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Apoptosis is induced in leishmanial cells by a novel protein kinase inhibitor withaferin A and is facilitated by apoptotic topoisomerase I–DNA complex

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Protein kinase C (PKC) is an important constituent of the signaling pathways involved in apoptosis. We report here that like staurosporine, withaferin A is a potent inhibitor of PKC. In *Leishmania donovani*, the inhibition of PKC by withaferin A causes depolarization of $\Delta\Psi_m$ and generates ROS inside cells. Loss of $\Delta\Psi_m$ leads to the release of cytochrome *c* into the cytosol and subsequently activates caspase-like proteases and oligonucleosomal DNA cleavage. Moreover, in treated cells, oxidative DNA lesions facilitate the stabilization of topoisomerase I-mediated cleavable complexes, which also contribute to DNA fragmentation. However, withaferin A and staurosporine cannot induce cleavable complex formation *in vitro* with recombinant topoisomerase I nor with nuclear extracts from control cells. Taken together, our results indicate that inhibition of PKC by withaferin A is a central event for the induction of apoptosis and that the stabilization of topoisomerase I–DNA complex is necessary to amplify apoptotic process.
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Apoptosis is thought to have evolved not only to regulate growth and development in multicellular organism, but also to guard against viral, bacterial, parasitic infection and the emergence of cancer.[1,2] It is postulated that in order to promote and maintain clonality within the population, the unicellular organism developed an altruistic mechanism to control growth via apoptosis.[3] The existence of apoptosis also could be useful to avoid killing by the host as suggested in the case of parasitic infection such as *Leishmania donovani*.[4]

Protein phosphorylation plays a key role in signal transduction in eucaryotic systems. Diacylglycerol activates Ca^{2+} and phospholipid-dependent protein kinase C (PKC) that is associated with many cellular processes in mammalian cells including differentiation, hormone and neurotransmitter release, and gene activation.[5,6] The protein kinase inhibitor staurosporine has been shown to induce programmed cell death (PCD) that involves alteration of the phosphorylation state of one or several cytosolic factors related to the signal transduction pathways.[7] Other biochemical alterations related to cell cycle control and DNA replication may also contribute to the staurosporine-induced apoptosis.[8,9] Staurosporine has been shown to inhibit cell cycle progression in a variety of cell lines. Staurosporine analogues (UCN-01[10–13] and CGP 41251[14]) with poor activity towards conventional PKC have been shown to interfere directly with the cell cycle machinery and to induce apoptosis. Staurosporine causes biochemical alterations in a variety of cell types irrespective of whether they expressed tumor suppressor genes p53 or Rb and leads to accumulations of CDK inhibitor $p^{27kip\ 1}$ and not of $p^{21Cip\ 10}$.

A steroidal lactone, Withaferin A (Figure 1), has been reported to have anti-tumor activity.[15,16] Earlier studies have shown that withaferin A at a concentration of 3 μM and above potently inhibits LPS-induced proliferation of B lymphocytes.[17] At 2 μM and above, withaferin A causes HUVECs (cell lines) to go into apoptosis as evidenced by the striking increase in intracellular pools of polyubiquinated proteins, which results in perturbation of many intracellular signaling pathways including activation of NF-$\kappa\beta$.[18,19] Withaferin A can alter cytoskeletal architecture by covalently binding annexin II and stimulating its basal F-actin crosslinking activity.[20]

Leishmania, a unicellular kinetoplastid protozoan parasite, is the causative agent of leishmaniasis. With the spread of human immunodeficiency virus (HIV), the parasites pose a much greater threat than before. To make the situation even worse, some parasite strains have developed resistance against the classical antimonial drugs, like sodium stibogluconate. The second line of drugs, amphotericin B and pentamidines although used clinically, are very toxic.[21] Therefore, improved drug therapy is still desirable and the need for newer intervention strategies is clear and justified. In search for such strategies, protein kinases of *Leishmania* offer the most attractive targets.

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Abbreviations: ROS, reactive oxygen species ; PI, propidium iodide; $\Delta\Psi$m, mitochondrial transmembrane potential; NAC, *N*-acetyl-cysteine; DMSO, dimethyl sulfoxide; CPT, camptothecin; DEVD-AFC, Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin; TUNEL, terminal deoxynucleotidyltransferase enzyme-mediated dUTP end labeling; VAD-fmk, Val-Ala-Asp-fluoromethyl ketone; FITC, fluorescein isothiocyanate; DTT, dithiothreitol; H_2DCFDA, 5-(and -6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate acetyl ester

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In the present study, we have shown for the first time that an anti-tumor agent withaferin A is a potent inhibitor of PKC like staurosporine. Unlike higher eucaryotes, inhibition of PKC inside leishmanial cells is sufficient to induce apoptosis like death in a previously unknown manner. We have analyzed nuclear, mitochondrial and cytosolic changes associated with apoptosis after inhibition of PKC. Finally, we provide evidences for the functional role of topoisomerase I–DNA complexes in chromatin fragmentation during withaferin A- and staurosporine-mediated apoptosis.

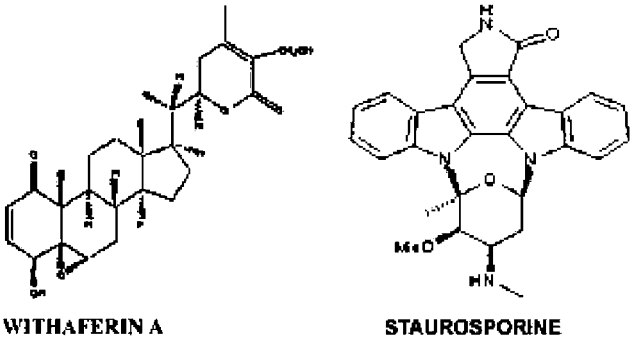

Figure 1 Structure of withaferin A and staurosporine

Results

Withaferin A inhibits PKC both *in vitro* and *in vivo*. Leishmanial cell extract prepared as described in Materials and Methods was tested for its activity to phosphorylate the peptide HCV (1487–1500).[22] It was observed that the phosphorylation of the peptide by leishmanial cell is decreased in a dose-dependent manner in the presence of withaferin A (Figure 2a). The extent of phosphorylation of the peptide by incorporation of ^{32}P-labeled Pi was monitored by scintillation counting and is shown in Figure 2b. At 5 μM concentration of withaferin A, the phosphorylation of the peptide was decreased to 50% (Figure 2a and b, lane 2) compared to untreated cell lysates (Figure 2a and b, lane 1), and at 10 μM, it was decreased to 74% (Figure 2a and b,

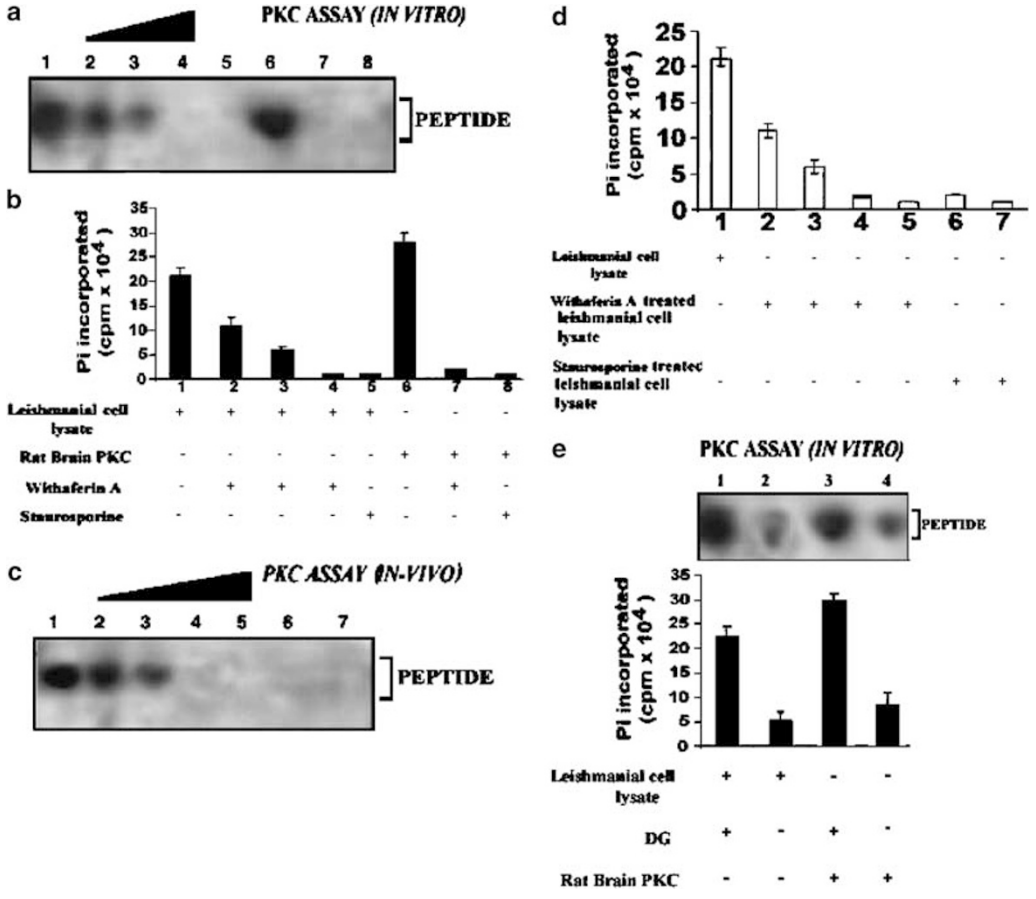

Figure 2 Inhibition of phosphorylation of the peptide (HCV (1487–1500)) by withaferin A and staurosporine. (**a**) Leishmanial cell lysate was analyzed for PKC activity in the absence of withaferin A (lane 1) and in the presence of 5 μM (lane 2), 10 μM (lane 3) and 15 μM (lane 4) of withaferin A and with 15 μM of staurosporine (lane 5). Kinase activity of rat brain PKC was assayed in the absence (lane 6) and in the presence of 15 μM withaferin A (lane 7) and 15 μM of staurosporine (lane 8) separately as described in Materials and Methods. (**b**) Incorporation of ^{32}P into the peptide was measured in differently treated cell lysates as mentioned in (**a**). (**c**) Leishmanial cells were treated with 0.2% DMSO for 14 h (lane 1), with 15 μM of withaferin A for 1 h (lane 2), 1.5 h (lane 3), 2 h (lane 4) and 3 h (lane 5) and with 15 μM of staurosporine for 1.5 h (lane 6) and 3 h (lane 7), and their cell lysates were used to measure kinase activities using the peptide substrate as described in Materials and Methods. (**d**) Incorporation of ^{32}P into the peptide was measured in differently treated cell lysates as mentioned in (**c**). (**e**) Incorporation of ^{32}P into the peptide by leishmanial cell lysates (lanes 1 and 2) and by rat brain PKC (lanes 3 and 4) in the presence (lanes 1 and 3) and in the absence (lanes 2 and 4) of DG

lane 3). At 15 μM concentration, no labeled peptide band was detected (Figure 2a and b, lane 4). As a positive control, we added staurosporine in leishmanial cell extracts and found that phosphorylation of the peptide is reduced to the extent of 92% (Figure 2a and b, lane 5). To prove whether withaferin A or staurosporine has direct inhibitory effects on rat brain PKC, we added withaferin A and staurosporine separately to the enzyme in the presence of this peptide substrate. It was observed that phosphorylation of the peptide is reduced by both withaferin A and staurosporine to the extent of 95% (Figure 2a and b, lane 7) and 98% (Figure 2a and b, lane 8), respectively, at 15 μM concentration compared to untreated cells (Figure 2a and b, lane 6).

To see whether withaferin A has the ability to inhibit PKC activity inside leishmanial cells, we treated exponentially growing leishmanial cells with withaferin A (15 μM) or staurosporine (15 μM) separately up to 8 h. At different time periods, cells were pelleted down and solubilized as described in Materials and Methods. The solubilized materials were tested for their activities to phosphorylate the peptide HCV (1487–1500) (Figure 2c and d). The phosphorylating ability of withaferin A-treated cell lysates was reduced to the extent of 50, 76, 84 and 92%, respectively, after 1 h (Figure 2c and d, lane 2), 1.5 h (Figure 2c and d, lane 3), 2 h (Figure 2c and d, lane 4) and 3 h (Figure 2c and d, lane 5) of treatment compared to healthy growing leishmanial cell extracts (Figure 2c and d, lane 1). Treatment with staurosporine reduced the phosphorylation of the peptide to the extent of 89% (Figure 2c and d, lane 6) and 95% (Figure 2c and d, lane 7) after 2 and 3 h, respectively. We found that the activity was reduced to the extent of 76% by leishmanial cell lysates and to the extent of 67% by rat brain PKC in the absence of DG in the reaction mixture (Figure 2e).

Withaferin A inhibits growth of *L. donovani* promastigotes, induces depolarization of mitochondrial membrane potential and releases cytochrome *c* into the cytosol. *L. donovani* AG83 promastigotes (2.5×10^6 cells/ml) were incubated with 15 μM of withaferin A for 7 h, following which the numbers of live promastigotes were counted by Trypan blue exclusion method. After 5 h, 70% growth was inhibited and this continued to the extent of 85% after 6 h of incubation and after 7 h only 2% live promastigotes were present (Figure 3a). To investigate the mode of cell death, we stained both treated and untreated cells with annexin V and PI. Apoptotic cells have condensed nuclei that are negative for PI and have integrated plasma membrane but PS translocates on the outer membrane, which can be detected by staining with annexin V. After 4 h of treatment with withaferin A, more than 25% cells undergo apoptosis and this was increased to the extent of 67% after 6 h. We also detected an increase in the number of annexin V- and PI-positive cells representing necrotic cells,

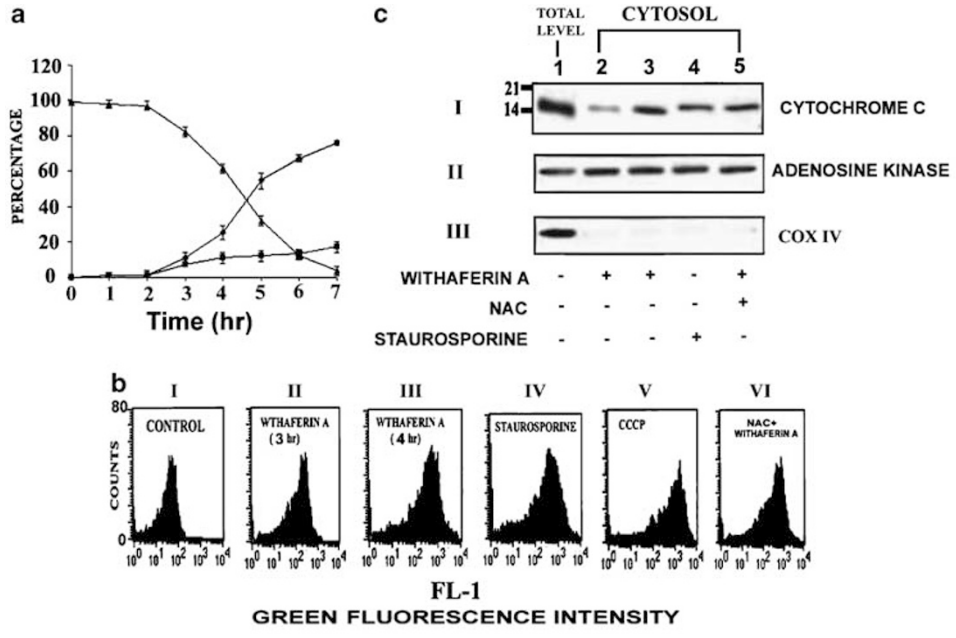

Figure 3 Treatment with withaferin A increases the number of apoptotic cells, and induces loss of mitochondrial membrane potential and release of cytochrome *c* into the cytosol. (**a**) *L. donovani* promastigotes were cultured in the presence of 15 μM withaferin A for 7 h. Percentage of viable promastigotes (▲) was measured by Trypan blue exclusion method. Percentage of apoptotic cells (◆) and necrotic cells (■) was measured during treatment with 15 μM of withaferin A for different time periods by double staining with annexin V and PI as described in Materials and Methods. Data are expressed as mean $\pm$ S.D. of three independent experiments. (**b**) Flow cytometry analysis of mitochondrial membrane potential after treatment with 0.2% DMSO alone (I), 15 μM withaferin A for 3 h (II) and 4 h (III). This was also determined after treatment with staurosporine (IV) and CCCP (V) alone and with antioxidant like NAC, before treatment with withaferin A (VI). FL1 channel indicates mean green fluorescence intensity of JC1. (**c**) Western blot analysis of the release of cytochrome *c* into the cytosol of differently treated cells. As loading controls, cytosolic fractions from differently treated cells were analyzed for the presence of adenosine kinase (II) and COX IV (III) by Western blotting. Lane 1: total level of cytochrome *c* (I), adenosine kinase (II) and COX IV (III) in untreated leishmanial cells; lanes 2 and 3: cells treated with withaferin A for 2 and 3 h, respectively; lane 4: cells treated with staurosporine for 2 h; lane 5: cells treated with NAC before treatment with withaferin A. Position of molecular weight markers ($\times 10^{-3}$) is shown in the left of top panel

concomitant with a decrease in the number of live (annexin V- and PI-negative) cells. The number of necrotic cells remained more or less 10% throughout the experiment (Figure 3a).

To investigate whether $\Delta\Psi_m$ is lost in withaferin A-treated cells during apoptosis, a time-course study of $\Delta\Psi_m$ was performed with withaferin A or staurosporine. $\Delta\Psi_m$ was measured with the mitochondrial membrane potential sensitive dye JC1, which undergoes a transition from molecular aggregation to molecular monomer formation that can be detected by a shift in fluorescence from red to green during depolarization of mitochondrial membrane potential.[23] As shown in Figure 3b, on exposure of leishmanial cells to 15 μM withaferin A for 3 h (II) and 4 h (III) and to 15 μM staurosporine for 3 h (IV), the uptake of this dye by the mitochondria decreased compared to control cells (I) and this was measured by an increase in green fluorescence intensity in the cytosol. When cells were treated with antioxidants like NAC, before the treatment with withaferin A, depolarization of $\Delta\Psi_m$ is not prevented (VI). This was evidenced by the fact that there is no shift in total cell population to the right side of FL-1 channel (Figure 3b). Our results suggest that ROS are not responsible for the loss of $\Delta\Psi_m$.

Cytochrome *c* is a component of the mitochondrial electron transport chain and is present in the inter-membrane space. Disruption of the outer mitochondrial membrane by apoptotic stimuli results in the release of cytochrome *c* into the cytoplasm where it initiates activation of caspases like proteases leading to apoptosis.[23] We have shown by Western blotting that withaferin A or staurosporine treatment causes cytochrome *c* accumulation in the cytoplasm (Figure 3c, lanes 2–4). In addition, treatment with antioxidants like NAC before the treatment with withaferin A cannot prevent the release of cytochrome *c* into the cytosol (lane 5). As controls we have checked the presence of adenosine kinase (a constitutive cytosolic protein) and the absence of COX IV (a mitochondrial protein) in the cytosol of *L. donovani* promastigotes treated with or without withaferin A and staurosporine.

Loss of mitochondrial membrane potential induces formation of ROS inside cells, which causes a decrease in cellular GSH level and induces oxidative DNA lesions. When *L. donovani* promastigotes were treated with withaferin A, ROS are generated inside cells, which can be measured fluorimetrically by conversion of H_2DCFDA to highly fluoroscent 2,7-dichlorofluorescein. The level of ROS in withaferin A-treated cells remains four-fold higher compared to the level of ROS in control cells throughout the experiment (Figure 4a). When cells were treated with NAC before the treatment with withaferin A, the level of ROS generation was reduced to the extent of 60% compared to that in DMSO-treated control cells.

GSH is an important molecule for protecting kinetoplastids from ROS or toxic compounds and may induce a loss of $\Delta\psi_m$. As shown in Figure 4b, withaferin A causes 30% decrease in GSH level after 2 h and the effect was more pronounced after 4 h of treatment with withaferin A. When cells were preincubated with NAC, before treatment with withaferin A, the decrease in GSH level was protected significantly. Time kinetics analysis reveals that loss of $\Delta\Psi_m$ leads to generation of ROS inside cells.

Generation of ROS inside cells causes oxidative DNA lesions such as formation of oxidized bases, abasic sites and strand breaks.[24,25] Here, we investigated the generation of such lesions by withaferin A. Leishmanial cells were permeabilized and exposed to formamidopyridine DNA glycosylase (Fpg), an enzyme that converts oxidized purines (e.g. 8-oxoguanosine) into DNA single-strand breaks. By using the alkaline comet assay, we observed that Fpg-induced single-strand breaks in withaferin A-treated cells were increased compared to control cells treated with Fpg. These results suggest that treatment with withaferin A in leishmanial cells causes oxidative DNA damage. Treatment with NAC before treatment with withaferin A decreased the amount of single-strand breaks induced by withaferin A (Figure 4c).

Withaferin A- or staurosporine-induced oxidative DNA lesions cause topoisomerase I–DNA complex formation inside cells, but they do not inhibit the catalytic activity of topoisomerase I nor do they form the cleavage complexes *in vitro*. In order to find out whether withaferin A and staurosporine can stabilize covalent complexes of topoisomerase I and DNA in intact cells, we carried out immunoband depletion experiments with *L. donovani* promastigotes. Nuclear fraction was prepared from untreated as well as from drug-treated promastigotes and subjected to SDS-PAGE. If topoisomerase I can form a covalent complex with genomic DNA inside cells, then the complex cannot enter the gel. On the other hand, if topoisomerase I does not form a complex with DNA and remains free, it will enter the gel. The presence of topoisomerase I was detected by immunoblotting as described in Materials and Methods. The immunoband depletion data are summarized in Figure 5a. It was observed that the immunoband of topoisomerase I gradually disappeared when withaferin A was applied to the medium at 20 μM concentration for 3, 4 and 5 h (lanes 2–4). The extent of topoisomerase I immunoband depletion was similar to that obtained by 20 μM of staurosporine (lane 5) and 50 μM of CPT for 3 h (lane 6). Thus, we can surmise that withaferin A induces cleavable complex stabilization inside *L. donovani* promastigote cells. Treatment with NAC before treatment with withaferin A causes reappearance of immunoband of LdTOPIS, as cleavable complex formation is prevented (lane 7). The result suggests that oxidative stress is responsible for the stabilization of topoisomerase I–DNA complex inside cells. It should be mentioned here that topoisomerase I of *Leishmania* is a heterodimer and the catalytic site (SKXXY) is present in the small subunit, which is involved in the formation of topoisomerase I–DNA covalent complex. So we have used the antibody raised against the small subunit of topoisomerase I to study immunoband depletion assay.

To know whether withaferin A or staurosporine has the ability to interact directly with topoisomerase I *in vitro,* we carried out DNA relaxation assay (Figure 5b).[26] When added together with DNA and enzyme, withaferin A could not inhibit the relaxation of supercoiled DNA at concentrations ranging from 20 to 80 μM (lanes 3–6). Lane 2 shows the relaxation of supercoiled pHOT1 DNA (lane 1) by 2 U of topoisomerase I.

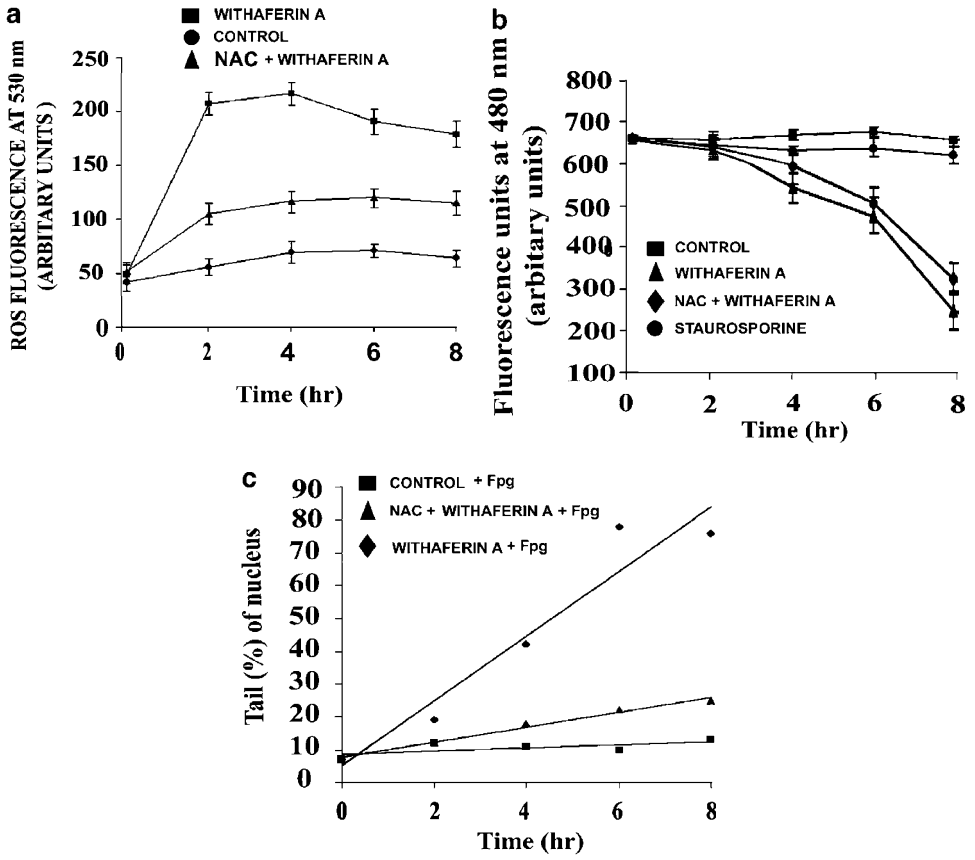

Figure 4 Withaferin A induces oxidative stress, causes decrease in GSH level and leads to subsequent DNA lesions. (**a**) Generation of ROS was measured using fluoroscent dye H_2DCFDA after treatment with 0.2% DMSO alone (●), 15μM withaferin A (■) and with NAC before treatment with withaferin A (▲). Data are expressed as mean $\pm$ S.D. of three independent experiments. (**b**) The intracellular GSH level was measured after treatment with 0.2% DMSO alone (■), 15μM withaferin A (▲) and 15μM staurosporine (●) separately and with NAC (◆) before treatment with withaferin A. (**c**) Leishmanial cells were treated with 0.2% DMSO (■), 15 μM of withaferin A for different time periods (◆) and with NAC before treatment with 15 μM of withaferin A (▲) and exposed to Fpg for 30 min. DNA single-strand breaks were analyzed by determining tail percentage using comet assay

Lanes 7 and 8 show that staurosporine also has no inhibitory effect on the activity of topoisomerase I. As a positive control, when CPT was added together with enzyme and DNA, the activity of topoisomerase I was inhibited almost to the extent of 80% (lane 9). CPT also has the ability to inhibit the relaxation of supercoiled DNA even in the presence of withaferin A (lane 10). This result suggests that withaferin A and staurosporine do not inhibit topoisomerase I *in vitro*.

To know whether withaferin A or staurosporine can stabilize covalent DNA–topoisomerase I complex *in vitro*, we performed cleavage assay as described in Materials and Methods. We found that withaferin A was unable to convert closed circular DNA (form I) to nicked circular DNA (form II) at concentrations ranging from 20 to 80 μM (Figure 5c, lanes 3–6). At 60 μM (lane 7) and 80 μM (lane 8) concentrations of staurosporine, form I DNA was not converted to form II DNA. Camptothecin, the well-studied topoisomerase I inhibitor, has been shown to stabilize the 'cleavable complex'.[27] At 50 μM concentration of CPT, closed circular DNA (form I) was converted to nicked circular DNA (form II) in the presence of 40 U of topoisomerase I (lane 9). CPT can also induce cleavable complex formation even in the presence of withaferin A (lane 10). Lane 2 shows the formation of nicked product when the covalent complex was trapped with SDS and proteinase K. This result shows that withaferin A or staurosporine cannot stabilize the 'cleavable complex' *in vitro.*

To understand whether withaferin A or staurosporine has any direct effect on LdTOPILS *in vivo,* we have isolated nuclear extracts from control cells as well as from withaferin A (15 μM)- or staurosporine (15 μM)-treated cells and relaxation assays were carried out with pHOT1 DNA (Figure 5d). It was found that each extract can relax the supercoiled DNA (lanes 2 and 3) and the enzyme activities are inhibited in the presence of CPT (lanes 4 and 5). However, CPT-treated nuclear extract was unable to relax supercoiled pHOT1 DNA (lane 6). The results suggest that withaferin A and staurosporine have no direct effect on topoisomerase I.

Both activation of caspase-like proteases and topoisomerase I–DNA complexes attribute to DNA fragmentation during apoptosis. The release of cytochrome *c* causes activation of caspases like proteases inside cells, as demonstrated by the ability of withaferin A-treated cell lysates to cleave DEVD-AFC (a fluoregenic peptide substrate) significantly, compared to untreated cell lysates (Figure 6a). Treatment with VAD-fmk (a caspase inhibitor)

before treatment with withaferin A inhibits DNA fragmentation to the extent of 47%, which further supports the involvement of caspase-like proteases in DNA fragmentation. From time kinetic analysis, we have found that the formation of the topoisomerase I–DNA complexes induced by withaferin A was coincident with the activation of caspase-like proteases inside cells. Furthermore, withaferin A-treated leishmanial cells exhibited an increase in the percentage of DNA fragmentation at the time when topoisomerase I–DNA complexes were detected. Treatment with NAC before treatment with withaferin A prevents stabilization of topoisomerase I–DNA complex (Figure 5a) and also decreases DNA fragmentation to the extent of 43% (Figure 6b). These results suggest that oxidative stress-induced topoisomerase I–DNA complex is also involved in DNA fragmentation along with caspase-like proteases.

To further establish the involvement of topoisomerase I in apoptosis, we treated topoisomerase I-deficient yeast cells (CY154) as well as wild-type cells (CY184) with withaferin A, staurosporine and CPT separately. CPT is a specific inhibitor of topoisomerase I; so in the absence of topoisomerase I in CY154 yeast cells, the sensitivity of this drug is reduced in these cells compared to wild-type CY184 cells as expected. But the sensitivity of PKC inhibitors towards topoisomerase I-deficient CY154 cells also remains low compared to wild-type CY184 cells (Figure 6c). Furthermore, it was observed that immediately after 2 h of treatment with withaferin A or staurosporine, the mitochondrial membrane potential was lost in the wild-type cells (CY184) compared to the topoisomerase I-deficient CY154 cells. This result is consistent with that obtained after treatment with CPT (Table 1). Together, these findings suggest that topoisomerase I cleavage complexes contribute to apoptosis.

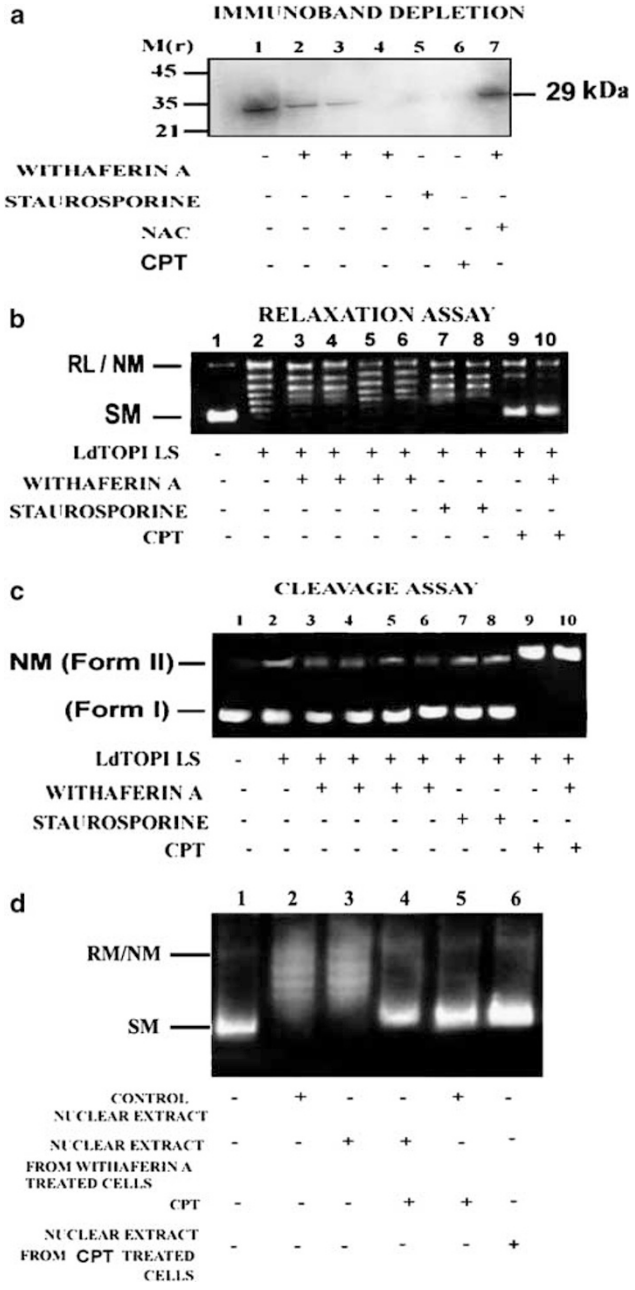

Figure 5 Treatment with withaferin A induces topoisomerase I–DNA complexes in cells but cannot interact with topoisomerase I directly. (**a**) Leishmanial cells were treated with 0.2% DMSO alone (lane 1), 15 μM of withaferin A for 3 h (lane 2), 4 h (lane 3), 5 h (lane 4), 15 μM of staurosporine for 4 h (lane 5), 50 μM of CPT for 3 h (lane 6) and with NAC before treatment with withaferin A (lane 7). Stabilization of topoisomerase I-mediated cleavable complex was determined by immunoband depletion assay. (**b**) Relaxation of supercoiled pBS (SK+) DNA (lane 1) with reconstituted LdTOPILS (lane 2) in the presence of 20 μM (lane 3), 40 μM (lane 4), 60 μM (lane 5) and 80 μM (lane 6) of withaferin A and 40 μM (lane 7) and 80 μM (lane 8) of staurosporine as well as with 50 μM of CPT (lane 9) separately. The relaxation assay was also carried out with CPT in the presence of withaferin A (lane 10). (**c**) LdTOPILS-mediated cleavage reaction was performed as described in Materials and Methods. Lane 1: 1 μg of supercoiled PHOT1 DNA; lane 2: with 40 U of LdTOPILS after SDS-proteinase K treatment; lanes 3–6: same as lane 2 but with 20, 40, 60 and 80 μM of withaferin A; lanes 7, 8, same as lane 2 but with 40 and 80 μM of staurosporine; lane 9: same as lane 2 but with 60 μM of CPT; lane 10: same as lane 2 but with both withaferin A and CPT. (**d**) Topoisomerase I activity was determined by relaxation of supercoiled pHOT1 DNA (400 ng) in the presence of nuclear extracts equivalent to 10^7 cells of differently treated cells. Lane 1:control DNA; lane 2: control nuclear extract; lane 3: nuclear extract from withaferin A-treated cells; lane 4: same as lane 3 but in the presence of 50 μM CPT; lane 5: control nuclear lysate in the presence of 50 μM CPT; lane 6: nuclear lysate from CPT-treated cells

Discussion

The molecular mechanism associated with PCD activation has been widely explored in mammalian cells, but not yet precisely understood in kinetoplastid parasites. Mounting evidences have indicated that changes in the phosphorylation state of proteins are important during PCD induced by different agents in higher eucaryotes.[28] Altogether, these observations and our present studies indicate that alterations of the phosphorylation state of cytosolic proteins like PKC can efficiently trigger PCD in a wide range of cell systems from higher eucaryotes to lower unicellular eucaryotes.

In this study, we established that a steroidal lactone withaferin A inhibits PKC both *in vitro* and *in vivo*. Here we have used leishmanial cell lysate as a source for PKC to phosphorylate the peptide substrate in the presence or absence of withaferin A. We provide evidences in support of the fact that withaferin A has a direct inhibitory effect on the

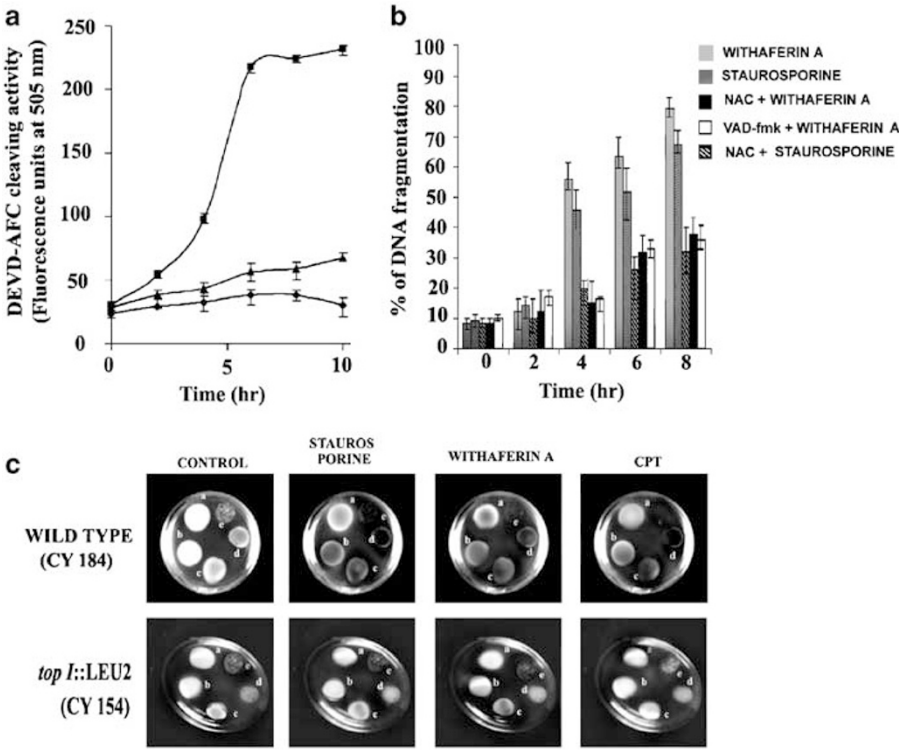

Figure 6 Determination of caspase-like protease activity and measurement of DNA fragmentation after treatment with withaferin A. (**a**) Cells were treated with 0.2% DMSO (◆), 15 μM of withaferin A (■) and with VAD-fmk before treatment with withaferin A (▲) and then DEVD-AFC cleavage activity was measured in these cells. (**b**) Percentage of DNA fragmentation was analyzed by TUNEL assay in differently treated cells. (**c**) Equal number of wild-type (CY184) and topoisomerase I-deficient yeast cells (CY 154) were serially diluted to 10-fold (**a**–**e**) and then spotted on plates containing 50 μg/ml CPT, 50 μg/ml staurosporine and 50 μg/ml withaferin A as described in Materials and Methods

Table 1 Effects of treatments with CPT, staurosporine and withaferin A on the mitochondrial membrane potential of yeast cells as measured by fluorescence of JC1 dye

Name of events	Ratio of 590/530
CY184	6.5±1.23
CY184+CPT	2.82±0.73
CY184+STAUROSPORINE	3.16±0.94
CY184+WITHAFERIN A	3.02±0.19
CY154	6.85±1.56
CY154+CPT	5.14±1.92
CY154+STAUROSPORINE	4.67±0.83
CY154+WITHAFERIN A	4.72±0.42

phosphorylation of the peptide substrate where rat brain PKC was used as the enzyme.

Cellular exposure to staurosporine or withaferin A induces mitochondrial outer membrane permeabilization. Downstream activation of proteases (caspases) causes DNA fragmentation. This was evident from the observation that treatment with VAD-fmk before treatment with withaferin A decreases the number of TUNEL-positive cells but only to the extent of 47%. This has led to the notion that there must be other mechanisms involved in DNA fragmentation (Figure 7). We found that topoisomerase I-mediated stabilization of cleavable complexes also contributes to DNA fragmentation. To interpret these observations, the following questions need to be addressed. First, how topoisomerase I–DNA cleavable complex is stabilized inside leishmanial cells during treatment with PKC inhibitors, and second, what is the probable mechanism by which topoisomerase I–DNA complex participates in DNA fragmentation.

Topoisomerase I–DNA cleavage complexes can be trapped by specific inhibitors such as CPT, which binds at the topoisomerase I–DNA interface and traps the cleavable complexes by preventing the DNA religation step.[29] Moreover, DNA modifications such as oxidative base lesions, mismatches, abasic sites and DNA single-strand breaks can also trap topoisomerase cleavage complexes by interfering with the nicking–closing activities of the enzyme.[25,30] Withaferin A or staurosporine does not interact directly with the enzyme. Here we have observed that treatment with withaferin A causes an increase in ROS production after inhibition of PKC inside cells. This in turn causes an increase in oxidative DNA lesions that can trap topoisomerase I–mediated cleavable complex formation during apoptosis. This is evident from the observation that treatment with NAC, before treatment with withaferin A, causes a decrease in oxidative stress as well as the reappearance of immunoband of topoisomerase I. Therefore, our result further confirms the fact that oxidative DNA lesion is a common mechanism for trapping of topoisomerase I cleavable complexes during apoptosis after treatment with PKC inhibitors.

But the major question regarding the role of topoisomerase I–DNA cleavable complex in apoptosis remains to be

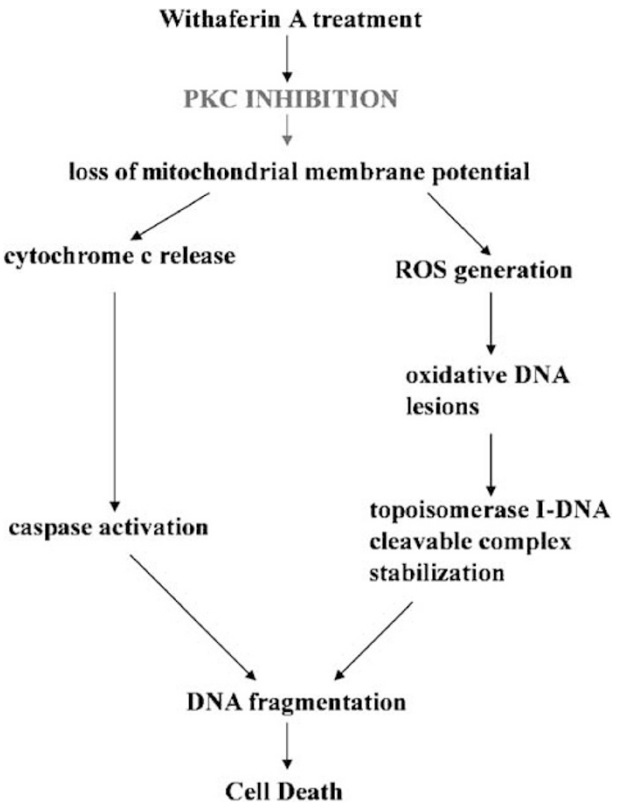

Figure 7 Proposed model for withaferin A-induced apoptosis-like death in leishmanial cells

answered. Topoisomerase I, although a ubiquitous enzyme in cells, possibly participates in apoptosis by directly generating DNA breaks like an 'apoptotic nuclease'. Moreover, we have found that wild-type yeast cells (CY184) lose the mitochondrial membrane potential very rapidly compared to the topoisomerase I-deficient yeast cells (CY154) after treatment with protein kinase inhibitors. This result suggests that expression of topoisomerase I confers sensitivity to PKC inhibitors and the absence of topoisomerase I only reduces but does not abrogate the apoptotic process. Therefore, topoisomerase I could act in concert with other apoptotic nucleases like caspase-activated DNase in leishmanial cells to propagate the process.

In conclusion, our study demonstrates for the first time that a novel PKC inhibitor, withaferin A, induces PCD in kinetoplastid parasites in a manner substantially different from higher eucaryotic cells. Inhibition of PKC by withaferin A likely represents an essential event responsible for the propagation of apoptosis and can act as a central regulator of the apoptotic machineries. Moreover, it was also established that both casapase-activated DNase and ROS-mediated formation of topoisomerase I–DNA suicidal complex are responsible for DNA fragmentation. So understanding the molecular mechanism of apoptotic cell death pathway provides the opportunities for discovering and evaluating molecular targets for drug designing, which now forms a rational basis for development of improved therapy against leishmaniasis.

Materials and Methods

Isolation of withaferin A. The crushed air-dried leaves of *Withania somnifera* Dun. (1.5 kg), defatted with petroleum-ether (60–80°C), were soxhleted with methanol for 48 h. The methanol extract was successively fractionated with chloroform and *n*-butanol. On removal of the solvent under reduced pressure, the *n*-butanol extract (220 g) was chomatographed over silica gel (60–120 mesh) and the column was eluted with 12% methanolic chloroform to yield a compound (1.2 g), crystallized from acetone–petroleum-ether as prisms, m.p. 252–253°C, M^+ 470, $[\alpha]_D^{28}$ +125° ($CHCl_3$,c 1.30). The homogeneity of the compound was determined by TLC and HPLC (reverse phase) and the structure of the compound was established by detailed spectral studies (IR, ^{1}H NMR, 2D NMR and MS). Finally, the compound was identified as withaferin A[16] by direct comparison with an authentic sample.

Parasite culture and maintenance. *L. donovani* strain AG 83 promastigotes were grown at 22°C in M199 liquid media supplemented with 10% fetal calf serum.

Preparation of cell lysates and PKC assay. Cell lysates of treated and untreated cells were prepared as described.[22] Briefly, the cells were washed with cold 150 mM NaCl and extracted with a buffer containing 1% (v/v) glycerol, 2 mM EDTA, 2 mM EGTA, 2 mM PMSF, 0.5 mg/ml leupeptin, 2 mM β mercaptoethanol and 25 mM Tris-HCl, pH 7.5. The material was homogenized with a glass/glass homogenizer and centrifuged at 15 000 $\times$ *g* for 10 min at 4°C. The supernatant was collected and investigated for kinase activity with HCV (1487–1500) as substrate. The activities of protein kinases towards peptide substrate were analyzed by SDS-PAGE as described.[22] The reaction mixture (50 μl) was incubated at 30°C for 30 min in a buffer containing 20 mM Tris-HCl, pH 7.5, 10% (v/v) glycerol, 0.05% (v/v) Triton X-100, 1 mM 2-mercaptoethanol, 6 mM $CaCl_2$, 0.1 mg/ml BSA, 50 μM [γ^{32}P]ATP (0.5 μCi), 20 μg/ml PS, 2 μg/ml DG, 0.2 mM PMSF and 1 μg of peptide substrate (Arg^{1487}-Arg-Gly-Arg-Th-Gly-Arg-Gly-Arg-Arg-Gly-Ile-Phe-Arg^{1500}) with cell extract equivalent to 5×10^7 cells. For assay with rat brain kinase (purchased from Calbiochem), addition of cell extract is omitted. The reaction was terminated by addition of 20 μl sample buffer and boiling for 3 min. The samples were applied to 20% SDS-PAGE. After staining with Coomassie brilliant blue, gels were dried and exposed to X-ray film at −70°C. Subsequently, peptides were cutout and ^{32}P incorporation was measured as Cerenkov radiation.

Detection of cytochrome *c* release. Treated and untreated cells were harvested and washed twice with 1 $\times$ PBS, suspended in cell fractionation buffer (Apo Alert^{TM} cell fractionation kit) and homogenized. After the separation of cytosolic and mitochondrial fraction, 50 μg each of cytosolic proteins was separated on 12% SDS-PAGE and immunoblotted with the rabbit polyclonal cytochrome *c* antibody. Alkaline phosphatase-conjugated secondary antibody was used and protein bands were visualized by NBT and BCIP color reaction.[23]

DNA fragmentation assay. To know the alterations in DNA content in the nucleus, treated and untreated *L. donovani* promastigote cells were fixed with 2% paraformaldehyde and incubated with 0.2% Triton X-100 for 5 min for permeabilization and layered with terminal deoxynucleotidyl transferase reaction mixture containing FITC-labeled dUTP for 1 h at 37°C according to the manufacturer's protocol (Apo Alert^{TM} DNA fragmentation assay kit). Cells were stained with propidium iodide and visualized with TCS-SP Leica confocal microscope using dual pass FITC/PI filter set. The number of TUNEL-positive cells was counted in each case.[23]

Double staining with Annexin V and propidium iodide. Externalization of phosphatidylserine on the outer membrane of untreated and withaferin A-treated promastigotes was measured by the binding of annexin V–FITC and PI using an annexin V-FLUOS staining kit (Roche Diagnostics). Cells were visualized with TCS-SP Leica confocal microscope using dual FITC/PI filter set. Total cells versus annexin V-labeled cells was calculated and data expressed as percentage of apoptotic cells. It should be noted that 100 cells per group with identical morphology were calculated for each condition.[23]

Purification of recombinant proteins and reconstitution of topoisomerase I activity. *Escherichia coli* BL21 (DE3) pLysS cells harboring *pET16bLdTOP1L* and *pET16bLdTOP1S* were separately induced at $OD_{600} = 0.6$ with 0.5 mM IPTG at 22°C for 12 h. Cells harvested from 1 l of culture

were separately lysed by lysozyme/sonication and the proteins were purified through Ni^{2+}-NTA agarose column (Qiagen) followed by phosphocellulose column (P11 cellulose, Whatman) as described previously.[26] Finally, the purified proteins LdTOP1L and LdTOP1S were stored at $-70^{\circ}C$. The concentrations of purified proteins were quantified by Bradford reaction using a Bio-Rad Protein Estimation Kit according to the manufacturer's protocol.

Purified LdTOP1L were mixed with purified LdTOP1S separately at a molar ratio of 1 : 1 at a total protein concentration of 0.5 mg/ml in reconstitution buffer (50 mM potassium phosphate, pH 7.5, 0.5 mM DTT, 1 mM EDTA, 0.1 mM PMSF, 10% glycerol). The mix was dialyzed overnight at $4^{\circ}C$ and the dialyzed fractions were used for the plasmid relaxation activity.[26]

Immunoband depletion assay. Leishmanial cells (1×10^7) were cultured for 3 days at $22^{\circ}C$ with or without drugs. Nuclear fraction was isolated as described.[31] Briefly, cells were suspended in hypotonic buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM EGTA, 1 mM PMSF, 1 mM benzamidine hydrochloride and 5 mM DTT) and homogenized. The homogenate was centrifuged for 10 min at $10\,000 \times g$. The pellets were washed and were the source of nuclear fraction. Then nuclear fraction was lysed by 1% SDS. Samples were subjected to SDS-PAGE (10%), and proteins that had entered the gel were electrophoretically transferred to nitrocellulose membranes. Immunoblotting of immobilized proteins was carried out using a mouse antibody raised against LdTOP1S.[26]

Plasmid relaxation assay. The type I DNA topoisomerase was assayed by decreased mobility of the relaxed isomers of supercoiled pBluescript (SK^+) DNA in an agarose gel. Relaxation assay was carried out as described[26] with LdTOP1LS in the relaxation buffer (25 mM Tris-HCl, pH 7.5, 5% glycerol, 0.5 mM DTT, 10 mM $MgCl_2$, 50 mM KCl, 2.5 mM EDTA and 150 μg/ml BSA), and supercoiled pBluescript (SK^+) DNA (85–95% were negatively supercoiled with the remaining being nicked circles). The relaxation assay was performed in the presence of CPT, staurosporine and withaferin A separately. The amount of supercoiled monomer DNA band fluorescence after EtBr staining was quantitated. Relaxation assay was also performed with nuclear extracts from differently treated leishmanial cells.

DNA cleavage assay. Reaction mixtures (50 μl) containing 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 50 mM KCl, 0.2 mM DTT, 0.5 mM EDTA, 30 μg/ml BSA, 20 μg/ml pHOT1 DNA, 40 U of *L. donovani* topoisomerase I and drugs were incubated at $37^{\circ}C$ for 30 min. The reactions were terminated by adding 1% SDS and 150 μg/ml proteinase K, and incubated further for 1 h at $37^{\circ}C$. DNA samples were electrophoresed in 1% agarose gel containing 0.5 μg/ml ethidium bromide to resolve the more slowly migrating nicked products (form II) from the supercoiled molecules (form I).[26]

Detection of oxidative DNA damage. After drug treatment, cells were permeabilized in hypotonic buffer (10 mM Tris-HCl, pH 7.8, 70 mM NaCl, 1 mM EDTA, 1 mM DTT) containing 0.05% Triton X-100 at $4^{\circ}C$ for 15 min by gentle mixing. Fpg (1 mg/ml) was added and further incubated for 30 min at $25^{\circ}C$. DNA breaks were analyzed by alkaline comet assay[32] with some modifications. Briefly 5000–10 000 cells were mixed with 100 μl of 0.75% low-melting agarose and kept at $37^{\circ}C$. The agarose cell suspension was spread on polylysine-coated coverslips. The preparations were left on a chilled plate for 5 min before lysis (0.03 M NaOH, 1 M NaCl, 2 mM EDTA, 0.5% *N*-lauryl sarcosyl) for 1.5 h and thereafter equilibrated (0.03 M NaOH, 2 mM EDTA) for 1 h. Electrophoresis of the agarose-imbedded cells was run at 0.67 V/cm for 20 min in the same solution. The agarose gel was neutralized in 0.4 M Tris-HCl, pH 7.5. Cells were then stained with EtBr. Analysis of the DNA that migrated from the nuclei, the tail moment, was carried out using an Olympus fluorescence microscope aided by the Kinetic Imaging Komet II system.

Drug sensitivity assays. Equal number of wild-type (CY184) and mutant top1::LEU2 (CY154) yeast cells (kindly provided by Michael F Chistman) were serially diluted to 10-fold and 5 μl aliquots were spotted on YPD plates containing 50 μg/ml CPT, 50 μg/ml staurosporine, 50 μg/ml withaferin A separately and buffered with 25 mM HEPES, pH 7.2. Control plates contained final concentrations of 0.25% DMSO and 25 mM HEPES, pH 7.2. Plates were incubated at $30^{\circ}C$ for 2 days.[33] The genetic descriptions of the yeast strains are as follows

Yeast strain	Description
CY184	*MATα ade 2-1 ura 3-1 his 3-11,15 trp 1-1 leu 2-3, 112 can 1-100 rDNA :: ADE 2*
CY154	*MATα top1-7 ::LEU2 in CY143*
CY143	*MATα ade 2-1 ura 3-1 his 3-11,15 trp 1-1 leu 2-3, 112 can 1-100 rDNA :: URA 3*

Measurement of mitochondrial membrane potential. Mitochondrial transmembrane potential was investigated using JC1 dye. Briefly, leishmanial cells after different treatments were harvested and washed with 1 $\times$ PBS. Cells were then incubated at $37^{\circ}C$ in 5% CO_2 incubator for 1 h with a final concentration of JC1 dye at 5 μg/μl (according to the manufacturer's protocol). Cells were then analyzed by flow cytometry, where FL-1 channel indicates the mean green fluorescence intensity. Flow cytometry data presented here are representative of two experiments.[23]

Mitochondrial membrane potential of yeast cells was measured as described.[34] Two milliliter of treated and untreated cell suspensions (OD 20.0) was pelleted down and washed with spheroplast buffer (1 M sorbitol, 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 10 mM DTT and 10 mM sodium azide). Cells were then pelleted, lyticase was added and kept in the dark at room temperature for 30 min. Cells were washed with water and incubated at $37^{\circ}C$ in 5% CO_2 incubator for 1 h with a final concentration of JC1 at 5 μg/μl (according to the manufacturer's protocol). Cells were then analyzed by spectrophotometer.

Measurement of ROS level and GSH level. Intracellular ROS level was measured in treated and untreated cells as described.[23] Briefly, cells (2×10^7) after different treatments were washed and resuspended in 500 μl of medium M199 and were loaded with a cell-permeant probe H_2DCFDA for 1 h. The green fluorescence of DCF was recorded at 515 nm by spectrofluorometer. GSH level was measured by monochlorobimane (MCB) dye.[23] *L. donovani* promastigotes (2.5×10^6 cells) were treated with or without withaferin A at different times. Cells were then pelleted down and lysed by cell lysis buffer according to the manufacturer's protocol (Apo Alert^{TM} glutathione assay kit). Cell lysates were incubated with MCB (2 mM) for 2 h at $22^{\circ}C$. The decrease in glutathione levels in the extracts of nonapoptotic and apoptotic cells was detected by fluorometer with 395 nm excitation and 480 nm emission wavelength.

Determination of caspase-like protease activity. *L. donovani* promastigotes (2.5×10^6) were treated with or without withaferin A for different time periods. Cells were then pelleted and lysed by cell lysis buffer according to the manufacturer's protocol (Apo Alert^{TM} caspase assay kit). Cell lysates were incubated with respective caspase buffers to detect CED3/CPP32 group of protease activity. A fluorogenic peptide substrate, DEVD-AFC at 100 μM and 1 $\times$ reaction buffer containing 100 mM DTT were added to corresponding cell lysates to measure the activity of CED3/CPP32 group of proteases. In a parallel set of reactions, 1 μl of CED3/CPP32 group of protease inhibitor was added to the reaction before the addition of cell lysates. AFC release was measured after incubating these samples at $37^{\circ}C$ for 2 h by fluorometer with 380 nm excitation and 460 nm emission.[23]

Statistical analysis. Data are expressed as mean $\pm$ S.D. unless mentioned otherwise. Comparisons were made between different treatments using unpaired Student's *t*-test.

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