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The farnesyltransferase inhibitor manumycin A is a novel trypanocide with a complex mode of action including major effects on mitochondria

Bassam R.S. Ali [a], Arun Pal [a], Simon L. Croft [b], Richard J.K. Taylor [c], Mark C. Field [a,*]

[a] *Wellcome Trust Laboratories for Molecular Parasitology, Imperial College of Science, Technology and Medicine, Department of Biochemistry, Exhibition Road, London, SW7 2AY, UK*

[b] *London School of Hygiene and Tropical Medicine (University of London, Keppel Street, London, WC1E 7HT, UK*

[c] *Department of Chemistry, University of York, York, UK*

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Abstract

Eukaryotes modify numerous proteins, including small GTPases of the ras superfamily, with isoprenes as a mechanism for membrane attachment. Inhibition of farnesylation of ras has been successfully exploited to control cell growth, with promise in the clinic for treatment of human tumours. Using an in vitro screen of mammalian farnesyltransferase inhibitors, we have identified manumycin A as potently active against growth of both bloodstream and procyclic forms of *Trypanosoma brucei*. Other structural classes of farnesyltransferase inhibitors were far less effective. Exposure of *T. brucei* for brief periods to lethal concentrations of manumycin A resulted in subsequent cell death whilst the concentration required to achieve killing was dependent on serum concentration, suggesting partitioning of manumycin A into hydrophobic cellular sites. Manumycin A did not affect trypanosomal protein and DNA synthesis or cell cycle progression but altered incorporation of prenyl groups into several polypeptides indicating a specific effect on the prenylation without effect on other mevalonate pathway products, most importantly prenyl pyrophosphate levels. Morphological analysis indicated that manumycin A caused significant mitochondrial damage suggesting an additional site of action. Structural analogues of manumycin A containing a quinone were also highly trypanocidal and altered mitochondrial morphology, suggesting interference with electron/proton transport systems. Furthermore, manumycin A also elicited mitochondrial alterations in mammalian cells indicating that the effect is not confined to lower eukaryotes. Manumycin A is well tolerated in vivo but failed to cure experimental trypanosomiasis in mice.

Keywords: Farnesyltransferase; Trypanosomiasis; Chemotherapy; Small GTPases

Abbreviations: Boc, *n*-tertbutyloxycarbonyl-; BSF, Bloodstream form; EM, electron microscopy; F, farnesyl; FPP, farnesyl pyrophosphate; FT, farnesyltransferase; FCS, foetal calf serum; GG, geranylgeranyl; GGT, geranylgeranyltransferase; MA, Manumycin A; PCF, procyclic culture form; TCA, trichloroacetic acid; TLC, thin layer chromatography; RP-HPTLC, reverse phase-high performance thin layer chromatography.

* Corresponding author. Tel.: +44-171-594-5277; fax: +44-171-594-5207.

E-mail address: m.field@ic.ac.uk (M.C. Field)

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1. Introduction

Protein prenylation is an essential posttranslational modification of all eukaryotes and is required for membrane localisation of the prenylated protein. The enzymology of the pathway is highly conserved [1,2]. Prenyl moieties attached to protein are of two types, farnesyl (F) and geranylgeranyl (GG), 15 and 20 carbon squaline derivatives respectively, and are products of the mevalonate pathway [3]. Several enzymes, collectively termed prenyl transferases, catalyse the attachment of a prenyl moiety to polypeptide at the C-terminus in a thioether linkage to a cysteine in a CAAX sequence [1]. Depending on substrate specificity, prenyltransferases are designated as either farnesyltransferases (FT) or geranylgeranyltransferases I or II (GGTI; GGTII) [1,2,4–6]. FT and GGTI are heterodimers, which share a common α-subunit. The observation that farnesylation is essential for the function of ras [7,8] suggests FT as a target for the control of malignant cell growth and this approach has shown considerable promise [9–13]. Significantly, FT inhibition is antihelminthic to *Schistosoma mansoni* [14] and prevents growth of *Giardia lamblia* [15]. FT inhibitors are based on a number of different strategies; the most successful are peptidomimetics based on the CAAX motif [11–13,16,17]. Alternatives are analogues of the FPP moiety [18] and several natural products resembling either farnesylpyrophosphate (FPP) or mevalonate pathway products [19].

Recently we demonstrated farnesylation and geranylgeranylation of several polypeptide species in *Trypanosoma brucei* by metabolic labelling with [^{3}H]mevalonic acid [20]. By analogy to higher eukaryotes, it is likely that the bulk of the GG is attached to small G proteins. CAAX-related prenylation signals have been identified at the C-termini of *T. brucei* small GTPases [21–23] and FT activity can be detected in trypanosome lysates [24]. Recently an FT inhibitor was reported to prevent growth of both *T. brucei* and *T. cruzi* in culture and the FT from *T. brucei* has been partially purified [25,48]. Based on both molecular mass and specificity, the enzyme appears highly divergent when compared to mammalian FT [48].

MA is a member of a closely related group of antibiotics [26] produced by *Streptomyces* [27] and is presumed to be a competitive inhibitor of FT by structural similarity to FPP [28] (Fig. 4). MA inhibits growth of transformed mammalian cells [29], reduces tumour size in mice in vivo [28,30] and suppresses ras mediated signalling [49]. Here we show that MA is a potent trypanocide in axenic culture. In addition MA has significant effects on mitochondrial morphology in both trypanosomes and mammalian cells; these data have important bearing on the mode of action of MA and the possible use of MA derivatives for the treatment of trypanosomiasis.

2. Materials and methods

2.1. Materials

Compactin (mevastatin), MA, limonene, perillyl alcohol, perillic acid were from Sigma, other FT inhibitors were from Calbiochem or Bachem. Manumycin related compounds were synthesised as previously described [31]. Tissue culture media and supplements were from either Sigma or GIBCO-BRL. [^{3}H]Mevalonolactone (20–40 Ci/mmol), [^{3}H]thymidine (10 Ci mmol^{-1}) and [^{35}S]methionine (>1000 Ci mmol^{-1}) from NEN-DuPont. MitoTracker™ green FM was from Molecular Probes. Solvents and buffer components were of analytical grade.

2.2. Cell culture

PCF *T. brucei* strain 427 was cultured at 27°C in SDM 79 [32] containing 10% heat inactivated FCS, 7.5 μg ml^{-1} haemin. BSF *T. brucei* (427 strain, originally expressing VSG 117) were grown at 37°C in a 5% CO_2 atmosphere in HMI-18 medium supplemented with 10% FCS. Mammalian cell cultures were grown in DMEM supplemented with 10% FCS in a 5% CO_2 atmosphere at 37°C.

2.3. Growth of trypanosomes in rodents

T. brucei brucei EATRO 1175, recently passaged through a Tsetse fly [33], were the kind gift of M. Carrington, Cambridge, UK. These cells were expanded in nude mice and harvested after 5 days before onset of an immune response. Parasites (3×10^6) were used to inoculate MFM1 mice and parasitaemia was monitored daily by tail bleed and counting with a hematocytometer as described [33]. A total of 18 mice were infected; three animals showed delayed parasitaemia and therefore were removed from the study. The remaining 15 animals were placed into three groups (five each), a control group and two test groups. After the initial wave of parasitaemia the test groups were given MA (2.5 or 5 mg kg^{-1}), dissolved in 10 μl DMSO diluted into 200 μl 0.3% carboxymethylcellulose (CMC), intraperitoneally daily for 6 days. The control group was given 200 μl 0.3% CMC.

2.4. Growth rate determination

Toxicity against trypanosomes was determined by adding drug from a stock solution in water, ethanol or DMSO (volume not exceeding 2 μl/ml) to a logarithmic-phase culture and assessing growth by a colorimetric assay [34] in combination with light microscopy. Dilution series were set up to cover a range of low nanomolar to millimolar in three-fold steps. For accurate determination of growth, cultures were set up in multiple well plates or tissue culture flasks and counted in a Coulter Z1 cell counter (Coulter) gated at 3–10 μm at least twice in each 24-h period. IC_{50} values were calculated from the primary growth curves as follows; periods of log-phase growth were selected and a curve fitted to the data to obtain the slope as a measure of relative growth rate during the log-phase. The slope of the untreated culture was set at 100% and the values from the treated cultures normalised. The IC_{50} was then obtained from a secondary plot of normalised slopes against drug concentration.

2.5. Metabolic labelling, lipid extraction and analysis

For [^{3}H]mevalonate labelling, cultures were pretreated with compactin [20]. MA or other drug was added after the preincubation with compactin and at the same time as the radiolabel. After the labelling period, cells were pelleted, washed twice with PBS and resuspended at 1×10^8 cells ml^{-1} in ice-cold acetone, vortexed for several minutes and incubated on ice for 30 min. The acetone extracts were taken for TLC and the pellets were rinsed once more with acetone, air-dried and resuspended at 4×10^8 cells ml^{-1} in SDS-PAGE sample buffer, boiled and separated by SDS-PAGE on 15% gels. Lipids were analysed by TLC in a saturated atmosphere either on 20 cm × 20 cm Si60 TLC plates (Merck) in *n*-propanol:ammonium hydroxide:water (60:30:10; v/v/v) (system A) or on 20 cm × 20 cm RP-HPTLC plates (KC18F Silica gel 60A; Whatman) in *n*-propanol:ammonium hydroxide:water (80:15:5; v/v/v) (system B). Cells were labelled with [^{35}S]methionine or [^{3}H]thymidine for up to 3 h in deficient media at 10 and 4 μCi ml^{-1}, respectively, pelleted, washed in PBS and incorporation of radiolabel into macromolecular material assessed by TCA precipitation onto glass fibre discs (Whatman) followed by scintillation counting in a Beckman LS50 or by SDS-PAGE.

2.6. FACS analysis

PCF trypanosomes were analysed for cell cycle behaviour after treatment with MA by FACS. Cells were fixed then stained with propidium iodide prior to analysis on a FACScan (Becton Dickinson) as described [35].

2.7. Microscopy

Trypanosome cells were prepared for microscopy using MitoTracker™ green FM and DAPI stains as described by Vassella et al. [36] with the exception that MitoTracker™ green FM was added at 2 μM concentration. African green monkey kidney cells (COS cells) were stained with MitoTracker™ following the manufacturer's in-

Table 1
Toxicity of FT inhibitors towards *T. brucei*[a]

Compound	Stage			
	BSF		Procyclic	
	MIC	LD_{95}	MIC	LD_{95}
(+)-Limonene	>1000	>1000	>1000	>3000
(*R*)-(+)-Perillyl alcohol	300	>1000	~1000	>3000
(*S*)-(−)-Perillyl alcohol	300	>1000	~1000	>3000
(−)-Perillic acid	300	>1000	>1000	>1000
N-Acetyl-farnesyl-*S*-cysteine	600	>1000	n.d.	n.d.
α-Hydroxyfarnesyl phosphonic acid	100	>300	n.d.	n.d.
PFT inhibitor I	85	~400	~400	>400
PFT inhibitor II	90	~400	~400	>400
PFT inhibitor III	80	~400	~150	>400
Manumycin A	3	10	2.2	15
H-Cys-4-Abz-Met-OH	>270	>270	~1000	>1500
H-Cys-Val-Phe-Met-OH	>210	>210	~700	>1000

[a] Compounds were tested over a range of concentrations, typically low nanomolar to millimolar, in three-fold dilution steps. Cell growth was monitored by acidification of the medium as detected by phenol red. Growth or killing was verified by microscopy. All compounds were tested in at least a duplicate dilution series. MIC, minimum inhibitory concentration of parasite growth; LD_{95}, dose required to obtain greater than 95% killing within 24 h; n.d., not determined. Concentrations are given in μM. PFT inhibitor I is {(E,E)-2-[(Dihydroxyphosphinyl)methyl]-3-oxo-3-[(3,7,11-trimethyl-2,6,10-dodecatrienyl)-amino]propanoic acid, sodium}; PFT inhibitor II is {(E,E)-2-[2-Oxo-2-[[(3,7,11-trimethyl-2,6,10-dodecatrienyl)oxy]amino]ethyl]phosphonic acid, sodium} and PFT inhibitor III is {(E,E)-[2-Oxo-2-[[(3,7,11-trimethyl-2,6,10-dodecatrienyl)oxy]amino] ethyl]phosphonic acid, (2,2-Dimethyl-1-oxopropoxy)methyl ester, sodium}.

structions. Cells were fixed and processed for immunofluorescence [40]. Logarithmically growing PCF cultures were treated with MA and prepared for electron microscopy as described [20].

3. Results

3.1. In vitro toxicity of farnesyltransferase inhibitors towards T. brucei

We screened broad range protein FT inhibitors, selected as representative of different structural classes; i.e. peptidomimetics based on the CAAX motif, isoprene analogues and natural products [41]. Using a simple colorimetric assay we found that most of the inhibitors had weak toxicity towards both BSF and PCF in culture (Table 1). The relative resistance of *T. brucei* towards these compounds was unexpected and may reflect divergence in the kinetoplastid FT enzyme [48] or poor uptake of these compounds into *T. brucei*. The sole exception was the *Streptomyces* natural product manumycin A (MA), which exhibited efficient growth-inhibitory activity in the low micromolar range (Table 1). Reexamination of the effect of this compound on trypanosome growth by a more accurate protocol (Section 2) allowed us to calculate an IC_{50} of 1.5 and 0.4 μM against BSF and PCF, respectively (Fig. 1).

Because of the potent effect of MA on trypanosome growth we investigated this compound further. MA has significant effects on the growth of transformed mammalian cells and is able to suppress ras-dependent signalling pathways in a variety of systems [46,47,49]. In addition there are structural features in MA which suggest that it can act as an FPP analogue [26]. Exposure of PCFs to high concentrations of MA for more than 5 min resulted in a significant reduction in viability of the cultures (Fig. 1C), suggesting that either MA activated an apoptotic programme or that the compound rapidly accumulated within the cells to lethal levels. This second possibility is

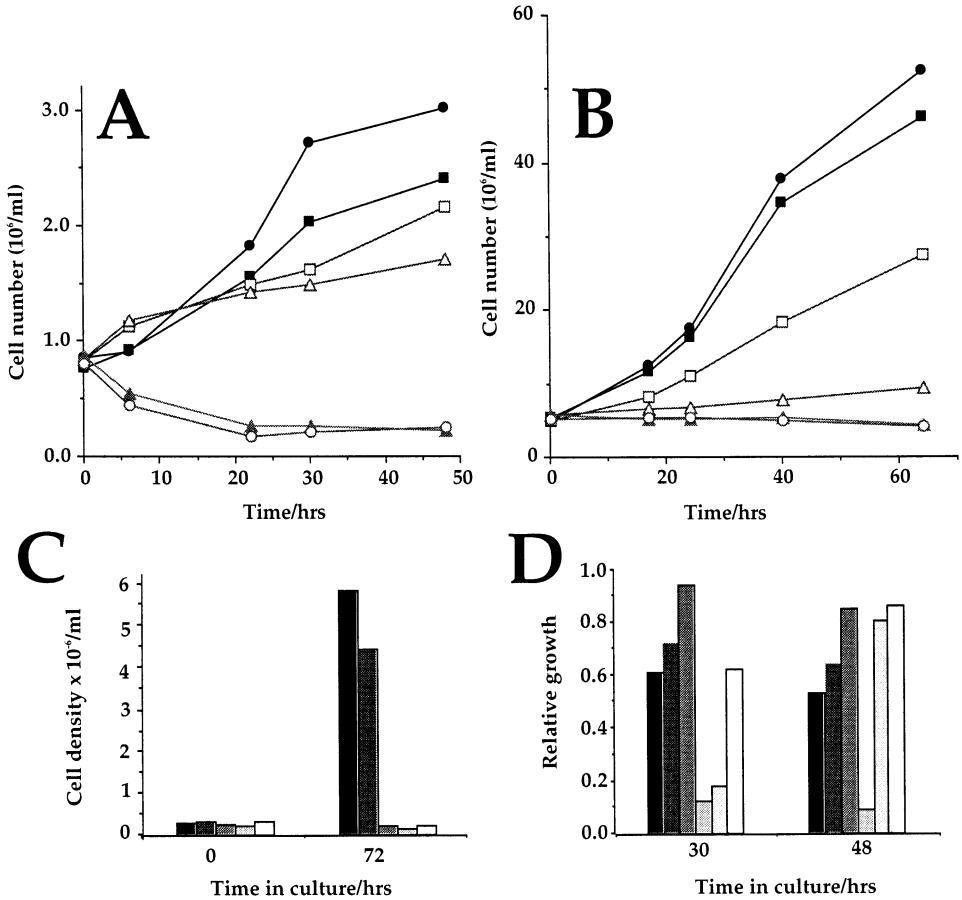

Fig. 1. Effect of MA on growth of *T. brucei* in vitro. Panels A and B: growth curves for BSF and PCF strain 427 *T. brucei*, respectively. Data are the mean of triplicate determinations and the experiment has been repeated twice with essentially identical results. MA concentrations in A; (●), 0; (■), 1 μM; (□), 2.5 μM; (△), 5 μM; (▲), 10 μM; (○), 25 μM and B; (●), 0; (■), 0.1 μM; (□), 0.5 μM; (△), 1 μM; (▲), 5 μM; (○), 25 μM. Panel C: effect of brief exposure of BSF to 16 μM MA. Following addition of MA aliquots of culture were rapidly diluted 20-fold into fresh media (final MA concentration 0.8 μM). Cell growth was assessed after 72 h. Bars correspond to time of exposure; left to right; unexposed, 5, 20, 60 and 90 min exposure. Panel D: effect of serum on toxicity of MA. BSF cells were grown in the presence of various concentrations of MA in 5, 10 and 20% FCS over 72 h. Data are normalised to control cultures and shown for 30 and 48 h. Conditions (left to right for each group of six bars) are bars 1–3 1 μM MA, serum at 5, 10 and 20%, bars 4–6 2.5 μM MA, serum at 5, 10 and 20%.

supported by the hydrophobic properties of MA [26]. The major protein component of serum is the lipid-binding protein albumin; we assessed accumulation by analysing the influence of serum on MA toxicity. High concentrations of serum in the medium resulted in significant elevation of the IC_{50} (Fig. 1D). A similar effect was seen with purified bovine serum albumin (data not shown).

3.2. Inhibition of protein prenylation in T. brucei by MA

We investigated the biochemical effects of MA on PCFs to determine a likely site of action. Metabolic labelling experiments indicated no effect on DNA and protein synthesis (data not shown). FACS analysis indicated that MA-treated

cultures contained a normal distribution of cells with diploid and tetraploid DNA content [35], indicating that mitotic processes were essentially unaltered (data not shown). In addition there was no evidence for subdiploid apoptotic cells (i.e. < 2 n) following MA treatment [50].

In contrast protein prenylation in PCFs, as visualised by metabolic labelling with ^{3}H-mevalonate was clearly altered (Fig. 2A). Effects were obvious at both 1 and 2 μM. Incorporation into several labelled species decreased, especially those at 67, 18 and 13 kDa, whilst incorporation into a prominent band at $\sim$46 kDa was augmented. By contrast, the peptidomimetic inhibitor H-Cys-Val-Phe-Met-OH (CVFM) had no effect (data not shown) whilst PFTIII at 200 μM decreased incorporation into the 46-kDa band as well as the 67-, 18- and 13-kDa species. The lack of effect on most of the polypeptides is consistent with the observation that $\sim$80% of protein-linked isoprene is GG [20]. Separation of ^{3}H-mevalonate-labelled acetone extracts by normal (Fig. 2B) and reverse phase TLC (data not shown) revealed only small alterations in the overall profile of mevalonate products with no significant change in FPP levels, indicating that the effect on protein incorporation is at the level of transfer and not synthesis of the isoprene donor. Therefore, MA perturbed the mevalonate pathway, but did not result in a global blockade to protein prenylation, manifest by either accumulation of precursor or a major decrease of incorporation of radiolabelled mevalonate into protein, suggesting a specific effect on FT. Attempts to study BSF protein prenylation profiles were less successful due to inability of this life-stage to survive prolonged compactin treatment, a requirement for efficient incorporation of ^{3}H-mevalonate [20].

3.3. Toxicity of MA related compounds

To gain insight into the essential structural features of MA we tested a series of related compounds on BSF and PCF trypanosomes (Table 2 and Fig. 3). The compounds tested retain the basic features of the MA core, but contain modifications or deletions of the side chains. With the exception of compound 4 the PCF was rather more sensitive to these compounds than the BSF. For both life stages, removal of the lower side chain and alterations to the structure of the upper side chain, predicted to affect the ability of the compound to act as an FPP analogue, resulted in decreased toxicity by approximately ten-fold (compare MA with compound 1). This would support the model that MA acts as an FT inhibitor. Further alteration, to produce an unsaturated core with two benzoketone substituents, resulted in a significant increase in toxicity (com-

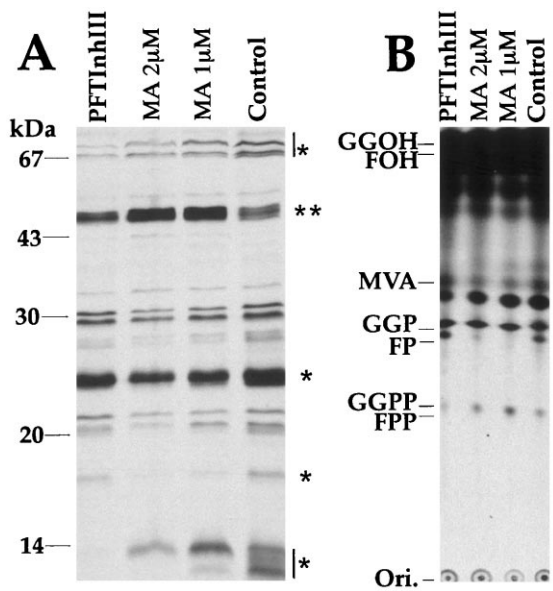

Fig. 2. Manumycin A alters mevalonate pathway metabolism. Logarithmically-growing PCF cultures were labelled with [^{3}H]mevalonate. The inhibitors were added at the following concentrations: MA, 1 and 2 μM and PFT III, 160 μM. After 24 h, cells were extracted (Section 2) and the equivalent of 5×10^6 cells from each culture were separated on a 15% SDS polyacrylamide gel or a TLC plate. Panel A: radioelectrophoretogram of [^{3}H]mevalonate-labelled PCF proteins. Positions of molecular mass standards are shown in kDa at left. Bands that significantly increased in intensity with MA are indicated with double asterisks, bands that decrease are indicated with a single asterisk. Panel B: autoradiogram of normal-phase TLC separation of acetone extracts corresponding to the protein samples in panel A. Migration positions of standards are indicated at left. Ori, origin; GGOH, geranylgeranitol; GGP, geranylgeranylphosphate; GGPP, geranylgeranylpyrophosphate; FOH, farnesol; FP, farnesylphosphate; FPP, farnesylpyrophosphate; MVA, mevalonic acid.

Table 2
Toxicity of manumycin A related compounds towards *T. brucei*[a]

Compound	Stage	
	BSF	PCF
Manumycin A	1.5	0.4
1	18	4.0
2	3.3	0.1
3	2.1	1.5
4	4.2	8.0
5	19	10
6	7.8	10

[a] See Fig. 3 for structures and Ref. [31] for synthetic details. Cell growth was monitored by counting cell numbers over 60 h in cultures after adding the drug at five different concentrations (see Section 2 for details). All compounds were tested in at least duplicate dilution series. Data were determined from secondary plots as described in Section 2 and are expressed as IC_{50} values, in μM.

pare compounds 2 and 3 with 1). These latter compounds have similar toxicities as MA itself, in the absence of the lower chain and the isoprene-like upper chain. Further modification, to create more minimal structures, resulted in some loss of potency; complete removal of the upper chain would be expected to preclude function as an FPP analogue, and may also affect the ability of the compounds to enter cells. Significantly, compounds 5 and 6 are less toxic than compound 4; the former contain a *n*-tertbutyloxycarbonyl (Boc) substituent, which is apolar and should increase membrane permeability; and therefore the decrease in potency probably correlates with alteration of the core region.

3.4. MA causes mitochondrial damage

Benzoquinones, which resemble ubiquinones, important electron-transport components of the mitochondria, can compromise energy production and the pH gradient in eukaryotic mitochondria [38]. Uncoupling electron transport is expected to lead to morphological alterations to the mitochondrion; we directly assessed the effect of MA using the PCF, as this stage has a more prominent mitochondrion and is more easily visualised. No changes to nuclear structure (DAPI), Golgi (TbRab31p [39]) or endoplasmic reticulum (TbRab2p [40]) were observed (data not shown). By contrast, the structure of the mitochondrion visualised with MitoTracker was rapidly and dramatically altered. In control cells the mitochondrion was visible as a threadlike structure in the cytoplasm but when treated with MA the mitochondrion became bloated and diffuse (Fig. 4A–D). Some effect was evident at the IC_{50} concentration (Fig. 4B), but exposure to higher concentrations for short periods illicited a dramatic effect (Fig. 4C). This was confirmed by EM (Fig. 4M–P). EM sections of cells from a time course of a culture following treatment with 7.5 μM MA confirmed the dramatic structural alterations after 2 h of exposure (compare M vs P); effects are visible within 30 min exposure (N and O, respectively). The presence of the kinetoplast at the base of the swollen structure confirms that it is the mitochondrion. No other structures were affected by this treatment.

We wished to determine if the effect on the trypanosome mitochondrion was specific for MA or a more general property of the group of compounds tested here. We chose to study the effects of compounds 2 and 4 (Fig. 3), both of which contain the quinone core structure. Effects were similar to MA (Fig. 4E–H and I–L for compounds 2 and 4, respectively); at the IC_{50} a small but detectable change to the MitoTracker staining was observed, which became far more severe at higher concentrations. Therefore, taken together, these data suggest that MA has a significant activity against the functioning of the trypanosome mitochondrion, in addition to its clear influence on protein farnesylation.

The effect of MA on the trypanosome mitochondrion prompted us to ask if similar effects occur in higher eukaryotes and we assessed this in COS cells. Control cells exhibited a network of MitoTracker-positive tubules distributed throughout the cytoplasm, typical of the higher eukaryote mitochondria (Fig. 4Q). By contrast, following exposure to MA at the IC_{50} (10 μM) the mitochondria retracted to a perinuclear region and appeared as a swollen membrane structure, a morphology which became more severe at higher

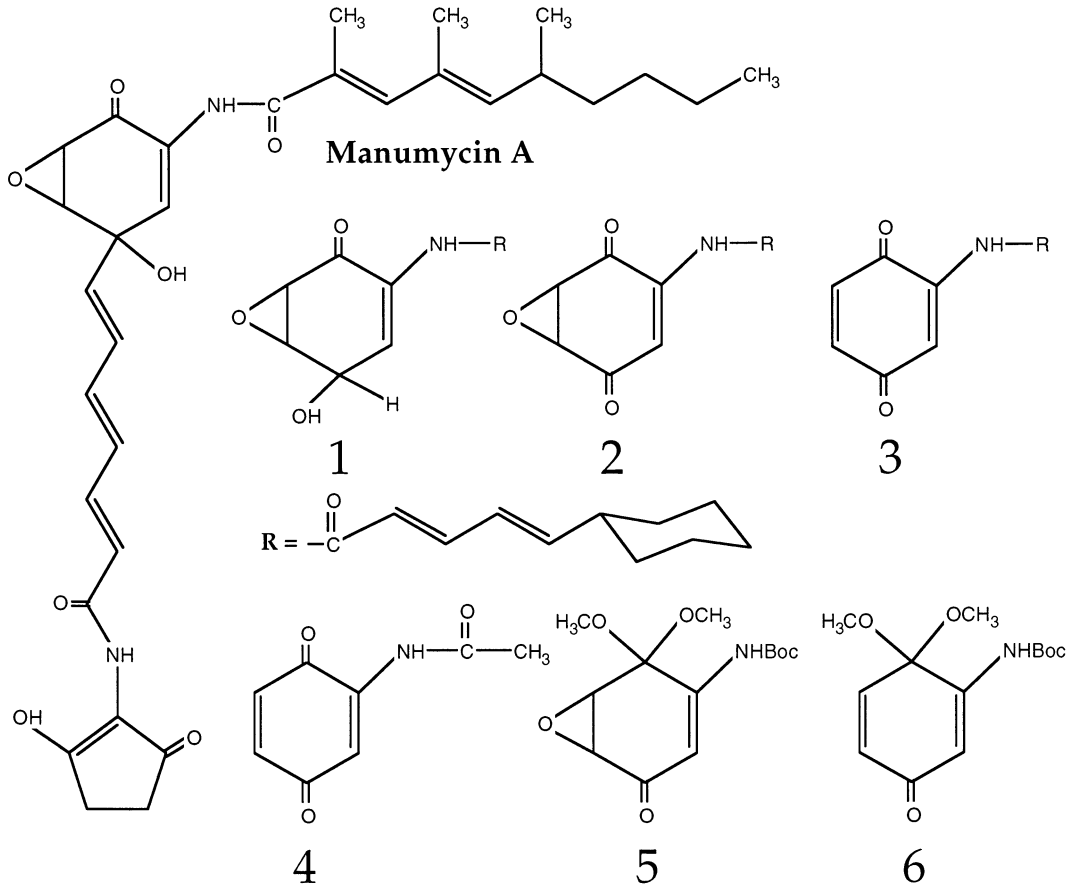

Fig. 3. Structure of manumycin A and several analogues. Structures of MA together with the analogues (compounds 1–6) used in this study. Synthesis routes are described in Ref. [31]. Conventionally, for the manumycin series of compounds, the branched aliphatic chain is termed the upper chain, and is considered to resemble the branched structure of an isoprene, whilst the substituted cyclopentene-terminating arm is called the lower side-chain. Boc, *n*-tertbutyloxycarbonyl-.

concentrations (Fig. 4R, S). These observations confirm that MA also has a potent effect on the mitochondria of higher eukaryotes.

3.5. MA is not effective for treatment of experimental trypanosomiasis

The data above indicated that MA is highly toxic to trypanosomes in axenic culture, and moreover, is significantly less toxic against mammalian cells. In addition, MA is reported well tolerated by mice in vivo [30]. We therefore tested the ability of MA to modulate experimental trypanosomiasis in mice. We used EATRO 1175 *T. brucei brucei* which has recently been passaged through a Tsetse fly, and can establish a cyclical parasitaemia within small rodents that is maintained for several weeks [33]. Mice were infected with 3×10^6 EATRO 1175 and the parasite burden in the bloodstream followed through the first peak until cell numbers began to decline due to immunological recognition. Animals were subsequently dosed daily with MA at concentrations reported to result in significant tumour regression (2.5 or 5 mg kg^{-1}) [30] and bloodstream parasite burden followed for a further 12 days. No significant difference was observed with either of the

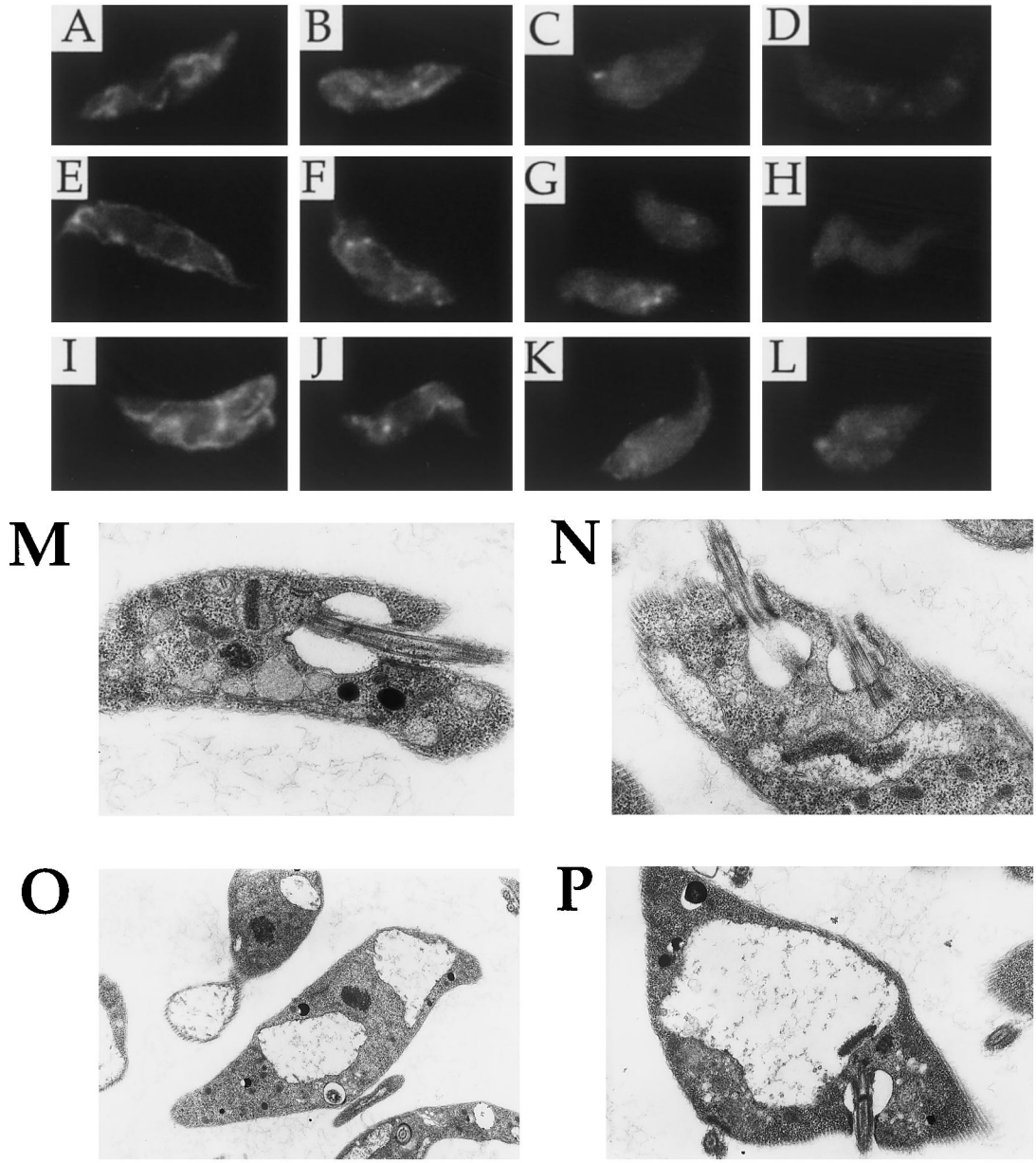

Fig. 4. Manumycin A causes severe mitochondrial damage in *T. brucei*. Panels A–L: PCF cells stained with MitoTracker™ green FM to visualise the mitochondrial membranes following 2-h exposure to various agents. A–D: treatment with MA; A: control; B: 0.5 μM (IC_{50}); C: 2.5 μM; D: 7.5 μM. E–H: treatment with compound 2; E: control; F: 0.1 μM (IC_{50}); G: 0.5 μM; H: 2.5 μM. I–L: treatment with compound 4; I: control; J: 2.5 μM; K: 7.5 μM (IC_{50}); L: 15 μM. Panels M–P: electron micrographs of PCF cultures exposed to MA (7.5 μM) prior to fixing and processing for EM for 0, 30, 60 and 120 min. Note the rapid swelling of the mitochondrion compartment and the presence of the position of the kinetoplast DNA in P. No other overt changes are seen in these sections. Panels Q–S: COS cells stained with MitoTracker™ green FM following treatment with MA at 0 (Q), 10 μM (R) or 50 μM (S) for 2 h. Note that 10 μM is below the IC_{50} for COS cells, and hence the mitochondrial effects are significant to the mode of action of MA in these cells. Under these conditions COS cells do not lose their morphology or structural integrity as evidenced by phase contrast (data not shown).

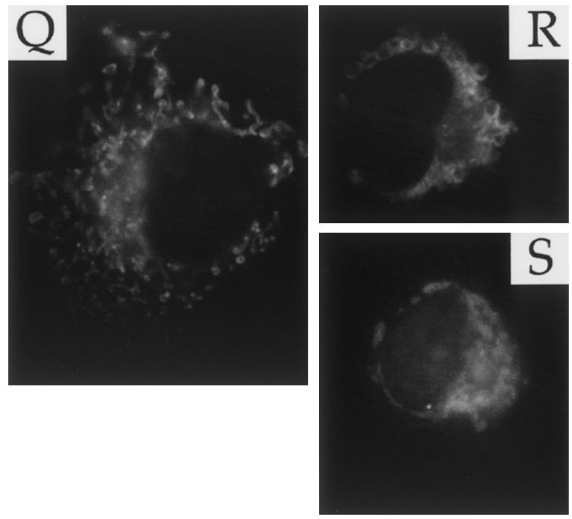

Fig. 4. (*Continued*)

doses of MA used compared with the control group, and the second parasitaemia wave in both the control and 5 mg kg^{-1} MA-treated mice emerged with identical kinetics and peak parasitaemia (Fig. 5). We consider emergence of a new wave of *T. brucei* resulting from an antigenic switching event as a sensitive test of efficacy as the number of parasites surviving from the first peak to establish the second wave is extremely small; the absence of a significant difference in the kinetics suggests essentially no growth disadvantage is experienced by the trypanosomes in the MA-treated mice compared with the untreated control animals.

4. Discussion

The identification of new drug targets for the treatment of protozoan diseases is a clear need. Based on the simple assumption that some life stages of these eukaryotic cells are comparatively fast growing compared with host cells, we have explored the potential of utilising a development in cancer chemotherapy for treatment of trypanosomiasis. Inhibition of FT, an essential modification of ras proteins, has been successfully exploited for the inhibition of tumour growth both in vitro and in vivo (see Ref. [41] for details). Screening a selection of FT inhibitors for growth inhibition of trypanosomes identified manumycin A as a good candidate. Unexpectedly, all the

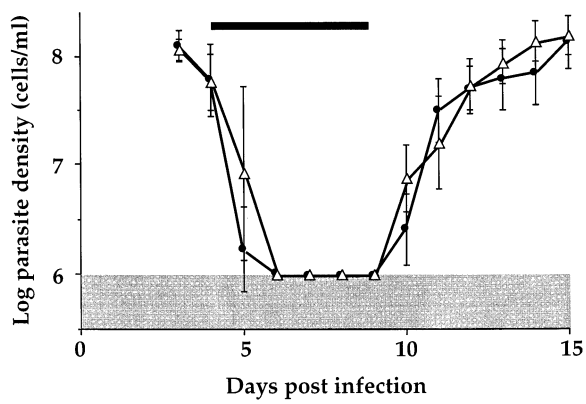

Fig. 5. MA is not protective against experimental trypanosomiasis. MFM1 male mice were infected with a recently Tsetse-passaged *T. brucei* strain EATRO 1175 and blood parasitaemia monitored daily by hematocytometer. MA at 5 mg/kg (△) or vehicle alone (●) was administered daily as indicated by the bar. Data are the mean (± standard deviation) parasitaemia from five mice for each group. A lower dose group (2.5 mg kg^{-1}) were indistinguishable from the 5-mg kg^{-1} group and data were therefore not plotted. Shading indicates the detection limit of 10^6 parasites ml^{-1}. Cell numbers are arbitrarily plotted at 10^6/ml for days 6–9 to aid clarity.

other compounds had poor trypanocidal activity below the 100-μM level. MA was highly toxic to both life stages of the parasite, with the PCF being approximately three-fold more sensitive than the BSF. The drug appears to be rapidly taken up by the cells, and is presumably incorporated into cellular membranes or other apolar compartments by virtue of its hydrophobicity.

Analysis of trypansomes following treatment with MA suggested complex interaction with the parasite. Specific defects were observed in the mevalonate pathway in the profile of prenylated proteins, without an effect on FT donor levels, confirming that MA acted selectively upon the trypanosome protein farnesylation system. We observed a loss of incorporation of mevalonate into proteins in the low molecular mass region which corresponds to the mobility of small G proteins, whilst we saw an increased incorporation into a protein of ~ 46 kDa. Almost identical effects have been recently reported for MA treated mammalian cells [49], suggesting that the biochemical response to MA is highly conserved.

Based on the criteria above FT inhibition appears as a promising target for design of trypanocidal therapeutics. Several MA analogues were also trypanocidal. The isoprenoid moiety of MA is important and isoprenoids themselves are toxic to trypanosomes [42]. This is similar to findings for mammalian cells where the isoprenoid upper chain appears to be an essential structural component for mediating cytotoxicity [37]. However, in trypanosomes, the presence of the quinone core structure appears to be the most significant structural element in terms of toxicity, which suggested that MA may affect mitochondrial function by interfering with ubiquinone electron and proton transport, and thus would not be a clean FT inhibitor. Indeed, modification of MA, by loss of the labile lower side chain [26] could allow subsequent formation of a quinone. MA exposure clearly elicited a profound effect on the trypanosome mitochondrion, causing the organelle to become swollen and presumably nonfunctional for electron transport and ATP production and these effects were observed at concentrations identical to those which significantly inhibit growth. The quinone-core containing analogues also affected the trypanosome mitochondrion, and MA itself produced clear damage to COS cell mitochondria, suggesting that this effect is indeed relevant to the growth-modulatory activity of MA. Such effects may also be important for tumour cells, presumably where high ATP synthesis levels are required for rapid growth. Therefore, whilst MA is clearly an FT inhibitor, these data suggest that there is an additional component to its activity, and may explain why this compound, out of a selection of FT inhibitors, is the only one to have potent trypanocidal activity. Unfortunately, in contrast to the reported efficacy of MA against tumours in animal systems [28,30], the compound was without effect on experimental trypanosomiasis; this is most probably ascribable to the high solubility of MA in apolar compartments, particularly lipid membranes.

With regard to the important structural features of MA, three main conclusions can be drawn. Firstly, modification of side chains decreases MA toxicity; however, in the examples studied here the relative contributions of membrane permeability and isoprene structural homology cannot be assessed. Secondly, the presence of a quinone core structure (compounds 2 versus 1) significantly augments toxicity, and thirdly, despite little structural homology with MA, some of these compounds (e.g. compound 4) retain potent trypanocidal activity. Therefore, whilst our data are consistent with MA action through FT, the possibility that related compounds with a quinone structure may also have trypanocidal activity, which is unlikely to be FT-mediated, is suggested from the behaviour of some of the analogues. Significantly, the lower side chain of MA is chemically labile in the presence of glutathiones [51], and therefore, removal and modification upon entry to the cell is a possible outcome in vivo.

The complexity of action of MA is paralleled by recent studies of other prenyltransferase inhibitors. For example, it is increasingly apparent that FT inhibitors do not completely block ras signalling, and indeed the ras protein may be alternatively modified by geranylgeranylation under conditions where the FT is fully inhibited, which would still facilitate membrane localisation

[43,44]. A number of other observations are also consistent with FT inhibitors mediating their toxicity towards transformed cells through a route distinct from prenylation, despite the clear potency of these compounds against FT [45,50]. Indeed, recent data have directly questioned whether ras is the true site of action for MA. Sensitivity towards MA does not correlate with the presence of an oncogenic ras allele, suggesting the involvement of additional components [49]. MA is remarkably specific for ras farnesylation as the compound does not appear to affect prenylation of the major nuclear farnesyl proteins, the lamins [29]. The similarity between the effects of MA on protein prenylation [49] and mitochondrial morphology in mammalian cells and trypanosomes suggests a close parallel between the molecular sites of action of this compound in both cell types. Most recently, FT inhibitors have been shown to activate a classical apoptosis signalling pathway when administered to transformed cells in the presence of low concentrations of serum; this includes caspase 3 activation and, critically, release of cytochrome c from the mitochondrion [50]. A potential implication of these data is that FT inhibitors may damage mitochondria sufficiently for release of intermembrane proteins including cytochrome *c*, and could be consistent with the MA-induced alterations seen here. However, we see no evidence for advanced apoptotic effects in trypanosomes, specifically decreased cellular DNA content or blebbing behaviour, and in this case mitochondrial damage may be sufficiently severe as to lead to rapid killing by non-apoptotic mechanisms.

The lack of efficacy of the other FT inhibitor compounds tested may be explained in part by the divergent specificity of the trypanosome FT for peptide substrates [48]. However, this cannot explain the resistance to isoprene analogues such as perillic alcohol, and whilst the additional mitochondrial effects of MA may explain its own potency, these observations clearly highlight the need for a more detailed study of the enzymology of trypanosome prenylation before the pathway can be fully utilised as a therapeutic target.

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