# A member of the Ras oncogene family, RAP1A, mediates antileishmanial activity of monastrol

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**Objectives:** To investigate the mode of action of monastrol in intracellular *Leishmania*.

**Methods:** Microarray experiments were conducted on an Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array, to determine the genes that encode proteins related to pathological alterations of cell signalling pathways in intracellular *Leishmania* amastigotes in response to monastrol treatment.

**Results:** Monastrol induced unprenylated Rap1A in intracellular *Leishmania* when exposed to this anticancer drug at the $IC_{50}$ (10 μM). Monastrol, known to cause mitotic arrest in cancer cells, inhibited Rap1A prenylation (geranylgeranylation) in intracellular *Leishmania*, which resulted in blockade at the G1 phase of the cell cycle. Growth inhibition, rather than apoptosis, was found to be the mechanism by which monastrol displays antileishmanial activity.

**Conclusions:** Prenylation inhibitors (unprenylation) of cell signalling pathways can be exploited in *Leishmania* parasites as novel therapeutic tools.

**Keywords:** antileishmanial agents, microarrays, chemotherapy, drug targets, signal transduction

## Introduction

Leishmaniasis is caused by parasitic protozoa of the genus *Leishmania*, of which >20 different species cause the disease. Humans are infected via the bite of phlebotomine sandflies. There are three main types of the disease: localized cutaneous, visceral (VL) and post-kala-azar dermal leishmaniasis. VL, also known as kala azar, is characterized by high fever, substantial weight loss, swelling of the spleen and liver and anaemia. Ninety percent of VL cases occur in seven countries: India, Bangladesh, Nepal, Sudan, Ethiopia, Kenya and Brazil.[1] The disease is fatal if left untreated. Discovering, developing and commercializing safe and affordable therapeutics for VL continues to engage the attention of researchers worldwide.[2]

Our laboratory is engaged in target-based drug discovery for leishmaniasis and has identified monastrol as having putative oral antileishmanial activity.[3] Monastrol, having a dihydropyrimidine pharmacophore, is a small inhibitor of mitotic kinesin Eg5 and is currently used in cancer therapy.[4,5]

In the present study, using Affymetrix U133 Plus 2.0 microarrays, we explored the genes that encode proteins related to pathological alterations of cell signalling pathways in intracellular *Leishmania* in response to monastrol. We identified the Ras-related protein Rap1A to be an effector signal for the antileishmanial activity of monastrol. The $IC_{50}$ of monastrol (10 μM) was sufficient to produce detectable inhibition of RAP1A prenylation and arrested *Leishmania* cells in the G1 phase of the cell cycle. This is the first report to demonstrate the effect of monastrol as a prenyl transferase inhibitor on the proliferation of *Leishmania* cells.

## Materials and methods

### Intracellular *Leishmania* infection and microarray

The maintenance of intracellular *Leishmania donovani* infection was essentially carried out as we described previously.[3] For the present study, RNA was isolated using Trizol reagent (Sigma) from four different samples: [A] mouse macrophage (J774A.1) cells; [B] macrophages treated for 48 h with monastrol; [C] macrophages infected with *Leishmania* amastigotes; and [D] amastigote-infected macrophages treated for 48 h with monastrol. Similar amounts of RNA from each of

the four samples (8 μg) were subjected to labelled cDNA synthesis and biotin-labelled cRNA samples were loaded onto an Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array. Target hybridization and scanning procedures were performed in accordance with the Affymetrix protocol detailed in our accession number: NCBI GEO 29246. Hybridization with each treated and control pair was performed on three different occasions, using different preparations of J774A.1 [A–D], and processed independently to give three replicates. We also performed a single hybridization with RNA derived from promastigotes as a control for cross-hybridization.

### Data analysis and preparation of gene lists

GeneChip analysis was performed with Microarray Analysis Suite 5.0 (MAS5), Data Mining Tool 2.0 and Microarray Database software (Affymetrix). The data were initially normalized by robust multiarray average (RMA) normalization algorithms in expression console software (Affymetrix). Genes on GeneChip were globally normalized and scaled to a signal intensity of 500. The MAS5 software used Wilcoxon's test to generate detected [present (P) or absent (A)] calls and used calls to statistically determine whether a transcript was expressed or not. After being filtered through a 'P' call ($P < 0.05$), the expression data were analysed using GeneChip Operating Software 1.2 (Affymetrix) and GeneSpring 6.1 (Silicon Genetics). Fold changes were calculated by comparing transcripts between samples [A–D]. Significantly altered genes between samples [A–D] were listed. The genes were filtered by scatter plots and the genes up- and down-regulated ≥2.5-fold were considered in preparing the gene lists. The gene lists were classified with respect to the 'biological processes' of the proteins encoded by the genes. Finally, the genes that encoded proteins related to pathological alterations of cell signalling pathways in intracellular amastigotes in response to monastrol were selected. The raw microarray data are available at NCBI GEO 29246. Pathway analysis was performed using Gene Set Enrichment Analysis v.2 (GSEA). The genes were ranked using a $\log_2$ ratio of gene expression in [C] and [D]. Network analysis was performed using MetaCore (GeneGo). Gene ontology analysis (Affymetrix) was performed to assess the content of differentially expressed gene lists in terms of their predominant biological themes. Microarray data were quantified using MAS5 software (Affymetrix). The MAS5 software compares the ratios of the signal hybridized to the specific and the mismatched probes to assess whether the signal intensity reliably reflects the abundance of target RNA. The results are called 'P' (present), 'M' (marginal) or 'A' (absent). Probe sets with statistically significant changes in signal intensity were further filtered to retain only those with at least two 'P' calls from the three repeats or one 'P' and two 'M' calls.

### Apoptosis determination

Apoptotic cell death was assessed using an annexin V-FITC apoptosis detection kit (catalogue no. APOAF, Sigma), in accordance with the manufacturer's recommendations. Data acquisition was carried out using a FACSCalibur flow cytometer and data were analysed using CellQuest Pro software.

### Cell cycle analysis

The DNA content analysis was assessed by flow cytometry. After 24 and 48 h of monastrol treatment, amastigotes were harvested, washed and fixed by incubation in 70% ethanol:30% PBS for 1 h at 4°C. Prior to analysis, fixed cells were washed and resuspended in PBS with RNase A (100 μg/mL) and propidium iodide (PI; catalogue no. 537059—50 mg, Calbiochem) at 10 μg/mL. Cells were incubated at 35°C for 45 min and analysed by flow cytometry. The cell cycle distribution was modelled using ModFit LT for Mac V3.0.

### Fluorescence/immunofluorescence microscopy

For total Rap1 (prenylated and unprenylated forms of both Rap1A and Rap1B) (SC-65, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and unprenylated Rap1A (SC-1482, Santa Cruz) colocalization studies, fluorescence microscopy was performed as described.[6] Cells were stained with antibodies to total Rap1 (1:100), Rap1A (1:100), P36 (1:100),[7] rK39 (1:100),[8] *Leishmania* actin (1:100),[9] coronin (1:100)[10] and ADF/cofilin (1:100).[11]

### Western blot analysis

To investigate the presence of total Rap1 (prenylated and unprenylated forms of both Rap1A and Rap1B) and unprenylated Rap1A proteins in intracellular amastigotes treated with monastrol, western blotting was performed as described previously.[12]

## Results

### Intracellular Leishmania infection and microarray

Microarray experiments were conducted using an Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array to determine the genes that encode proteins related to pathological alterations of cell signalling pathways in intracellular amastigotes in response to monastrol. There were four sample sets: [A] mouse macrophage (J774A.1) cells; [B] macrophages treated with monastrol; [C] macrophages infected with *Leishmania* amastigotes; and [D] amastigote-infected macrophages treated with monastrol. Light microscopy on Giemsa-stained slides of J774A.1 cells infected with amastigotes confirmed that both the percentages of cells infected and the mean amastigote loads were comparable in each of the three experimental replicates used in this study. Comparison of the signal intensities of *Leishmania*-specific (16S and 23S) peaks in the RNA extracts between [A–D] revealed similar peak heights, confirming that parasite loads were comparable. To assess the effect of cross-hybridized *Leishmania* RNA, we hybridized RNA from *L. donovani* to a single Human Genome U133 Plus 2.0 Array.

Using the four sample sets [A–D], six possible analytical combinations can be obtained: [AB], [AC], [AD], [BD], [BC] and [CD]. The combinations [AB] and [BD] were analysed solely with the intent to reconfirm that the specified concentration of monastrol did not cause any host cytotoxicity. Our previous publication[3] has already established that the test drug, monastrol, has been checked for host cytotoxicity on J774A.1 cells by MTT assay using different concentrations (2–50 μg/mL) and was found to be 8–9 times higher $CC_{50}$ (85 μM) than the $IC_{50}$ (10 μM) dose. The combinations [AD] and [BC] comprised irrelevant sets. In the present study, we were interested in identifying signalling molecules found to be expressed in response to monastrol in intracellular amastigotes [D] in comparison with infected macrophages [C]; therefore, the combination [CD] was analysed further.

The data from this combination set [CD] were normalized by RMA normalization algorithms in expression console software (Affymetrix). The data were normalized across a set of hybridizations, at the probe level. These RMA absolute signals ($\log_2$ values) of the microarray data are a result of hybridization of host macrophage RNA regulated by RNA derived from amastigotes treated with monastrol. The results from a representative
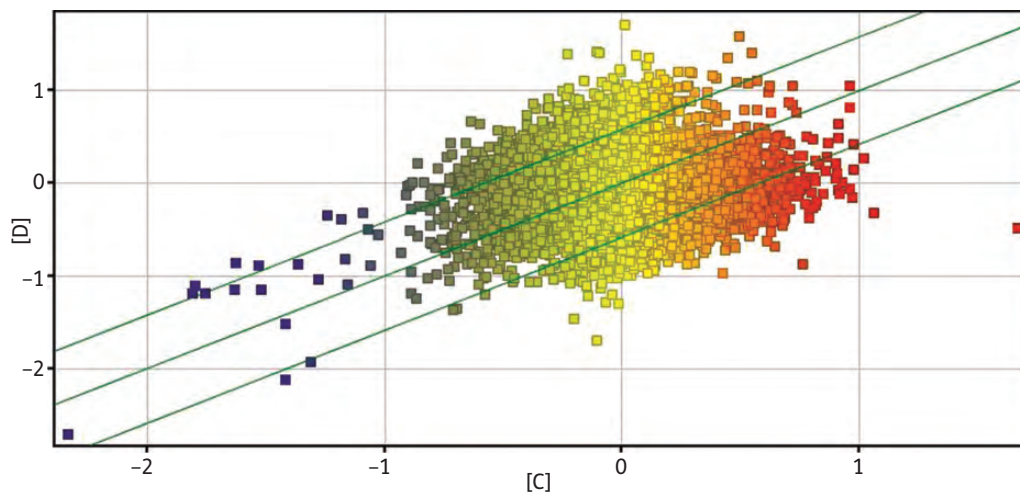
**Figure 1.** Gene expression profiling by microarray of mouse macrophages infected with *L. donovani* clinical isolate [C] versus infected macrophages treated with monastrol [D] transcripts. Results of three representative experiments comparing [C] versus [D]. Distribution of $log_2$-transformed normalized ratios for the microarray experiment. The line of best fit is shown. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

hybridization experiment comparing [C] and [D] are summarized in Figure 1. This scatter plot shows that most of the spots fit a predominantly linear relationship. Out of the 2354 differentially expressed genes, 1454 (62%) were found to be up-regulated and 900 (38%) down-regulated (Figure S1, available as Supplementary data at *JAC* Online). To identify the regulated pathways between [C] and [D], we investigated the interactions of the 2354 differentially expressed genes identified in this study using the MetaCore analysis tool. As expected, we observed a subnetwork centred on signal transduction. We then investigated whether the 2354 genes interacted biologically; GSEA was carried out on the gene expression ratios between [C] and [D], to examine if there was any enrichment of functional genes whose abundance can correlate with the expression of signalling proteins in intracellular amastigotes treated with monastrol. The top-ranked gene set (8-fold change) contained RAP1A (Table S1, available as Supplementary data at *JAC* Online). The product of this gene belongs to the family of Ras-related proteins. Ras family GTPases regulate signalling pathways that control multiple biological processes, allowing a cell to respond to its microenvironment. Rap1A is thought to contribute to cell proliferation and survival.[13] Our microarray results corroborate that increased expression of Rap1 in *Leishmania* parasites upon exposure to monastrol is linked to the inhibition of proliferation.

### Immunofluorescence microscopy to confirm the localization of Rap1A in Leishmania parasites

Immunofluorescence microscopic experiments were carried out to establish the presence of signalling molecules, total Rap1 (prenylated and unprenylated forms) and unprenylated Rap1A, in *Leishmania* cells. Subcellular localization results showed that the two forms of the Rap1 protein are distinct in the *Leishmania* parasite. 4′-6-Diamidino-2-phenylindole (DAPI) staining was included in the overlay to show the relationship of the staining of the nucleus (arrow) and kinetoplast (arrowhead). DAPI is

known to form fluorescent complexes with natural double-stranded DNA, showing fluorescence specificity for AT, AU and IC clusters (Figure 2a–f). Our results confirmed the presence of Rap1 in intracellular structures and on the cell membrane of the parasite (Figure 2a), similar to that reported in mammalian cells.[14,15] Unexpectedly, Rap1A was detected on the cellular membrane instead of its previously reported cytoplasmic localization as in other cell types.[16] Partial colocalization of Rap1 with Rap1A was also noted, as expected since our Rap1 antibody recognizes both prenylated and unprenylated forms of both Rap1A and Rap1B (Figure 2a, enlarged and partial overlap of line scan trace for Rap1 and Rap1A). The reasons for the membrane localization of unprenylated Rap1A in *Leishmania* require further study. One possible hypothesis is that Rap proteins in *Leishmania* might lack the Ras family's characteristic CAAX prenylation signal and instead contain a cluster of basic amino acids, as reported for certain membrane-localized unprenylated proteins.[17]

In order to reaffirm our results, we used *Leishmania*-specific antisera targeted against intracellular and membrane proteins, i.e. P36, K39, ADF/cofilin, actin and coronin (obtained as gifts). Using anti-P36 (goat polyclonal antiserum against recombinant P36) and anti-K39, cytosolic proteins of *Leishmania* spp.[7,8] (Figure 2b and c), we confirmed the discrete localization of unprenylated Rap1A on the cell and flagellar membrane (Figure 2b and c). As it is known that Rap signalling regulates the dynamics of the actin cytoskeleton,[18] further colocalization experiments using antisera against well-established *Leishmania* cytoskeletal proteins (actin,[9] coronin[10] and ADH/cofilin[11]) were carried out (Figure 2d–f).

### Western blotting for detection of prenylated/unprenylated Rap1A in intracellular amastigotes

We attempted to correlate the changes in gene expression with protein function. The presumed involvement of unprenylated
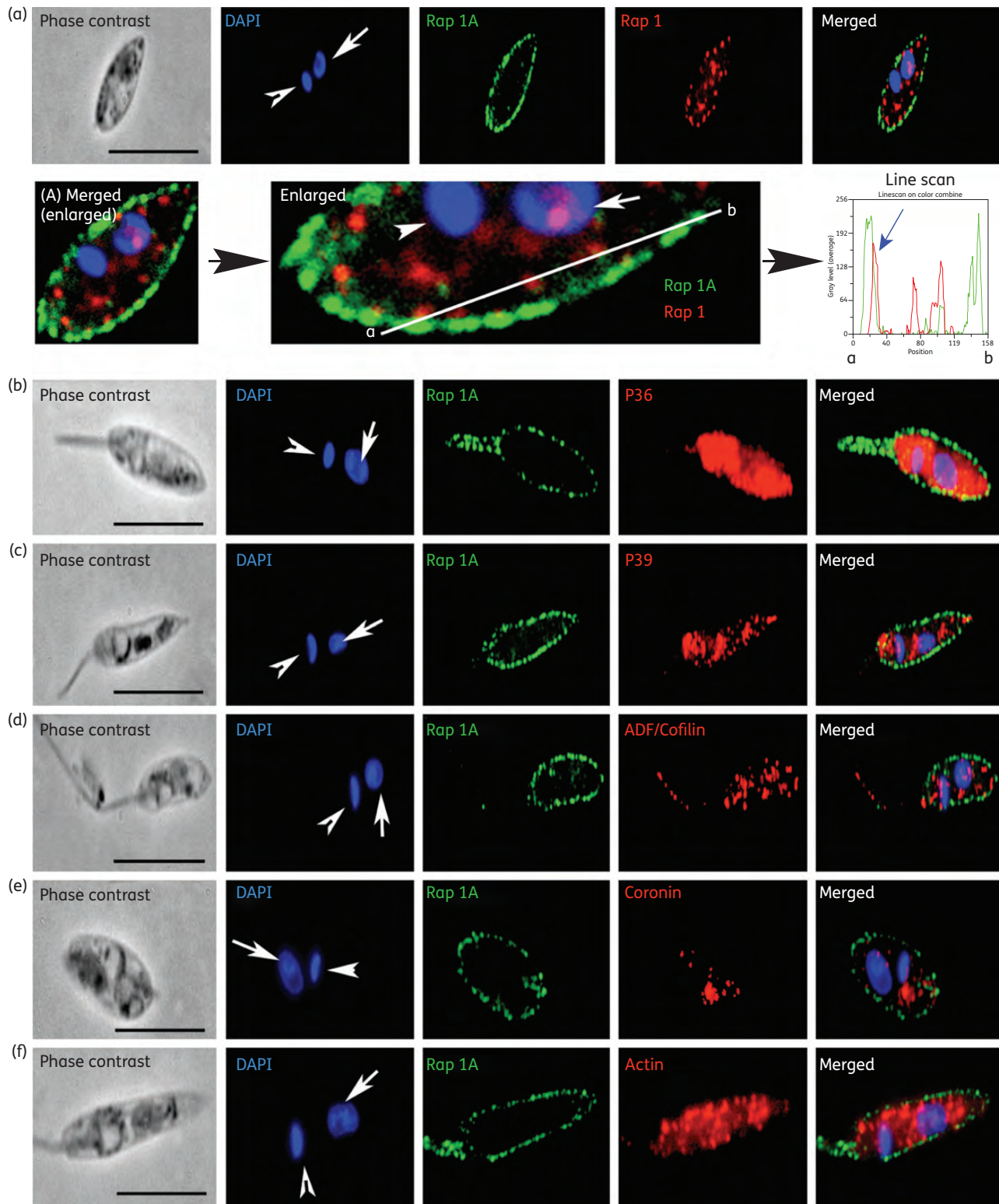
**Figure 2.** Immunofluorescence microscopy showing the localization of Rap1A in *L. donovani* parasites. The figure shows the colocalization of total Rap 1 (red) and Rap 1A (green) (a), P36 (b), K39 (c), actin (d), coronin (e) and ADF/cofilin (f). Fixed cells were permeabilized and labelled with polyclonal antibody against the respective proteins (a–f). Panels show (from left to right) phase-contrast, DAPI staining (blue), Rap1A antibody view, antibodies to respective proteins (a–f) view and overlay. DAPI staining was included in the overlay to show the relationship of the staining pattern of the nucleus (arrow) and kinetoplast (arrowhead). Scale bar=10 μm. The data presented are representative images of >50 microscopic fields analysed from three independent experiments.

Rap1A in the antileishmanial effect of monastrol, as identified by microarray, was given further credence through the results obtained by western blotting. Prenylated (P) and unprenylated (UP) forms of Rap1A were detected with commercially available Rap1 polyclonal antibody (SC-65) raised against a peptide mapping near the C-terminus of Rap1 of human origin. With total Rap1 antibody, endogenous Rap1A (P) was readily detected in all of the samples [A–D] (Figure 3). Representative data from three separate experiments are shown in Figure 3. The specificity of this antibody for unprenylated Rap1A (URap1A) in our study originating from parasite and not from host macrophage is demonstrated by the finding that URap1A immunoreactivity was only seen when infected macrophages were treated with monastrol (experimental set [D]) (Figure 3). Experimental set [D] (Figure 3) was more prone to accumulating unprenylated Rap1A (URap1A) in comparison with untreated intracellular amastigotes from experimental set [C]. Two bands were seen in monastrol-treated infected macrophages [D] and almost all of this protein was in the unprenylated form (Figure 3). A shift to a higher mobility band represents unprenylated forms of protein. As a loading control, samples were blotted concurrently with an antibody specific for β-tubulin (Figure 3). Rap1A prenylation was inhibited by monastrol at the $IC_{50}$ of 10 μM; the accumulation of unprenylated Rap1A, resulting in parasite cell death, contributes to the therapeutic activity of monastrol.[3]

As reported earlier, the top-ranked gene obtained from our microarray analysis in the infected macrophages treated with monastrol, experimental set [D] contained 8-fold up-regulated Rap1A and now our western blot results confirm that this Rap1A is in the unprenylated form. Also in confirmation from the western blot (Figure 3) we can see that the host macrophages, experimental set [A], did not show the presence of any unprenylated Rap1A (Figure 3). The same is true for experimental set [B], in which macrophages were treated with monastrol; thereby, we can convincingly affirm URap1A originating from *Leishmania* parasites on treatment with monastrol. It is well established that the growth of cancer cells expressing prenylated Rap1A can be curtailed by directing therapy with inhibitors
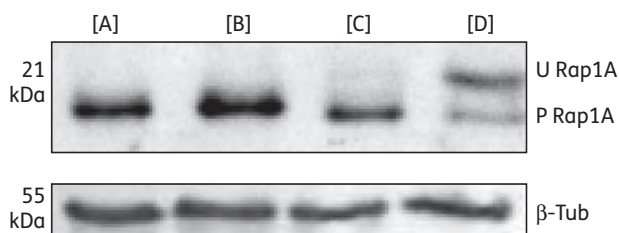


**Figure 3.** Inhibiting protein prenylation blocks the processing of Rap1A in intracellular *Leishmania* amastigotes. [A] Mouse macrophage cells (J774A.1). [B] Macrophages treated with 10 μM monastrol. [C] Macrophages infected with *L. donovani* clinical isolate. [D] Infected macrophages treated with 10 μM monastrol. The cell lysates from experimental sets [A–D] were immunoblotted with anti-Rap1A antibody as described in the Materials and methods section. Bottom band, prenylated (P) form of the Rap1A protein; top band, unprenylated (U) form of the Rap1A protein. P Rap1A was observed in all the samples [A–D]. U Rap1A was only observed for [D]. The levels of β-tubulin (β-Tub) were analysed to assure equal loading. The data are representative of three independent experiments.
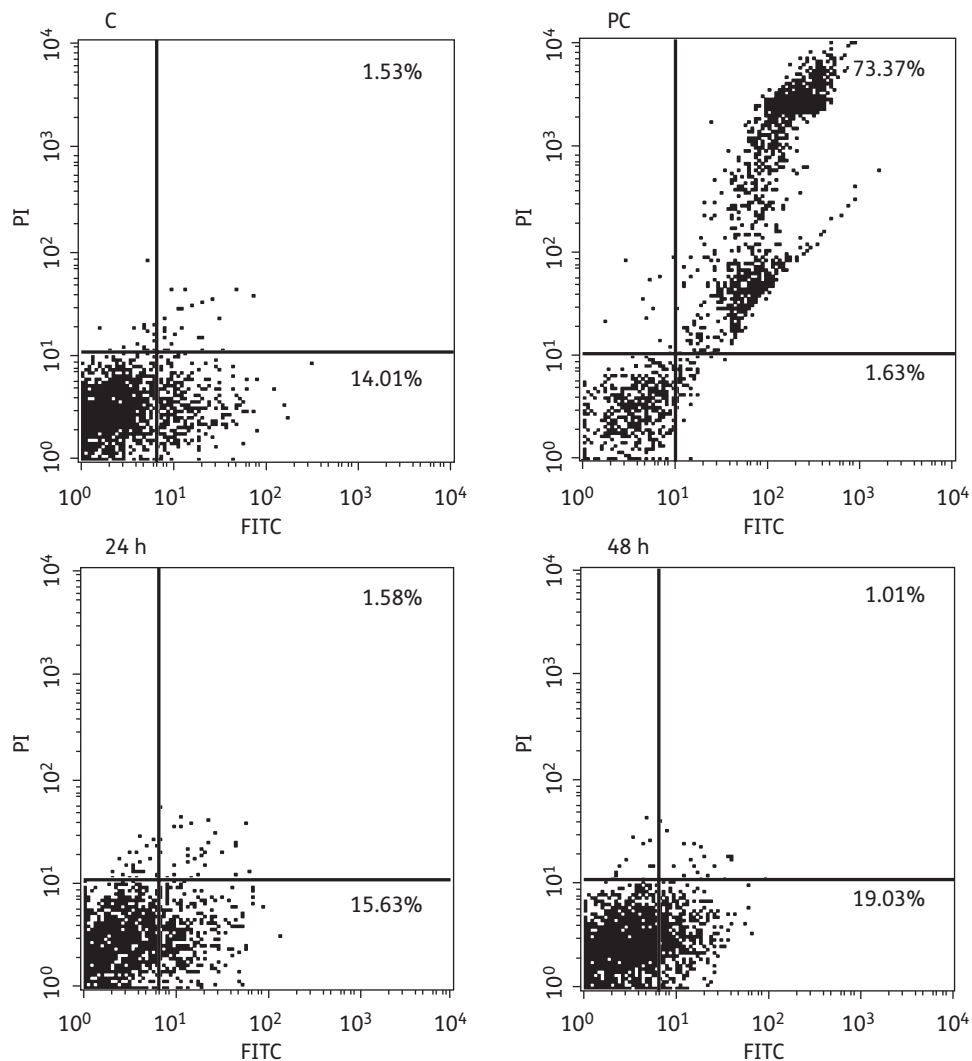
inducing unprenylation.[19] Similarly, the $IC_{50}$ of monastrol induced unprenylated 8-fold up-regulated Rap1A causing the leishmanicidal effect and this has also biologically been proven by us in our earlier publication.[3] In correlation with our microarray results of infection of host macrophages with *Leishmania* parasite (experimental set [C]), the western blot analysis also detected Rap1A (P) immunoreactivity (Figure 3). This prenylated Rap1A of the host macrophage is essential for the intracellular establishment of parasite infection.

### Monastrol-treated L. donovani amastigotes fail to induce apoptosis

Translocation of phosphatidylserine from the inner side to the outer layer of the plasma membrane is a common alteration during PCD.[20] Annexin V, a $Ca^{2+}$-dependent phospholipid-binding protein with a special affinity for phosphatidylserine, is a general reagent used to detect the externalization of phosphatidylserine, thus labelling cells that have lost their membrane integrity. Accordingly, to determine whether monastrol-triggered cell death followed a similar course, amastigotes treated with monastrol (10 μM for 0–48 h) were double-stained with annexin V-FITC and PI. There was no significant change in the number of annexin V-FITC-positive amastigotes after monastrol treatment (14.01%, 15.63% and 19.03% after 0, 24 and 48 h of monastrol treatment, respectively) (Figure 4). However, the number of cells that were both annexin V-FITC-positive and PI-positive (top right quadrant) did not change (1.53%, 1.58% and 1.01% after 0, 24 and 48 h of drug treatment, respectively) (Figure 4). On the other hand, 73.37% of amastigotes were both annexin V-FITC-positive and PI-positive when treated with staurosporine, a known inducer of apoptosis (Figure 4). These observations showed no apoptosis-like cell death. The number of cells positive for PI only was negligible at all timepoints.

### Monastrol induces G1 arrest in intracellular amastigotes

To assess the role of monastrol in mediating G0/G1 arrest, we performed a cell cycle analysis by flow cytometry after PI staining of the parasites incubated for 24 and 48 h with the $IC_{50}$ of monastrol (10 μM).[3] As can be seen from Figure 5, after 24 h, monastrol induced an increase in the number of cells in the G0/G1 phase, a moderate decrease in the number of cells in the S phase and no change in the number of G2/M cells compared with the untreated control (G1: 51.43% versus 59.12%; S: 48.57% versus 38.97%; and G2/M: 0.00% versus 1.92% for control and monastrol-treated amastigotes, respectively). After another 24 h, there was a further increase in the number of cells in the G0/G1 phase, a decrease in the number in the S phase and no change in the number of G2/M cells compared with the untreated control (G1: 51.43% versus 64.32%; S: 48.57% versus 35.68%; G2/M: 0.00% versus 0.00% for control and monastrol-treated amastigotes, respectively). The time-dependent effect on G0/G1 arrest in amastigotes was largely at the expense of S-phase cells, with a non-significant change in the G2/M-phase cell population compared with the untreated amastigotes. The drug-induced cell cycle perturbations, such as an increase in the number of cells in the G1 phase, have been reported to correlate with a response to chemotherapy.[21] The sensitivity of *Leishmania* parasites to monastrol was shown by growth arrest, with an increase in the

**Figure 4.** Externalization of phosphatidylserine in monastrol-treated intracellular *L. donovani* parasites. Cells were incubated with 10 μM monastrol for 0–48 h and analysed by flow cytometry. Undamaged cells were not stained with annexin V-FITC/PI (bottom left quadrant). After incubation for 24 h, a very small number of cells were stained positive with annexin V-FITC and negative with PI (bottom right quadrant). After incubation for 48 h, there was no increase in the number of cells stained positive with annexin V-FITC and with PI (top right quadrant). Cells treated with staurosporine [positive control (PC)] advanced apoptotic cells stained positive with annexin V-FITC and with PI (top right quadrant). The data are presented as means ± SD from triplicates; two other independent experiments produced similar results. C, control; PC, positive control.

number of diploid cells (G0/G1) and had activation of unprenylated Rap1A. We presume that unprenylated Rap1A interacts with downstream effector proteins and switches the balance from cell proliferation to growth inhibition of *Leishmania* cells.

## Discussion

We have earlier reported that monastrol showed potential for therapeutic application against VL.[3] In the present study, using an Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array, we attempted to measure the signalling pathways that regulate monastrol metabolism in intracellular amastigotes of an *L. donovani* clinical isolate. Monastrol, a small cell-permeable drug candidate for cancer, is a known kinesin spindle protein (KSP) inhibitor.[4] We used a human genome array to investigate

*Leishmania* transcripts from infected mouse macrophages. We thought this experimental design would be more conducive to a clinical situation than isolating the amastigotes from the infected macrophages. Our results showed significant signal intensities of *Leishmania*-specific (16S and 23S) peaks in the RNA extracts of macrophages. We analysed altered gene expression in host macrophages infected with the *Leishmania* parasite [C] and compared this with the parasite when exposed to monastrol within the host macrophage [D]. The focus of this study is on the mode of action of monastrol in intracellular *Leishmania* parasites in experimental set [D].

Monastrol triggered an alteration of gene expression in [D] when compared with [C]. The IC$_{50}$ of monastrol (10 μM) was sufficient to produce a detectable inhibition of RAP1A prenylation, which is the effect of the antileishmanial activity of monastrol
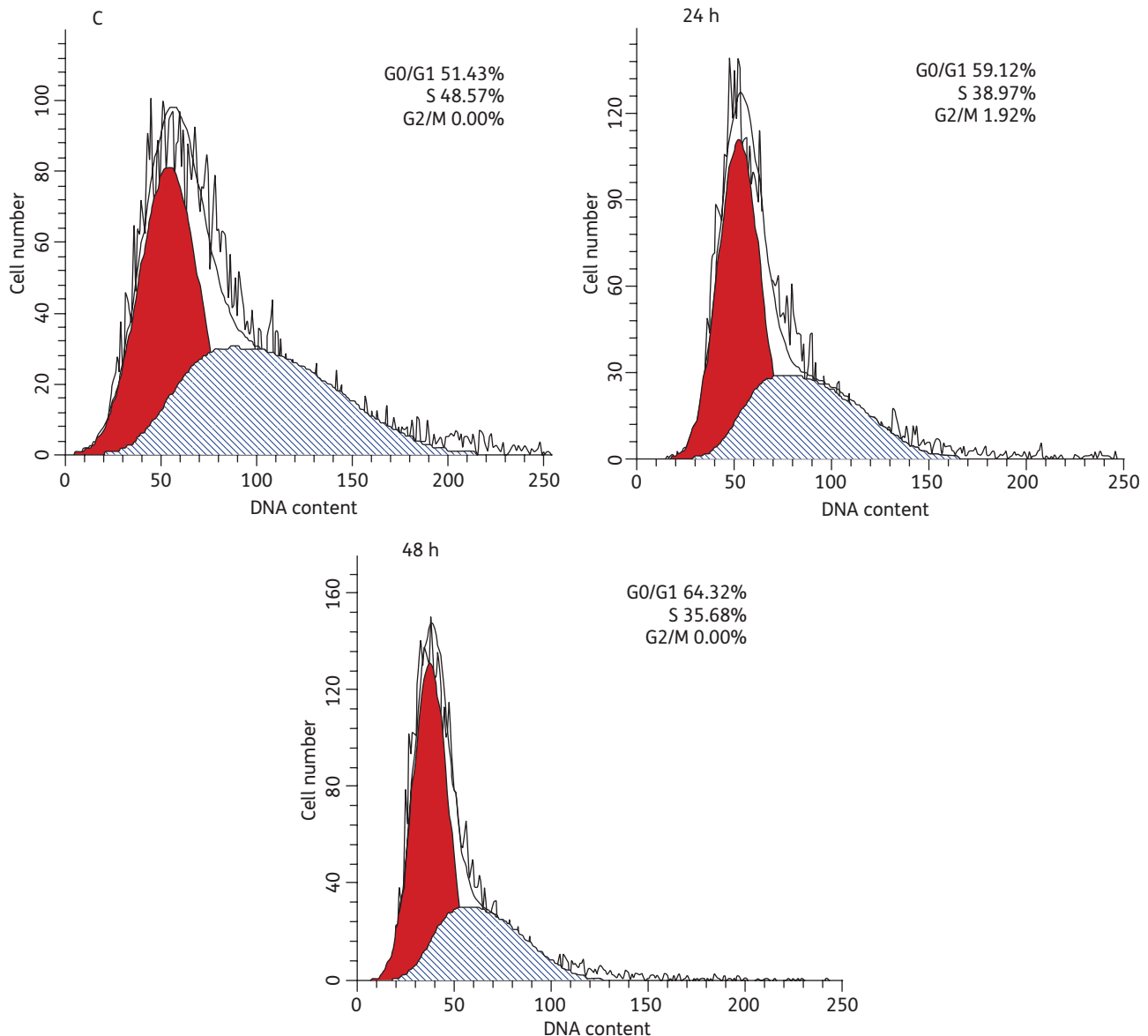
**Figure 5.** Monastrol-induced arrest in the G0/G1 phase of intracellular *L. donovani* parasites. After treatment with 10 μM monastrol for 0–48 h and staining with PI, the DNA content was analysed by flow cytometry. The differently shaded areas represent the G0/G1 (red/solid), S (diagonal shading) and G2M (no shading) phases of the cell cycle. The data are presented as means ± SD from triplicates; two other independent experiments produced similar results. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

in the *Leishmania* parasite (Figure 6), after exposure to the drug for 48 h and corroborate our earlier findings.[3] We hypothesize that the monastrol-induced Rap1A is in the unprenylated form in intracellular parasites, thereby causing the antileishmanial effect. Functional roles of unprenylated proteins causing cell death in parasites have been reported.[22–24] Rap1A is a substrate of geranylgeranyltransferase (GGTase-I). GGTase-I has also been proposed as a target for countering parasitic infections, such as malaria, by selective inhibition of the parasite enzyme.[25] Inhibition of protein geranylgeranylation associated with accumulation of unprenylated RAP1A is a predominant molecular mechanism exerted by certain bisphosphonates directed anti-tumour activity.[16,26,27]

To further confirm that monastrol inhibits the prenylation of substrates for GGTase-I, cell lysates of J774A.1 cells, comprising sample sets [A–D], were analysed by western blotting using an antibody that specifically recognizes the unprenylated form of Rap1A, a substrate of GGTase-I. Unprenylated Rap1A was absent in monastrol-untreated mouse macrophage (J774A.1) cells, but accumulated markedly in parasite-infected host cells after treatment with monastrol. There was a shift from the processed form to a slower-migrating unprocessed form. This unequivocally established the origin of Rap as being from *Leishmania* parasite and not host macrophages.

It has been reported that Ras-like small GTP-binding proteins (LmjF36.1820), involved in cell signalling and/or membrane
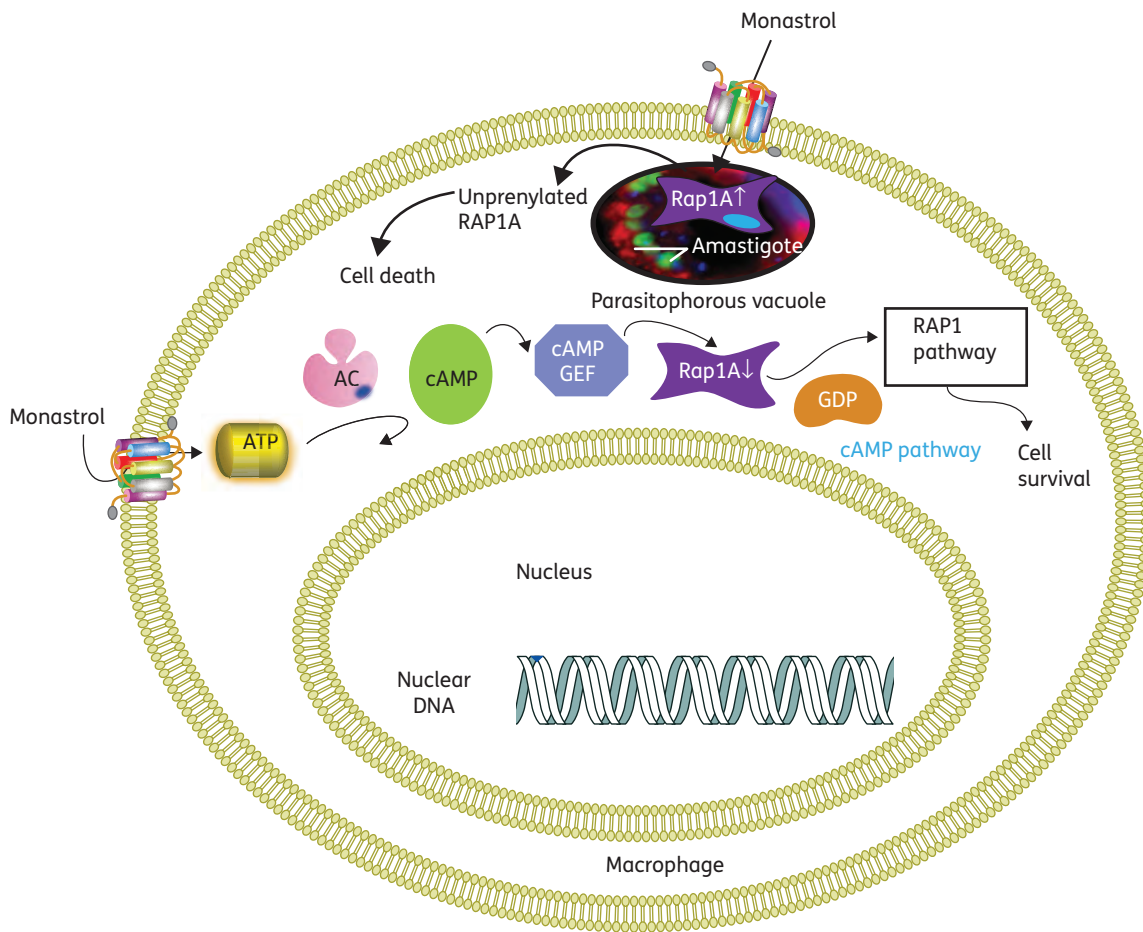
**Figure 6.** Proposed mechanism for monastrol-induced unprenylation of Rap1A in intracellular *L. donovani* parasites. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*. AC, adenylyl cyclases; GEF, guanine nucleotide exchange factors.

trafficking/transport pathways and exocytosis, are present in pathogenic *Leishmania* but absent in non-pathogenic *Leishmania*, or present in a lower copy number.[28,29] A Rap1 homologue exists in yeast.[30] The lower eukaryote *Dictyostelium*[31] has been shown to have Rap1 that is different from human rap proteins at the extreme amino terminus. We were unable to detect RAP1 in the genome of *L. donovani*, although other *ras* genes, such as Rab6, could readily be detected by PCR in the *L. donovani* genome. *Trypanosoma brucei* lacks *QDPR* in its genome, despite maintaining apparent QDPR activity (NADH-dependent quinonoid dihydropteridine reductase enzyme required to salvage and maintain intracellular pools of tetrahydropterin).[32]

As a KSP inhibitor, monastrol induces apoptosis with a prolonged G2/M mitotic arrest.[33] However, we failed to detect G2/M arrest in intracellular *Leishmania* on exposure to monastrol. We found that after 48 h of treatment of *Leishmania* cells with monastrol, there was an accumulation of cells in the G0/G1 phase (64.32%) of the cell cycle (Figure 5). This was probably because of induction of unprenylated Rap1A in *Leishmania* cells. Unprenylation is highly dependent on the rate of cell proliferation.[34] The actively proliferating *Leishmania* cells (48.57% S phase) are suitable for the monitoring of Rap prenylation on

treatment with monastrol (Figure 5). The inhibition of Rap1A prenylation in *Leishmania*-infected macrophages was associated with a decrease in the viable cell number, as a result of decreased cell proliferation rather than increased cell death. Monastrol had no effect on mouse macrophage (J774A.1) host-cell apoptosis at concentrations that inhibited Rap1A prenylation in the parasite. The inhibition of Rap1A prenylation in mouse fibroblasts leads to blockade of the cells in the G1 phase of the cell cycle.[35] Human tumour cell lines, when exposed to a GGTase inhibitor, typically arrest in the G0/G1 phase.[36] G1 arrest can also occur via inhibition of the proteasome and up-regulation of p21, as seen in the case of the lactone moiety of isoprenoid inhibitors.[21] Rap1 has a ubiquitin fold, thereby implicating its degradation via the proteasome. Based on our earlier work,[3] we speculate that monastrol is capable of modulating cell cycle progression in intracellular amastigotes by inhibiting the proteasome-mediated degradation of the pteridine reductase enzyme (AY547305).

In conclusion, we identified a distinct RAP1A in intracellular *Leishmania* parasites that is induced in response to the antileishmanial effect of monastrol. The disruption of the prenylation pathway within the *Leishmania* parasite by this RAP1A is responsible for the growth inhibition of the parasite. Increasing

endogenous Rap1A in infected macrophages treated with monastrol is a pharmacological means to influence the prenylation status of Rap1A in *Leishmania* parasites, causing cell death.

## Transparency declarations

None to declare.

## Supplementary data

Figure S1 and Table S1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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