcium ionophore A23187 (Sigma-Aldrich), and the negative control was the assay buffer. The plate was then incubated in a FLUOstar OPTIMA fluorometer 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 seconds/cycle).

The plasma membrane potential was recorded using the fluorescent probe bisbenzimid, as described by de Koning and Jarvis (1997). Briefly, 100 μM of a cell suspension of 106 cells/ml was added to 2.9 ml of 0.1 μM bisbenzimid in a quartz cuvette loaded in a PerkinElmer (Waltham, MA) LS55 luminescence spectrometer. Fluorescence was monitored at an excitation wavelength of 540 nm and emission at 580 nm. Test compounds were added 140 seconds after the start of the recording to establish a stable baseline. Control traces without cells and added compound were recorded as well.

The cellular CAMP content of trypanosome was determined using the Direct Cyclic AMP Enzyme Immunoassay kit (Assay Designs, Exeter, UK), as described by de Koning et al. (2012) and Gould et al. (2013), following the manufacturer's instructions. The CAMP phosphodiesterase inhibitor CpdA (de Koning et al., 2012) was used as a 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-Fluorouracil (Ali et al., 2013). Briefly, log-phase trypanosomes (Lister 427 or AS-HK014-adapted cells [TA014]) were resuspended at 2 × 106 cells/ml in 50 ml HMI-9/FBS and incubated for 30 minutes with or without 0.75 μM 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, 1,250g, 10 minutes), and the supernatant was removed. For mass spectrometry (MS) analysis of thiols and other metabolites, we used the modified method for thiol derivatization with the thiol specific reagent C6(2-hydroxyethyl)piperazine-N'-3-propanesulfonic acid, 2 mM EDTA, pH 8.0, and 50 μl of 2 mM mBBr in absolute ethanol were added to the pellet and vigorously mixed. Each solution was incubated at 70°C for 5 minutes and cooled on ice for a minute. Then, 100 μl of 100% acetonitrile precooled at 4°C was added and incubated on ice for 10 minutes. Precipitated proteins and cellular debris were removed by centrifugation (13,000g, 3 minutes), and metabolite extracts were stored at −80°C. Control samples included untreated cells grown in parallel, unused growth medium, test compound dissolved in HMI-9/FBS, and extraction solvent blanks.

Analysis was by hydrophilic interaction liquid chromatography coupled to high resolution MS. Liquid chromatography separation used a zwitterionic ZIC-hydric hydrophilic interaction liquid chromatography column (Merck Sequant, Watford, UK) with a formic acid gradient, as previously described (Zhang et al., 2012). The method was performed on a Dionex U3000 RSLC (Thermo Fisher, Loughborough, UK) liquid chromatography system coupled to an Exactive Orbitrap (Thermo Fisher) operating at 50,000 resolution in positive and negative mode electrospray ionization (rapid switching), with MS parameters as described (Creek et al., 2011). Metabolomics data were 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 (Thermo Fisher). All determinations were in triplicate. Real-time cell survival was monitored over 500 minutes 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 (λexc = 544 nm; λem = 620 nm) generates an output proportionate to the number of 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 10^5 cells/ml in a FLUOstar Optima fluorimeter fitted with environmental controls (37°C, 5% CO2) (Ibrahim et al., 2011). Western Blotting. Log-phase bloodstream forms of T. b. brucei were resuspended in phosphate-buffered saline at 10^6 ml^-1, lyed in 300-μl lysis buffer (20 mM 4-morpholinopropanesulfonic acid, pH 8.0, 4 mM MgCl2 plus Roche complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany)), vigorously mixed, and flash frozen in liquid nitrogen. Freeze/thaw cycles were repeated three times before cell debris was removed by centrifugation (13,000g, 3 minutes). 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 minutes in a 4× sample buffer for SDS polyacrylamide gel electrophoresis. A 15-μl sample was separated on a 4–12% NuPAGE Novex 2-ibis2-hydroxyethylaminol-2-hydroxyethylpropane-1,3-diol (Bis-Tris) gradient gel using 4-morpholineethanesulfonic acid/SDS running buffer (both from Invitrogen) at 180 V. The reaction product of glutathione and AS-HK014 yielded a white amorphous solid, which separated out, was filtered, washed with water/acetone (2 ml) was added, and the solution was left to stand. The colorless solid with a melting point of 170°C (foam) that decomposed at 210°C. It was amorphous solid, which separated out, was filtered, washed with water/dichloromethane (3 times), and the aqueous solution was washed with dichloromethane. An apparent reaction product was detected by thin layer chromatography, and stirring continued for 7 hours. 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%).

A 15-μl sample was separated on a 4–12% NuPAGE Novex 2-ibis2-hydroxyethylaminol-2-hydroxyethylpropane-1,3-diol (Bis-Tris) gradient gel using 4-morpholineethanesulfonic acid/SDS running buffer (both from Invitrogen) at 180 V. The reaction product of glutathione and AS-HK014 yielded a white 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 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 a 1.5-ml phosphate buffer, pH 7.4, and 130 mg (0.37 mmol) of AS-HK014 in 3-ml ethanol was added. The reaction was stirred at an ambient temperature (30–32°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 to visualize spots. After 15 minutes of stirring, an apparent reaction product was detected by thin layer chromatography, and stirring continued for 7 hours. 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: infrared νmax: 3393, 3067, 2937, 2860, 1705, 1651, 1602, 1519, 1398, 1274, 1154, 1033, 817, and 544 cm⁻¹; 1H NMR (400 MHz, D2O): 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 (observed signal, 1H, H-1''b), 2.78 (observed signal, 1H, H-5'), 3.60 (observed signal, 3H, 4''-OCH3), 3.63 (br, s, 2 × 3H, CH2O-3' and CH2O-3''), 4.32 (br dd, J = 8.3, 5.3 Hz, 1H, H-7'), 6.40 and 6.45 (each d, J = 7.3 Hz, 2 × 1H, H-5'' and H-6''), 6.65–6.68 (m, 4H, H-2', H-3', H-5, and H-5''), 13C NMR (100 MHz, D2O): 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-1''), 53.3 (C-2''), 54.1 (C-9''), 55.8 (OCH3α × 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-3), 142.9 (C-4'), 143.1 (C-4''), 147.22 and 147.29 (C-9', C-3''), 171.5 (C-3''), 173.8 and 174.5 (C-6'', C-10''), 175.8 (C-5''), and 213.3 (C-3); and electrospray mass spectrum (–ve): m/z (percentage relative abundance) 662 [M–H]+ (100).

Results

Production and Characterization of a Trypanosoma brucei Cell Line Resistant to AS-HK014

Two adapted cell lines were established by exposure to curcin and AS-HK014, with initial drug concentrations of 5 μM and 75 nM for curcin and AS-HK014, respectively (structures and EC50 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 curcin reached only 8 μM, indicating an inability of T. brucei to meaningfully adapt to curcin. For AS-HK014, the maximum tolerated concentration in culture eventually reached 4.5 μM (Supplemental Fig. 1A). Figure 1 shows that whereas the long-term exposure to curcin did not significantly change the curcin EC50 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 wild-type (WT) 427 and TA014 trypanosomes were identical (Supplemental Fig. 1B).

Interestingly, the TA014 line was not cross-resistant to curcin (P > 0.05; Table 1) and indeed only showed cross-resistance to other analogs bearing the exact same monooenone 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 monooenone analogs have a mode of action that is distinct from all other the curcin analogs.

Effects of ASHK014 on T. b. brucei Growth and Survival

To investigate the mechanism of action of the monooenone curcin analogs, it is necessary to establish how 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 or 500 nM AS-HK014 or without drug (control), and samples were taken over a 24-hour 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 hours) 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 hours using an assay based on the cellular penetration of fluorophore propidium iodide (Gould et al., 2008). This allowed us to establish that s427WT cells succumb to AS-HK014 at concentrations ≥500 μM in approximately 40 minutes and to as little as 250 μM in 3 hours, whereas 100 nM had no measurable effect on cellular integrity over the course of the 500-minute experiment (Fig. 2C).

TA014 cells were vulnerable to 4 μM, with lysis completed in approximately 3 hours, and partly sensitive to 1.5 μM AS-HK014, with effects becoming apparent after 4 hours (Fig. 2D). Curcin only started to affect plasma membrane integrity after 4 hours and only at concentrations ≥25 μM (Fig. 2E).
TABLE 1
EC₅₀ values of curcumin and its analogs against wild-type trypanosomes (Lister s427) and the stain adapted to AS-HK014 (TA014)
All values are average and S.E.M. of at least three independent determinations, with EC₅₀ values against the two strains determined in parallel. Also shown is the resistance factor, which is the ratio EC₅₀ TA014/EC₅₀ s427. When more than one linker structure is given, it represents a mixture of both isomers. Significance was determined using a paired Student’s t test. Some of the HEK EC₅₀ values listed here were previously reported by Changtam et al. (2010a).

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<th>Compound</th>
<th>s427 EC₅₀</th>
<th>TA014 EC₅₀</th>
<th>Resistance Factor</th>
<th>t Test</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
<th>HEK EC₅₀</th>
<th>SI</th>
<th>Linker Structure</th>
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<td>Curcumin</td>
<td>2.7 ± 0.4</td>
<td>2.9 ± 0.13</td>
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<td>NS</td>
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<td>AS-HK014</td>
<td>0.11 ± 0.01</td>
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<td>28.2</td>
<td>P &lt; 0.001</td>
<td>OCH₃</td>
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<td>8.1 ± 1.0</td>
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<td>OCH₃</td>
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<td>Pentamidine</td>
<td>0.005 ± 0.001</td>
<td>0.003 ± 0.001</td>
<td>0.6</td>
<td>NS</td>
<td></td>
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<tr>
<td>Eflornithine</td>
<td>39.5 ± 3.76</td>
<td>43.17 ± 3.64</td>
<td>1.1</td>
<td>NS</td>
<td></td>
<td></td>
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<tr>
<td>Cymelarsan</td>
<td>0.0059 ± 0.0015</td>
<td>0.0078 ± 0.0021</td>
<td>1.33</td>
<td>NS</td>
<td></td>
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<tr>
<td>Nifurtimox</td>
<td>9.27 ± 1.09</td>
<td>7.21 ± 1.38</td>
<td>0.78</td>
<td>NS</td>
<td></td>
<td></td>
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<tr>
<td>Suramin</td>
<td>0.025 ± 0.005</td>
<td>0.036 ± 0.006</td>
<td>1.44</td>
<td>NS</td>
<td></td>
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HEK, human embryonic kidney; NS, not significant; OBz, benzylic; SI, selectivity index being the ratio of the EC_{50} values against HEK cells and wild-type trypanosomes.
without 200 nM AS-HK014 for 30 minutes. This is a sufficient

Assessment of the Effects of Curcumin and AS-HK014 on Plasma Membrane Conductance and Signaling in Wild-Type T. brucei. It has been previously reported that curcumin and several other common phytochemicals, such as epigallocatechin gallate from green tea, exert a nonspecific effect on biologic 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 have an impact on membrane integrity at therapeutic levels. The effects of curcumin and the monoene 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 of signaling events that could be triggered by nonspecific activation of membrane proteins) were assessed. None of the test compounds had any effect on calcium conductance (Fig. 3) or plasma membrane potential (Supplemental Fig. S2), although the concentrations used were much higher than the EC_{50} 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 (Supplemental Fig. 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 (Supplemental Fig. S3).

Metabolomic Analysis of the Action of AS-HK014 on T. b. brucei

To measure the 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 the cells, but the incubation was terminated, and the metabolites were extracted well before the test compound had a measurable effect on cell survival or plasma membrane integrity (Fig. 2, A and 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.

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 the most changed metabolites shows that AS-HK014 impacted the intracellular thiol levels strongly (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 nontreated 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 (Faiyeh and Newton, 1983; Fairlamb et al., 1987). This method can derivatize intracellular sulfhydryls to prevent their oxidation and allow detection of both oxidized and reduced forms of intracellular thiols.

The relevant pathway (Supplemental 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 minutes led to very clear reductions in thiol levels (Fig. 5). Trypanothione levels, both in the reduced form (detected as di-monobromobimane adduct) and as disulfide, were dramatically reduced under these conditions [to 12 ± 3% (P < 0.01) and 6 ± 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 glycine (both P < 0.05), but there was no change in glutamate levels (Fig. 5). All 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 (Supplemental Fig. S5) was analyzed. There was no change in spermidine levels upon treatment with AS-HK014, nor in any of the metabolites of its biosynthesis pathway: ornithine, S-adenosyl methionine, 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-adenosyl methionine in the synthesis of spermidine.

The above shows that the only metabolic 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 AS-HK014, 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 minutes at 37°C, but also when AS-HK014 was added after 30-minute drug-free incubation and the cells had been rapidly cooled to 4°C in a dry ice/ethanol bath. These cells were then immediately

Fig. 2. Effect of different concentrations of AS-HK014 on the growth and integrity of bloodstream-form T. brucei brucei s427WT and TA014 lines. 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 (C) (s427WT) and (D) (TA014), fluorescence of 9 μM propidium iodide was monitored in real time, with cells in HIT-9/FBS (37°C, 5% CO2) incubated in the presence or absence of various concentrations of AS-HK014. (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 μM; (b) 4 μM; (c) 1.50 μM; (d) 500 nM; (e) 250 nM; (f) 100 nM; (g) control, no drug; (h) 50 μM; (i) 25 μM; (j) 12.5 μM; (k) 3.1 μM; (l) 0.78 μM.
processed for metabolite extraction (quenched cells). Indeed, the levels of adduct were similar in the incubated and quenched cells, clearly showing that the AS-HK014 reaction with thiols is a rapid chemical reaction with free thiols rather than an enzymatic reaction.

**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, which is 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 (Supplemental 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.

**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-hour incubation time. AS-HK014 was used as a positive control. Whereas AS-HK014 again had profound effects on thiol levels, particularly glutathione and trypanothione, curcumin had no effect on thiol levels. 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 a 31% yield (Fig. 7). It should be noted that without the buffer,
only a very small quantity of adduct 2 was detected after 7 hours of stirring at the same temperature. The rate of the addition reaction increased with increased temperature. The structure of adduct 2 was elucidated by spectroscopic methods. A negative ion electrospray mass spectrum showed the [M–H]\(^{-}\) peak at m/z 662, which was compatible with the molecular formula of C\(_{31}\)H\(_{41}\)N\(_{3}\)O\(_{11}\)S of adduct 2. The \(^1\)H 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 \(^1\)H and \(^13\)C NMR data were assigned based on the correlation spectroscopy, heteronuclear multiple-quantum correlation spectroscopy, and heteronuclear multiple bond correlation spectroscopy experiments. Apart from four carbonyl carbon resonances of the glutathione part at \(\delta\) 171.5, 173.8, 174.5, and 175.8, the carbon resonance of the non-conjugated keto function of the curcuminoid part appeared at \(\delta\) 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 heteronuclear multiple bond correlation spectroscopy correlation between H-5 (\(\delta\) 2.78) and C-1 (\(\delta\) 31.4); H-1\(^{\prime}\)a (\(\delta\) 2.64) and H-1\(^{\prime}\)b (\(\delta\) 2.77); and C-5 (\(\delta\) 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, and 1000 \(\mu\)M). Whereas extracellular glutathione up to 1 mM had no effect on the activity of curcumin on WT trypanosomes (Fig. 8A), coinubcation 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 \(\mu\)M glutathione significantly increased the AS-HK014 EC\(_{50}\) value against WT cells (\(P < 0.05\)); however, the EC\(_{50}\) 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-GCS (Tb427.10.12370) and GS (Tb427.07.4000) (Supplemental Fig. S4). As the adaptation in TA014 giving protection to AS-HK014 was connected with thiol levels, we used a polymerase chain reaction to verify the presence of both genes in trypanosome wild-type and AS-HK014–resistant lines. Bands of the expected size were amplified from both strains (Fig. 9A). The polymerase chain reaction products were cloned and amplified in *Escherichia coli*. For each reaction, nine colonies were picked and the inserts were sequenced. Both genes were heterozygous in the wild-type and 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 the 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 minutes, with untreated cells incubated in parallel serving as controls. Protein extracts were separated by polyacrylamide gel electrophoresis and probed by 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).

**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; Fan et al., 2013; Norris 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, as curcumin itself has insufficient anti-parasitic 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 curcmins 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, AS-HK014, displayed an EC_{50} value of 0.053 ± 0.007 μM against T. b. brucei bloodstream forms (Changtam et al., 2010a), which is approximately 50-fold better than curcumin and 2-fold better than diminazene aceturate. AS-HK014 displayed an in vitro selectivity index of 221 versus human embryonic kidney cells compared with only 13.7 for curcumin itself. Other curcuminoids with the same monoenone linker displayed similar activity. However, a realistic optimization strategy would require knowledge of the mechanism of action, both for curcumin and the enone analogs.

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; Ali et al., 2013; Gould et al., 2013; A. Eze and H. de Koning, unpublished data), and we were able to induce resistance to AS-HK014 without problems. More importantly, the resultant AS-HK014–resistant strain was not cross-resistant with curcumin, which is 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, glutathionylperoximide, 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 an 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) 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 (Saleheen 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 × EC_{50} concentrations. Although curcumin did affect plasma membrane permeability in the propidium iodide fluorescence assay (>5 × EC_{50}, >4 hours), we cannot exclude the possibility that this reflects cell death by a different mechanism unrelated to membrane fluidity. We also find no evidence that monoenone curcuminoids have a deleterious effect on plasma membrane stability or permeability, especially at the midnanomolar concentrations at which they exert their trypanocidal effects. Indeed, a nonspecific 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).

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**Fig. 9.** (A) Polymerase chain reaction amplification of G1 (γ-GCS) and G2 (GS) for both strains of bloodstream-form T. b. brucei WT and the TA014-resistant line. NG, negative control that consists of all polymerase chain reaction components except genomic DNA. (B) Western blot of the T. b. brucei WT and TA014 lines at 1 × 10⁶ cells/ml. The cells were incubated for 30 minutes in the presence or absence of 200 nM of AS-HK014. 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.
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 an 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 monoenoine curcuminoids like AS-HK014 may react nonselectively with glutathione, the 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, Waltham, MA), 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 do not provide any direct evidence for either pathway, as no novel metabolite signals were specifically enriched in cells exposed to the 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 led to accumulation of the enzyme substrates (Supplemental Fig. S4). However, L-γ-glutamyleysteine, 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 minutes), 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 we find evidence for mutations in the open reading frames of the main enzymes involved in glutathione synthesis, glutathione synthetase, and γ-glutamyleysteine synthetase, nor evidence of differential expression in TA014. Yet, the amount of the 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 the adduct, this would help maintain total free glutathione and TSH pools within the cell.

In summary, we have established that monoenoine 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 curcuminoids in the sense of improved action on a common target.

Authorship Contributions

Participated in research design: Creek, Barrett, de Koning.
Conducted experiments: Alkhaldi, Creek, Ibrahim, Kim, Burgess, Changtam, Quashe.
Contributed new reagents or analytic tools: Alkhaldi.
Performed data analysis: Creek.
Wrote or contributed to the writing of the manuscript: Creek, Barrett, Suksamarrn, de Koning.

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Conducted experiments: Alkhaldi, Creek, Barrett, de Koning.
Participated in research design: Creek.
Wrote or contributed to the writing of the manuscript: Creek, Barrett, Suksamarrn, de Koning.

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