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

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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

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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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

2.1. Reagents and plasmids

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

2.2. PCR amplification, cloning and expression

The *P. falciparum* 3D7 αI- and β-tubulin were previously cloned into the pMAL-c2x vector [7]. These genes were sub-cloned into the pMAL-c2 g vector using primers listed in Supplementary Table S1.

A "Hotstart" PCR was done using 0.5 μM primer, ~100 ng DNA template, 2 mM dNTPs (Roche), 0.5 mM $MgCl_2$ (Promega), 1 unit *Pfu* Turbo® DNA polymerase (Stratagene, La Jolla, California, USA) and *Pfu* Turbo® buffer (Stratagene) in a 50-μl reaction volume with a Techne TC-300 (Techne, Burlington, NJ, USA) thermocycler. 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 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.

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