Antimitotic herbicides bind to an unidentified site on malarial parasite tubulin and block development of liver-stage *Plasmodium* parasites

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Abbreviations: APM, amiprophosmethyl; BSA, bovine serum albumin; GFP, green fluorescent protein; MBP, *Escherichia coli* maltose-binding protein; MCAC, metal-chelate affinity chromatography; MT, microtubule; PIPES, 1,4-piperazinediethanesulphonic acid; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

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ABSTRACT

Malarial parasites are exquisitely susceptible to a number of microtubule inhibitors but most of these compounds also affect human microtubules. Herbicides of the dinitroaniline and phosphorothioamidate classes however affect some plant and protozoal cells but not mammalian ones. We have previously shown that these herbicides block schizogony in erythrocytic parasites of the most lethal human malaria, *Plasmodium falciparum*, disrupt their mitotic spindles, and bind selectively to parasite tubulin. Here we show for the first time that the antimitotic herbicides also block the development of malarial parasites in the liver stage. Structure-based design of novel antimalarial agents binding to tubulin at the herbicide site, which presumably exists on (some) parasite and plant tubulins but not mammalian ones, can therefore constitute an important transmission blocking approach. The nature of this binding site is controversial, with three overlapping but non-identical locations on α-tubulin proposed in the literature. We tested the validity of the three sites by (i) using site-directed mutagenesis to introduce six amino acid changes designed to occlude them, (ii) producing the resulting tubulins recombinantly in *Escherichia coli* and (iii) measuring the affinity of the herbicides amiprophosmethyl and oryzalin for these proteins in comparison with wild-type tubulins by fluorescence quenching. The changes had little or no effect, with dissociation constants (K_d) no more than 1.3-fold (amiprophosmethyl) or 1.6-fold (oryzalin) higher than wild-type. We conclude that the herbicides impair *Plasmodium* liver stage as well as blood stage development but that the location of their binding site on malarial parasite tubulin remains to be proven.

1. Introduction

The ability of microtubules (MT) to assemble and disassemble reversibly and in a non-equilibrium fashion is key to their many functions in eukaryotic cells [1]. This dynamic instability is frequently targeted by tubulin-binding MT poisons, as small perturbations in the process can be lethal [2]. This is especially true for fast-dividing cells, as mitotic division is exquisitely susceptible to disruption of the microtubular spindle. As a result, tubulin-binding compounds have proven to be successful anti-cancer agents [3, 4]. However, they have also proven effective against slower growing cells, such as helminth parasites [4, 5].

The use of MT inhibitors as drugs for protozoal infections has been more limited, but a number of MT inhibitors have potent activity on a range of protozoal pathogens [4]. The malarial parasite *Plasmodium* is highly susceptible to a number of MT inhibitors, with 50% inhibitory concentrations (IC_{50}) on cultured, asexual, blood-stage parasites of the most lethal malaria species *P. falciparum* as low as 100 pM [6], well below those of most antimalarial drugs in use. The major target for MT inhibitors in this parasite stage appears to be the series of mitotic divisions that occur in the schizont, just before formation and release of new merozoites [7]. There have also been reports of effects of MT inhibitors on invasion of erythrocytes by merozoites [8] and on the development of pre-sexual blood stages (gametocytes) [9]. As mitotic inhibitors, they might also be expected to be effective on the liver stage that precedes the blood stage, where multiple divisions occur before the release of the first merozoites, but this has never been reported. This issue is potentially important because of the paucity of proven inhibitors of liver-stage parasites [10, 11].

Unfortunately, blood-stage *Plasmodium* parasites are no more susceptible than mammalian cells to the classical MT inhibitors colchicine, vinblastine and taxol nor to other anticancer agents tested to date [9, 12]. This lack of selectivity has so far precluded the development of novel antimalarial drugs from these compounds. The dinitroaniline and phosphorothioamidate herbicide classes are however an exception in that they are active against *Plasmodium* as well as certain other protozoa, fungi and plants, but ineffective against mammalian cells [13, 14]. This distinction appears to be based at least in part on differences in binding affinities for the tubulins of these different organisms. Like colchicine, vinblastine and taxol, these compounds are known to bind to tubulin, but their binding sites (or site, as the two classes may bind in the same location [15]) have so far not been established.

Purified tubulins from a number of cell types have been used to measure the binding affinities of a range of MT inhibitors and the effects of these inhibitors on MT assembly and disassembly [16]. The ability to purify tubulin directly from a cell is however largely dependent upon its initial concentration, especially because purification strategies usually rely on cycles of assembly and disassembly of MT from tubulin in cell extracts, and this requires a minimum 'critical concentration' of tubulin for success [17]. As a result, a recombinant strategy has been adopted for tubulin-poor organisms [7, 18-28], including *Plasmodium*, in which tubulin constitutes <1% of cellular protein [4]. Despite the complex chaperone machinery necessary for tubulin folding in intact cells [29], several groups have generated recombinant tubulins from bacterial hosts that are capable of ligand binding, recognition by conformation-dependent antibodies and in some cases assembly and disassembly [7, 18-27, 30-32]. Blood-stage *P. falciparum* are known to produce two α-tubulins, with αI-tubulin predominating in asexual parasites and αII-tubulin in gametocytes, but only one β-tubulin [4]. We previously reported the

recombinant production of soluble *P. falciparum* tubulins in *E. coli* as fusions to *E. coli* maltose-binding protein (MBP) [7]. These tubulins could be bound by a radiolabelled version of the dinitroaniline herbicide trifluralin. Although binding to the tubulins was much higher than to MBP alone or to an irrelevant protein, it was not possible, using this system, to measure the affinities of trifluralin for the α- and β-tubulin subunits. Moreover, the MBP–tubulins were not assembly competent when combined together, possibly because of the bulky (42-kDa) MBP tag.

In this paper we have adapted the MBP–tubulin system to measure the affinities of two tubulin-binding herbicides for *P. falciparum* tubulin. We have also addressed the question of the binding site of the herbicides. At least three overlapping but distinct sites of dinitroaniline binding on α-tubulins of different species have been proposed in the literature [33-37]. These putative sites were proposed using *in silico* modelling and were based around known tubulin mutations that confer resistance to the herbicides. To date, only one study has used altered tubulin from the ciliate *Tetrahymena thermophila* in an attempt to validate one of these sites experimentally [38]. However, it is still not known whether any of the proposed sites is applicable to *Plasmodium*. We therefore specifically engineered αI-tubulin with novel amino acids and measured herbicide-binding affinity in order to validate one or other of the putative sites. We were unable to find evidence to support any of the sites in *P. falciparum* tubulin and believe that the herbicides more than likely bind at a novel site yet to be determined. Finally, we demonstrate here the activity of tubulin-binding herbicides on liver-stage *Plasmodium* parasites, the first report of MT inhibitors active on this stage of the parasite's life cycle.

2. Materials and methods

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114 *2.1. Reagents and plasmids*

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116 All chemicals and reagents were from Sigma-Aldrich (Dublin, Ireland) and were of
117 analytical grade unless otherwise stated. Vinblastine, oryzalin and amiprophosmethyl (APM)
118 (Fluka Chemie AG, Buchs, Switzerland) were all dissolved in dimethylsulphoxide. Primers were
119 synthesized by IDT DNA Technology Inc. (Coralville, IA, USA). The pMAL-c2g (New England
120 Biolabs, Hertfordshire, UK) vector was used for the majority of the cloning work. Recombinant
121 plasmids were transformed into *E. coli* strains XL-1 Blue (Stratagene, CA, USA) and TB1 (New
122 England Biolabs). Bovine brain tubulin (α/β) was obtained from Cytoskeleton (Denver, CO,
123 USA).

124

125 *2.2 PCR amplification, cloning and expression*

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127 The *P. falciparum* 3D7 αI- and β-tubulin were previously cloned into the pMAL-c2x
128 vector [7]. These genes were sub-cloned into the pMAL-c2g vector using primers listed in
129 Supplementary Table S1.

130 A "Hotstart" PCR was done using 0.5 μM primer, ~ 100 ng DNA template, 2 mM dNTPs
131 (Roche), 0.5 mM $MgCl_2$ (Promega), 1 unit *Pfu* Turbo® DNA polymerase (Stratagene, La Jolla,
132 California, USA) and *Pfu* Turbo® buffer (Stratagene) in a 50-μl reaction volume with a Techne
133 TC-300 (Techne, Burlington, N.J., U.S.A.) thermocycler. The programme used for the PCR was:
134 denaturation at 95°C for 3 min; followed by 28 cycles of 95°C for 30 s, annealing at 55°C for 1
135 min and extension at 72°C for 3 min; with a final extension at 72°C for 7 min. The amplified

fragments were separated on 1% agarose gels, excised and purified using a High Pure® PCR kit (Promega). The fragments were cloned into the pMAL-c2g vector using T4 DNA ligase (Roche Diagnostics Ltd., Lewes, East Sussex, UK) and transformed into $CaCl_2$-competent XL-1 Blue cells by a heat shock method [39]. Clones containing the construct of interest were identified by an increase in plasmid size using agarose gel electrophoresis and confirmed by DNA sequencing.

Nucleotide changes were introduced into the αI-tubulin gene by inverse PCR using the same conditions as above with the following exceptions. The programme used for the PCR was: denaturation at 95°C for 3 min; followed by 28 cycles of 95°C for 30 s, annealing at 55°C for 1 min and extension at 72°C for 9 min; with a final extension at 72°C for 10 min. One unit of *Dpn*I (NEB) was added to the PCR reaction and incubated at 37°C for 3 h. The sample was cleaned up using a High Pure® PCR kit and transformed into XL-1 Blue cells as previously described.

The C-terminal His_6-tag encoding region was inserted into the tubulin genes using a modified inverse PCR strategy. This was done using 1 unit of KAPA HiFi® DNA polymerase (Kapa Biosystems, MA, USA), 0.3 μM primer and 50 ng DNA template in a 25-μl reaction volume using a Techne TC-300 thermocycler. The programme used for the PCR was: 95°C for 3 min, followed by 30 cycles of 95°C for 30 s, annealing at 60°C for 20 s and extension at 72°C for 5 min; with a final extension at 72°C for 10 min. The sample was purified using a High Pure® PCR kit and digested with 1 unit of *Dpn*I (NEB) and 1 unit of *Xho*I. The DNA was purified with a High Pure® PCR kit, ligated with T4 DNA ligase and transformed into XL-1 Blue cells as previously described.

2.3. Production and purification of recombinant proteins

The MBP-tagged tubulin fusion proteins were generated as previously described [7]. The MBP- and His_6-tagged tubulin fusions were also partially purified using a metal chelate affinity column and then subsequently purified on an amylose column. Briefly, the recombinant proteins were produced by inoculating L-broth/ampicillin (100 μg/ml) in baffled flasks with overnight cultures of the strain of *E. coli* harbouring the construct of interest, and growing at 37^{o}C with agitation at 200 rpm to an A_{600} ~0.5. Recombinant gene expression was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (Melford Laboratories, UK) and the culture was incubated for a further 1–3 h. Cells were harvested by centrifugation at 10,000 x g for 5 min at 4^{o}C and were stored at -20^{o}C until required. The cells were resuspended in MCAC-0 buffer (20 mM Tris-HCl, 200 mM NaCl, pH 7.4) supplemented with Complete Mini EDTA-free protease inhibitor tablets (Roche) and were lysed by 2–3 passages through a pre-cooled French pressure cell at 2000 psi. Following clarification of the lysate by centrifugation at 40,000 x g in a Sorvall RC50 Plus centrifuge using a SS-34 rotor for 1 h at 4^{o}C, the His_6-containing fusion proteins were purified from the soluble fraction on a Ni^{2+}-loaded metal affinity column. The soluble fraction was loaded at 1 ml/min, washed with 20 column volumes of MCAC-60 buffer (MCAC-0 buffer supplemented with 60 mM imidazole) and eluted with 20 column volumes MCAC-150 buffer (MCAC-0 buffer supplemented with 150 mM imidazole). The eluate was loaded onto an amylose resin (NEB) at 1 ml/min, washed with 20 column volumes of amylose buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA pH 7.4) and eluted with elution buffer (amylose buffer supplemented with 10 mM maltose). The eluate was concentrated by ultra-filtration through Amicon centrifugal filter devices (Millipore) and the purity assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined using the assay of Bradford with bovine serum albumin (BSA) as the standard.

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183 *2.4. Co-sedimentation of the tubulin fusions with bovine tubulin*

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185 Decreasing concentrations of bovine tubulin (24 μM, 16 μM and 12 μM) were mixed
186 with increasing concentrations of tubulin fusion protein (12 μM, 16 μM and 18 μM) so that the
187 monomer ratio was 1:1, 1:2 and 1:3 respectively. The samples were made up as follows: bovine
188 tubulin (12–24 μM), tubulin fusion protein (12–18 μM), GTP (2 mM), taxol (30 μM) 2-
189 mercaptoethanol (1 mM) and GPEMG buffer (1 mM GTP, 80 mM 1,4-
190 piperazinediethanesulphonic acid [PIPES]-NaOH, pH 6.9, 1 mM EGTA, 1 mM $MgCl_2$, 10%
191 [v/v] glycerol). Taxol and 2-mercaptoethanol were not always included. BSA and purified MBP
192 were used in place of the tubulin fusions as controls. Vinblastine (20 μM) was used in place of
193 taxol to inhibit MT formation. The samples were incubated on ice for 30 min and then
194 transferred to a 37^{o}C water bath for 1 h. Afterwards, the samples were overlaid on a pre-warmed
195 (~ 37^{o}C) 60% (v/v) glycerol cushion in PEM buffer (80 mM PIPES-NaOH, pH 6.9, 1 mM
196 EGTA, 1 mM $MgCl_2$) in sterile microcentrifuge tubes. MT were sedimented by the method of
197 Kumar [40] through a glycerol cushion by centrifugation at 40,000 x g at 37^{o}C in a Sorvall RC50
198 Plus centrifuge using a SS-34 rotor with specially outfitted Sorvall 408 adapter cones.
199 Supernatant and cushion fractions were transferred to new microcentrifuge tubes containing equal
200 volumes of 2 x SDS-PAGE loading buffer (0.125 M Tris-HCl, pH 6.8, 2.3% (v/v) SDS, 10%
201 (v/v) glycerol, 10% (v/v) 2-mercaptoethanol and 0.009% (w/v) bromophenol blue) and boiled for
202 10 min at 100^{o}C. The sedimented fraction was carefully rinsed using warm GPEMG buffer and
203 re-centrifuged at 40,000 x g, 37^{o}C for 10 min. This wash step was repeated 2–3 times. The
204 rinsed, sedimented fraction was resuspended in GPEMG buffer and 2 x loading buffer and boiled

205 at 100^{o}C for 10 min also. Equal quantities of protein sample were resolved by SDS-PAGE and
206 densitometry was performed using the Quantity One® software (Bio-Rad Laboratories Inc.,
207 California, U.S.A).
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209 *2.5. Protein affinity assay using the tubulin fusion proteins*
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211 The affinity of the tubulin fusions for other proteins was determined by their ability to
212 retain the protein(s) after a purification step using a metal affinity column. Sixteen μM of non-
213 His_6-tagged proteins (MBP–αI-tubulin or MBP), were added alone or with 16 μM of MBP–β-
214 tubulin–His_6 to 1 mM GTP and GPEM buffer in a final volume of 300 μl. MBP–β-tubulin–His_6
215 was pre-incubated with 1 mM 2-mercaptoethanol before being mixed with the other sample
216 components (final 2-mercaptoethanol concentration 0.25 μM). The samples were incubated on
217 ice for 30 min, in a 37^{o}C water bath for 30 min and then on ice for 30 min. Nine hundred μl of
218 cold MCAC-0 were added to the sample and it was then loaded onto a 1-ml Ni^{2+}-chelate column
219 at a rate of 1 ml/min. The column was washed with exactly 6 ml MCAC-0, 6 ml MCAC-60 and
220 6 ml MCAC-500 (MCAC-0 supplemented with 500 mM imidazole). The eluate (4.5 ml) was
221 concentrated to exactly 50 μl, mixed with 2 x SDS loading buffer and boiled for 10 min at 100^{o}C
222 before being resolved by SDS-PAGE.
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224 *2.6. Gel electrophoresis, western blotting and densitometry*
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226 SDS-PAGE and western immunoblotting were done by standard methods as described
227 previously [41]. Bands on gels were quantified using densitometry software Quantity-One

(BioRad). Known protein concentrations were used as standards to quantify the unknown protein amounts. Bovine brain tubulin was separated on a modified SDS-PAGE using the procedure outlined by Banerjee *et al.* [42].

2.7. Alignments and modelling

Multiple sequence alignments were performed using the ClustalW program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Three-dimensional structures of αI-tubulin were obtained using the Molecular Operating Environment program (MOE 2008.10 release of Chemical Computing Group Inc., Quebec, Canada) with the assistance of C. Flood (School of Biochemistry & Immunology, Trinity College Dublin) to create homology models using as a template the *Bos taurus* α/β-tubulin dimer (PDB accession 1tub, [43]) which has a completely resolved N-loop structure.

2.8. Fluorescence measurements by fluorescence spectroscopy

Tubulin or tryptophan samples were prepared in MME buffer (0.1 M 2-(N-morpholino)ethanesulphonic acid pH 6.9, 1 mM $MgCl_2$ and 1 mM EGTA). Tubulin samples had a final concentration of either 0.15 μM for the mixture of MBP–α/β-tubulin or 0.3 μM for the monomer alone. The tryptophan samples had a final concentration of 3.6 μM which corresponds to molar amount of tryptophans of the tubulin samples. Five μl of tubulin ligand (APM or oryzalin with a concentration of 0–20 mM) were added to the sample (final volume 500 μl). The sample was incubated at 37^{o}C for 5 min, then transferred to a thoroughly cleaned luminescence

cuvette (Perkin Elmer, Waltham, MA, USA) and read using a spectrofluorimeter (Perkin Elmer LS-50B). The excitation and emission wavelengths were 280 nm and 300–400 nm respectively and both had a slit width of 4 nm. The absorbance of the sample was measured using a spectrophotometer (Shimadzu Scientific Instruments Inc., Maryland, U.S.A.). The sample was scanned every 0.5 nm from 280–400 nm. Inner filter effects were corrected for by using the Lakowicz equation, $F_{corr} = F_{obs}\text{antilog}[(A_{ex} + A_{em})/2]$ (where F_{corr} is corrected fluorescence, F_{obs} is observed fluorescence, A_{ex} is absorbance at the excitation wavelength and A_{em} is absorbance at the emission wavelength) [44]. In some cases, inner filter effects were corrected by measuring the quenching of N-acetyl-L-tryptophanamide (3.6 μM) in the presence of the ligands. The dissociation constant (K_d) was determined using the following equation originally described by Acharya *et al.*: $F_{max}/(F_o\text{-}F) = 1+K_d/L_f$, where L_f represents the free ligand equilibrium concentration in the reaction mixture and was determined by $L_f = C – X [Y]$ [45]. C represents the total concentration of ligand in the sample and Y is the molar concentration of ligand-binding sites. One high affinity binding site was assumed for all the calculations. The fraction of binding sites (X) occupied by a ligand were determined using the equation $X=(F_o\text{-}F)/F_{max}$, where F is the corrected fluorescence intensity of tubulin in the absence of a ligand, F_o is the tubulin fluorescence in the presence of a ligand and F_{max} was calculated by plotting $1/(F_o\text{-}F)$ vs 1/[ligand] and extrapolating 1/[ligand] to zero.

2.9. Measurement of susceptibility of liver-stage parasites

To assess the effects of APM and oryzalin on liver stage *Plasmodium* infection in culture, Huh7 cells, a human hepatoma cell line, were treated with defined amounts of each inhibitor and

infected with luciferase-expressing or green fluorescent protein (GFP)-expressing *P. berghei* parasites, freshly extracted from the salivary glands of infected *Anopheles* mosquitoes. Solvent-treated infected cells were used as controls. Infection loads were measured 48 h after infection by bioluminescence readings or flow cytometry, as previously described [46, 47]. Briefly, luminescence measurements were carried out on infected cell lysates following addition of the luciferin substrate and flow cytometry analysis was performed on cells collected by trypsinization and resuspended in 2% fetal calf serum in PBS. Cells intended for luminescence measurements were infected with 10,000 sporozoites on 96-well plates and cells intended for flow cytometry analysis were infected with 30,000 sporozoites on 24-well plates. Inhibitors were added to the cells 1 h prior to sporozoite addition, for evaluation of their effect on overall infection, or 2 h after addition of sporozoites for evaluation of their effect on post-invasion parasite development. For microscopic observations, cells on glass coverslips were infected with 30,000 sporozoites on 24-well plates. Cells were fixed 48 h after infection with ice-cold methanol and stained with the 2E6 antibody (1/300 dilution) against the parasite's Hsp70, affinity-purified anti-*P. falciparum* β-tubulin antibody (1/50 dilution) (41) and the nuclear dye diaminophenylindole (DAPI). Anti-mouse Ig–AlexaFluor 594 and anti-rabbit Ig–AlexaFluor 488 (both at 1/400 dilution) were used as secondary antibodies for detection of the parasite's Hsp70 and MT, respectively. Infected cells were imaged on a Zeiss LSM 510 META confocal microscope (Zeiss, Oberkochen, Germany).

3. Results

297 *3.1. Assessment of functionality of MBP-tagged tubulins*

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299 In a previous study [7], we used recombinant *P. falciparum* tubulins fused at their N-
300 termini to MBP in order to obtain usable quantities of soluble tubulin from this organism.
301 Recombinant approaches to tubulin production have been questioned on the grounds that if the
302 proteins cannot be shown to be assembly competent, they may be improperly or incompletely
303 folded and data obtained on ligand affinity, for example, may be misleading [38]. Combinations
304 of α- and β-tubulins can form MT *in vitro* in the absence of other proteins under what are called
305 'assembly promoting' conditions. The MBP–tubulins together proved unable to form MT but
306 this was not unexpected given the presence of the bulky MBP tags on both subunits. We
307 believed that the MBP-attached tubulins were likely to be correctly folded on the basis of (i) their
308 high solubility, compared with the insolubility of untagged *P. falciparum* tubulins and (ii) their
309 ability to ligate [^{14}C] trifluralin, whereas neither MBP alone nor bovine tubulins could do this [7].
310 Nonetheless, assembly competence remains the 'gold standard' for assessing tubulin
311 functionality. Therefore, before developing a quantitative ligand binding assay for *P. falciparum*
312 tubulins using the MBP-fusion proteins, we sought further reassurance of their correct folding.
313 Extensive efforts to produce significant quantities of soluble untagged tubulin, to cleave the MBP
314 tag or to co-express the two proteins had proved fruitless (data not shown).

315 In view of the high amino acid sequence conservation of tubulins and the ability of
316 tubulins from different species to co-assemble [20, 48, 49], we attempted co-assembly of MBP–
317 tubulins with bovine brain tubulin using a previously described co-sedimentation assay [50]. In
318 this assay, MT are allowed to form in assembly-promoting conditions before being sedimented
319 by high-speed centrifugation (Fig. 1). Unassembled tubulin is found in the supernatant. The

supernatant and pellet fractions were resolved by SDS-PAGE and densitometry was used to determine the relative amounts of tubulin sedimented (Fig. 1). Under such conditions, bovine tubulin was found predominantly in the pellet, as expected, but a shift to the supernatant was seen upon the addition of the MT disassembling agent vinblastine (79% of tubulin in supernatant: data not shown). When either MBP–αI/β *P. falciparum* tubulin was centrifuged alone, it was absent from the pellet, but when mixed with bovine brain tubulin, the MBP–αI/β tubulins were detected in the pellets. Altering the ratios of *P. falciparum* to bovine tubulin showed that the MBP–tubulin fusions were co-sedimented with bovine brain tubulin in a concentration-dependent fashion, presumably by being incorporated into growing MT. For MBP–β-tubulin to be capable of co-assembly it required pre-treatment with mercaptoethanol, indicating that some of its cysteine residues were possibly oxidised. Mercaptoethanol had little observable effect on MBP–αI-tubulin (data not shown). The control protein BSA did not demonstrate any co-sedimentation with bovine tubulin (Supplementary Fig. S1). We attempted to determine if the recombinant *Plasmodium* tubulin could displace the bovine brain monomers in the experiment shown in Fig. 1. The bovine brain tubulin monomers were separated into distinct bands by using a modified SDS-PAGE which had impure SDS and more basic buffers, a technique devised by Banerjee *et al.* [42]. Using densitometry, it was possible to determine that the relative concentrations of the tubulin monomers were similar, indicating that no detectable displacement had been caused by the presence of the recombinant tubulin (Supplementary Fig. S2).

Since the α/β tubulin dimer is the most physiologically relevant form of soluble (unassembled) tubulin, we wanted to carry out ligand binding studies using dimers, if possible. MBP–αI-tubulin or MBP alone were incubated in the presence or absence of MBP–β-tubulin–His_6 at 37^{o}C before being passed through a nickel-chelate column to determine whether they

interacted. The tubulin or MBP that had not interacted with the MBP–β-tubulin–His_6 was readily washed off the column by a low imidazole concentration (60 mM). However, in the case of a real interaction, the protein would co-elute with MBP–β-tubulin–His_6 following the high imidazole concentration (500 mM) elution step. Only negligible amounts (<0.1 μg) of MBP or MBP–αI-tubulin were recovered in the absence of MBP–β-His_6 tubulin as determined by densitometry (Fig. 2 A and C). However, in its presence, there was an obvious band representing MBP–αI-tubulin that was not present in the case of MBP alone (compare Fig. 2 D and B). This indicated that MBP–αI-tubulin is capable of specifically interacting with MBP–β-His_6 tubulin, suggesting that dimerisation is possible.

3.2 Validation of proposed herbicide binding sites in P. falciparum *α-tubulin*

α-tubulin genes from a wide cross-section of herbicide-resistant and -sensitive organisms were aligned (Supplementary Fig. S3). This alignment highlights the amino acids comprising the three putative dinitroaniline herbicide binding sites of Mitra & Sept [36] from *Toxoplasma gondii*, Délye *et al.* [34] from green foxtail and Nyporko *et al.* [37] from *Eleusine indica*, and identifies the amino acid overlaps. Although the proposed binding sites are distinct, with only L136 common to them all, they were found to exist in the same region of the tubulin molecule. However, none of the amino acids predicted by any of the sites was unique to either resistant or sensitive organisms. In fact only half of all the amino acids that comprise the putative 'Mitra & Sept' site are completely conserved. Therefore we set about introducing amino acid changes that would be expected to occlude the sites, concentrating mainly on the 'Mitra & Sept' site due to the close relationship between *T. gondii* and *Plasmodium* [36]. Furthermore, there is more

substantial experimental support for this site than for the 'Délye' or 'Nyporko' sites [34, 37]. Six separate point mutations in the *P. falciparum* αI-tubulin gene were chosen. *In silico* models were generated to demonstrate that the altered residues were central to the sites and that they were expected dramatically to alter the structure of the putative binding pocket compared with the wild type protein (Fig. 3). The six altered MBP–αI-tubulins were purified to near homogeneity with minimal degradation observed by either SDS-PAGE or western blotting (Supplementary Fig. S4).

To determine the affinity of the herbicides for the recombinant *P. falciparum* tubulins, a fluorescence assay was developed. Essentially, tubulin-ligand interactions were reported by the quenching of tryptophan fluorescence (Fig 4A). A double reciprocal plot with the fluorescence reduction versus ligand concentration was graphed and from this it was possible to determine the dissociation constant (K_d) by using the formula described by Acharya *et al.* [45] (Fig. 4B). As a positive control, the K_d of vinblastine for bovine tubulin was determined to be 0.91 ± 0.29 μM, which was consistent with previous reports [51]. The MBP–tubulins were measured as monomers (0.3 μM) or as a 1:1 mixture of αI- and β-tubulins (0.15 μM each). An equi-molar concentration of tryptophans was present in the samples to minimise error caused by using different tryptophan controls, which was the reason for using twice the molar concentration of the monomer compared with the mixture. At least five different ligand concentrations were used for determining the K_d. The phosphorothioamidate APM was able to quench the MBP–αI/β-tubulin mixture significantly over a range of concentrations (Fig. 4A). MBP–αI-tubulin had greater affinity for APM than MBP–β-tubulin but the mixture had the greatest affinity (Table 1 A). Therefore, only the tubulin mixture was used for subsequent binding analysis. Oryzalin, a dinitroaniline, was demonstrated to bind with almost 3-fold higher affinity (15.51 μM ± 3.55) to the MBP–αI/β-tubulin mixture than APM (44.14 μM ± 7.15) (Table 1 A). K_d values were also

generated for each of the six altered tubulins (mixed 1:1 with MBP–β-tubulin) for both APM and oryzalin (Table 1 B). Surprisingly, none of the mutations had the dramatic effect expected if the binding site had been occluded. For APM, the maximum increase in K_d relative to the wild type was a mere ~1.3-fold and only 2/6 alterations (Cys65Ala and Thr238Ile) reached statistical significance. For oryzalin, the K_d increased by no more than 1.6-fold and of the five K_d determined only Val4Cys and Leu136Phe were significantly higher than the wild type. Taken together, these results cast doubt on the idea that the putative 'Mitra & Sept' binding site is applicable to *Plasmodium* tubulin. Regarding the 'Délye' and 'Nyporko' sites, each of these should be occluded by 3 of the 6 mutations chosen so it also seems unlikely that either of these sites represents the actual location of herbicide binding in *Plasmodium* tubulin. It should be borne in mind however that these results were obtained with recombinant tubulins containing MBP tags.

3.3. Activity of herbicides against liver-stage malarial parasites

The activities of APM and oryzalin against *P. berghei* liver stages in culture were evaluated using established methods for measurement of infection loads in hepatoma cells [52]. Our results show that both compounds inhibit overall infection, as measured by bioluminescence readings of cells infected with luciferase-expressing parasites [46] with IC_{50} values of ~4.6 and ~3.5 µM, respectively (Fig 5A). We further observed that both APM and oryzalin inhibited infection to similar extents whether they were added to the cells before infection or after the parasites had invaded the cells, suggesting that the compounds act by inhibiting intracellular parasite replication (data not shown). To verify this conclusion, we employed GFP-expressing *P.*

berghei parasites and monitored the extent of their development by flow cytometry, as previously described [47]. These results show that incubation of both cells with either compound significantly decreases GFP fluorescence intensity of the infected cells ($p<0.001$; Fig. 5B), confirming that the compounds act by impairing the parasite's ability to replicate inside its host cells. Finally, we sought to investigate the effect of the two compounds on the microtubular network of liver stage *Plasmodium* parasites. To this end, infected cells incubated with each compound or mock-treated with solvent were stained using an antibody against the parasite's MT and observed by confocal fluorescence microscopy. These observations indicated that treatment with either compound disrupted the filamentous microtubular pattern inside the parasite (Fig. 5C), consistent with both inhibitors interfering with normal tubulin assembly and, hence, parasite replication. Overall, our data are consistent with the notion that the two herbicides used in this study inhibit hepatic stage *Plasmodium* infection by acting on the parasites' microtubular structures to impair their development/replication inside host cells.

4. Discussion

The recent emergence of malarial parasites with reduced artemisinin susceptibility [53] underlines the still urgent need to identify new drug targets in and new lead compounds against the disease-associated, asexual blood-stage malarial parasites. In addition, primaquine is the only licensed drug for the liver stages of the disease and it suffers from drawbacks in terms of toxicity [11]. Antimitotic herbicides of the dinitroaniline and phosphorothioamidate classes offer potential starting points for the design of new drugs targeting MT-dependent processes in

malarial parasites [54]. In this study we have extended previous observations on blood-stage parasites to those of the liver stage of the rodent parasite *P. berghei*. We observed that the two compounds assayed in this study, APM and oryzalin, inhibited *Plasmodium* hepatic infection with single-digit micromolar IC_{50} values comparable to those on blood stages [7] and lower than that of primaquine (~11 μM) measured by the same method [46]. We further established that the compounds disrupted the microtubular network of the exoerythrocytic parasite. It appears that MT inhibitors may be a promising class of agents for liver-stage malaria.

While the dinitroaniline herbicides themselves had unsuitable pharmacokinetics and/or toxicity in rodent malaria models [55], numerous more drug-like derivatives have been reported [4, 56]. None of these was superior to the dinitroanilines themselves against *Plasmodium*, but Mara *et al*. [54] recently achieved modest reductions in IC_{50} against cultured *P. falciparum* in some members of a series of compounds related to APM. Greater improvements in activity have been obtained against trypanosomes [27] and *Leishmania* spp. [56] but these protozoa are quite unrelated to *Plasmodium* and their structure–activity relationships are apparently very different.

A significant barrier to structure-based drug design based on this theme is the lack of a known 3-D structure for any tubulin of a herbicide-susceptible organism. This necessitates modelling of plant and protozoal tubulins using mammalian tubulins as a template, yet the mammalian tubulins evidently lack the site [4]. The three modelled herbicide-binding sites proposed in the literature [33-37] must therefore be treated with some scepticism. A notable exception has been the recent work by Lyons-Abbott *et al.* who demonstrated that Leu136Phe and Ile252Leu mutations affect binding of oryzalin to *Tetrahymena* α-tubulin [38].

In order to validate the proposed herbicide-binding sites in *Plasmodium* tubulin and to evaluate the binding affinities of novel herbicide derivatives, we required a reasonably abundant

source of soluble parasite tubulins. To date, it has not been possible to isolate tubulin directly from *Plasmodium* due to the limited amounts of starting material and low concentrations of tubulin in cultured parasites (~ 0.1 mg/l in a culture of 10% parasitaemia and 5% haematocrit). Therefore, we opted for a recombinant strategy using *E. coli* as a host. While lacking the post-translational modifications expected in eukaryotic cells, tubulins produced in this way have the advantages of lack of intrinsic tubulin contamination, high protein yields and freedom to alter the tubulin gene in a way that could be lethal for a eukaryotic cell. Tubulins from several other organisms produced in *E. coli* have been shown to bind small-molecule ligands and antibodies and in some cases to dimerise and even to assemble into MT [7, 18-27, 30].

Several groups have argued that tubulin requires specific machinery in order to fold correctly [57-59] but other reports demonstrated MT formation by isolated recombinant tubulins [21, 26, 28, 60, 61]. Success using this method seems to depend on the source of the tubulin, and subtle but significant differences between these proteins may explain the disparity. Bacterial tubulins have been recently discovered in some *Prosthecobacter* species, and recombinant proteins made in *E. coli* formed functional MT [62]. In our case, following extensive investigation of various expression systems the only one that produced useful quantities of soluble *P. falciparum* tubulins was one in which the tubulins were fused to MBP tags [7]. The exact reason why the MBP tag causes an improvement to the solubility of the passenger protein is unknown [23] but MBP is thought to act as a general molecular chaperone [63]. A previous study by Yaffe *et al.* determined that an α-tubulin monomer can be stable without its partner protein β-tubulin but it was also capable of interacting with other preformed dimers in a co-polymerisation assay [50]. Since the MBP–tubulins described in the present study could not polymerise in the absence of other proteins, we determined that they could be incorporated into

bovine MT, as shown by dose-dependent co-sedimentation with bovine tubulins after incubation under assembly-promoting conditions. These results were specific as they were not replicated by unrelated proteins such as MBP or BSA. The exact mechanism for the incorporation of our MBP–tubulins into the bovine MT is unclear. Yaffe *et al.* argued that their refolded α-tubulin formed heterodimers [50]. However, in our case, we think this is unlikely. We separated the bovine tubulin that had bound to our MBP–αI-tubulin and MBP–β-tubulin into its monomers and determined that they were in approximately equal concentration, indicating that no major displacement had occurred. Instead we propose that the MBP–tubulins would probably bind at the extreme ends of the MT's so that a partner protein would not be required for the interaction to occur. Binding here may also limit the steric hindrance effect that the MBP tag may have on the overall structure. Another possibility is that the MBP–tubulins are incorporated at low frequency along the shaft of the MT. It was also possible to demonstrate that the monomers had a significant affinity for each other over other unrelated proteins by their co-eluting after being trapped on a Ni^{2+}-chelate column. We have not been able to show that 100% of our MBP–tubulin molecules are correctly folded. However, Wampande *et al*. managed successfully to calculate binding affinities for native and chemically refolded tubulin [31]. They determined that although the native tubulin had a lower K_d, the binding capacity and the rank order of affinity for several different ligands were the same [31]. Taken together, the results suggested that the MBP–tubulins had the potential to be useful for ligand-binding studies provided that the affinities obtained were comparable with those in the literature.

It was previously shown that radiolabelled trifluralin had much greater binding to purified tubulin from *Plasmodium* than that of bovine brain but an exact measurement of affinity was not obtained [7]. We addressed this issue here by using intrinsic tryptophan fluorescence to detect

perturbations of the tubulin protein by small ligands such as the herbicides APM and oryzalin. We found that APM was able to bind to both monomers, but with ~2 fold greater affinity to MBP–αI-tubulin than to MBP–β-tubulin, indicating that the binding site may exist on both tubulins. This result has been reported before in the literature for other herbicide-based compounds [7, 27, 37]. However, we found that our αI/β-tubulin mixture had the strongest affinity for APM. Furthermore, we found that oryzalin had almost a 3-fold greater affinity than APM for our tubulin, with a K_d of 15.51 ± 3.55 μM. The affinity of oryzalin has been examined using a range of diverse organisms and reported binding affinities range from 0.1 μM for plant tubulin to 17 μM for *Leishmania* tubulin [64, 65]. Therefore, our results fit closely with the recorded affinity for this ligand albeit at the higher end of the scale. This is not surprising as *Plasmodium* in culture is susceptible to this compound only in the low micromolar range and above (IC_{50} 6.1 μM). APM is a slightly more potent inhibitor than oryzalin against cultured parasites (IC_{50} 3.5 μM), perhaps because oryzalin, like trifluralin, may be more susceptible to sequestration in cell membranes [66]. This is the first time the interaction between small ligands and *Plasmodium* tubulin has been quantified.

Currently, at least three overlapping but distinct putative herbicide-binding sites, all of which reside on α-tubulin, have been proposed [34-37]. To date, there have been no *in vitro* ligand-tubulin binding or any other data demonstrating that any of them applies to *Plasmodium*. However, Mitra and Sept have argued that their refined site can be modelled in *Plasmodium* tubulin so we focused primarily on it [36]. We altered 6 different residues on αI-tubulin based on amino acids that lined the proposed binding pocket but also on previously generated mutations [67]. To confirm that our alterations would prevent or substantially reduce binding, we made molecular models of all the changes to ensure that they would be central in the 'Mitra & Sept'

site on *Plasmodium* tubulin. However, for neither APM nor oryzalin were substantial decreases in binding affinity, compared with wild type, apparent. The small changes observed for some alterations were probably due to slight allosteric effects. These results are in contrast to previous work that demonstrated that L136F was responsible for almost 20-fold decreased binding of oryzalin to the mutated tubulin [38]. There are several possible explanations for this incongruity. Our recombinant tubulins may not be forming the correct binding pocket owing to improper folding. We think this possible but unlikely, based on the functional characterisation reported here and in Fennell *et al*. [7] and the fact that the K_d values for the wild-type tubulins are in line with previous reports from other species. Another possibility is that the 'Mitra & Sept' site may be present in *T. gondii* but not in *Plasmodium*. *T. gondii* cultures are approximately 10-fold more sensitive to oryzalin than *Plasmodium* [7, 68] and this may be illustrative of different binding affinities of this ligand to the tubulin from these two organisms. The existence in such a highly conserved protein of two or more distinct sites among α-tubulins of the herbicide-susceptible organisms, but absent from those of the herbicide-resistant ones, seems however an unlikely prospect. Even if this were so, one might expect two organisms as closely related as *Plasmodium* and *Toxoplasma* to share the same site. A third possibility, and the one we consider most probable, is that the level of sophistication of molecular modelling does not yet permit sufficiently accurate estimation of the structure of the herbicide-binding site, given that it by definition lies outside the region of sequence identity with the template (mammalian) tubulin. In this case, while it may be in the general area highlighted by the 'Mitra & Sept', 'Délye' and 'Nyporko' sites, the exact location and architecture of the binding pocket still remains to be determined. Obtaining this information may open the door to structure-based approaches to new, potent and selective antimalarial MT inhibitors active on both blood- and liver-stage parasites.

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Figure legends

Fig. 1. Co-sedimentation of MBP–tubulins and bovine brain tubulin. Various concentrations of MBP–αI-tubulin or MBP–β-tubulin, in the presence or absence of bovine brain tubulin, were incubated at 37°C in the presence or absence of 30 μM taxol and centrifuged through a glycerol cushion. The concentrations used were: A, ~24 μM bovine tubulin + 12 μM MBP–αI-tubulin; B, ~ 16 μM bovine tubulin + 16 μM MBP–αI-tubulin; C, ~ 12 μM bovine tubulin + 18 μM MBP–αI-tubulin; F ~24 μM bovine tubulin + 12 μM MBP–β-tubulin; G ~ 16 μM bovine tubulin + 16 μM MBP–β-tubulin; H ~ 12 μM bovine tubulin + 18 μM MBP–β-tubulin. The resulting pellets were resuspended in SDS-loading buffer and equal proportions from the pellet fractions of the control (MBP–tubulin fusion alone) (X) and bovine brain tubulin + MBP–tubulin mixture (Y) were resolved by SDS-10% PAGE and stained with Coomassie Blue. D, I: Equal proportions from the supernatant (S) and pellet (P) fractions from experiments A and F respectively (MBP–tubulin + bovine tubulin) are shown. The extents of the MBP–tubulin incorporation into polymerised bovine brain tubulin for experiments A–C and F–H were quantified by densitometry (E and J respectively): x-axis values are concentrations of MBP–tubulin. The running positions of molecular weight markers are shown in kDa on the left of each gel. Upper arrows indicate MBP–tubulins and lower arrows bovine tubulins.

Fig. 2. Purification of the MBP–tubulin–His_6 by metal affinity chromatography after incubation with other tubulins or MBP. Proteins were separated by SDS-PAGE and stained with Coomassie Blue. A. MBP incubated alone. B. MBP–β-tubulin–His_6 and MBP incubated together. C. MBP–αI-tubulin incubated alone. For panels A–C: 1) sample applied to the metal affinity column, 2)

flow-through sample, 3) 60 mM imidazole wash sample and 4) 500 mM imidazole eluate. D. MBP–β-His_6-tubulin incubated with MBP–αI-tubulin. 1) MBP–αI-tubulin (5 μg), 2) MBP–β-His_6-tubulin (5 μg), 3) Sample applied to the metal affinity column, 4) 60 mM imidazole wash sample and 5) 500 mM imidazole eluate. The data shown are representative of experiments carried out in triplicate. White arrows indicate MBP, grey arrows indicate MBP–αI-tubulin and black arrows indicate MBP–β-tubulin–His_6.

Fig. 3. Models of the putative 'Mitra & Sept' binding site on WT and altered *P. falciparum* αI-tubulin. Only the 'Mitra & Sept' site is highlighted. The models were displayed using either a surface molecular map (Connolly analytic) (A, B, D, F, H, J, L and N) or space-filled amino acids (C, E, G, I, K and M). The surface molecular map highlights predicted hydrophobic (green), polar (blue) and hydrogen-bonding (purple) regions. The space-filled models highlight the 'Mitra & Sept' site residues (aqua) or the specific alteration (red). A, *B. taurus* α-tubulin. B, Homology model of the *P. falciparum* αI-tubulin wild type; C and D, Val4Cys alteration; E and F, Phe24His alteration; G and H, Cys65Ala alteration; I and J, Leu136Phe alteration; K and L, Thr239Ile alteration; M and N, Arg243Ser alteration.

Fig. 4. Analysis of the intrinsic fluorescence of MBP–αI/β-tubulin mixture in the presence of different concentrations of APM. A. The MBP–αI/β-tubulin mixture (0.15 μM) was incubated with either DMSO (control) or different concentrations of APM as indicated for 5 min at 37^{o}C. Three independent samples were used for each concentration. For reasons of clarity, the error bars have been omitted and the uncorrected fluorescence was graphed. B. Double reciprocal plot of the corrected fluorescence change and ligand concentration. A best fit line was used to plot the graph (R^2 value = 0.9712). The F_{max} (the point of interception at the Y-axis) was used to predict the maximum fluorescence quench achievable. F_o represents the observed fluorescence in the absence of a ligand (after correction). F represents the observed fluorescence in the presence of a ligand (after correction).

Fig. 5. Activity of herbicides against *Plasmodium* liver stages. A. Sigmoidal inhibition curves and IC_{50} determination for APM (red) and oryzalin (green). Huh7 cells were infected with luciferase-expressing *P. berghei* sporozoites and incubated with 1, 5, 10 or 25 μM of each compound for 48 hours, prior to measurement of parasite load by bioluminescence. B. Effect of 25 μM APM (red) and 10 μM oryzalin (green) on parasite development. The geometrical mean of the GFP intensity of Huh7 cells 48 h after infection with GFP-expressing *P. berghei* sporozoites is a measure of the extent of parasite replication [47]. Vertical bars show standard errors. C. Representative maximum intensity projections of confocal fluorescence microscopy images of *P. berghei*-infected Huh7 cells. Cells were solvent-treated or treated with 25 μM APM or 10 μM oryzalin, fixed 48 h after infection, and stained for *Plasmodium* Hsp70 (red), β-tubulin (green), and nuclei (blue). The scale bar in the top left panel applies at all panels and corresponds to 10 μm.

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