

# Tolerance to drug-induced cell death favours the acquisition of multidrug resistance in *Leishmania*

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The control of the protozoan parasite *Leishmania* relies on few drugs with unknown cellular targets and unclear mode of action. Several antileishmanials, however, were shown to induce apoptosis in *Leishmania* and this death mechanism was further studied in drug-sensitive and drug-resistant *Leishmania infantum*. In sensitive parasites, antimonials (SbIII), miltefosine (MF) and amphotericin B (AMB), but not paromomycin (PARO), triggered apoptotic cell death associated with reactive oxygen species (ROS). In contrast, *Leishmania* mutants resistant to SbIII, MF or AMB not only failed to undergo apoptosis following exposure to their respective drugs, but also were more tolerant towards apoptosis induced by other antileishmanials, provided that these killed *Leishmania* via ROS production. Such tolerance favored the rapid acquisition of multidrug resistance. PARO killed *Leishmania* in a non-apoptotic manner and failed to produce ROS. PARO resistance neither protected against drug-induced apoptosis nor provided an increased rate of acquisition of resistance to other antileishmanials. However, the PARO-resistant mutant, but not SbIII-, MF- or AMB-resistant mutants, became rapidly cross-resistant to methotrexate, a model drug also not producing ROS. Our results therefore link the mode of killing of drugs to tolerance to cell death and to a facilitated emergence of multidrug resistance. These findings may have fundamental implications in the field of chemotherapeutic interventions.

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The bactericidal mode of action of antibiotics has recently been shown to depend less on their primary target, and more on the induction of common downstream effector mechanisms leading to cell death. Indeed, the generation of oxidants has been a unifying step of such common death mechanisms (reviewed in Kohanski *et al.*<sup>1</sup>). Similarly, the induction of apoptosis by several anticancer drugs has been suggested to occur through the generation of oxidants, and a characteristic feature of drug-resistant neoplastic cells seems to be their ability to evade drug-induced apoptosis.<sup>2,3</sup> These studies suggested a causal link between evolutionary-conserved cellular events leading to death and the mode of action of cytotoxic compounds, and highlighted the importance of understanding the mode of action of drugs in order to understand and prevent the emergence of drug resistance.

Apoptotic features have also been reported in the eukaryotic parasite *Leishmania* following exposure to several drugs.<sup>4–8</sup> *Leishmania* are unicellular parasites responsible for a wide range of human diseases ranging from self-healing cutaneous lesions to fatal visceral infections. Anti-*Leishmania* therapy relies on the use of a handful number of drugs including pentavalent antimony (SbV)—a prodrug that needs to be reduced to its trivalent state (SbIII) in order to acquire antiparasitic activity, the polyene antibiotic amphotericin B (AMB) and the phosphocholine analog miltefosine (MF).<sup>9</sup> A phase 3 clinical trial has also been successfully completed for the aminoglycoside paromomycin (PARO),<sup>10</sup> leading to its

approval for the treatment of visceral leishmaniasis in India. Although the mechanisms responsible for the anti-*Leishmania* activities of these compounds have not yet been clearly established, their primary targets are thought to differ substantially. Indeed, evidence suggests that antimony have a rather complex mode of action, acting at the level of multiple cellular targets.<sup>11–18</sup> The leishmanicidal activity of MF has been associated with perturbation of the metabolism of alkyl-phospholipids, and the biosynthesis of alkyl-anchored glycolipids and glycoproteins.<sup>19,20</sup> The cytotoxic activity of AMB has been associated with its high affinity for ergosterol,<sup>21</sup> the main sterol in the plasma membrane of fungi and *Leishmania*.<sup>22</sup> Finally, PARO, which interferes with protein synthesis by binding to ribosomes in bacteria, was also shown to affect translation in *Leishmania*,<sup>23</sup> in addition to alter the composition and fluidity of the plasma membrane of the parasite.<sup>24,25</sup>

Most antileishmanials were shown to induce apoptosis features in *Leishmania*, which include an increase in intracellular calcium levels, an accumulation of reactive oxygen species (ROS), a drop of the mitochondrial membrane potential ( $\Delta\psi_m$ ), genomic DNA degradation, exposure of phosphatidyl serine and induction of caspase-like activity.<sup>7,16,17,26,27</sup> Interestingly, the antimony-resistant phenotype of field isolates was reported to be accompanied by a decreased propensity to undergo apoptosis following exposure to either SbIII, MF or AMB.<sup>7</sup> Therefore, it is possible that the common mechanisms involved in cell killing described for

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**Abbreviations:**  $\Delta\psi_m$ , mitochondrial membrane potential; AMB, amphotericin B; DCFDA, dichlorofluorescein diacetate; GSH, glutathione; MF, miltefosine; MitoSOX, red mitochondrial superoxide indicator; MTX, methotrexate; PARO, paromomycin; ROS, reactive oxygen species; TMRE, tetramethylrhodamine ethyl ester

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bactericidal antibiotics might hold true for leishmanicidal compounds and that a decrease in sensitivity to one drug could favor the development of resistance against other drugs.

In this study, we sought to determine the putative apoptotic features induced by SbIII, MF, AMB and PARO in *Leishmania infantum* drug-sensitive and drug-resistant parasites, and to assess whether an increased tolerance to apoptosis might accompany the selection of drug resistance. Our results are suggesting two types of drug-induced death in *Leishmania*, and are linking the mode of killing to tolerance to cell death and to a facilitated emergence of multidrug resistance.

## Results

**Drug-induced accumulation of ROS in the *Leishmania* mitochondria.** As several drugs were shown to induce apoptosis in *Leishmania*, we first studied the accumulation of ROS upon drug exposure in the context of drug sensitivity and resistance. The *L. infantum* Sb2000.1, MF200.5, AMB1000.1 and PARO1200.1 cell lines were selected for resistance to SbIII, MF, AMB and PARO, respectively, in a stepwise manner until they reached at least a 10-fold increased resistance compared with the parental clonal strain (Table 1). None of the mutants showed marked cross-resistance, with the exception of the SbIII- and PARO-resistant mutants, which were five to six times hypersensitive to MF (Table 1). The accumulation of ROS was measured using the dichlorofluorescein diacetate (DCFDA) dye whose fluorescence intensity is indicative of the levels of intracellular ROS.<sup>28</sup> *L. infantum*-sensitive parasites subjected to increasing concentrations of SbIII, MF and AMB exhibited a dose-dependent increase in DCFDA fluorescence signals (Figure 1), confirming increased ROS production upon exposure to these antileishmanials. Pentamidine, another anti-*Leishmania* drug, also induced the accumulation of ROS in sensitive *L. infantum* (Supplementary Figure S1A) and so was the classical apoptosis-inducing drug staurosporine<sup>29</sup> (Supplementary Figure S1C). In contrast, no ROS production could be measured by DCFDA fluorescence in the resistant mutants Sb2000.1, MF200.5 and AMB1000.1 exposed to the drug defining their resistance phenotype (Figure 1). PARO behave differently as it failed to induce ROS accumulation irrespective of the PARO susceptibility status of the parasites (Figure 1). This was not unique to

PARO as we found that methotrexate (MTX), a model drug often studied in *Leishmania*,<sup>30,31</sup> also failed to induce ROS in *Leishmania* (Supplementary Figure S1B).

As the major source of oxidants in eukaryotes is the mitochondria, we used the MitoSox dye (red mitochondrial superoxide indicator) to measure the mitochondrial accumulation of superoxide, which is an indicative measure of ROS level in the mitochondria.<sup>32</sup> As expected, SbIII, MF and AMB induced an increase in MitoSox fluorescence levels in sensitive but not resistant parasites (Figure 2a). Colocalization experiments between DCFDA and mitotracker fluorescence signals further confirmed that the mitochondria were the site of drug-induced ROS accumulation (Figure 2b). The accumulation of superoxide within the mitochondria usually results in a misregulation of cellular iron homeostasis through the oxidation of iron-sulfur clusters, which translates into the production of highly active hydroxyl radicals via the Fenton reaction.<sup>4,33</sup> The role of iron in the drug-induced accumulation of oxidants was confirmed by the reduced level of ROS produced within the mitochondria of sensitive *L. infantum* pretreated with the iron-chelating agent deferoxamine before being exposed to increasing concentrations of SbIII, MF and AMB (Figure 2c). The same protective effect against the accumulation of ROS was observed in *L. infantum* parasites pretreated with the antioxidant glutathione (GSH) (Figure 2c).

Every experiments presented so far were performed with the more experimentally tractable promastigote insect stage of the parasite. Accordingly, we tested whether a similar drug-induced ROS accumulation could be observed with the more clinically relevant intracellular amastigotes. MitoSOX-preloaded promastigotes were used to infect macrophages and the resulting intracellular sensitive parasites, but not their resistant counterparts, accumulated ROS when exposed to SbV (the prodrug that is converted to SbIII), MF or AMB (Figure 2d).

**Tolerance to drug-induced apoptosis in drug-resistant *Leishmania*.** As the accumulation of oxidants within the mitochondria is one of the hallmarks of apoptosis including in parasites,<sup>34,35</sup> we tested whether other apoptotic features could be observed following exposure to different antileishmanials. We monitored changes in the  $\Delta\psi_m$  using the tetramethylrhodamine ethyl ester (TMRE) dye,<sup>7</sup> a cationic fluorescent molecule whose accumulation within the mitochondria is an indicative measure of the  $\Delta\psi_m$ , and tested for genomic DNA degradation by gel electrophoresis.

**Table 1** Susceptibility of *L. infantum* WT and drug-resistant mutants to SbIII, MF, AMB, PARO and MTX

	SbIII ( $\mu$ M)	MF ( $\mu$ M)	AMB ( $\mu$ M)	PARO ( $\mu$ M)	MTX ( $\mu$ M)
WT	49.6 $\pm$ 9.6	16.3 $\pm$ 5.4	0.040 $\pm$ 0.013	80.9 $\pm$ 39.3	255 $\pm$ 23
Sb2000.1	>2000**	3.3 $\pm$ 0.5*	0.045 $\pm$ 0.028	142.8 $\pm$ 15.7*	ND
MF200.5	116 $\pm$ 24*	>200**	0.055 $\pm$ 0.017	104.3 $\pm$ 21.4	ND
AMB1000.1	97 $\pm$ 3*	21 $\pm$ 2.6	>1000**	110.7 $\pm$ 24.4	ND
PARO1200.1	50 $\pm$ 1.4	2.8 $\pm$ 0.4*	0.040 $\pm$ 0.012	>1680**	212 $\pm$ 18
MTX1000.3	ND	ND	ND	60 $\pm$ 17.6	>1000**

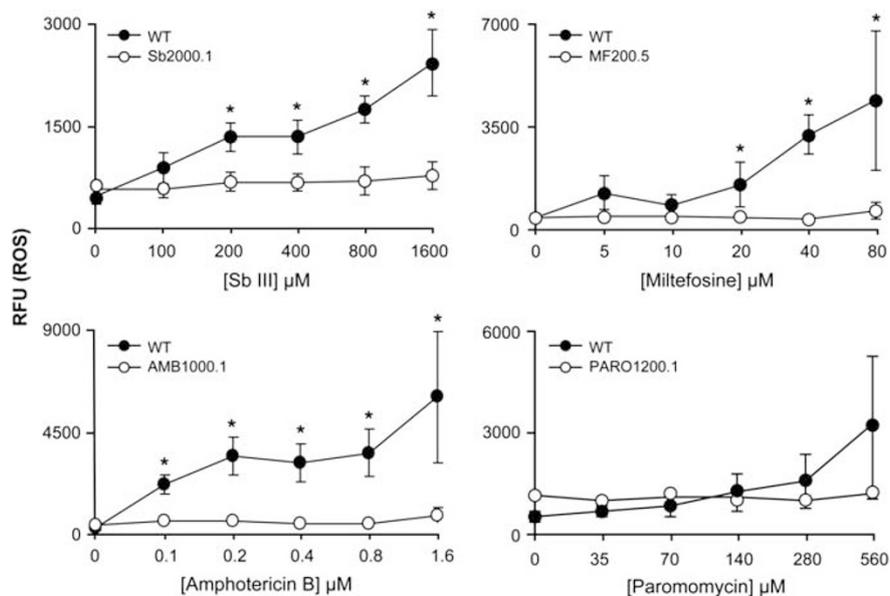
Abbreviations: AMB, amphotericin B; MTX, methotrexate; MF, miltefosine; ND, not determined; PARO, paromomycin; WT, wild-type

\* $P < 0.05$ ; \*\* $P < 0.01$

EC<sub>50</sub> were determined after 72 h of culture in the presence of various drug concentrations as described<sup>28</sup>

Results are the average of at least three independent experiments

Paired Student's *t*-test (\* $P < 0.05$ ; \*\* $P < 0.01$ )



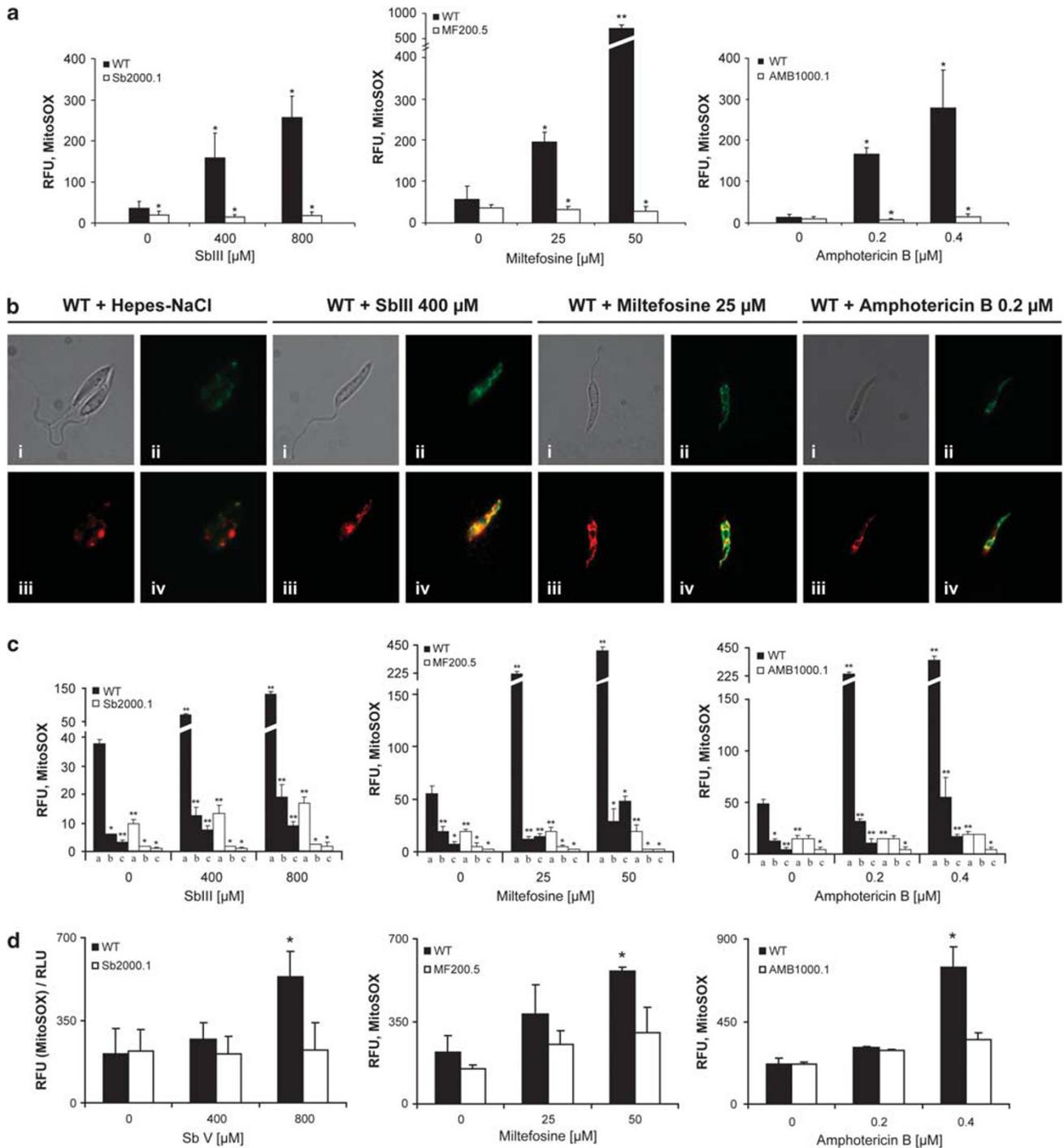
**Figure 1** Drug-induced ROS in *Leishmania infantum* wild-type and drug-resistant mutants. Drug-induced ROS accumulation in *L. infantum* cells. *L. infantum* WT or *L. infantum* Sb.2000.1, MF200.5, AMB1000.1 and PARO1200.1 log-phase cultures, selected for resistance to SbIII, MF, AMB and PARO, respectively, were exposed to increasing concentrations of drugs for 48 h. Aliquots (500  $\mu$ l) were washed and incubated in the presence of DCFDA for 30 min. Fluorescence was measured at 530 nm and normalized according to protein concentrations. Results are the average of three independent experiments. \* $P < 0.05$

A significant drop in the  $\Delta\psi_m$  was observed in sensitive cells exposed to SbIII, MF and AMB, but not with PARO (Figure 3a). Similarly, drug-induced DNA laddering occurred in *L. infantum* wild-type cells treated with SbIII, MF and AMB, but not with PARO (Figure 3b). Interestingly, neither a decrease in  $\Delta\psi_m$  nor a degradation of genomic DNA could be observed in the drug-resistant mutants exposed to their selective drugs (Figures 3a and b). In contrast, the protonophore carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone remained potent at inducing a significant loss in  $\Delta\psi_m$  in these mutants (Figure 3a), suggesting that the proton gradient was still intact in the resistant parasites.

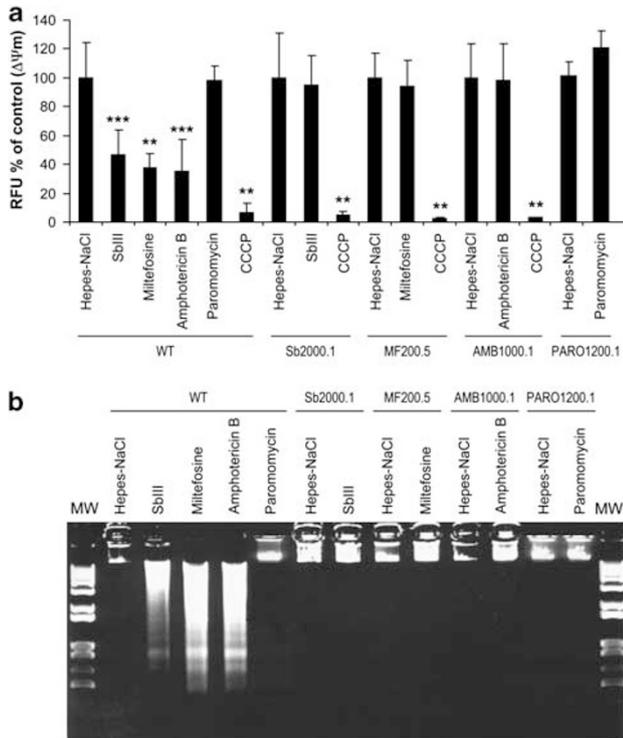
Having found that SbIII, MF, AMB, but not PARO, induced several apoptosis features in drug-sensitive parasites but not in the Sb2000.1-, MF200.5- and AMB1000.1-resistant lines, respectively, we sought to understand whether a non-specific tolerance to drug-induced apoptosis occurred in these mutants. Interestingly, the Sb2000.1-, MF200.5- or AMB1000.1-resistant mutants failed to produce ROS when challenged with the drug used for their selection, and there was also a significant lack of ROS production in these mutants upon challenge with any of the ROS-inducing drugs (Figure 4a). Again, the PARO1200.1 mutant behave differently as ROS levels comparable to wild-type cells were observed in this mutant after a challenge with SbIII and even higher levels were observed after a challenge with MF or AMB (Figure 4a). The drop in  $\Delta\psi_m$  following exposure either to SbIII, MF or AMB was also lower in the Sb2000.1, MF200.5 and AMB1000.1 mutants than in the sensitive cells or the PARO1200.1 mutant (Figure 4b). The modulation in ROS production and change in  $\Delta\psi_m$  were further correlated with drug-induced laddering of genomic DNA. Indeed, no DNA laddering was induced in the Sb2000.1, MF200.5 and

AMB1000.1 mutants following exposure either to SbIII, MF or AMB, whereas the same drugs induced oligonucleosomal fragmentation in the PARO1200.1 mutant (Figure 4c). We also tested whether this phenomenon of cross-tolerance to drug-induced apoptosis occurred with intracellular amastigotes. We measured ROS in parasites infecting macrophages and we found that the Sb2000.1, MF200.5 and AMB1000.1, but not PARO1200.1, failed to produce more ROS than the control, when challenged with ROS-inducing drugs (Figure 4d). As GSH was shown to protect cells against drug-induced ROS (Figure 2c) and that GSH has been linked to SbIII resistance,<sup>36–39</sup> we tested whether cells resistant to ROS-inducing drugs had increased levels of reduced thiols. However, analysis by HPLC failed to show a significant increase of reduced thiols in the drug-resistant mutants (results not shown).

**Tolerance to drug-induced cell death favors the acquisition of multidrug resistance.** We showed that the tolerance to drug-induced apoptosis in the Sb2000.1, MF200.5 and AMB1000.1 lines occurred not only against the drug initially used for the selection of resistance but also extended to any apoptosis-inducing drugs. We hypothesized that this phenomenon may facilitate the emergence of multi-resistance against other drugs, provided that these shared similar killing effector mechanisms. We thus compared the rates of selection of resistant parasites by plating  $5 \times 10^6$  drug-sensitive or drug-resistant parasites in the presence of MF, AMB or PARO at a concentration inhibiting the growth of *L. infantum* wild-type parasites. SbIII was omitted as the susceptibility to this drug varied between mutants (Table 1). The AMB1000.1, MF200.5, PARO1200.1 and MTX1000.3 mutants yielded from  $6.9 \times 10^4$  to  $1.3 \times 10^5$  colonies, and a plating efficiency of  $\sim 10\%$  when plated with their respective



**Figure 2** Drug-induced ROS accumulation in *Leishmania infantum* mitochondria. (a) Log-phase cultures of *L. infantum* WT or *L. infantum* Sb.2000.1, MF200.5 and AMB1000.1 drug-resistant mutants were preloaded with MitoSOX for 2 h, washed and exposed to two concentrations of SbIII, MF or AMB for 48 h. Aliquots (500  $\mu$ l) were taken, washed and the fluorescence was measured at 580 nm. (b) ROS accumulation in *Leishmania* cells was visualized with the fluorescent dye DCFDA and the mitochondria-specific dye Mitotracker. Log-phase cultures were exposed to drugs for 48 h, washed and incubated with DCFDA and Mitotracker for 30 min each. Fluorescence microscopy showed colocalization of the oxidant-dependent DCFDA and the mitochondria-specific Mitotracker in drug-treated cells. (i) Brightfield, (ii) DCFDA fluorescence, (iii) Mitotracker fluorescence, (iv) Merge. (c) Protection against drug-induced mitochondrial ROS accumulation. Log-phase cultures of WT and drug-resistant mutants were preloaded with MitoSOX for 2 h, washed, preincubated with Hesper-NaCl (bars a), 500  $\mu$ M of deferoxamine (bars b), or with 2 mM GSH (bars c) for 24 h, washed and exposed to two concentrations of SbIII, MF or AMB for 48 h. Aliquots (500  $\mu$ l) were taken, washed and the fluorescence was measured at 580 nm. (d) Drug-induced ROS accumulation within intramacrophagic *L. infantum*. *L. infantum* WT (black bars), Sb2000.1 (white bars, left panel), MF200.5 (white bars, central panel) and AMB1000.1 (white bars, right panel) promastigotes containing a luciferase expression vector were preloaded with MitoSOX, and subsequently used to infect J774 macrophages. The drug-induced ROS accumulation in intracellular parasites was measured 48 h post infection, and fluorescence (RFU) was normalized to luciferase activity level (RLU). Results are the average of at least three independent experiments. \* $P < 0.05$ ; \*\* $P < 0.01$



**Figure 3** Drug-induced apoptosis in *Leishmania*. Drug-induced loss of  $\Delta\psi_m$  (a) and gDNA laddering (b) in sensitive and resistant *Leishmania* cells. Log-phase cultures were exposed to drugs (SbIII, 400  $\mu$ M; MF, 25  $\mu$ M; AMB, 0.2  $\mu$ M; and PARO, 280  $\mu$ M) or to saline buffer (control) for 48 h. (a) For measuring  $\Delta\psi_m$ , a 500- $\mu$ l aliquot was washed, incubated with TMRE for 30 min, washed and the fluorescence was measured (545 nm). Fluorescence was normalized to protein concentrations. (b) Genomic DNA degradation was investigated by analyzing the isolated gDNA on 1.3% agarose gels. Results represent the average of at least three independent experiments and are expressed as percentage of the control. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . MW: 1 kb Plus Ladder (Invitrogen)

drugs (Figure 5). In contrast, the wild-type cells did not yield any colony when plated in the presence of MF or PARO, and on an average 14 colonies were counted on the AMB plates (Figure 5a). Remarkably, the *L. infantum* Sb2000.1 and MF200.5 mutants generated significantly more colonies on the AMB plate than the *L. infantum* wild-type strain or the PARO1200.1 mutant (Figure 5a). Similarly, the AMB1000.1 mutant yielded significantly more colonies under MF pressure than wild-type parasites or the PARO 1200.1 mutant (Figure 5a). However, the Sb2000.1 mutant behaved unexpectedly, as few colonies were observed on MF-containing plates (Figure 5a). This is probably due to the fact that Sb2000.1 is fivefold hypersensitive to MF (Table 1).

In the presence of PARO, the MF200.5 and AMB1000.1 mutants did not yield significantly more colonies than wild-type parasites (Figure 5a). Again, the Sb2000.1 behaved in an unexpected manner, producing a very high number of colonies on PARO plates (Figure 5a). This observation might be explained in part by the fact that the Sb2000.1 mutant initially exhibited a slight but significant cross-resistance to PARO (Table 1). Although PARO and MTX did not induce ROS in wild-type parasites (Figure 1 and Supplementary Figure S1), it is not clear whether similar killing effector

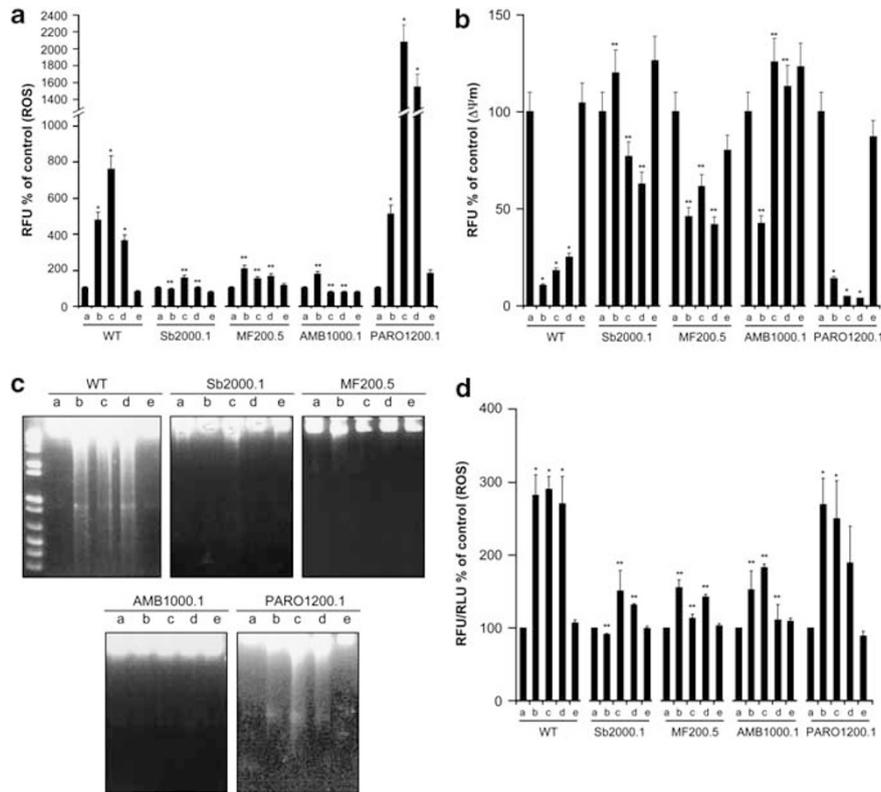
mechanisms are shared by both drugs. Nonetheless, it is intriguing that the MTX-resistant mutant yielded more colonies than the MF200.5 mutant or wild-type parasites in the presence of PARO (Figure 5b), and that the PARO1200.1 mutant also yielded a higher number of colonies on MTX-containing plates than wild-type or MF200.5 cells (Figure 5b).

## Discussion

Our study has revealed that anti-*Leishmania* compounds kill by at least two different mechanisms, with SbIII, AMB, MF and most likely pentamidine killing by apoptosis, and PARO and the model drug MTX acting through another lethal mechanism. In order to be classified as apoptosis-inducing drugs, compounds were required to fulfill a number of criteria, including the induction of mitochondrial ROS, a drop in  $\Delta\psi_m$  and genomic DNA degradation. Several studies had already reported on the role of redox pathways in the increased survival of *Leishmania* in the presence of SbIII,<sup>18,38,39</sup> with iron potentiating the antileishmanial activity of SbIII.<sup>4</sup> Our results indicate that this is probably valid for all drugs inducing ROS, as experiments under iron-depleted conditions demonstrated a key role for iron in drug-induced ROS production (Figure 2c).

The dichotomy in the mode of killing between antileishmanials was further reinforced by the observation that cells resistant to SbIII, MF and AMB were tolerant to apoptosis not only against the initial selective drug but also against all drugs sharing a similar mode of killing. This tolerance is likely to arise by alterations in shared death pathways. Obviously, alterations in these pathways do not lead to high-level cross-resistance (Table 1) but can contribute to a decreased propensity to undergo apoptosis, thereby favouring the emergence of more specific resistance mechanisms. For example, although no specific mutations leading to a high level of AMB resistance are present in Sb2000.1 or MF200.5 cells (Table 1), an alteration in a death pathway allowing tolerance to AMB-induced apoptosis may likely be shared by these mutants (Figure 4). In contrast, as PARO kills through a different mechanism, we did not observe tolerance to SbIII-, MF- or AMB-induced apoptosis in the PARO-resistant mutant (Figure 4). We are currently searching for phenotypic features related to PARO and MTX killing as this would allow to test whether non-ROS-producing drugs constitute a distinct class of unrelated compounds that also share similar killing mechanisms.

Such tolerance to drug-induced killing accelerated the emergence of cross-resistance. Indeed, the SbIII-, MF- and AMB-resistant mutants, but not the PARO-resistant mutant, became more easily cross-resistant to the apoptosis-inducing drugs SbIII, MF and AMB. Conversely, the PARO- and MTX-resistant mutants acquired cross-resistance to the non-ROS-producing drugs more easily. Two exceptions were noted, both involving the Sb2000.1 mutant when plated with either MF or PARO. This could possibly be explained by collateral hypersusceptibility or cross-resistance already present in this mutant (Table 1), where a mutation leading to resistance or susceptibility may supersede alteration(s) in a death pathway when cross-resistance is selected. As SbIII interferes with the thiol redox metabolism,<sup>18</sup> it is possible that oxidant-induced



**Figure 4** Cross-tolerance to drug-induced apoptosis in *Leishmania* drug-resistant mutants. The accumulation of ROS (**a** and **d**), loss of  $\Delta\psi_m$  (**c**) and gDNA degradation (**b**) were measured in *Leishmania* WT and resistant mutants (Sb2000.1, MF200.5, AMB1000.1 and PARO1200.1). Promastigotes log-phase cultures (**a**, **b** and **c**) were left untreated (controls, bars a) or exposed to 400  $\mu\text{M}$  SbIII (bars b), 25  $\mu\text{M}$  MF (bars c), 0.2  $\mu\text{M}$  AMB (bars d) or 280  $\mu\text{M}$  PARO (bars e) for 48 h. Aliquots were washed and incubated in the presence of (**a**) DCFDA (ROS) or (**b**) TMRE ( $\Delta\psi_m$ ) for 30 min. Fluorescence was measured at 530 nm (DCFDA) or 545 nm (TMRE) and normalized to protein concentrations. (**c**) Genomic DNA was analyzed on 1.3% agarose gels MW: 1 kb Plus Ladder (Invitrogen). (**d**) Accumulation of ROS were measured in intracellular *Leishmania* wild-type cells and drug-resistant mutants. Purified metacyclics were preloaded with DCFDA and used to infect THP-1 macrophages. Infected cells were then left untreated (controls, bars a) or treated with 150  $\mu\text{M}$  of Pentostam (SbV) (bars b), 1  $\mu\text{M}$  of MF (bars c), 0.05  $\mu\text{M}$  of AMB (bars d) or 30  $\mu\text{M}$  of PARO (bars e). Results are the average of three independent experiments and are expressed as percentage of controls (**a**, **b** and **d**). \* $P < 0.01$ ; \*\*statistically different from \* with  $P < 0.01$

mutational events occurred specifically in the Sb2000.1 mutant and led to increased PARO resistance (Figure 5a). This would be reminiscent of bactericidal antibiotic-induced mutagenesis in bacteria.<sup>40</sup>

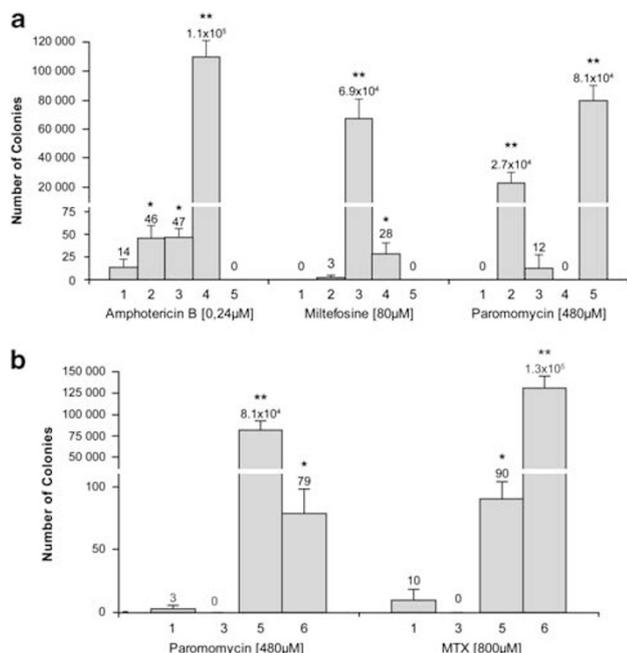
The present results suggest that *Leishmania* parasites resistant to SbIII, MF or AMB in the field could be more prone at acquiring multidrug resistance to these antileishmanials, while retaining sensitivity to PARO. These conclusions are supported by the observation that field isolates resistant to SbIII were less prone to undergo apoptosis following exposure to either SbIII, MF or AMB.<sup>7</sup> A recent study also suggested the possible cross-resistance of *L. donovani* field isolates to SbIII, MF and AMB in areas of endemicity.<sup>41</sup> Drug combination is now an important strategy in our fight against infectious diseases and this has now recently been shown to be effective in *Leishmania*.<sup>42</sup> Our data provide a rationale for combining drugs with different modes of killing.

In summary, there are at least two effector mechanisms for anti-*Leishmania* drugs. Resistance is accompanied by tolerance to drug-induced apoptosis not only against the selective drug but also against drugs sharing a similar mode of killing. This cross-tolerance leads to a facilitated emergence of cross-resistance against other drugs that have different cellular targets but similar mode of killing.

## Materials and Methods

**Leishmania strains and culture conditions.** *L. infantum* (MHOM/MA/67/ITMAP-263) and the drug-resistant mutants *L. infantum* Sb2000.1, *L. infantum* MF200.5, *L. infantum* AMB1000.1 and *L. infantum* PARO1200.1 (resistant to 2000  $\mu\text{M}$  SbIII, 200  $\mu\text{M}$  MF, 1 mM AMB and 1680  $\mu\text{M}$  PARO, respectively) were grown in SDM-79 medium supplemented with 10% fetal bovine serum at 25 °C. Drug susceptibilities were obtained as described. Log-phase parasites were preincubated with deferoxamine (500  $\mu\text{M}$ ) or GSH (2 mM) for 24 h before being washed and subsequently exposed to drugs for 48 h. To determine apoptotic features (ROS,  $\Delta\psi_m$  and gDNA laddering), log-phase cultures were exposed to toxic drug concentrations for 48 h in order to trigger apoptosis.

**Measurement of ROS accumulation.** Intracellular ROS accumulation was measured using the DCFDA dye (Invitrogen, Burlington, Ontario, Canada) as described.<sup>28</sup> Briefly, DCFDA is cell permeant and non-fluorescent. Upon its entry into live cells, the diacetate groups are cleaved by cellular esterases. Oxidation of the reduced dye occurs in the presence of ROIs, causing fluorescence. After drug exposure (48 h), parasites were centrifuged at 3000 r.p.m., washed, resuspended in 500  $\mu\text{l}$  of HEPES-NaCl (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 6 mM glucose, pH 7.4) and incubated for 30 min in presence of DCFDA (40 nM). After washing, 200  $\mu\text{l}$  of the promastigote resuspension was analyzed with a Victor fluorometer (Perkin-Elmer, Turku, Finland) at 485 nm excitation and 535 nm emission wavelengths. Fluorescence was normalized with proteins concentration measured using the 2D QuantKit (GE Healthcare, Quebec, Canada). A similar procedure was used with the MitoSOX dye (Invitrogen, Ontario, Canada) to determine the ROS accumulation occurring within the mitochondrion. MitoSOX is a live-cell permeant, and rapidly and selectively targets the



**Figure 5** Accelerated selection of multidrug-resistant *Leishmania* cells. Wild-type parasites or drug-resistant mutants ( $5 \times 10^6$  cells) were plated on drug-containing SDM-agar plates and colonies were counted after 14 days. (a) Wild-type (WT) cells and mutant cell lines Sb2000.1, MF200.5, AMB1000.1 and PARO1200.1 were plated on AMB-, MF- and PARO-containing plates. (b) Wild-type cells and mutant cell lines MF200.5, PARO1200.1 and MTX1000.3 were plated on PARO- and MTX-containing plates. Results are the average of three independent experiments. 1, WT; 2, Sb2000.1; 3, MF200.5; 4, AMB1000.1; 5, PARO1200.1; 6, MTX1000.1. \* $P < 0.05$ ; \*\* $P < 0.01$

mitochondria. Once in the mitochondria, the MitoSOX probe is oxidized by superoxide, hence, exhibiting a red fluorescence (580 nm).

**Measurement of ROS accumulation in intracellular amastigotes.** Luciferase-containing promastigotes were harvested in stationary growth phase (6 days), and metacyclics were purified using the peanut agglutinin assay.<sup>43</sup> Briefly, 10 ml of stationary-phase cultures were centrifuged at 3000 r.p.m., washed and resuspended in 10 ml Hepes–NaCl containing 100 μg/ml of the lectin peanut agglutinin (Sigma-Aldrich, Ontario, Canada), and incubated with agitation for 30 min at room temperature. The mixture was then centrifuged at 900 r.p.m., the metacyclic-containing supernatants were collected and centrifuged at 3000 r.p.m., and the pellet was washed and resuspended in appropriate Hepes–NaCl volume. Purified metacyclics were preincubated with 40 nM of DCFDA for 20 min, washed and used for subsequent macrophages infection as described.<sup>44</sup> At 3 h after infection, macrophages were washed to remove non-phagocytized parasites. Drugs at EC<sub>50</sub> levels: Pentostam (SbV, 150 μM), MF (1 μM), AMB (0.05 μM) or PARO (30 μM) were added. After 2 days, DCFDA fluorescence was taken to measure oxidants accumulation within the amastigotes parasites. Macrophages were then lysed and processed in order to measure luminescence from luciferase-expressing amastigotes as a measure of survival to normalize oxidants-induced fluorescence.

**Measurement of Δψ<sub>m</sub> and gDNA laddering.** Δψ<sub>m</sub> was measured using the cationic lipophilic fluorescent TMRE dye as described.<sup>7</sup> Fluorescence was normalized with proteins concentrations as described above. gDNA was harvested from untreated and treated parasites using a method previously described.<sup>7</sup> In all, 10 μg of gDNA was loaded on a 1.3% agarose gel and was allowed to migrate at 90 V for 1 h.

**Mitochondrial localization of drug-induced ROS using fluorescence microscopy.** After drug exposure (48 h), parasites were centrifuged at 3000 r.p.m., washed and resuspended in 500 μl of Hepes–NaCl, and were coincubated for 30 min in presence of both DCFDA (40 nM) and Mitotracker

(40 nM). Parasites were subsequently centrifuged at 3000 r.p.m., washed and resuspended in 500 μl of Hepes–NaCl, and 20 μl of parasite suspension was layered on poly-L-lysine-coated microscope slides under a cover slip. Slides were analyzed using a fluorescence microscope (Nikon eclipse TE300 fluorescence microscope; Nikon, Ontario, Canada) using typical green and red filters. Images were captured using Image Pro Plus 5.0 software (Media Cybernetics, Inc., Bethesda, MD, USA). At least 20 microscopic fields were observed for each sample.

**Cross-resistance selection on drug-containing SDM-agar plates.** Equal number ( $5 \times 10^6$ ) of wild-type or drug-resistant parasites were centrifuged at 3500 r.p.m., washed two times in Hepes–NaCl and resuspended in 50 μl Hepes–NaCl, and subsequently plated on SDM-agar plates containing either 0.24 μM of AMB, 80 μM of MF or 480 μM of PARO. These concentrations, which corresponded to six times the EC<sub>50</sub> of the wild-type strain, were the lowest allowing minimal growth of wild-type parasites. Colonies were counted after 14 days. Plating was repeated at least three independent times.

### Conflict of Interest

The authors declare no conflict of interest.

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