# A Proteomics Screen Implicates HSP83 and a Small Kinetoplastid Calpain-related Protein in Drug Resistance in *Leishmania donovani* Clinical Field Isolates by Modulating Drug-induced Programmed Cell Death*

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**The therapeutic mainstay against the protozoan parasite *Leishmania* is still based on the antiquated pentavalent antimonials (Sb(V)), but resistance is increasing in several parts of the world. Resistance is now partly understood in laboratory isolates, but our understanding of resistance in field isolates is lagging behind. We describe here a comparative analysis of a genetically related pair of Sb(V)-sensitive and -resistant *Leishmania donovani* strains isolated from kala-azar patients. The resistant isolate exhibited cross-resistance to other unrelated *Leishmania* drugs including miltefosine and amphotericin B. A comparative proteomics screen has highlighted a number of proteins differentially expressed suggesting that programmed cell death (PCD) is modified in the resistant parasite. Indeed drug-induced PCD progression was altered in the Sb(V)-resistant strain as determined using early and late markers of apoptosis. Two proteins, the heat shock protein HSP83 and the small kinetoplastid calpain-related protein (SKCRP14.1) were shown to be intimately implicated in the drug-induced PCD phenotype. HSP83 increased drug resistance and reduced drug-mediated PCD activation by interfering with the mitochondrial membrane potential, whereas SKCRP14.1 promoted antimonial-induced PCD but protected against miltefosine-induced PCD. This study highlights the important role of PCD in drug susceptibility/resistance in the protozoan parasite *Leishmania*. *Molecular & Cellular Proteomics 6:88–101, 2007.***

The protozoan parasite *Leishmania* is endemic in large parts of the world with 600,000 new clinical cases reported annually and possibly more unreported. Visceral leishmaniasis is fatal if not treated and is caused mostly by *Leishmania donovani*, *Leishmania infantum*, and *Leishmania chagasi* (1–3). Given the difficulties linked to vector (sandfly) control and the lack of an effective vaccine, the control of leishmaniasis relies mostly on chemotherapy. Unfortunately the prevalence of parasites becoming resistant to the first line drug pentavalent antimony (Sb(V)) is increasing in several parts of the world, including South America (4, 5), Europe (6, 7), the Middle East (8), and most notably in India (9–12). Indeed in the endemic region of Bihar, treatment with Sb(V) fails in more than 60% of cases. Few alternative drugs are available (13), and the short list includes amphotericin B, pentamidine, and the oral drug miltefosine, which is in phase IV clinical trial in India. Already a decrease in efficacy has been noted against this novel molecule (14).

Sb(V) is believed to be a prodrug converted to active Sb(III) in either the macrophage and/or the parasites (for reviews, see Refs. 14 and 15). Although a single cellular target cannot yet be discounted, it is believed that Sb(V)/Sb(III) may interact with several targets including trypanothione, the main reduced cellular thiol of the parasite (16). Antimonials appear to kill cells by a process with several features characterizing programmed cell death (PCD)[1] (17–19). Current evidence suggests that miltefosine (20, 21) and amphotericin B (18) also kill *Leishmania* cells by a process reminiscent of PCD. The cellular process of PCD clearly occurs at the phenotypic level in *Leishmania* with several cytoplasmic, mitochondrial, and nuclear features of apoptosis having been demonstrated. For example, cell shrinkage, phosphatidylserine exposure, a decrease in the mitochondrial membrane potential ($\Delta\Psi m$), cytochrome *c* release, nuclear condensation, DNA fragmentation,

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[1] The abbreviations used are: PCD, programmed cell death; HSP, heat shock protein; SKCRP, small kinetoplastid calpain-related protein; MRPA, multidrug resistance protein A (formerly known as PGPA); GSH1, γ-glutamylcysteine synthetase; AQP1, aquaglyceroporin 1; TMRE, tetramethylrhodamine ethylester perchlorate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PI, propidium iodide; $\Delta\Psi m$, mitochondrial membrane potential; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 2D, two-dimensional; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

and protease activation have all been associated with PCD in *Leishmania* (17, 18, 20, 22, 23). However, even if *Leishmania* (and related parasites) share phenotypic similarities with PCD of multicellular organisms, it is evident that the cellular and molecular details in *Leishmania* are different from metazoans, and it is still not clear that every *Leishmania* species or even life stage uses similar PCD pathway(s) (for reviews, see Refs. 24–27).

Laboratory-induced antimonial resistance is now partly understood in *Leishmania*. The high level resistance to antimony observed in *Leishmania* can be due to simultaneous selection of loss in metal reduction, decreased drug uptake, increased glutathione and trypanothione synthesis, and increased transport (sequestration or efflux) of thiol-metal conjugates (for reviews, see Refs. 14 and 15). This does not exclude that the parasite could have other mechanisms that confer metal resistance. In contrast to *in vitro* selected strains, resistance to Sb(V) in *Leishmania* field isolates is not well understood. Using intracellular susceptibility testing, parasites isolated from Sb(V)-unresponsive patients were found to be more resistant to Sb(V) as compared with parasites isolated from Sb(V)-responsive patients (6, 8, 9). Recent studies with field isolates[2] (8, 28, 29) suggest similarities and differences with laboratory resistant isolates, but it is not yet clear whether any of the highlighted mechanisms is central to the resistance phenotype of field isolates. Understanding how antimonial drugs work and why they sometimes fail is fundamental to the optimal use of the existing formulations and is also likely to be instructive in the development of new therapies.

In this study, we used a comparative proteomics screen for comparing a genetically related pair of Sb(V)-resistant and -sensitive Indian *L. donovani* field isolates. This permitted us to pinpoint at least two proteins, the heat shock protein HSP83 and a small kinetoplastid calpain-related protein (SKCRP14.1), that modulate susceptibility to antimonials and other drugs by interfering with drug-induced PCD pathways.

## MATERIALS AND METHODS

*Parasite Cultures and Characterization*—The *L. donovani* field isolates 9518 and 9551 were isolated from kala-azar patients in Bihar, India, and have been described previously (9, 31). Promastigotes were maintained in SDM79 medium supplemented with 10% heat-inactivated fetal calf serum at 25 °C. Growth assays of promastigotes were done as described previously (32). For assays with intracellular amastigotes, parasites transfected with the firefly luciferase (33) were used at a ratio of 10:1 to infect the human leukemia monocyte cell line THP-1 as described and validated previously (8, 34, 35). Pulsed field gel electrophoresis karyotyping was done as described previously (8, 36).

*Real Time PCR Analysis of Gene Expression in L. donovani Cells*—Primers for the *MRPA*, *GSH1*, and *AQP1* genes were designed using the Gene Runner software (Hastings Software, Inc., Hastings, NY). These three genes have been implicated in antimonial resistance in *Leishmania* (for a review, see Ref. 15). Complementary DNA synthesis and real time PCR were performed in triplicate exactly as described previously (37). The relative amount of PCR product generated from each primer set was determined based on the threshold cycle (Ct) value and amplification efficiency and was normalized by dividing the values by the relative amount of the *GAPDH* gene used as a control. Primers used were: GAPDH forward, 5′-GAAGTACACGGTGGAGGCTG-3′; GAPDH reverse, 5′-CGCTGATCACGACCTTCTTC-3′; MRPA forward, 5′-GCGCAGCCGTTTGTGCTTGTGG-3′; MRPA reverse, 5′-TTGCCGTACGTCGCGATGGTGC-3′; GSH1 forward, 5′-CATTGGCTGGCGCGTTGAGTTC-3′; GSH1 reverse, 5′-ATGTGCGCGGCCCATATTCTCG-3′; AQP1 forward, 5′-TTTGGAACCGGCGTCGTTGC-3′; and AQP1 reverse, 5′-ACACAGTTCGCCAGCGTTACGG-3′.

*Protein Extraction, Separation, Detection, and Identification*—Parasite cultures were grown to late log phase as determined by optical density at 600 nm. Cells were harvested by centrifugation at 2500 × *g*, washed twice in HEPES-NaCl buffer, resuspended, and disrupted by sonication in hypotonic buffer (10 mM Hepes, pH 7.5, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 1× protease inhibition mixture). The cell lysate was centrifuged at 3000 × *g* to pellet unbroken cells and nuclear material. The supernatant was ultracentrifuged at 65,000 rpm, the membrane protein pellet was discarded, and the supernatant soluble proteins were precipitated using TCA (40%, v/v) and resuspended in 2D lysis buffer (7 M urea, 2 M thiourea, 40 mM Tris, 4% CHAPS, 0.1 mg/ml PMSF) as described previously (38). Protein concentration was assayed using the 2D Quant kit (Amersham Biosciences), and 250 $\mu$g of protein were loaded on the first dimension on 24-cm Immobiline DryStrips of narrow pH ranges of 4–5, 4.5–5.5, and 5–6 (Amersham Biosciences). Second dimension SDS-PAGE was run, and proteins were visualized by SYPRO Ruby fluorescence as described previously (39). 2D gels of four independent samples per strain and condition were averaged and compared using Progenesis software (Nonlinear Dynamics). Significance levels of differences observed were determined by Progenesis software using Student's two-tailed *t* test. Gel plugs containing the proteins of interest were excised from the gel, processed, and characterized as described previously (40). Resulting peptide MS/MS spectra were interpreted using Mascot Daemon version 1.9 (Matrix Science; Ref. 41). Peak lists were created automatically by Mascot Daemon (with lcqdta.exe) using a scan range of 500–2000 and a mass range of 700–3500. No scan grouping was allowed, and precursor charge state was set to auto. Searches were carried out in-house against proteins in the Leishpep database (version dating from April 27, 2004, containing 8081 sequences and 5,170,567 residues) generated by annotation of *Leishmania major* Friedlin sequence (GeneDB, www.genedb.org). Carbamidomethylation of cysteine, partial oxidation of methionine, up to two missed cleavages, and an error tolerance of 2.0 Da for peptides and 0.5 Da for fragments were considered in the search. A peptide was considered a good match if it produced a Mascot score greater than 33 (identity or extensive homology at $p < 0.05$; scores significant for homology varied between peptides). A minimum of two peptide matches (at least one with a score >33, the other significant at the level of homology, and no other significant hits) were required for protein identification. Homologous peptides were considered because the species being studied is not the sequenced species. Peptide identifications close to the score threshold or proteins with a minimum number of matched peptides were confirmed by inspection of the spectra.

*DNA Constructs*—Genes of interest were amplified by PCR using the Expand High Fidelity polymerase (Roche Applied Science). PCR amplifications were performed using modified primers bearing XbaI and HindIII restriction sites on forward and reverse primers, respectively: HSP83 forward, 5′-GCTCTAGAGCATGACGGAGACGTTCGCGTT-3′; HSP83 reverse, 5′-CCCAAGCTTGGGTCAGTCCACCTGCT-

[2] A. Mukherjee, P. K. Padmanabhan, S. Singh, G. Roy, I. Girard, M. Chatterjee, M. Ouellette, and R. Madhubala, submitted manuscript.

CCATGC-3′; 14-3-3 forward, 5′-GCTCTAGAGCATGACCAACGTCT-TCAAGGT-3′; 14-3-3 reverse, 5′-CCCAAGCTTGGGTTACTCGAGC-TCCTCCATGG-3′; SKCRP14.1 forward, 5′-GGCTCTAGAGATGTCT-GAGATCAAATAC-3′; and SKCRP14.1 reverse, 5′-CAAGCTTGTCA-CGCCTTCGCAATCGG-3′. The *14-3-3* and *HSP83* genes were amplified from *L. donovani* 9551 genomic DNA, whereas *SKCRP14.1* gene amplification was performed on *L. donovani* 9518 genomic DNA. The PCR fragments were first cloned into pGEMT-easy (Invitrogen) and then digested by XbaI and HindIII enzymes to be subcloned into XbaI/HindIII-digested *Leishmania* expression vector pSP72$\alpha$NEO$\alpha$ encoding paromomycin/G418 resistance (42). These constructs were electroporated into the relevant strains as described elsewhere (43).

*DNA Fragmentation Assay*—For DNA fragmentation analysis, parasites were seeded at $5{\cdot}10^5$ cells/ml and grown for 48 h. After cell counting, drugs were added to the culture medium for different times depending on the drug used. Total DNA was extracted following the "salting-out DNA extraction" method (44). Briefly parasites were pelleted and treated with lysis buffer (10 mM Tris-HCl, 5 mM EDTA, pH 8, 0.5% SDS, 200 mM NaCl, 100 $\mu$g/ml proteinase K) for 1 h at 65 °C. Two volumes of ice-cold absolute ethanol were added, and DNA was centrifuged for 15 min at 13,000 rpm. Supernatants were discarded, and dried pellets were resuspended in 100 $\mu$l of 10 mM Tris-HCl, 0.1 mM EDTA and treated with RNase A (0.3$\mu$g/ml) for 1 h at 37 °C.

*Measurement of* $\Delta\Psi m$—Tetramethylrhodamine ethylester perchlorate (TMRE; Molecular Probes-Invitrogen) is a cationic lipophilic dye that accumulates in the negatively charged mitochondrial matrix according to the Nernst equation potential (45). To follow $\Delta\Psi$m variations, $10^6$ *L. donovani* promastigotes were washed once in PBS and resuspended in PBS containing 100 nM TMRE. Cells were incubated for 30 min at room temperature and directly analyzed on the FL2-H channel using a Beckman Coulter EPICS XL flow cytometer and Expo 32 ADC software. Base-line TMRE was recorded and expressed as mean fluorescence in arbitrary units. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, Sigma) was used to serve as a control of depolarized mitochondria and was added to the cells (100 $\mu$M) at the same time as TMRE. The experiments were made at least in triplicate.

*Measurement of Phosphatidylserine Exposure*—All apoptotic assays were done with an equal number of parasites ($5 \times 10^5$ cells/ml) grown for 48 h after which drug treatments were performed and apoptotic markers were analyzed. Externalization of phosphatidylserine on the outer membrane was measured by cytometer using the annexin V-Alexa Fluor® 488 conjugate kit according to the manufacturer's instructions (Molecular Probes-Invitrogen). Fluorescence intensity was measured on the FL1-H channel for Annexin V and FL3-H for propidium iodide (PI). Analyses were performed on 10,000 gated events, and data were represented as dot plots using WinMDI software.

*In Situ Labeling of DNA Fragments by TUNEL*—*In situ* detection of DNA fragmentation in intracellular amastigotes was performed using the APO-BrdU™ TUNEL assay kit (Molecular Probes). TUNEL labeling detects DNA fragmentation in apoptotic cells by exploiting the 3′-hydroxyl ends of DNA breaks. THP-1 macrophages were infected with either 9518 or 9551 stationary phase *L. donovani* promastigotes at a parasite:cell ratio of 15:1 in RPMI 1640 medium supplemented with 10% fetal calf serum. After 24 h, new medium containing 300 $\mu$g/ml Pentostam was added for an additional 96 h. After 4 days of infection, slides were washed twice in PBS and fixed for 20 min at room temperature in PBS containing 4% paraformaldehyde. After two further washes in PBS, cells were permeabilized in PBS containing 0.2% Triton X-100 for 10 min at room temperature, and slides were left in 70% ethanol for at least 30 min. Slides were then processed with the TUNEL labeling reaction using the manufacturer's instructions. The samples were counterstained with propidium iodide (10 $\mu$g/ml) for an additional 30 min and visualized under a Nikon eclipse TE300 fluorescence microscope. Images were captured using Image Pro Plus 5.0 software. At least 20 microscopic fields were observed for each sample.

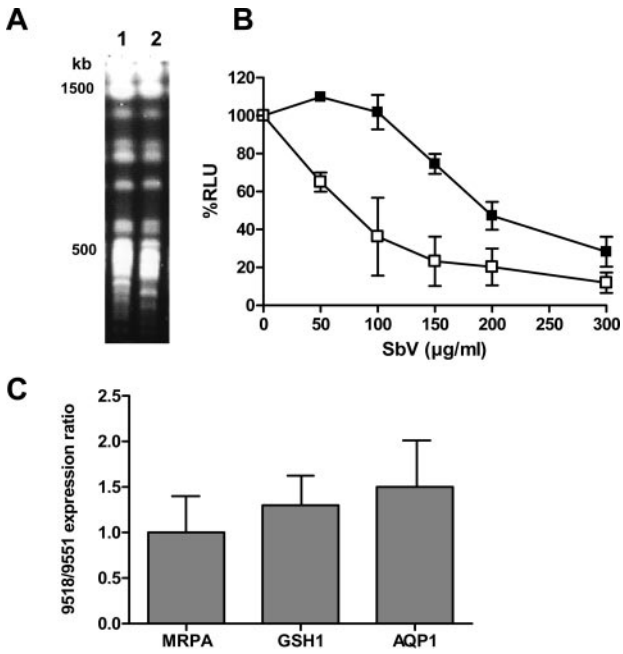



FIG. 1. **Characterization of a pair of *L. donovani* clinical isolates.** *A*, pulse field gels of *L. donovani* 9518 (*lane 1*) and *L. donovani* 9551 (*lane 2*) clinical isolates coming, respectively, from patients not responding and responding to Pentostam treatment. Size was estimated from a *Saccharomyces cerevisiae* molecular weight marker (Bio-Rad). *B*, susceptibility to Pentostam (Sb(V)) of intracellular *L. donovani* field isolates 9551 (□) and 9518 (■) expressing the luciferase reporter gene in THP-1 cells. Each data point represents the average of three separate assays. *RLU*, relative light units. *C*, expression ratios of MRPA, GSH1, and AQP1 analyzed by real time RT-PCR on RNA derived from 9518 resistant and 9551 sensitive *L. donovani* isolates. Ratios were normalized with GAPDH. The mean of experiments performed from three different RNA preparations is shown.

## RESULTS

*Characterization of L. donovani Field Isolates*—Lira *et al.* (9) were the first to show that *L. donovani* parasites isolated from Sb(V)-unresponsive patients were resistant to Sb(V). A selection of the same *L. donovani* isolates were further characterized by pulse-field gels in an attempt to find genetically related parasites that differed in their response to Sb(V) treatment. Considerable differences were seen between the PFGE profiles of these *L. donovani* isolates (result not shown), but two strains, *L. donovani* strain 9551 (isolated from a patient responsive to Sb(V)) and strain 9518 (isolated from a patient unresponsive to Sb(V)), were related (Fig. 1*A*). The pair 9518/9551 was used as a paradigm for this study. Sb(V) susceptibility was monitored in intracellular THP-1 monocytes using the well established luciferase-expressing parasite assay (33), and indeed as reported earlier (9), 9518 was shown to be more resistant to Sb(V) than 9551 (Fig. 1*B*). Sb(V) sus-

TABLE I
*Drug susceptibility values in L. donovani clinical isolates*

n.d., not done.

| strains | $EC_{50}$ | | | |
|---|---|---|---|---|
| | SbV[a] (μg/ml) | SbIII[b] (μM) | Miltefosine[b] (μM) | Ampho B[b] (μM) |
| L.d 9551 | 85 ± 25 | 150 ± 75 | 5 ± 1 | 0.38 ± 0.05 |
| L.d 9518 | >300* | >400 * | 10 ± 2 * | 0.65 ± 0.05 * |
| L.d 9551 – NEO | 95 ± 25 | 140 ± 75 | 4 ± 0.5 | n.d |
| L.d 9551 – 14-3-3 | 85 ± 25 | 200 ± 30 | n.d | n.d |
| L.d 9551 – HSP83 | † | 300 ± 50 * | 8 ± 2 * | n.d |
| L.d 9518 – NEO | >300 | >400 | 11 ± 2 | n.d |
| L.d 9518 – SKCRP14.1 | 140 ± 25* | 200 ± 50 * | >16 * | n.d |

[a] As determined in intracellular amastigotes with Pentostam.
[b] As determined in the promastigote stage.
† Highly variable results. See text for possible explanation.
* $p$ value $< 0.05$ within each group separated by a line.

ceptibility cannot be measured with promastigotes as these are insensitive to this drug (46, 47). However, Sb(V)-resistant amastigotes are often, although not always, cross-resistant to Sb(III) (the active form of the metal), and parasites selected for Sb(III) resistance are also cross-resistant to Sb(V) as intracellular parasites (34, 35). We found that 9518 was significantly more resistant to Sb(III) compared with 9551 (Table I), and thus the easier susceptibility assays with promastigotes were used preferentially for studying phenomena associated with antimonial resistance.

Several genes are already known to contribute to antimonial resistance in *Leishmania* cells. Decreased drug uptake can be mediated by a decrease in the expression of the aquaglyceroporin gene *AQP1* (31, 48); a modulation in thiol levels is correlated to resistance[2] (29, 35, 49) and is often, but not always, due to an alteration in the expression of *GSH1* coding for the rate-limiting step in glutathione biosynthesis; and finally the overexpression of the ATP-binding cassette gene *MRPA* is implicated in Sb(V) resistance[2] (35, 50). However, neither *AQP1*, *GSH1*, nor *MRPA* were differentially expressed in the strain 9518 compared with 9551 as determined by real time RT-PCR analysis (Fig. 1*C*).

*Comparative Two-dimensional Gel Electrophoresis Analysis of L. donovani Field Strains*—Proteomics studies have been useful to study resistance mechanisms in *Leishmania* (38, 39, 51), and a comparative 2D gel approach of soluble proteins was used with the pair 9518/9551 in the hope of finding new insight into resistance mechanisms. Gels of four independent samples were run, averaged, and compared using the 2D gel analysis software package Progenesis. Representative gels of one pH range analyzed, pH 4–5, are shown in Fig. 2*A*. Differences of greater than 2-fold identified by the software were individually validated, and spots of interest were excised from the gels and identified by mass spectrometry and are listed in Table II. Several of the spots contained more than one protein, whereas others, in particular spots 398, 1250, and 1606, contained only one identified protein (Fig. 2*B*). Interestingly all these unique proteins identified have published roles within PCD in a number of organisms. For example, the 14-3-3 protein (spot 1250) that is overexpressed in 9518 is part of a conserved family of proteins capable of binding numerous phosphorylated proteins implicated in several cellular processes, including apoptosis (for a review, see Ref. 52). Most differentially expressed proteins identified in Table II are heat shock proteins (HSPs), a class of proteins known to prevent PCD activation by modulating multiple events within apoptotic pathways (for a review, see Ref. 53). The small kinetoplastid calpain-related protein SKCRP14.1 (spot 1606), which is down-regulated in the resistant strain 9518, is often linked to calpain-related proteins in kinetoplastid parasites (54), and calpains are key effectors of PCD (for a review, see Ref. 55).

The above results raised the intriguing possibility that PCD is altered in resistant field isolates. Because most clinically useful *Leishmania* drugs act by a phenomenon sharing several features with PCD, we tested the susceptibility of our pair of isolates as promastigotes to other drugs. Interestingly strain 9518 was found to be more resistant to both miltefosine and amphotericin B when compared with the Sb(V)-sensitive 9551 strain (Table I).

*L. donovani 9518 Is Less Susceptible to Drug-mediated Apoptosis*—The proteomics screen described above raised the possibility that strain 9518 was less susceptible to drug-mediated PCD compared with 9551. We therefore carried out a number of assays to test whether the pair 9518/9551 responded differently to drug-mediated PCD using Sb(III) and miltefosine. DNA fragmentation is an end result of apoptosis in numerous organisms. Sb(III) treatment indeed led to DNA ladder formation, and interestingly the DNA ladder formation was repeatedly more intense in the case of the sensitive strain 9551 when compared with the resistant strain 9518 (Fig. 3*A*). The same trend was obtained with cells treated with miltefosine (Fig. 3*B*) or with amphotericin B (results not shown).

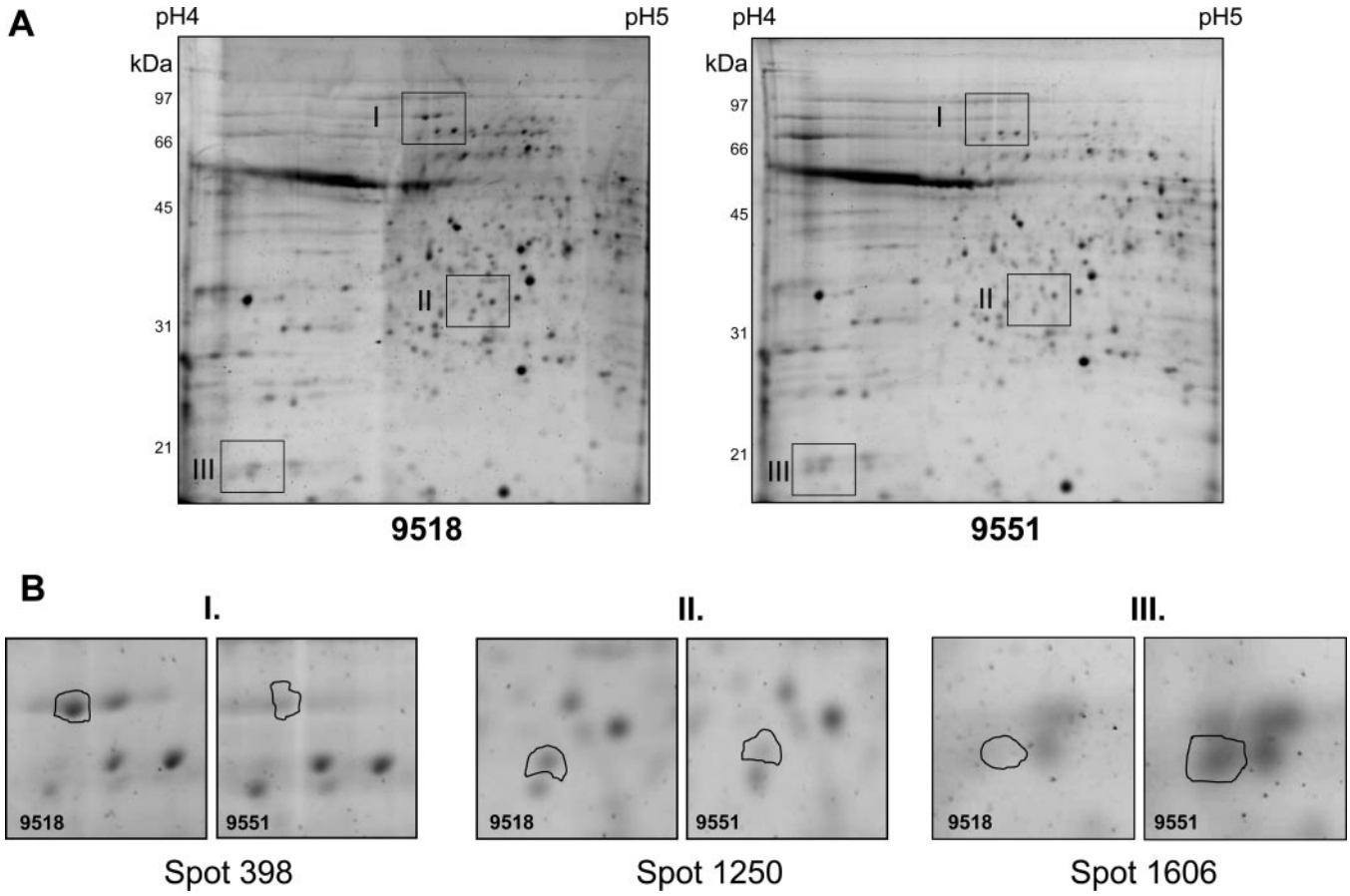



FIG. 2. **Proteomics analyses of resistance in *L. donovani*.** *A*, representative 2D gels (pH 4–5) comparing *L. donovani* 9518 resistant and *L. donovani* 9551 sensitive strains. *B*, *insets* (*I–III*) corresponding to zoomed gel areas with spots showing a difference in protein expression between the two strains.

We next investigated earlier events of the apoptotic process by analyzing changes in $\Delta\Psi$m upon drug treatment with the mitochondrial dye TMRE. Changes in $\Delta\Psi$m are central events of PCD in metazoans and *Leishmania* (18, 22, 56, 57). As a control, TMRE-loaded promastigotes were co-incubated with the protonophore CCCP (100 $\mu$M), which induces the complete dissipation of mitochondrial potential (Fig. 3, *C* and *D*). Sb(III) was shown to induce a progressive decline in $\Delta\Psi$m that initiates a few hours after treatment, but this was less pronounced for resistant parasites (Fig. 3*C*). Consistent with the work of Lee *et al.* (18), we noted that membrane permeabilization and PI labeling occurred only when the $\Delta\Psi$m reached background levels (not shown). Miltefosine induces PCD in *Leishmania* (20, 21), but unexpectedly and reported here for the first time, a 10 or 20 $\mu$M miltefosine treatment induced an initial high increase of $\Delta\Psi$m during the first hours of treatment that was followed by a rapid depolarization of the mitochondria (Fig. 3*D*). Although the mitochondrion hyperpolarization phase is similar between strains 9551 and 9518, the subsequent depolarization occurred faster in the sensitive 9551 parasites (Fig. 3*D*).

A last measure of drug-induced PCD in these *Leishmania* clinical isolates consisted of the analysis of phosphatidylserine residue externalization using Annexin V and subsequent membrane permeabilization following drug treatment as measured by PI labeling. Although control untreated parasites were negative for both Annexin V and PI labeling (Fig. 3, *E* and *F*), phosphatidylserine externalization was detected in ~13% of both Sb(V)-sensitive and -resistant parasites treated with 500 $\mu$M Sb(III) after 48 h (Annexin V-positive and PI-negative cells). However, at higher Sb(III) concentration, most of the Annexin V-positive cells also became positive for PI labeling in strain 9551, whereas the 9518 resistant strain remained impermeable to PI (Fig. 3*E*). Similarly treatment of the Sb(V)-sensitive strain 9551 with 10 or 20 $\mu$M miltefosine over 15 h led to cell death (late apoptotic cells: Annexin V-positive and PI-positive), whereas the same treatment of the Sb(V)-resistant 9518 cells led to significant Annexin V binding (about 25 and 30% for 10 and 20 $\mu$M, respectively) but no significant PI labeling (Fig. 3*F*).

The results presented above were obtained using the more tractable Sb(III)/promastigote assay. Drug-induced PCD has been demonstrated in intracellular parasites using the TUNEL labeling assay (19, 21). Consequently we showed that Sb(V)-

TABLE II
*Protein identification of 2D gel spots differentially expressed in a comparative screen between Sb(V)-resistant 9518 versus sensitive 9551 L. donovani isolates*

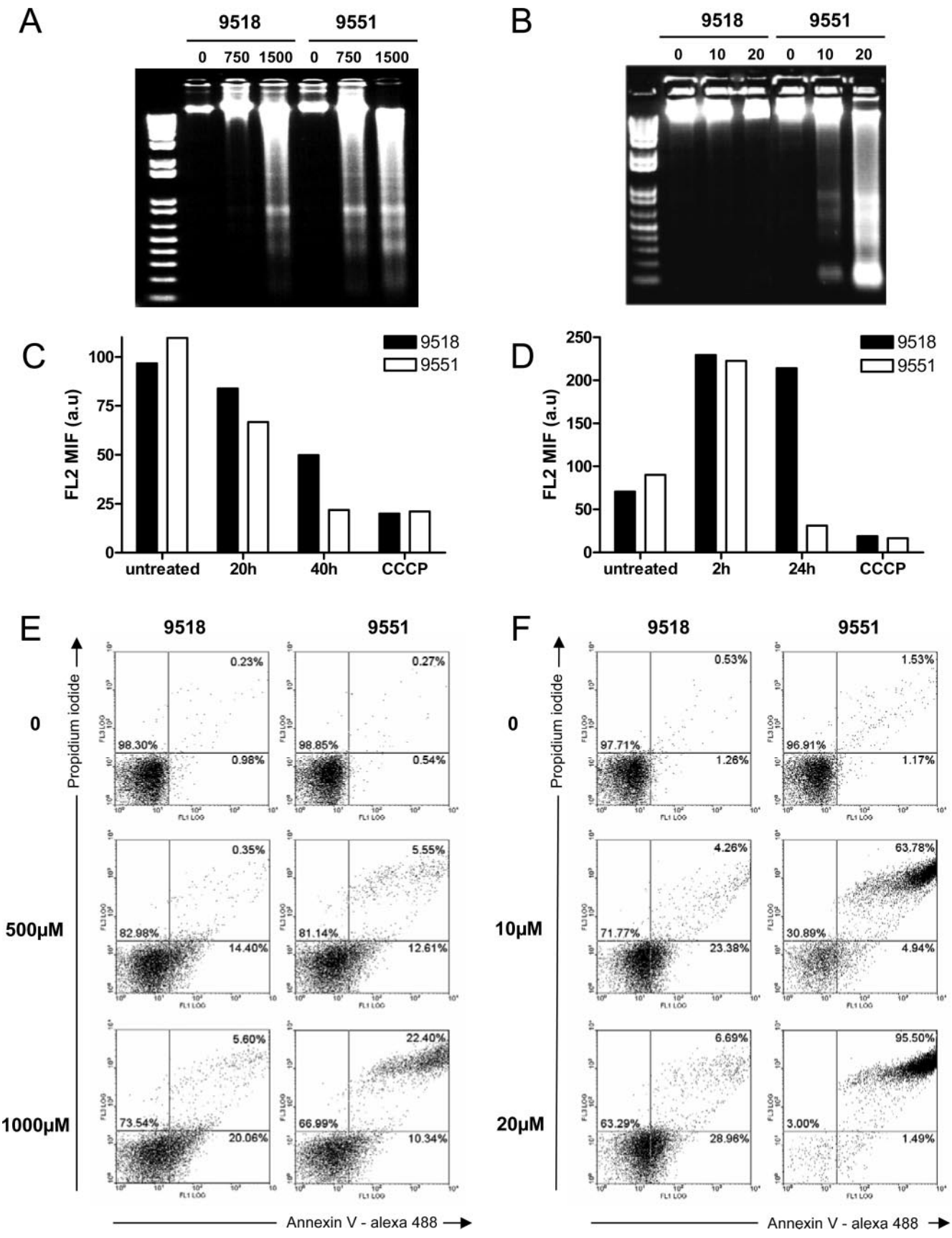
| Spot no. | -Fold change[a] | Accession no.[b] | Protein name/function | Mascot score[c] | Coverage | No. peptides identified |
|---|---|---|---|---|---|---|
| | | | | | % | |
| 398 | +3.56 | LmjF33.0312 +16 homologues | Heat shock protein 83–1 | 613 | 19 | 14 |
| 618 | +3.56 | LmjF30.2470 LmjF30.2480 LmjF30.2490 LmjF30.2550 | HSP70-related protein 1, mitochondrial precursor, putative | 261 | 13 | 6 |
| | | LmjF36.6910 | Chaperonin containing tcp1, subunit 8, putative | 96 | 4 | 2 |
| 1250 | +2.1 | LmjF11.0350 | 14-3-3 protein, putative | 281 | 25 | 7 |
| 2446 | +4.4 | LmjF33.0312 + 16 homologues | Heat shock protein 83–1 | 520 | 17 | 13 |
| | | LmjF32.0400 | ATP-dependent RNA helicase, putative | 278 | 11 | 6 |
| | | LmjF28.2770 | Heat shock protein HSP70, putative | 273 | 11 | 6 |
| | | LmjF05.0960 | Dipeptidyl-peptidase III, putative | 84 | 3 | 2 |
| 584 | −2.13 | LmjF30.2470 LmjF30.2480 LmjF30.2490 LmjF30.2550 | HSP70-related protein 1, mitochondrial precursor, putative | 467 | 18 | 11 |
| | | LmjF11.0820 | Hypothetical protein, unknown function | 109 | 7 | 2 |
| 1258 | −2.94 | LmjF14.1160 | Enolase | 172 | 10 | 4 |
| | | LmjF21.1830 | 20 S proteasome $\alpha$5 subunit, putative | 125 | 14 | 4 |
| 1606 | −3.67 | LmjF14.0850 | SKCRP14.1 (small kinetoplastid calpain-related protein) | 155 | 30 | 4 |

[a] In the resistant isolate 9518 *versus* the sensitive isolate 9551.
[b] Accession numbers are from the *L. major* GeneDB (www.genedb.org) as of April 27, 2004.
[c] Mascot scores above 33 for individual peptides were considered significant for identity ($p < 0.05$). Scores significant for homology varied depending on the peptide.

induced PCD in intracellular parasites was also more prevalent in the 9551 strain than in the 9518 strain as indicated by a more intense TUNEL labeling observed for the 9551 sensitive strain (Fig. 4). This is in line with Pentostam susceptibility assays carried out with intracellular amastigotes (Fig. 1*B*).

*Gene Products Implicated in Drug-mediated PCD in Leishmania*—The comparative proteomics screen (Table II) suggested a number of likely candidates implicated in PCD that are differentially expressed between strains 9518 and 9551 (*e.g.* HSP83, 14-3-3, and SKCRP14.1). Indeed several HSPs were overexpressed in the resistant strain 9518, and the protein most down-regulated in strain 9518 was SKCRP14.1 (Table II). Two HSPs were increased, HSP70 and HSP83 (Table II), but the (minor) role of HSP70 in Sb(III) tolerance has already been established in *Leishmania* (58). The genes coding for HSP83, SKCRP14.1, and 14-3-3 were cloned into the *Leishmania* Psp$\alpha$NEO$\alpha$ vector. Both 14-3-3 and HSP83 constructs were transfected individually into the 9551 sensitive strain, whereas the SKCRP14.1 construct was transfected into the 9518 resistant line. Transfectant controls consisted of the same strains electroporated with Psp$\alpha$NEO$\alpha$. Growth inhibition assays performed with strain 9551 promastigotes transfected with the 14-3-3 (LmjF11.0350) construct did not result in any increase of resistance to Sb(III) when compared with control transfectants (Table I). These parasites were also not more resistant to Sb(V) as intracellular amastigotes (Table I).

Growth inhibition assays performed on promastigotes showed that the sensitive 9551 strain overexpressing HSP83 was more than 2-fold resistant to Sb(III) compared with 9551 control parasites (Table I). Interestingly the 9551-HSP83 transfectants were also cross-resistant to miltefosine (Table I). Infection of macrophages and subsequent susceptibility testing of the intracellular HSP83 transfectant has led to irreproducible results likely because of the key role of HSP83 in parasite differentiation. Indeed HSP83 inhibition induces promastigote to amastigote transformation (59), and thus HSP83-overexpressing parasites may have difficulty differentiating. The mechanism by which HSP83 confers Sb(III) resistance was further investigated by using the TMRE mitochondrial dye and by DNA fragmentation assays. The $\Delta\Psi$m in HSP83-overexpressing parasites was found to be higher than in control cells (Fig. 5*A*). When Sb(III) was added to logarith-



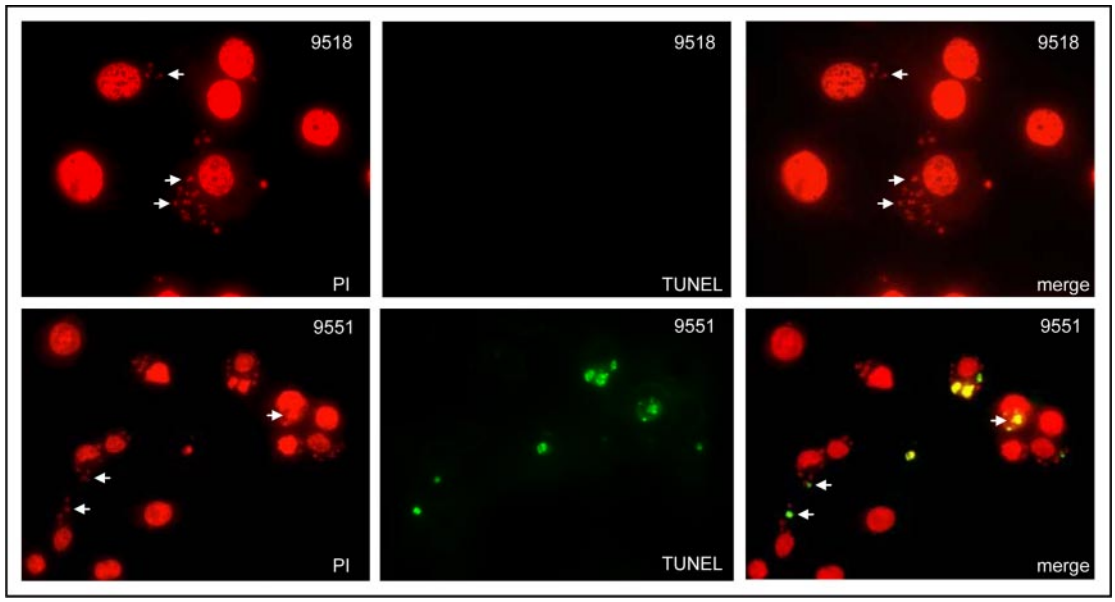



FIG. 4. ***In situ* analysis of drug-induced programmed cell death in *L. donovani* intracellular parasites.** THP-1 macrophages infected with either 9518 or 9551 stationary phase *L. donovani* promastigotes were treated with 300 $\mu$g/ml Pentostam for 96 h. Nuclei of macrophages and intracellular amastigotes (*arrows*) are visualized as *red dots* with PI staining, whereas nuclei of apoptotic, intramacrophagic amastigotes show *green* fluorescence with TUNEL staining. Amastigotes were visualized at 100× magnification. Results are representative of two similar experiments done in duplicate.

mic phase parasites, we observed that HSP83-overexpressing parasites were protected against a drop of $\Delta\Psi$m (Fig. 5*A*). This protection against a decrease in $\Delta\Psi$m in the HSP83 transfectant was also paralleled by a decrease in Sb(III)-mediated DNA fragmentation (Fig. 5*B*). As reported, miltefosine induced a rapid increase of $\Delta\Psi$m in the first hours of treatment, and HSP83 overexpression further accelerated the rise in $\Delta\Psi$m following miltefosine treatment in HSP83-transfected parasites compared with control strains (results not shown). The subsequent drop in $\Delta\Psi$m analyzed 24 h after treatment occurred in a much higher proportion of 10 $\mu$M miltefosine-treated control parasites (30%) than HSP83 transfectants (13%) (Fig. 5*C*). At a higher drug concentration (20 $\mu$M), HSP83 overexpression, however, did not protect against depolarization of the mitochondrial membrane potential (Fig. 5*C*). Finally HSP83 overexpression also provided increased protection against miltefosine-induced DNA fragmentation (Fig. 5*D*).

The SKCRP14.1 protein was down-regulated in the 9518 resistant parasites, and its overexpression significantly increased the sensitivity of this strain to Sb(III) as promastigotes and also to Sb(V) as intracellular amastigotes (Table I). The difference in Sb(III)-induced $\Delta\Psi$m variations between SKCRP14.1 and Psp$\alpha$NEO$\alpha$ transfectants was small (if any) (Fig. 6*A*). Furthermore the SKCRP14.1 transfectant was much more sensitive to Sb(III)-induced DNA fragmentation (Fig. 6*B*). Unexpectedly SKCRP14.1 overexpression had the opposite effect on miltefosine-treated 9518 parasites. Indeed growth inhibition assays indicated that 9518 parasites transfected with SKCRP14.1 became more resistant to miltefosine compared with the 9518-NEO control (Table I). Monitoring $\Delta\Psi$m variations after drug addition showed that SKCRP14.1 protected significantly against miltefosine-induced mitochondrial depolarization (Fig. 6*C*). Even after 40 h of treatment with 20 $\mu$M miltefosine, almost half of the SKCRP14.1 transfectants still had polarized mitochondrial membranes (Fig. 6*C*). Furthermore overexpression of SKCRP14.1 led to resistance against miltefosine-mediated DNA fragmentation as compared with control (Fig. 6*D*).

Fig. 3. **Differential drug-induced programmed cell death in *L. donovani* clinical isolates.** Representative agarose gels showing DNA fragmentation between *L. donovani* 9518 and *L. donovani* 9551 strains 48 h after treatment with 750 and 1500 $\mu$M Sb(III) (*A*) or 24 h after treatment with 10 and 20 $\mu$M miltefosine (*B*) are shown. Genomic DNA from treated parasites was extracted as described under "Materials and Methods," and 7 $\mu$g were loaded on a 1.5% agarose gel and visualized by ethidium bromide staining. Dissipation of the $\Delta\Psi$m in *L. donovani* 9518 and *L. donovani* 9551 promastigotes with time following treatment with 750 $\mu$M Sb(III) (*C*) or with 20 $\mu$M miltefosine (*D*) is shown. The histogram represents the mean intensity fluorescence (*MIF*) of TMRE-loaded parasites expressed in arbitrary units (*a.u.*) and recorded by fluorescence-activated cell sorting. This is a representative experiment that was repeated at least four times with similar results. Untreated parasites co-incubated with TMRE and 100 $\mu$M CCCP were used as controls for completely depolarized mitochondria. Flow cytometry analysis of both phosphatidylserine exposure (Annexin V-positive cells) and subsequent plasma membrane permeabilization events (Annexin V- and PI-positive cells) following treatment with Sb(III) (*E*) for 48 h or miltefosine (*F*) for 15 h is shown. Dot plots are representative of three independent analyses.

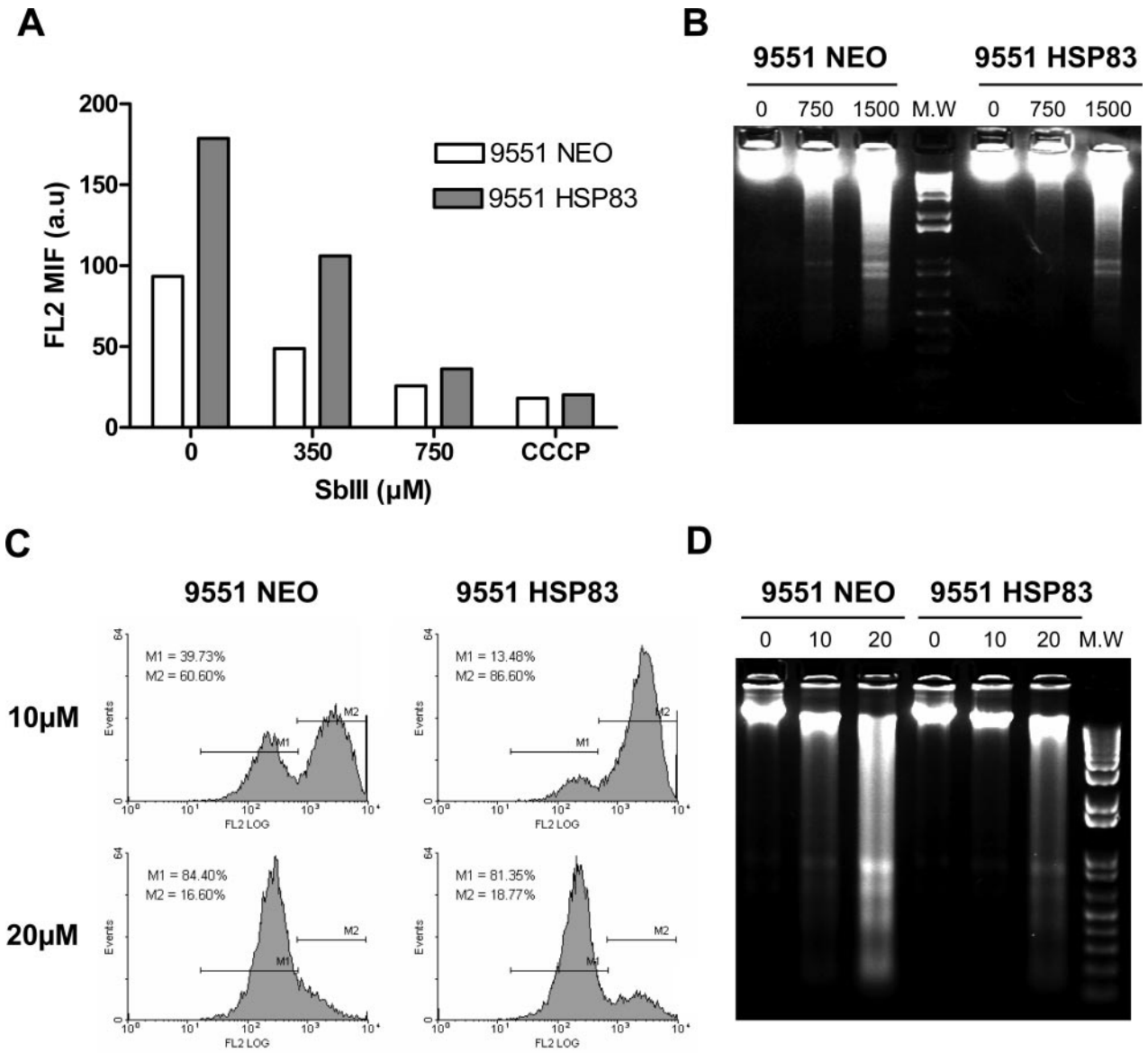



FIG. 5. **HSP83 is involved in protection against drug-induced programmed cell death.** *A*, histograms representing variations in $\Delta\Psi$m observed in both control (*9551 NEO*) and HSP83-overexpressing (*9551 HSP83*) parasites 24 h after treatment with 350 or 750 $\mu$M Sb(III). *B*, DNA laddering in both control and HSP83-overexpressing *L. donovani* 9551 parasites after treatment with different concentrations of Sb(III) for 48 h. *C*, depolarization phase of the $\Delta\Psi$m in both control and HSP83-overexpressing parasites observed 24 h after treatment with 10 or 20 $\mu$M miltefosine. The higher fluorescence intensity peaks are marked as *M2*, whereas the lower fluorescence intensity peaks, corresponding to parasites with depolarized mitochondria, are marked as *M1*. *D*, DNA laddering in both control and HSP83-overexpressing parasites 24 h after treatment with 10 or 20 $\mu$M miltefosine. All data shown, either on mitochondrial potential measurements or DNA laddering, were consistently observed in all replicates (at least three) done. *a.u.*, arbitrary units.

## DISCUSSION

The control of *Leishmania* infections relies on a few chemotherapeutic drugs including Sb(V), amphotericin B, miltefosine, and pentamidine. Despite years of usage, the mode of action of several of these drugs is not known, although recent evidence suggests that they kill the parasite by an apoptotic-like process. Resistance to Sb(V) is now widespread in several geographic regions and has reached epidemic proportions in some parts of India. Although treatment failure can be due to several factors, including host immune status (60, 61), parasites with decreased drug susceptibility have been associated with treatment failure (5, 8, 9).[2] Antimonial resistance mechanisms are starting to be understood in laboratory isolates but are less clear in clinical isolates. The expression levels of several genes known to be altered in *in vitro* resistant isolates were unchanged in the selected pair of sensitive and resistant clinical isolates (see Fig. 1*C*) suggesting that the resistance mechanisms in these field parasites may differ from those of

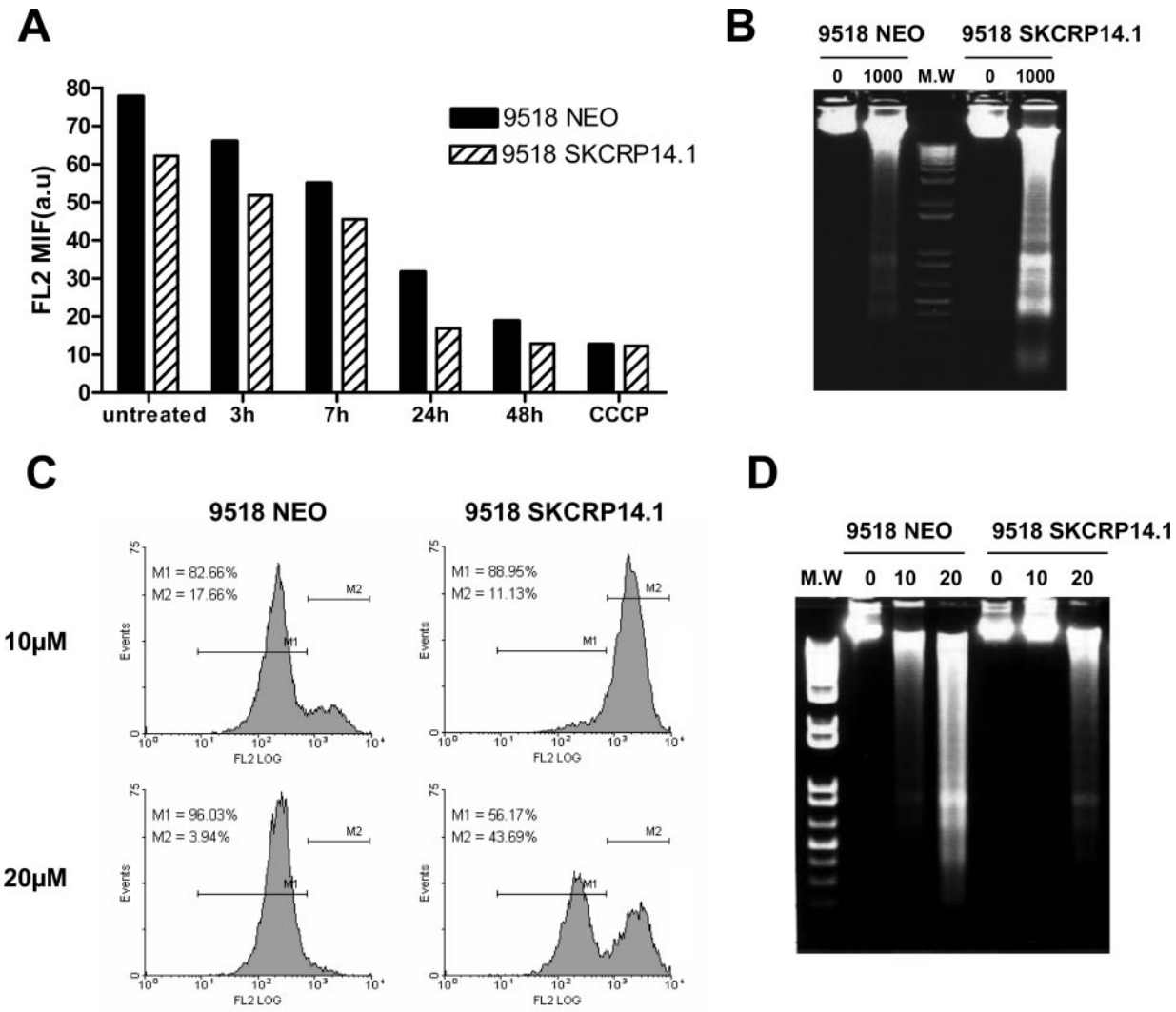



FIG. 6. **The dual role of SKCRP14.1 in drug-mediated programmed cell death.** *A*, change in $\Delta\Psi$m over time observed in both control (*9518 NEO*) and SKCRP14.1-overexpressing (*9518 SKCRP14.1*) parasites after treatment with 750 $\mu$M Sb(III). *B*, differences in DNA laddering between control and SKCRP14.1-overexpressing parasites after treatment with 1000 $\mu$M Sb(III) for 48 h. *C*, depolarization phase of the $\Delta\Psi$m in control and SKCRP14.1-overexpressing parasites observed 40 h after treatment with 10 or 20 $\mu$M miltefosine. The higher fluorescence intensity peaks are marked as *M2*, whereas the lower fluorescence intensity peaks, corresponding to parasites with depolarized mitochondrion, are marked as *M1*. *D*, DNA laddering in both control and SKCRP14.1-overexpressing parasites 24 h after treatment with 10 or 20 $\mu$M miltefosine. *a.u.*, arbitrary units.

laboratory resistant mutants. This is in contrast to *L. donovani* field isolates from a different geographic region where *MRPA* and *GSH1* expression levels were increased[2] or *AQP1* expression was decreased (29) in resistant parasites.

Proteomics screens have been useful in pinpointing known and novel resistance mechanisms to antimicrobial agents (38, 39, 62–65), and part of the soluble proteome of the 9518/9551 *L. donovani* pair was thus compared. Comparative proteomics analyses of genetically linked parasites differing in Sb(V) susceptibility highlighted the differential expression of proteins potentially implicated in PCD. Interestingly strain 9518, first isolated from an Sb(V)-unresponsive patient, exhibited cross-resistance to miltefosine and amphotericin B (Table I). Consistent with this cross-resistance, 9518 was not only more resistant to Sb(III)- and Sb(V)-induced PCD (Figs. 3*A* and 4) but was also more resistant to miltefosine-induced PCD (Fig. 3*B*). Thus, 9518 is clearly less susceptible to drug-induced PCD compared with 9551. The phenomenon of PCD has been recently established in *Leishmania* spp. (for reviews, see Refs. 24–27), but the mechanisms and gene products involved in this process are not known. The proteomics screen highlighted a number of differentially expressed proteins (Table II), which included several potentially interesting candidates: two overexpressed proteins (14-3-3 and HSP83) and one down-regulated (SKCRP14.1) protein in the resistant strain 9518.

Transfection of the 14-3-3 construct (LmjF11.0350) did not lead to a readily measurable phenotype related to either drug-mediated PCD or resistance (Table I and results not shown).

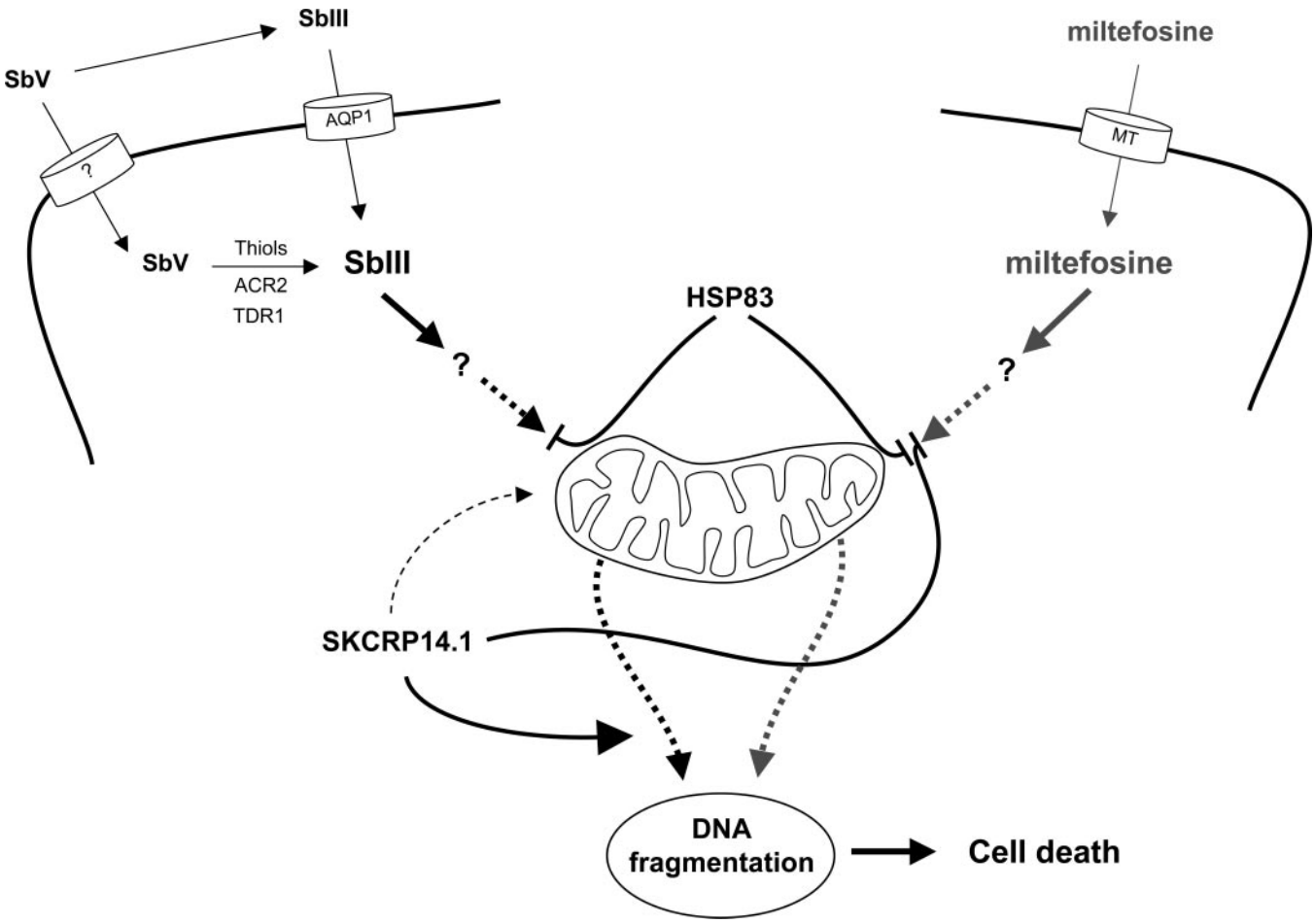



FIG. 7. **Drug-induced programmed cell death in *Leishmania.*** The pentavalent antimony importer is not known, but the trivalent form of antimony enters by the aquaglyceroporin AQP1 (31). Miltefosine enters the cell through an amino-phospholipid translocase (30). Sb(III) and miltefosine interact either directly or indirectly with the mitochondrion, eventually leading to its depolarization. Note that at early time points, miltefosine leads to hyperpolarization of $\Delta\Psi$m. HSP83, either directly or most likely by interaction with other partners, provides protection against drug-induced PCD. The SKCRP14.1 protein also protects against miltefosine-induced PCD by preventing depolarization of the $\Delta\Psi$m. However, SKCRP14.1 exacerbates Sb(III)-induced apoptosis mostly by inducing downstream events leading to DNA fragmentation, although we cannot exclude that it also modestly increases mitochondrial membrane depolarization. This dual effect of SKCRP14.1 may arise by interactions with different protein partners. Not shown here are phosphatidylserine exposure and membrane permeabilization, which are also associated with PCD in *Leishmania*, and potentially many other unknown factors involved in PCD. *MT*, miltefosine transporter.

Either the 14-3-3 protein is not well expressed in the transfected cells, or the 14-3-3 protein requires additional factors absent in 9551 to lead to a phenotype. We cannot exclude that this protein was overexpressed spuriously in 9518 and is not implicated in the resistance phenotype studied. Overexpression of HSP83 had a clear phenotype where transfection of its gene in the sensitive 9551 line conferred increased resistance to both Sb(III) and miltefosine (Table I). This resistance phenotype is associated with a decrease in drug-mediated PCD as measured by $\Delta\Psi$m measurements and DNA fragmentation (Fig. 5). The *Leishmania* HSP83 corresponds to the mammalian HSP90 (GeneDB, www.genedb.org). HSP90 was found to be a negative regulator of the mitochondrial cytochrome *c*-dependent apoptosis pathway (66). The association of HSP90 to Bcl-2 is required to prevent mitochondrial apoptotic cascades (67), whereas in another type of cell, HSP90 was found to interact with proapoptotic proteins inducing mitochondrial apoptotic cascades (68). The involvement of HSP90 in PCD is consistent with our proposed role for HSP83 involvement in drug-induced PCD in *Leishmania*. Interestingly HSP83 overexpression indeed appears to allow the cell to maintain a higher mitochondrial potential (Fig. 5*A*), and thus HSP83 may interact with other proteins to negatively regulate the *Leishmania* mitochondria-dependent apoptotic pathway (Fig. 7). Recently HSP90 was shown to help fungi resist cytotoxic drugs before specific resistance mechanisms emerge (69). It is thus possible that the *Leishmania* HSP90 orthologue (HSP83) overexpression in the 9518 field isolate is a relic of a general first line of defense that remained despite the emergence of more specific resistance mechanisms. We have previously suggested a similar role for HSP70 where it was proposed to serve as a first nonspecific stress response

allowing the cell to develop more specific and efficient resistance mechanisms against Sb(III) (58).

The last protein characterized here is SKCRP14.1. Calpains constitute a large family of calcium-dependent cysteine proteases usually containing four conserved domains (I–IV). However, several unconventional calpains have been identified and are referred to as calpain-like proteins. *Leishmania* and other kinetoplastid parasites, an early diverging branch of the eukaryotic lineage, have several of these calpain-like proteins (54). These *Leishmania* calpain-like proteins contain four putative domains: domain I, which is unique to kinetoplastid parasites; domain II, which contains the catalytic domains; domain III, which is homologous to domain III of conventional calpains; and a last domain called C, which is also unique to kinetoplastids. SKCRP14.1, like all other SKCRPs found exclusively in kinetoplastid parasites, contains only domain I (54). This novel protein was down-regulated in the 9518 strain (Fig. 2*B*), and overexpression of the SKCRP14.1 gene in 9518 resensitized cells to Sb(III) and Sb(V) (Table I). Consistent with these phenotypes, SKCRP14.1 overexpression increased the susceptibility of 9518 to Sb(III)-mediated PCD as measured by DNA fragmentation (Fig. 6*B*). SKCRP14.1 overexpression appears to modify $\Delta\Psi$m in the presence of Sb(III) only modestly. Overexpression of SKCRP14.1 increased antimonial susceptibility in 9518 but surprisingly led to an increased resistance to miltefosine (Table I). Consistent with this resistance phenotype, SKCRP14.1 overexpression led to increased protection against miltefosine-induced PCD (Fig. 6, *C* and *D*). Thus, an alteration of SKCRP14.1 expression can have opposite effects on antimonials and miltefosine susceptibility (Fig. 7). SKCRP14.1 is likely to be a regulator of PCD and must interact with other calpain-like proteins having domains II and III. Clearly, however, other gene products are implicated in miltefosine resistance. Indeed despite the lower expression of SKCRP14.1 in strain 9518 compared with 9551 (Table II), it is nonetheless more resistant to miltefosine (Table I).

Because an altered expression of the same protein can have such different outcomes on drug-induced PCD, there may be more than one PCD pathway in *Leishmania* (Fig. 7), and the current literature indeed supports this view (23, 25). Although both antimonials and miltefosine led to PCD (Fig. 3), it seems to be achieved by different mechanisms because mitochondrial membrane depolarization kinetics caused by the two drugs are very different (Figs. 3, 5, and 6).

The proteomics screen carried out on clinical isolates has pinpointed novel resistance mechanisms to Sb(V), although it remains to be seen whether these comprise general protective mechanisms allowing the emergence of new mutations or whether they constitute primary resistance mechanisms. Indeed the increased levels of resistance provided by HSP83 and SKCRP14.1 are low, and possibly other resistance mechanisms are also present. However, it cannot be excluded that a combination of reduced expression of SKCRP14.1 and increased expression of HSP83 could lead to high levels of resistance to Sb(V). It also cannot be excluded that a 2-fold decrease in susceptibility can lead to treatment failure as the therapeutic window for Sb(V) is small. Ongoing analyses of other paired clinical isolates have nonetheless showed that Sb(V)-resistant isolates are indeed less susceptible to Sb(III)-induced PCD,[3] suggesting that this may be a more general phenomenon, although the proteins implicated may vary. The proteomics screen was also instrumental in finding novel functions for HSP83 in *Leishmania* and to find a role for a member of a novel family of protein (SKCRP). Both proteins appear to be involved in the mode of action of several anti-*Leishmania* drugs and this new understanding could certainly be exploited to find much needed novel treatment strategies against leishmaniasis.

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