Antimonal-induced Increase in Intracellular Ca$^{2+}$ through Non-selective Cation Channels in the Host and the Parasite Is Responsible for Apoptosis of Intracellular Leishmania donovani Amastigotes*

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The capability of the obligate intracellular parasites like Leishmania donovani to survive within the host cell parasitophorous vacuoles as nonmotile amastigotes determines disease pathogenesis, but the mechanism of elimination of the parasites from these vacuoles are not well understood. By using the anti-leishmanial drug potassium antimony tartrate, we demonstrate that, upon drug exposure, intracellular L. donovani amastigotes undergo apoptotic death characterized by nuclear DNA fragmentation and externalization of phosphatidylserine. Changes upstream of DNA fragmentation included generation of reactive oxygen species like superoxide, nitric oxide, and hydrogen peroxide that were primarily concentrated in the parasitophorous vacuoles. In the presence of antioxidants like N-acetylcysteine or Mn(III) tetrakis(4-benzoic acid)porphyrin chloride, an inhibitor of inducible nitric-oxide synthase, a diminution of reactive oxygen species generation and improvement of amastigote survival were observed, suggesting a close link between drug-induced oxidative stress and amastigote death. Changes downstream to reactive oxygen species increase involved elevation of intracellular Ca$^{2+}$ concentrations in both the parasite and the host that was preventable by antioxidants. Flufenamic acid, a non-selective cation channel blocker, decreased the elevation of Ca$^{2+}$ in both the cell types and reduced amastigote death, thus establishing a central role of Ca$^{2+}$ in intracellular parasite clearance. This influx of Ca$^{2+}$ was preceded by a fall in the amastigote mitochondrial membrane potential. Therefore, this study projects the importance of flufenamic acid-sensitive non-selective cation channels as important modulators of antimonal efficacy and lends credence to the suggestion that, within the host cell, apoptosis is the preferred mode of death for the parasites.

Apoptosis, a morphologically distinct form of cell death that is crucial for embryogenesis, tissue homeostasis, and disease control in metazoa, has also been demonstrated in unicellular organisms including yeast and protozoan parasites (1–6). The protozoan parasites have a digenic life cycle, residing as flagellated extracellular promastigotes in the gut of the insect vector or as obligatory intracellular amastigotes found in the parasitophorous vacuoles of mammalian macrophages. Within the protozoan parasite group, Leishmania spp., the causative agents of various forms of leishmaniasis (7) are one of the most well studied genera for expression of apoptosis-like phenotypes in extracellular forms induced by exposure to various death-inducing stimuli. For example, stationary phase promastigotes and axenic amastigotes of Leishmania donovani, Leishmania major, and Leishmania mexicana show a DNA fragmentation pattern in multiples of oligonucleosomal length units when undergoing death in culture, thus demonstrating the expression of a hallmark of apoptosis (5, 6). Exposure of axenic amastigotes of Leishmania infantum to an antimonal compound (SbIII) yields similar results (8). Hydrogen peroxide ($H_2O_2$) induces nuclear condensation, DNA ladder formation, and activation of caspase-like activity in L. donovani promastigotes that occur through a Ca$^{2+}$-dependent mechanism (4, 9). The protein kinase inhibitor staurosporine, known to induce apoptosis in all mammalian nucleated cells (10), stimulates apoptosis-like death in the promastigotes of L. major (11). Not only the Leishmania spp. but also other closely related kinetoplastid parasites of the group Trypanosoma are known to use apoptotic death to control cell proliferation in the insect vector mid gut and $in vitro$ (12). All the above studies describe apoptotic phenotype in promastigote forms that are either encountered in the insect gut or in the mammalian blood stream. Investigations on promastigotes yield important information, but their utility is limited because the promastigote form is not a clinically relevant stage although they transmit the disease. The actual survival of intracellular amastigotes encountered in the mammalian host determines disease pathogenesis. Importantly, promastigote and amastigote forms surviving in two disparate biological environments are very different in terms of metabolic pathways and their susceptibility to anti-leishmanial compounds (13, 14). Therefore, observations with intracellular amastigotes addressing questions related to parasite survival are more relevant and could lay the foundation for rational strategies of drug development. However, mechanisms of parasite death inside the macrophages remain elusive, and there is no clear consensus in the literature regarding the manner by which the intracellular protozoan parasites are killed. To our knowledge, two recent reports suggest that intracellular amastigotes of the kinetoplastid parasites upon exposure to death-inducing stimuli show DNA breakage, but the kinetics of DNA fragmentation or the mechanisms inducing such changes are not known (6, 15).

Intracellular pathogen survival depends upon the efficiency of host-defense mechanisms like the respiratory burst (16, 17) that involves generation of large amounts of superoxide anion.

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(\text{O}_2^\bullet-) and related reactive oxygen species (ROS), such as hydrogen peroxide (H\text{}_2\text{O}_2), nitric oxide (NO), hydroxyl radicals, and singlet oxygen (18, 19). ROS is known to induce apoptosis in metaonals (20) and in unicellular organisms existing outside the host cell (4, 9). Therefore, it is possible that ROS generation might have a direct bearing on apoptotic death of intracellular parasites, but this phenomenon has not been examined adequately. ROS is also generated by anti-leishmanial drugs like antimonials in blood (21), but it is not known whether this is directly linked to apoptotic death of amastigote forms of \textit{Leishmania} spp. It remains to be shown whether apoptotic pathway of death is an inherent feature of intracellular parasitic life style because the features and mechanisms of apoptotic death in intracellular parasitic forms have not been sufficiently described.

The idea that, while existing in closely knit groups, unicellular organisms acquire a new level of individuality and can express features of apoptosis under situations of stress prompted us to explore whether or not, while living in close association inside the macrophages, the parasites have the capability to undergo apoptosis. Also, if there is apoptotic death, what are the associative mechanisms of the process? Earlier studies from this laboratory have shown that the amastigotes of \textit{L. donovani} undergo apoptosis in response to H\text{}_2\text{O}_2 via a Ca\textsuperscript{2+}-mediated mechanism induced by oxidative stress (4, 9); therefore, we asked whether intracellular amastigote death involves similar features or uses different mechanisms. To address this issue, we used a model of drug-induced intracellular amastigote death to study the changes associated with amastigote clearance. The findings project a new possibility that, under conditions of drug exposure, non-selective cation channels in both the host and the parasite are important modulators of drug efficacy. Importantly, it lends acceptance to the suggestion that, in situations of close existence of the parasites, such as within the host cell, apoptosis is the preferred mode of death.

EXPERIMENTAL PROCEDURES

Materials

The Apoptosis Detection System was procured from Promega (Madison, WI). Proteinase K was from Roche Molecular Biochemicals GmbH (Mannheim, Germany). 5',5',6',6'-Tetrachlorol1,1,3',3'-tetraethylen-benzimidazole carbocyanide iodide (JC-1), fluo-3 acetyloxyethyl ester (fluo-3/AM), 4-amino-5-methylamino-2',7'-difluorescein diacetate (DAF-FM), bis-N-methylacridinium nitrate (lucigenin), and 2',7',di-chlorodihydrofluorescein diacetate (H\text{}_2\text{DCFDA}) were obtained from Molecular Probes (Eugene, OR). Mn(III) tetakis(4-benzoic acid)porphyrin chloride (MnTBAP) was obtained from Calbiochem (La Jolla, CA). Fluorescein isothiocyanate (FITC)-conjugated annexin V was from Pharmingen (San Diego, CA). N-Acetylcyesteine (NAC), aminoguanidine, and all other chemicals were purchased from Sigma.

Cells

Promastigotes—Culture of promastigotes of \textit{L. donovani} of URL6 strain was carried out as described previously (4, 9). Briefly, the cultures were grown on slants containing 1% glucose, 5.2% brain heart infusion agar, and rabbit blood (6%, v/v) at 25 °C.

Macrophages—\textit{J774A.1} macrophage cell line (ATCC no. TIB-67) was maintained in Dulbecco's modified Eagle's medium (DMEM) with 4 mm \text{t}-glutamine adjusted to contain 1.5 g/liter sodium bicarbonate, 4.5 \text{g} / \text{l}iter glucose, and 10% fetal calf serum (FCS) at 37 °C in an atmosphere of 5% CO\textsubscript{2} and air.

Infected Macrophages—\textit{J774A.1} cells (5 × 10\textsuperscript{5} cells/ml) were seeded on coverslips and incubated overnight using DMEM containing 10% FCS. After 24 h, these cells were incubated with \textit{L. donovani} promastigotes (5 × 10\textsuperscript{6}) in the log phase of growth in serum-free media for 2–6 h. The infected cells were then maintained in DMEM containing 10% FCS from 1–18 h at 37 °C with 5% CO\textsubscript{2} and air. Before use, loosely adherent promastigotes were removed from the macrophages by incubation of the cells for 3 min in cold distilled water.

Purified Amastigotes—\textit{J774A.1} cells grown in 125-mm tissue culture flasks infected with \textit{L. donovani} promastigotes (as per procedures described above) were used to purify amastigotes by the method of Chang and Hendricks (22) with a few modifications. Briefly, flasks containing infected \textit{J774A.1} were flushed, and cells were harvested and centrifuged. The cells were then subjected to freezing in liquid nitrogen with subsequent thawing at 37 °C for four cycles. The released amastigotes and disrupted macrophages were spun at 400 × g for 5 min, and the pellet was suspended in DMEM containing 10% FCS. This suspension was loaded onto a Percoll gradient (20–40%) and spun in a swing rotor at 1200 g for 90 min. The amastigotes were collected using a Pasteur pipette from the appropriate layer and viewed under a phase contrast microscope for purity check.

Treatments

To rule out any toxic effect of the drug on the host cell, potassium antimony tartrate (PAT) was used at different doses (10–50 \text{μg/ml}) to incubate uninfected macrophages and cell viability was determined at 36 h. For assessment of anti-leishmanial activity of the drug, infected macrophages were incubated with selected doses of PAT from 3 to 36 h. For cell treatments in Ca\textsuperscript{2+}-related studies, nifedipine was used as a nitric oxide synthase (iNOS) (23), was used as a nitric oxide inhibitor at a concentration of 200 \text{μM} and flufenamic acid (FFA) was used at a concentration of 200 \text{μM}. For cell–Ca\textsuperscript{2+} chelation, 10 \text{μM} 3,3'-dihaloacetfluorescein diacetate (H\text{}_2\text{EDTA}) was used in the extracellular media. Antioxidants, NAC and MnTBAP were used at concentrations of 20 \text{μM} and 100 \text{μM}, respectively. Aminoguanidine, known to be a fairly specific inhibitor of cytokine inducible nitric-oxide synthase (iNOS) (23), was used as a nitric oxide inhibitor at the doses of 50 and 100 \text{μM}.

Cytotoxicity Measurements

To visualize the infection rate of the macrophages, coverslips containing infected \textit{J774A.1} cells were treated with cold water for 3 min with mild shaking to dislodge loosely bound promastigotes. Coverslips were then washed thoroughly with phosphate-buffered saline and stained with propidium iodide (PI) (7.5 \text{μg/ml}) containing digitonin (0.05%) for permeabilization for 5 min so that both the macrophage and amastigote nuclei take up PI to make counting comfortable. The coverslips were mounted in 10% glycerol and infection was checked either with a Nikon Optiphot fluorescence microscope or a Zeiss LSM 510 confocal microscope (Zeiss Inc, Thornwood, NY). For confocal microscopy, a 488-nm argon ion laser was used to excite the cells and a 560-nm long pass filter was used to collect images for the PI stain. Phase contrast images were collected in the transmission mode, both images were overlapped when necessary, and the number of amastigotes counted. Only well rounded nuclei showing PI stain were counted as surviving amastigotes because irregular structures may represent disrupted nuclei of cells undergoing death. At least 200 cells of four independent experiments were counted. All counts were carried out on coverslips. Measures of both the number of infected cells and the number of amastigotes/100 cells were taken at different intervals to arrive at a time point that could be used as time 0 for drug treatment. Following drug treatment, drug activity was estimated by calculating percentage of growth inhibition by estimating the number of amastigotes/100 infected macrophages in treated wells divided by the number of amastigotes/100 infected macrophages in untreated wells × 100.

Detection of DNA Fragmentation

Terminal Deoxynucleotidyltransferase Enzyme (TdT)-mediated dUTP Nick End Labeling (TUNEL)—Detection of DNA fragmentation by TUNEL was carried out using a TUNEL assay kit according to instructions from the manufacturer and as described previously (4, 9).
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Briefly, cells were fixed in 4% formaldehyde and postfixation permeabilization was carried out with 0.2% (v/v) Triton X-100 for 10 min at room temperature followed by incubation with buffer containing nucleotide mix (50 μM fluorescein-12-DUTP, 100 μM dATP, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 7.6) for 1 h at 37 °C. Detection was carried out by both flow cytometry and microscopy.

Flow Cytometry—Formaldehyde fixed cells (10^6) stained for TUNEL were analyzed on an Elite ESP flow cytometer (Beckman Coulter, Fullerton, CA) using an argon ion laser tuned to 488 nm. Green fluorescence gated on forward and side light scatter was collected using a band-pass filter (525 ± 10 nm) and displayed using logarithmic amplification.

Microscopy—TUNEL labeling was visualized with a Zeiss LSM 510 confocal microscope using plan-neofluor objectives of 40× or 100× with numerical apertures of 0.75 and 1.3, respectively. Pinhole was set at 100 μm. For detection of green TUNEL-stained nuclei and red PI counterstain, cells were simultaneously illuminated with 488- and 563-nm laser lines and fluorescence was collected with a 500–550-nm band pass filter for green fluorescence detection and a 560-nm long pass filter for red fluorescence. Individual frames were averaged over four to eight frames. Laser was used at 13% power. Serial z-sections of each field were captured sequentially, and the sections were collected at 0.5-μm intervals in the z axis through a 40× objective.

Agarose Gel DNA Analysis—Isolation of DNA from amastigotes and agarose gel electrophoresis was carried out as described previously (4, 9).

Visualization of Phosphatidylserine Exposure

Amastigotes harvested from infected macrophages and subsequently Percoll purified were incubated with annexin V conjugated to fluorescein isothiocyanate (1:50) for 30 min at room temperature in dark. Cells were then lightly permeabilized and stained by a solution containing digitonin (0.01%) and PI (7.5 μg/ml), respectively. The staining was visualized through a confocal microscope by simultaneously illuminating the cells with 488- and 563-nm laser lines, and fluorescence was collected with a 500–550-nm band pass filter for green fluorescence and a 560-nm long pass filter for red fluorescence detection.

Measurement of Reactive Oxygen Species

To monitor the level of ROS, the cell permeant probe H_2DCFDA was used as described previously (9). H_2DCFDA is a nonpolar compound that readily diffuses into cells, where it is hydrolyzed to the nonfluorescent derivative dichlorodihydrofluorescein and trapped within the cells. In the presence of a proper oxidant, dichlorodihydrofluorescein is oxidized to the highly fluorescent 2,7-dichlorofluorescein. Cells from that readily diffuses into cells, where it is hydrolyzed to the nonfluorescent derivative dichlorodihydrofluorescein and trapped within the cells. In the presence of a proper oxidant, dichlorodihydrofluorescein is oxidized to the highly fluorescent 2,7-dichlorofluorescein. Cells from

Measurement of Intracellular L. donovani Amastigotes Residing within J774A.1 Cells—Host cell entry by promastigote protozoa involves the formation of membrane-bound PVs, where they transform into the immotile amastigote forms (25). The success of these organisms in surviving within the PVs determines pathogenesis of a disease. To arrive at a time point with optimum infection that could be used as a model system to study amastigote changes induced by the anti-leishmanicidal drug used, macrophages were checked at different time points after termination of co-infection with the promastigotes. It was observed that, after 18 h in serum-rich media, ~18 amastigotes/macroage were visible (no. of amastigotes/macroage at 0 h, 2 ± 0.01; at 6 h, 8 ± 1; at 12 h, 14 ± 2; at 18 h, 18 ± 2 (n = 5)). The percentages of macroages that could be infected in a given experiment varied between 92 and 95%. The last time point (18 h) was used as time 0 for initiation of drug treatment.

For combating visceral leishmaniasis, the most commonly used anti-leishmanicidal drug is sodium stibogluconate, a pentavalent antimonial that is targeted to destroy parasites within the PVs (26). We chose PAT, a trivalent antimonial, because pentavalent antimony is converted to trivalent antimony inside macrophages before it can be cytotoxic to the parasites; therefore, trivalent antimony is the active form of pentavalent antimony (27, 28). Prior to assessing the anti-leishmanicidal activities of PAT toward L. donovani amastigotes residing within J774A.1 cells, toxicity of the drug (10, 15, 20, 25, and 50 μg/ml) on untreated J774A.1 cells was evaluated to rule out doses
that would affect the host cell. With 50 μg of PAT, within 12 h -45% macrophages were dead, a feature reported for pentavalent antimony that is toxic at high doses to THP-1 cells (8). Although 20 and 25 μg/ml amounts of the drug did not have significant effect on macrophages in terms of cell death, the adhering capacity of the macrophages was compromised by 36 h. The doses of 10 and 15 μg/ml PAT were neither cytotoxic to macrophages nor did they reduce their adhering capacity by 36 h. Therefore, we tested these two doses for their cytotoxic activity on amastigotes to arrive at a suitable time frame within which the effects of the drug on the amastigotes could be studied. To determine the rate of clearance, permeabilized PI-stained treated and untreated infected macrophages were used for counting the number of amastigotes in the host cell by visualization using both phase contrast and fluorescence images (Fig. 1, inset ii). The dose of 15 μg/ml was found to be the appropriate dose because that could reduce the number of amastigotes significantly within a 24-h treatment period (Fig. 1). Percentage of growth inhibition by the dose of 15 μg/ml PAT is represented in Fig. 1 (inset i). Thus, treatment of the infected macrophages with 15 μg/ml PAT provided a time frame of 24 h for mechanistic studies on changes associated with amastigote clearance.

Anastigote Nuclear DNA Fragmentation Occurs when Infected Macrophages Are Treated with PAT—Having established the dose of the drug and the time required for its cytotoxic effect on amastigotes, we checked amastigote DNA after exposure of the macrophages to PAT. DNA fragmentation, recognized as a hallmark of metazoan apoptosis (29), is reported to occur in free-swimming protozoan parasites and intracellular amastigotes after exposure to various apoptosis-inducing stimuli (4, 5, 15). Flow cytometric analysis of amastigotes probed for TUNEL isolated from treated and untreated macrophages showed an increase in the number of cells staining positive for TUNEL, represented by increased forward scatter in the drug-treated groups indicating greater degree of staining for fragmented DNA with advancing time of exposure (Fig. 2, A (a), B (a), and C (a)). Analysis of optical sections through the TUNEL-labeled treated and untreated macrophages with a laser-scanning confocal microscope showed insignificant numbers of TUNEL-positive amastigote nuclei in untreated macrophages (Fig. 2A (b)). The number of TUNEL-positive cells gradually increased with time of exposure to the drug (Fig. 2, B (b) and C (b)). Late time points like 12 h showed larger masses of green (Fig. 2C (b)), possibly representing large communal PVs harboring multiple TUNEL-positive amastigotes. Macrophage nuclei did not stain positive for TUNEL in any of the groups, and the cells showed intact morphology demonstrating that they were not affected during the treatment. It is pertinent to mention here that, while scanning a sizable number of infected macrophages not exposed to drug, 1 in 50 macrophages would show occasional green nuclei of 1 or 2 amastigotes. Therefore, it is possible that, during normal growth within the macrophages, a few cells do undergo death that is preceded by DNA fragmentation. Actual counts of the TUNEL-positive amastigotes within the macrophages in different treatment groups showed a significant increase of TUNEL-positive parasites by 12 h of drug treatment (Fig. 3A (a)). Analysis of the DNA from amastigotes recovered from treated (Fig. 3A (b)) and untreated macrophages on agarose gels showed a clear breakdown in the treated groups (Fig. 3A (c), lane 2) as compared with controls (Fig. 3A (c), lane 1) although no oligonucleosomal ladder was visible. This observation differs from previous studies with free-swimming kinetoplastid parasites, where DNA fragmentation in multiples of oligonucleosomal fragments were reported during apoptotic death induced by various stimuli (1, 5, 4, 30). Our observations are similar to DNA fragmentation in staurosorine-induced apoptotic death of L. major, where no DNA breakage into
oligonucleosomal fragments was detected (11). PAT is known to induce DNA fragmentation in axenically grown amastigotes of L. infantum (8), but its effect on intracellular amastigote DNA is not known. Therefore, the data presented in this part of the study provide clear evidence that intracellular protozoan parasites respond to appropriate doses of antimonial treatment by expressing the apoptotic phenotype of DNA fragmentation.

Externalization of Phosphatidylserine Occurs in the Amastigotes after Exposure to PAT—Externalization of phosphatidylserine is a feature of metazoan apoptosis (1) that has also been demonstrated in staurosporine-treated promastigotes of Leishmania spp. (11). In our experiments, when amastigotes were harvested from treated and untreated infected macrophages and stained with annexin V conjugated to FITC, a significant number of amastigotes recovered from treated cells stained positive for annexin V (Fig. 3B (a–c)). The percentage cells staining positive for annexin-V was significantly high (6 h, 30 ± 4, n = 3) (12 h, 82 ± 10, n = 3), as compared with controls (0 h, 3 ± 0.2, n = 3). Therefore, externalization of phosphatidylserine occurs in the amastigotes when exposed to anti-leishmanial drugs while residing within the PVs of the host cell.

**PAT Induces an Increase in the Generation of ROS within the Macrophages**—Because DNA fragmentation is a relatively late event in apoptotic death, changes upstream to DNA fragmentation induced by the drug would then have occurred at a much earlier time point. To investigate early changes induced by PAT, we checked ROS generation within the macrophages as these cells can ingest and kill invading microorganisms using microbicidal mechanisms that include generation of ROS (31) and PAT is known to induce ROS production in blood (21). Three different fluorescent probes were used for the investigations, namely H2DCFDA, which primarily detects H2O2 and hydroxyl radical, and lucigenin and DAF-FM, which are probes for O2− and NO, respectively. Increase in signals was detected with all three probes, indicating an augmentation in H2O2, hydroxyl radical, O2−, and NO with increasing time of exposure.
to the drug (Fig. 4A (a–c)). Note that DAF-FM- and H$_2$DCFDA-sensitive fluorescence increased significantly within the first 6 h. Studies at earlier time points on the level of H$_2$DCFDA-sensitive fluorescence showed that as early as 1 h after drug treatment there was an increase in ROS (FIU at 530 nm; control, 22 ± 3; 1 h, 75 ± 3; 3 h, 127 ± 24; n = 3). This was further checked by microscopy where cells labeled with H$_2$DCFDA were probed with a laser-scanning confocal microscope and a rise in the total number of cells showing H$_2$DCFDA fluorescence, with an enhancement in general ROS levels with increasing time of exposure to the drug, was detected (Fig. 4B (a–h)). It was therefore clear that PAT was inducing an increase in ROS levels. The next question was whether the site of generation of ROS was within the macrophage cytoplasm or within the PVs harboring the parasites, as that would indicate the possible site of action of the ROS. It is known that Leishmania-containing phagosomes fuse with lysosomes or late endosomes of the macrophages to form PVs (32); therefore, to pinpoint the site of ROS generation, we used a lysosome-specific lysotracker red dye to identify the PVs, whereas H$_2$DCFDA was used to detect ROS. Overlapping of two images with differentially colored labels showed that ROS was primarily localized within the PVs, although some basal staining with H$_2$DCFDA was present within the cytosol (Fig. 4C (a–c)). Similar phenomenon has been shown in compartments harboring Staphylococcus aureus (33). Our studies with uninfected macrophages show that PAT is able to generate ROS in uninfected J774A.1 cells as well (FIU at 530 nm: control, 9.25; drug-treated, 57.1; result is representative of three replicates); however, the rate of generation was much less as compared with infected macrophages. The high generation of ROS within the PVs may have collectively contributed to high ROS observed in infected cells. Therefore, it can be inferred that PAT induces ROS generation within the infected macrophages with maximal concentration occurring within the PVs.

Preincubation of Cells with Antioxidants Reduces Intracellular Parasite Death Induced by PAT—To explore further the role of ROS in the induction of apoptotic death, we studied the impact of a thiol and a non-thiol antioxidant like NAC and MnTBAP on amastigote death induced by PAT. Arguably, if ROS were the cause of death, then scavenging the ROS with antioxidants would reduce the clearance of amastigotes from the macrophage. NAC, an aminothiol, is a unique compound that can raise intracellular GSH levels by acting as a synthetic precursor to intracellular cysteine and GSH and thereby protect cells from adverse effects of ROS. In addition, the chemical properties of the cysteinyl thiol of NAC provide antioxidant properties (34). MnTBAP, a stable metalloporphyrin that is a cell-permeable low molecular weight superoxide dismutase mimetic and peroxynitrite scavenger (35), also has the capability to scavenge H$_2$O$_2$ in certain cell types (36).

Total cell death percentages as determined by PI staining at 12 h showed that both NAC and MnTBAP were able to provide protection against the cytotoxic effects of PAT (Fig. 5A). The efficacy of protection was significantly more when cells were preincubated with a combination of MnTBAP and NAC, the percentage of cell death being only ~15% (percentage of cell death: drug-treated, 51 ± 4; drug + MnTBAP + NAC, 8 ± 1) as compared with independent treatments where the percentage of cell death varied between 30 and 40% (Fig. 5A). This suggested that the scavenging action of the two antioxidants was partially independent of each other. Flow cytometric analyses of TUNEL-positive amastigotes in groups treated with or without NAC showed a decrease in the percentage of TUNEL-positive cells (percentage of TUNEL-positive cells: infected macrophages treated with drug (0 h), 15%; infected macrophages treated with drug (3 h), 62%; infected macrophages treated with drug (6 h), 70.3%; infected macrophages pretreated with 20 mM NAC + drug (0 h), 8%; infected macrophages pretreated with 20 mM NAC + drug (3 h), 27%; infected macrophages pretreated with 20 mM NAC and drug (6 h), 41%; results are representative of four experiments) showing that apoptotic death was reduced by NAC known to interfere with the efficacy of PAT to eliminate intracellular parasites (14). Even though the protective effect of NAC on kinetoplastid parasite apoptosis is not known, it protects several types of mammalian cells from undergoing apoptosis (37, 38). Therefore, there was a clear relationship between cell survival and antioxidant treatment. To ensure that the effects of the antioxidants observed were actually through the scavenging of ROS, the capability of the above compounds to reduce ROS levels was checked. Both MnTBAP and NAC were able to reduce H$_2$DCFDA-sensitive fluorescence during the period of 6 h monitored (Fig. 5B). Next, the possibility of NAC acting through the increase in cellular GSH was probed and we found a decrease in GSH induced by PAT, which could be inhibited if NAC was present (nmol of GSH/10$^6$ cells: control, 0.680; PAT (6 h), 0.262; PAT (6 h) + NAC pretreatment (20 mM), 0.546; data are representative of three experiments). Even a partial depletion of GSH, an important molecule for protecting organisms including kinetoplastids and metazoans from ROS and other toxic chemicals, would be detrimental to the cell (39). Therefore, one of the possible mechanisms by which NAC provided protection could be through an increase in GSH synthesis that would alter the redox state of the cell; however, the relative contribution of the different mechanisms of NAC action is not

**Fig. 3.** DNA fragmentation and phosphatidylserine exposure in amastigotes isolated from treated and untreated macrophages. A, counts of amastigote nuclei within the macrophages showing DNA fragmentation. a, the number of TUNEL-positive cells (TdT + ve) inside macrophages at different time points after initiation of drug treatment. Asterisks represent level of significant variance compared with control group (p < 0.001). Data represent mean ± S.E., n = 3. b, microphotograph of one drug-treated macrophage representing cells from which amastigotes (green staining bodies) were isolated and DNA extracted at 12 h. c, DNA analysis of amastigotes isolated from untreated and drug-treated macrophages (12 h) on agarose gel. Note DNA shearing in treated amastigotes (lane 2). B, annexin V labeling of isolated amastigotes. Amastigotes isolated at 12 h from untreated (a) and from treated macrophages at 6 h (b) and 12 h (c) showing labeling with FITC-conjugated annexin V.
possible to specify. Taken together, the ability of the antioxidants to reduce ROS and inhibit amastigote death by PAT links drug-induced ROS generation to the cytotoxic effect of the drug and indicates the existence of a regulated mechanism used for the clearance of *L. donovani*-infected macrophages stimulated to produce ROS by oxidative stress generating drugs like PAT.

**Preincubation with Aminoguanidine Reduces Cell Death**—The significant increase in DAF-FM-sensitive fluorescence after drug treatment indicating generation of NO suggested the involvement of the reactive species in amastigote death. NO generated *in vitro* by NO-donating compounds have been shown to induce DNA fragmentation (15). To investigate whether NO was actually responsible in PAT-induced killing, we used aminoguanidine, an inducible nitric-oxide synthase inhibitor (23) during treatment. There was no significant improvement of amastigote growth within the macrophages with 50 μM aminoguanidine; however, with 100 μM amount of this drug there was a significant increase in amastigote survival within the drug-treated macrophages (Fig. 6A). A reduction in DAF-FM-sensitive fluorescence (Fig. 6B) by aminoguanidine indicated a decrease in NO levels. This observation, coupled with the partial prevention of amastigote death observed, suggests that inhibition of NO is in part responsible for prevention of parasite death.

**Isolated Amastigotes Show Increased Cell Death when Exposed to PAT and a Decrease in Mitochondrial Membrane Potential**—Having shown that intracellular amastigote apoptosis was linked to ROS, we wanted to further look at changes downstream to ROS generation and upstream of DNA fragmentation. Our earlier studies have established a close link between ROS-induced apoptosis in promastigotes and the maintenance of Δψm (9); however, measurement of amastigote mitochondrial potential was not possible, as macrophage mitochondria would have interfered with the measurements. Conversely, the other possibility was to isolate the amastigotes from macrophages after drug treatment and measure Δψm; however, in this case the preparation time would have contributed to alterations in the Δψm. Therefore, amastigotes were isolated and purified (Fig. 7, inset a) to carry out the studies on PAT-induced cellular changes. Treatment of PAT to isolated amastigotes had a significant effect on Δψm by 3 h (Fig. 7A) that could be rescued if these cells were preincubated with either MnTAP or NAC (Fig. 7A). A 20% fall in Δψm occurred by 1 h after drug treatment (ratio at 590/530 nm: control, 9.7; drug...

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**Fig. 4.** PAT-induced generation of reactive oxygen species within infected macrophages. *A,* bar graphs showing increase in ROS generation in macrophages after exposure to PAT. ROS levels were measured using specific fluorescent probes. a, total ROS was measured using H$_2$DCFDA; b, superoxide was measured using lucigenin; c, nitric oxide was measured using DAF-FM. Asterisks represent level of significant variance compared with 0 h group (*p* < 0.001). Data represent mean ± S.E., *n* = 4. *B,* photomicrographs showing increase in ROS generation in macrophages after exposure to PAT at different time points. Note the increase in the number of cells generating ROS with increasing time of drug exposure. *C,* ROS generation in PVs at 3 h. Confocal microphotographs of drug-treated macrophages stained with a lysosomal marker lysotracker red (red) and a fluorescent probe for ROS, H$_2$DCFDA (green). Colocalization of both the red and the green stains showing orange-yellow color indicates PVs as primary sites of ROS generation. Arrow indicates location of the PVs.
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1 h, 7.79; results are representative of three experiments) reaching to above 40% by 3 h as shown in Fig. 6A. One might reasonably assume that, if mitochondria undergo a major depolarization, the cells will proceed to die, and if this depolarization is linked to death, prevention of depolarization should prevent death. An increase in amastigote death was recorded after treatment with PAT, and this drug-induced death of the amastigotes was preventable if the cells were pretreated with the antioxidants (Fig. 7B). Therefore, drug-induced mitochondrial depolarization was a possible step leading to the death of the isolated amastigotes. Because PAT was inducing an apoptotic type of death in the amastigotes inside the macrophages, the next question was whether the death in isolated amastigotes occurred in a similar manner or not. It was necessary to do this because, to link mitochondrial depolarization to amastigote death, we had to be sure that isolated amastigote death showed similar features to those inside macrophages. TUNEL labeling studies with isolated amastigotes and analysis of DNA on agarose gels showed that DNA fragmentation occurred in response to PAT in isolated forms as well (Fig. 7B, inset a, b, and c). Experiments described in the earlier section indicate that the generation of ROS was high inside the PVs. To find out whether the amastigotes could themselves generate ROS or whether ROS observed in the PVs was a contribution from the macrophage, we determined whether PAT was able to induce ROS generation in the isolated amastigotes. A significant increase in H$_2$DCFDA fluorescence that could be salvaged by antioxidants was noted (Fig. 7C). However, DAF-FM-positive fluorescence signifying NO levels showed an increase with drug but no significant quench with MnTBAP (Fig. 7D). This is understandable because MnTBAP is primarily a scavenger of superoxide and peroxinitrite; therefore, it would effectively not reduce the NO levels. Because MnTBAP was able to salvage cell death, its primary action was possibly through scavenging of the peroxinitrite or reduction of O$_2^-$ level. Although intracellular amastigote forms have not been directly shown to be susceptible to PAT in vitro, axenic amastigotes of *L. donovani* grown in vitro without any contact with macrophages are known to be killed by PAT (27). As the amastigotes were susceptible to drug action even when they were outside the macrophages, it raises the possibility that the drug action inside the macrophages could possibly be host cell-independent. Taken together, studies with isolated amastigotes recovered from macrophages demonstrate that $\Delta \psi_m$ loss was an upstream event to DNA fragmentation induced by PAT.

Amastigotes Show an Increase in Cytosolic Ca$^{2+}$ in Response to PAT, and Inhibition of Non-selective Cation Channels Could Block the Increase in Ca$^{2+}$—In the metazoans it is established that ROS can impair Ca$^{2+}$ transport systems (40), and our studies with promastigotes of *L. donovani* show that they undergo apoptosis by a Ca$^{2+}$-mediated mechanism when exposed to oxidative stress (9). In this part of the study, we used both...
the isolated amastigotes and amastigotes residing within the macrophages to define the role of Ca$^{2+}$. When intact infected macrophages were exposed to PAT, a clear increase in fluo-3 fluorescence representing increase in Ca$^{2+}$ was recorded at 3 h. This could be inhibited in the presence of MnTBAP and NAC and a combination of both (Fig. 8A). This was evident that antioxidants were able to inhibit events leading to increase of intracellular Ca$^{2+}$ levels. To see whether the changes in Ca$^{2+}$ translated into apoptotic death, the number of apoptotic cells under manipulated Ca$^{2+}$ conditions was examined and a reduction in apoptotic cells was observed (no. of TdT-positive cells: control, 2%; PAT- treated cells, 62%; PAT-treated cells in the presence of 3 mM EGTA, 9%; data are representative of three experiments), thus linking Ca$^{2+}$ to apoptotic death. When isolated amastigotes were exposed to PAT, there was an increase in cytosolic Ca$^{2+}$. Presence of extracellular EGTA prevented the cytosolic Ca$^{2+}$ increase, showing that Ca$^{2+}$ was entering the cells from extracellular sources (Fig. 8B). Because the above data clearly indicated that the primary source of increased Ca$^{2+}$ was from extracellular sources, experiments were conducted to establish the route of entry. We used nife- dipine, a blocker of voltage gated L-type Ca$^{2+}$ channel, which could reduce the Ca$^{2+}$ increase by 10% only and was unable to reduce drug-induced cell death (PAT treatment, 50% at 12 h) significantly (PAT + nifedipine, 43% at 12 h). Because entry of Ca$^{2+}$ through the non-selective cation channels was a possibility (9), isolated amastigotes were incubated with FFA prior to exposure to PAT and an increase in amastigote viability was recorded in the presence of FFA (Fig. 8C (a)). The question was whether non-selective cation channels were important in isolated amastigote death: were these channels active when the amastigotes were residing within the macrophages? To address this question, we pre-incubated infected macrophages with FFA prior to exposure to PAT, and a count of intracellular amastigotes at different time points after drug exposure was estimated. There was a significant reduction in the percentage of amastigotes dying within the macrophages if the macrophages were preincubated with FFA (Fig. 8C (b)). To relate whether this was because of a decrease in Ca$^{2+}$ levels within the macrophages, we measured cytosolic Ca$^{2+}$ levels of the FFA-treated infected macrophages and found that there was an inhibition of PAT-induced increase in cytosolic Ca$^{2+}$ within the macrophages (nmol of Ca$^{2+}$; control, 83.2 ± 19; drug-treated, 419 ± 32; FFA + drug-treated, 153.4 ± 7; data ± S.E. of three experiments, $p < 0.05$, drug-treated versus FFA + drug-treated), thus linking decreased Ca$^{2+}$ influx to increased...
survival of the amastigotes. However, it was not clear in this scenario whether a decrease of Ca\(^{2+}\) occurred within the macrophages or a decline in the amastigotes was responsible for improved survival. Therefore, to delineate whether FFA was affecting the macrophage Ca\(^{2+}\) or not, which would imply the involvement of macrophage channels, we labeled drug-treated infected macrophages with fluo-3 and subsequently lysed the cells, following which separation of macrophage cytosol, cell debris, and amastigotes was carried out. The results show that Ca\(^{2+}\) levels in both the cell cytosol of the macrophages and the amastigotes increase after PAT treatment; however, if EGTA or FFA was present, the increase in both cells was inhibited (Fig. 8D). The fact that FFA was able to decrease macrophage Ca\(^{2+}\) made it clear that FFA-sensitive channels were also operative in macrophages. Because spectrofluorometric results represent an averaged or integrated estimate, we analyzed Ca\(^{2+}\) localization by confocal microscopy. Visual examination of macrophages labeled with fluo-3 showed that fluo-3 labeling was strong in the intracellular amastigotes exposed to drug (Fig. 9B) as compared with controls (Fig. 9A). Amastigotes appeared as distinctive strong fluo-3 spots (Fig. 9B) because the amastigote cell bodies being small concentrated Ca\(^{2+}\) in small areas as compared with macrophage cytosol where Ca\(^{2+}\) was distributed over a much larger surface area. Presence of FFA reduced the number of fluo-3-positive cells (Fig. 9C). This experiment confirmed that there was a significant increase in Ca\(^{2+}\) in the macrophages as well as the amastigotes induced by PAT that was inhibited by FFA. In combination, these observations confirm the importance of non-selective cation channels in modulation of drug activity in both the parasite and the host. The presence of FFA inhibits Ca\(^{2+}\) increase in the cytosol of both cell types that translates into increased amastigote survival.

**DISCUSSION**

By synthesizing the data obtained from in vitro experiments using intact infected macrophages and amastigote forms isolated from the macrophages, we show that (i) intracellular *Leishmania donovani* amastigotes undergo apoptotic death in response to PAT that is accompanied by DNA fragmentation and externalization of phosphatidylserine; (ii) PAT induces the formation of ROS within the infected macrophages, and, when these ROS are scavenged by antioxidants, amastigote death is prevented; (iii) intracellular amastigote death is associated
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![Image of macrophages and amastigotes](image)

**Fig. 9.** Localization of Ca$^{2+}$ in the intracellular amastigotes by confocal microscopy at 3 h. A, control cells. Panel shows control image of macrophages and intracellular amastigotes showing no fluo-3 reactivity. a, control; b, fluo-3 image of a; c, overlap of a and b. B, cells treated with PAT. d, image of macrophages; e, fluo-3 image of d; f, overlap of d and e. Note fluo-3-positive spots representing amastigotes. C, cells treated with PAT after preincubation with FFA. g, macrophages preincubated with FFA prior to treatment with drug; h, fluo-3 image of g; i, overlap of g and h. Note decrease in the number of fluo-3-positive spots in i. Bar represents 5 μm.

with increases in Ca$^{2+}$ concentrations in both the parasite and the host; (iv) increase in Ca$^{2+}$ occurs from extracellular sources via non-selective cation channels present in both the macrophages and the amastigotes; (v) DNA fragmentation induced by PAT is preceded by a fall in the amastigote ΔΨ\text{m}.

The J774A.1 cells infected with the *L. donovani* amastigotes was an ideal system to demonstrate drug-induced changes in the parasites because resident macrophages, which fail to restrict *Leishmania* growth, do not mount a significant anti-leishmanicidal response like ROS generation as compared with activated macrophages from mouse peritoneum (41). Therefore, the J774A.1 cells harboring *Leishmania* promastigotes would have minimal background ROS generation, and this was suitable because measurement of ROS was a significant component of the study. Fragmentation of DNA is a hallmark of apoptosis that occur in metazoa (29) as well as unicellular organisms (4, 5) in response to various apoptosis-inducing stimuli. PAT-induced fragmentation of amastigote DNA provided evidence that amastigote death was a consequence of induction of apoptosis-like death process. Interestingly, we observed few occurrences of DNA fragmentation in amastigotes existing within untreated macrophages, showing that death induced in the continuing struggle between the parasite and the host cell also occurs through apoptosis. Unpublished data\textsuperscript{a} from this laboratory demonstrate that there is a larger number of TUNEL-positive amastigotes within the untreated activated mouse peritoneal macrophages as compared with infected J774A.1 cells, suggesting that the normal response of macrophages toward the pathogens with oxidative burst (51) also induce pathogen death accompanied by DNA fragmentation. Therefore, the very low rate of amastigote DNA fragmentation observed in the untreated infected J774A.1 macrophages may be the result of low reactivity of the host cell toward the parasites. The apoptotic nature of death was further confirmed by the exposure of phosphatidylserine (1) in the amastigotes recovered from treated macrophages, a feature detected during staurosporine-induced death of *Leishmania* promastigotes (11). Certainly, a mode of death similar to apoptosis would be preferable for intracellular parasites because products generated by necrotic death would adversely affect other parasite members residing in close proximity and the host cell as well.

To illustrate the mechanistic details leading to DNA fragmentation, several cellular parameters were checked. Even though there is no clear consensus in the literature as to how the ROS brings about pathogen death inside the host cell, it is known that anti-leishmanicidal drugs generate ROS in blood (21). Therefore, it was checked whether the cellular response to the drug was generation of ROS. The increase in several species of ROS after exposure to PAT followed by amastigote clearance suggested drug action via the ROS. Interestingly, higher level of ROS was actually being generated within the PVs, a site where amastigotes were lodged. This indicated two possibilities; either the macrophage-generated ROS (31) was being concentrated in the PVs because host-generated ROS can enter the PVs (32), or the drug was able to generate ROS within the PVs or in the parasites located within the PVs. Because PAT could induce ROS in isolated amastigotes, it was possible that ROS generation in response to the drug in the PVs was a macrophase-independent event. The difference between the amount of ROS generated by the uninfected and infected macrophages treated with PAT was possibly caused by the additive effect of the ROS generated by the amastigotes residing within the PVs. In this connection, it is instructive to note that several reactive oxygen intermediates have been implicated in *Leishmania* killing including O$_2^\bullet$, H$_2$O$_2$, *OH*, and NO (41, 42). Because PAT was able to generate all the above species, on the basis of existing literature they can be linked to amastigote death via ROS generation. ROS are known to induce apoptosis in mammalian cells (43, 44); therefore, it was entirely possible that, when amastigotes were exposed to appropriate levels of these species, apoptotic death ensued. This concept of ROS-induced amastigote death was further validated by the use of antioxidants. Arguably, if oxidants were responsible for the cytotoxic effect of the drug, antioxidants should have protective action. The ability of one thiol and one non-thiol antioxidant and an iNOS inhibitor to significantly inhibit parasitic death provided clues to a direct link between ROS generation and amastigote death. The ability of NAC to reduce DAF-FM- and H$_2$DCFDA-sensitive fluorescence and apoptotic death indicated that it either acted via inhibition of NO through interference with iNOS activation (45) or through direct scavenging of the radicals. NO has a significant role in amastigote killing, as shown by data with iNOS knockout mice (19) and its ability to induce DNA fragmentation in intracellular amastigotes (15). It is pertinent to mention that the possibility of the protective effect of NAC through the increase in GSH levels cannot be ruled out because the depletion of GSH that occurred concurrently with the increase in ROS (46) was restored by NAC. Therefore, the ability of NAC to act as an intracellular antioxidant (37), coupled with its capability to function as a modifier of intracellular thiol levels (38), was important. Involvement of NO in this particular system was evident from the ability of a NO inhibitor, aminoguanidine (23), to provide protection against cell death through inhibition of NO. MnTBA was ineffective in reducing NO levels because it does not scavenge NO but it scavenges peroxinitrite, a product generated by reaction between NO and superoxide (47). Our earlier studies have shown that promastigotes were susceptible to ROS at high doses (9); similarly, when we compared the susceptibility of the promastigotes and amastigotes to withstand PAT-induced stress, we found that amastigotes isolated

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\textsuperscript{a} G. Sudhindharan and C. Shaha, unpublished observations.
show that, under certain conditions, nifedipine can block Ca\(^{2+}\) influx. We provided two pieces of evidence, first that Ca\(^{2+}\) influx was linked to the Ca\(^{2+}\)-selective cation channel blocker (48), to see whether these results were consistent with our earlier observations in promastigotes (9), we used FFA, a non-selective cation channel blocker, during drug treatment because unpublished data\(^3\) from this laboratory show that, under certain conditions, nifedipine can block Ca\(^{2+}\) influx in the \textit{L. donovani} promastigotes. Nifedipine was unable to inhibit cell death and Ca\(^{2+}\) entry, indicating non-involvement of Ca\(^{2+}\)-selective cation channels in the amastigotes. Based on our earlier observations in promastigotes (9), we used FFA, a non-selective cation channel blocker (48), to see whether these channels were involved in the Ca\(^{2+}\) entry. The ability of FFA to reduce Ca\(^{2+}\) influx (49) and restore cell viability clearly provided two pieces of evidence, first that Ca\(^{2+}\) was involved in the cell death process and second that non-selective cation channels were involved. At this point, the primary question was whether the intracellular Ca\(^{2+}\) increase took place only within the amastigotes or in the macrophages as well. Both the lysed macrophage supernatants and amastigotes showed increased Ca\(^{2+}\) levels, providing evidence that Ca\(^{2+}\) increase took place in both the cell types. The ability of FFA to reduce Ca\(^{2+}\) influx in both the macrophages and the parasite was suggested by the observations that the amastigotes were drawing Ca\(^{2+}\) from the macrophage cytosol and the macrophage was in turn drawing Ca\(^{2+}\) from the extracellular media, both via non-selective cation channels. Because ROS generation occurred prior to Ca\(^{2+}\) increase and antioxidants could prevent Ca\(^{2+}\) entry into the cell, it can be concluded that Ca\(^{2+}\) increase was a consequence of drug-induced ROS action. To examine the relationship of ROS and Ca\(^{2+}\) further, we measured ROS generation after FFA treatment and found that FFA did not alter ROS generation (data not shown). Therefore, there was no secondary ROS increase after Ca\(^{2+}\) influx. These results suggest that Ca\(^{2+}\) plays a central role in the mechanism of PAT-induced death. This is the first demonstration that an anti-leishmanicidal drug induces the opening of FFA-sensitive channels in both the host and the parasite. Our earlier studies have implicated the involvement of promastigote mitochondria in H\(_2\)O\(_2\)-induced death (9); therefore, the generation of ROS by PAT suggested that amastigote mitochondria might be a possible target. Because it was not possible to measure amastigote \(\Delta:\psi_m\) while they were lodged inside the macrophages, this was confirmed in studies with isolated amastigotes, where an actual loss of potential was observed after drug treatment and this loss could be salvaged by antioxidants that reduced ROS levels and cell death. Therefore, there was no secondary ROS increase after application of oxidative stress. Evidently, PAT did not interfere with macrophage mitochondria, as our tests with uninfected J774A.1 cells did not show any potential fall (data not shown). Because we do not know that exact ROS content per cell in the macrophages versus the amastigotes, we are unable to comment on the susceptibility of the macrophage mitochondria versus the amastigote mitochondria. Because macrophages use oxidative stress to kill pathogens, their mitochondria may be comparatively more resistant to oxidative stress than the parasite mitochondria. Thus, the temporal relationship between increase in ROS generation, reduction in \(\Delta:\psi_m\), and augmentation of Ca\(^{2+}\) levels followed by DNA fragmentation clearly shows that amastigote apoptosis is a consequence of changes in Ca\(^{2+}\) homeostasis brought about by ROS generation induced by the antimicrobial.

Interpreting the above results, we propose that influx of Ca\(^{2+}\) through FFA-sensitive channels in both the macrophages and amastigotes represents a novel way by which pathogen death occurs in response to an anti-parasitic drug. Because opening of these channels brought about by ROS generated by the drug could be a critical event in drug efficacy, it would be interesting to design drugs with improved ability to open these channels to precipitate apoptotic death. Finally, the identification of parasite-specific modulators of Ca\(^{2+}\), effective in the phagosomal environment, may lead to improved methods for inducing parasite clearance.

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\(^3\) S. B. Mukherjee and C. Shaha, unpublished observations.
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Antimonial-induced Increase in Intracellular Ca\textsuperscript{2+} through Non-selective Cation Channels in the Host and the Parasite Is Responsible for Apoptosis of Intracellular Leishmania donovani Amastigotes

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