Altered tubulin dynamics, localization and post-translational modifications in sodium arsenite resistant *Leishmania donovani* in response to paclitaxel, trifluralin and a combination of both and induction of apoptosis-like cell death

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SUMMARY

In this study the anti-leishmanial activity and anti-microtubule effects of paclitaxel, trifluralin and a combination of paclitaxel and trifluralin have been tested in a wild type and sodium arsenite-resistant strain of *Leishmania donovani*. Both paclitaxel and trifluralin have been shown to be effective in limiting parasite growth. Specific alterations in morphology, tubulin polymerization dynamics, post-translational modifications and cellular distribution of the tubulins have been confirmed to be a part of the intracellular anti-microtubule-events that occur in arsenite-resistant *L. donovani* in response to these agents, ultimately leading to death of the parasite. DNA analyses of the drug-treated wild type and arsenite-resistant strains revealed an apoptosis-like death in response to paclitaxel and the combination but not to trifluralin. Data provide valuable information for further development of chemotherapeutic strategies based on anti-microtubule agents against drug resistant *Leishmania* parasites.

Key words: *Leishmania*, tubulins, paclitaxel, trifluralin, combination, apoptosis, arsenite resistance.

INTRODUCTION

Leishmaniases currently affect more than 12 million people in 88 countries (Havens *et al*. 2000). Most of the fatalities are due to visceral leishmaniasis for which the parasite *Leishmania donovani* is a causative organism (Moulay *et al*. 1996; Handman, 2001). The success of chemotherapy against leishmaniasis has been limited due to the high toxicity of conventional drugs, lack of efficacy in immuno-compromised individuals and the generation of drug-resistant parasites (Berhe, 1999).

The search for drugs to inhibit parasite growth may reasonably include agents that affect the parasite's complex microtubule networks since microtubules, the major component of the cytoskeleton, are essential for the growth and differentiation of the kinetoplastid protozoans (Gull *et al*. 2001). Paclitaxel is known to be active on protozoan parasites (Havens *et al*. 2000). It is a potent inhibitor of cell replication and enhances the polymerization of tubulin into stable bundles of microtubules (Ojima *et al*. 1995). Tubulin in a taxol-resistant strain of *Leishmania* was more resistant to *in vitro* polymerization than the wild type strain (Kapoor, Gosh & Madhubala, 1999).

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We have previously reported differences in the expression of α- and β-tubulins in response to paclitaxel in an *in vitro*-developed sodium *m*-arsenite resistant strain of *L. donovani* (Prasad, Kumar & Dey, 2000*b*; Jayanarayan & Dey, 2002). Altered expression, polymerization and cellular distribution of α-/β-tubulins in response to treatment with sodium arsenite have been reported recently in the same strain (Jayanarayan & Dey, 2004). The strain is shown to be hypersensitive to paclitaxel (Prasad *et al*. 2000*b*). The anti-microtubule effects of paclitaxel on the sodium arsenite-resistant *L. donovani*, which is hypersensitive to paclitaxel, have not yet been clearly defined. Trifluralin, another experimental anti-leishmanial compound, has anti-microtubule properties that are opposite to paclitaxel. Trifluralin binds to leishmanial tubulin and affects microtubule assembly and stability, inhibits promastigote proliferation, decreases amastigote-to-promastigote transformation and reduces infectivity of amastigotes (Chan *et al*. 1993; Werbovetz, Brendle & Sackett, 1999). Of particular interest with respect to the development of selective anti-parasitic agents is the report that trifluralin binds better to partially purified leishmanial tubulin than to rat brain tubulin (Chan & Fong, 1990). A number of trifluralin analogues are currently being investigated for optimum tubulin binding and anti-leishmanial activity

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(Werbovetz *et al*. 2003). However, the susceptibility or cross-resistance of a drug-resistant *L. donovani* strain, such as the arsenite-resistant *L. donovani* used in this study, to trifluralin and the cellular events associated with trifluralin activity in this strain are also yet to be fully defined. In the current study we have sought to investigate the anti-microtubule effects of two oppositely acting agents, namely, paclitaxel, which promotes microtubule assembly and stabilization and trifluralin, which affects microtubule assembly, in the sodium arsenite-resistant *L. donovani*.

A number of investigators have studied the effect of combinations of oppositely acting anti-microtubule agents such as the vincas and taxanes (Dumontet, 2000). Synergy between paclitaxel and vinorelbine against a human melanoma cell line has been reported by Photiou *et al*. (1997). A combination of vinorelbine and paclitaxel has been reported to show reduced toxicity and a significant percentage of long-term cures in the P388 murine model (Knick, Eberwein & Miller, 1995). Preliminary reports of combinations of vinorelbine with docetaxel in patients with advanced breast cancer or lung cancer suggest promising activity with no substantial increase in toxicity (Dieras *et al*. 1997). Combination treatments of docetaxel and vinorelbine have been reported to be beneficial with no substantial increase in associated toxicity in non-small-cell lung carcinoma (Dieras *et al*. 1997). In the current study we have also investigated the anti-microtubule effects of a combination of paclitaxel and trifluralin on the wild type and drug-resistant strain.

MATERIALS AND METHODS

Chemicals

Paclitaxel was obtained from Dr Rama Mukherjee, Dabur Research Foundation, India. Trifluralin was purchased from Fluka Chemicals (St Louis, MO, USA). Mouse monoclonal anti-α-tubulin antibody was purchased from NeoMarkers (Fremont, CA, USA). Rabbit polyclonal anti-β-tubulin and rabbit polyclonal anti-γ-tubulin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Mouse monoclonal anti-acetylated tubulin antibody (clone 6-11B-1) and mouse monoclonal anti-tyrosinated tubulin antibody (TUB-1A2) were purchased from Sigma Chemical Company (St Louis, MO, USA). ApoAlert DNA fragmentation assay kit was purchased from BD Biosciences Clontech (Palo Alto, CA, USA). All other chemicals, unless specified, were from Sigma Chemical Company (St Louis, MO, USA).

Parasite culture and treatments

Wild type *L. donovani* promastigotes (Ld-Wt) and *L. donovani* promastigotes (Ld-As20) resistant to 20 μM sodium arsenite ($NaAsO_2$) were cultured in RPMI-1640 medium (pH 7·2, containing 25 mM HEPES, supplemented with 10% FCS and antibiotics) at 24 °C as described by Kaur & Dey (2000). The arsenite-resistant strain, developed by sequential exposure to increasing $NaAsO_2$ concentration, was grown under similar conditions as the wild type, except that the medium was supplemented with 20 μM $NaAsO_2$. Similar growth kinetics, under the above-mentioned culture conditions, was determined for the two strains by use of the MTT-formazan assay (Kaur & Dey, 2000). The IC_{50} of $NaAsO_2$ was reported to be 5·0 μM and 65·0 μM in the wild type and arsenite-resistant strain respectively (Prasad, Kaur & Dey, 2000*a*). Ld-As20 promastigotes were passaged in $NaAsO_2$ free medium when being used for experiments and was not found to revert the resistant phenotype for at least five generations under these conditions (Prasad *et al*. 2000*a*). The IC_{50} concentration of paclitaxel has been previously reported to be 63·0 μM in Ld-Wt and 25·0 μM in Ld-As20 (Prasad *et al*. 2000*b*). The IC_{50} concentration of trifluralin was determined in the current study and was found to be 30·0 μM in both the strains.

For all the experiments, Ld-Wt and Ld-As20 were grown for 48 h in culture. Ld-Wt cells were then treated with 31·5 μM of paclitaxel, 63·0 μM of paclitaxel, 15·0 μM of trifluralin, 30·0 μM of trifluralin, or a combination of 31·5 μM paclitaxel and 15·0 μM trifluralin as indicated. Ld-As20 cells were treated with 12·5 μM of paclitaxel, 25·0 μM of paclitaxel, 15·0 μM of trifluralin, 30·0 μM of trifluralin, or a combination of 12·5 μM paclitaxel and 15·0 μM trifluralin as indicated. The samples were incubated for a further 48 h at 24 °C in the presence of the drugs (paclitaxel, trifluralin or a combination of both) and processed as described. Paclitaxel and trifluralin were dissolved in dimethyl sulphoxide (Me_2SO). The stock solutions of paclitaxel and trifluralin were prepared such that the final concentration of Me_2SO in the treated samples was always <1% (v/v). The untreated (control) samples in all the experiments contained an equivalent concentration of Me_2SO as the treated samples.

Determination of drug cytotoxicity

The cytotoxicity of trifluralin or a combination of paclitaxel and trifluralin in Ld-Wt and Ld-As20 was determined by the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as described by Verma & Dey (2004). The cells were treated with 10·0, 20·0, 30·0, 40·0 and 50·0 μM of trifluralin in experiments to determine the IC_{50} of trifluralin for Ld-Wt and Ld-As20. The concentration of trifluralin that allowed 50% survival of the parasites was taken as its IC_{50}. Ld-Wt was treated with 31·5 μM paclitaxel, 15·0 μM trifluralin or a combination of 31·5 μM paclitaxel plus 15·0 μM

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trifluralin and Ld-As20 with 12·5 μM paclitaxel, 15·0 μM trifluralin or a combination of 12·5 μM paclitaxel plus 15·0 μM trifluralin to determine the percentage survival of the parasites when treated with these anti-microtubule agents. The results were expressed as percentage survival of the treated parasites as compared to untreated parasites in response to treatment with known concentrations of the drugs.

Determination of cell morphology

For microscopic determination of cell morphology, treated and untreated cells were processed as described by Jayanarayan & Dey (2004). Cells were observed under 100 × objective on a Nikon E-600 microscope equipped with differential interference contrast (DIC) module (Nikon, Tokyo, Japan) and a digital camera DXM 1200 (Nikon Tokyo, Japan). Images were processed using Image-Pro Express (Media Cybernetics, Madison, U.S.A.) and Adobe Photoshop 5.5 (Adobe Systems Inc., Mountain View, CA) softwares.

Preparation of cell lysates

Whole cell lysates were prepared as described by Prasad & Dey (2000*b*). Protein concentrations were estimated by the bicinchoninic acid method according to the manufacturer's instruction, using BSA as a protein standard.

SDS-PAGE and Western blot analysis

For determination of α-, β- and γ-tubulin expression and the concentration of acetylated and tyrosinated tubulins, cells were treated as described above and lysed. Aliquots of the cell lysate containing equal concentration of proteins (10 μg) were separated by 8% SDS-PAGE (Laemmli, 1970; Snapp & Landfear, 1997) and subjected to Western blot analysis as described previously (Jayanarayan & Dey, 2002). Mouse anti-α-tubulin antibody, rabbit anti-β-tubulin antibody or rabbit anti-γ-tubulin antibody were used as primary antibodies at 1 : 1000 dilution. Mouse anti-acetylated and mouse anti-tyrosinated tubulin antibodies were used at 1 : 4000 dilution. Alkaline phosphatase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG antibodies, as appropriate were used at 1 : 10 000 dilution. Reactive bands were detected by using 5-bromo-4-chloro-3-indolyl-phosphate and 4-nitroblue tetrazolium chloride. Densitometry was performed using a gel documentation system (Gel Doc 2000, Bio-Rad) with Quantity One® software (Bio-Rad). The density of the untreated samples was arbitrarily given a value 1·0 and relative densities of the other samples were derived.

Determination of unpolymerized and polymerized tubulins in vivo

To determine the effect of trifluralin and paclitaxel on the tubulin assembly *in vivo*, the detergent soluble and insoluble tubulin fractions, representing the unpolymerized and polymerized tubulin respectively, were separated and estimated as described by Jayanarayan & Dey (2004). Briefly, control and treated promastigotes ($1–2 \times 10^7$ cells) were harvested by centrifugation and resuspended in buffer containing 10 mM MOPS, pH 6·9, 0·1 mM EGTA, 1 mM $MgSO_4$ and 0·1% Triton X-100 plus protease inhibitors (1 μM leupeptin and 1 μM aprotinin). The cells were centrifuged at 3000 ***g*** to separate the supernatant. Protein concentration in the supernatant was estimated. The pellets were resuspended in PBS/SDS-PAGE sample buffer in an equal volume to that of the supernatant. Equal volumes of the both supernatant and pellet fractions were subjected to 8% SDS-PAGE.

Distribution of α-, β-*, acetylated and tyrosinated tubulins*

To observe changes in cellular distribution of α-, β-, acetylated and tyrosinated tubulins, samples were processed as described by Snapp & Landfear (1997) with some modifications as mentioned by Jayanarayan & Dey (2004). Mouse anti-α-tubulin antibody (1 : 50 dilution), rabbit anti-β-tubulin antibody (1 : 50 dilution), mouse anti-acetylated tubulin antibody (1 : 100 dilution) or mouse anti-tyrosinated tubulin antibody (1 : 100 dilution) was prepared in PBS containing 2% goat serum. Affinity-isolated goat anti-mouse IgG FITC-conjugate (1 : 50 dilution) or affinity isolated goat anti-rabbit IgG FITC conjugate (1 : 100 dilution) was used as secondary antibody appropriately. Slides were analysed by DIC coupled epifluorescence microscopy using a Nikon E-600 microscope. Images were processed by Image-Pro Express and Adobe Photoshop 5.5 softwares.

DNA fragmentation assay

To determine the sizes of DNA fragments generated during cell death, total cellular DNA was isolated according to Sambrook, Fritsch & Maniatis (1989) and analysed by agarose gel electrophoresis as described by Das *et al.* (2001).

In situ *labelling of DNA fragments*

Cells undergoing apoptosis generate abundant DNA fragments in their nuclei. *In situ* detection of DNA fragments by terminal deoxyribonucleotide transferase (TdT)-mediated dUTP nick end labelling (TUNEL) was performed as described by

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Jayanarayan & Dey (2004). The samples were counter stained with 10 μg/ml propidium iodide and visualized under a Nikon E600 fluorescence microscope. Images were processed by Adobe Photoshop 5.5 software.

Statistical analysis

A paired two-tailed Student's *t*-test was performed for statistical analysis and data sets were said to be significantly different for $P<0{\cdot}01$.

RESULTS

Cytotoxicity of paclitaxel, trifluralin and a combination of paclitaxel and trifluralin

The IC_{50} concentration of paclitaxel has been previously reported to be 63·0 μM in Ld-Wt and 25·0 μM in Ld-As20 (Prasad *et al*. 2000*b*). In this study, we sought to determine the activity of trifluralin on the Ld-As20 strain compared with the Ld-Wt. The MTT cytotoxicity assay was used to determine the IC_{50} of trifluralin for Ld-Wt and Ld-As20. Results (Fig. 1A) show that the IC_{50} of trifluralin in Ld-Wt was $30{\cdot}0 \pm 2{\cdot}02$ μM and in Ld-As20 it was found to be $30{\cdot}0 \pm 3{\cdot}75$ μM, suggesting that both Ld-Wt and Ld-As20 were equally susceptible to trifluralin, without any cross-resistance. Based on these data, 30·0 μM was chosen as the IC_{50} of trifluralin for both Ld-Wt and Ld-As20 promastigotes.

We next explored the possibility of using a combination of paclitaxel and trifluralin at lower individual concentrations (half of IC_{50} concentrations of each) against Ld-Wt and Ld-As20. Ld-Wt showed ~20–25% relative inhibition of growth when treated with 31·5 μM paclitaxel or 15·0 μM trifluralin compared to untreated cells (Fig. 1B) as determined by the MTT cytotoxicity assay. Ld-As20 showed ~35% inhibition when treated with 12·5 μM paclitaxel and ~25% inhibition when treated with 15·0 μM trifluralin (Fig. 1B). However, both Ld-Wt and Ld-As20 showed ~55% inhibition when treated with a combination of respective half of IC_{50} concentrations of trifluralin and paclitaxel (31·5 μM paclitaxel plus 15·0 μM trifluralin in case of Ld-Wt and 12·5 μM paclitaxel plus 15·0 μM trifluralin in case of Ld-As20) (Fig. 1B). Data show that a combination of paclitaxel and trifluralin induces additive cytotoxic effect in both Ld-Wt and Ld-As20. More importantly, no cross-resistance to the combination was exhibited by Ld-As20. Since a combination of paclitaxel and trifluralin was observed to be effective against both Ld-Wt and Ld-As20, we sought to provide further insights into the mechanism of action of the combination in relation to the changes in microtubules and compare it with the effects induced by paclitaxel or trifluralin.

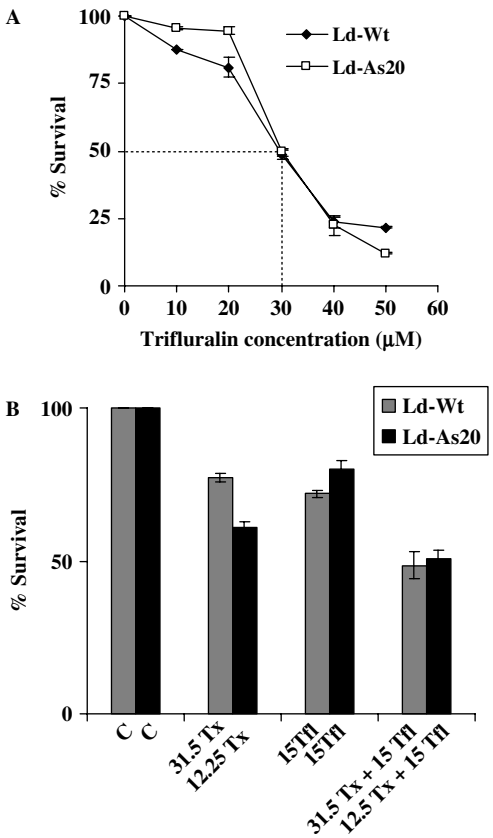

Fig. 1. (A) Determination of IC_{50} of trifluralin against Ld-Wt and Ld-As20. (B) Determination of cytotoxicity of a combination of paclitaxel and trifluralin against Ld-Wt and Ld-As20. Cell viability of promastigotes treated with specific concentrations of paclitaxel, trifluralin or a combination of paclitaxel and trifluralin was measured by MTT assay as described in the Materials and Methods section. Results are expressed as the mean ± S.E.M. of 3 independent experiments. C, untreated Ld-Wt or Ld-As20; 31·5Tx: Ld-Wt treated with 31·5 μM paclitaxel; 12·5Tx: Ld-As20 treated with 12·5 μM paclitaxel; 15Tx: Ld-Wt or Ld-As20 treated with 15·0 μM trifluralin; 31·5Tx + 15Tfl: Ld-Wt treated with 31·5 μM paclitaxel plus 15·0 μM trifluralin; 12·5Tx + 15Tfl: Ld-As20 treated with 12·5 μM paclitaxel plus 15·0 μM trifluralin.

Effects of paclitaxel, trifluralin and a combination of paclitaxel and trifluralin on Ld-Wt and Ld-As20

Effects on parasite morphology. Previous reports have shown that paclitaxel induced changes in the normal morphology of *L. donovani* promastigotes (Havens *et al*. 2000). We have recently reported the occurrence of abnormal morphology in Ld-As20 in response to arsenite treatment (Jayanarayan & Dey, 2004). In this study we sought to find out the morphological changes, if any, induced by paclitaxel, trifluralin and a combination of paclitaxel and trifluralin in Ld-As20 and compare it to the wild type. Microscopic observation of Ld-Wt exposed to 63·0 μM paclitaxel revealed drastic morphological changes (Fig. 2, 63Tx) consistent with previous reports (Havens *et al*. 2000). Ld-As20 treated with

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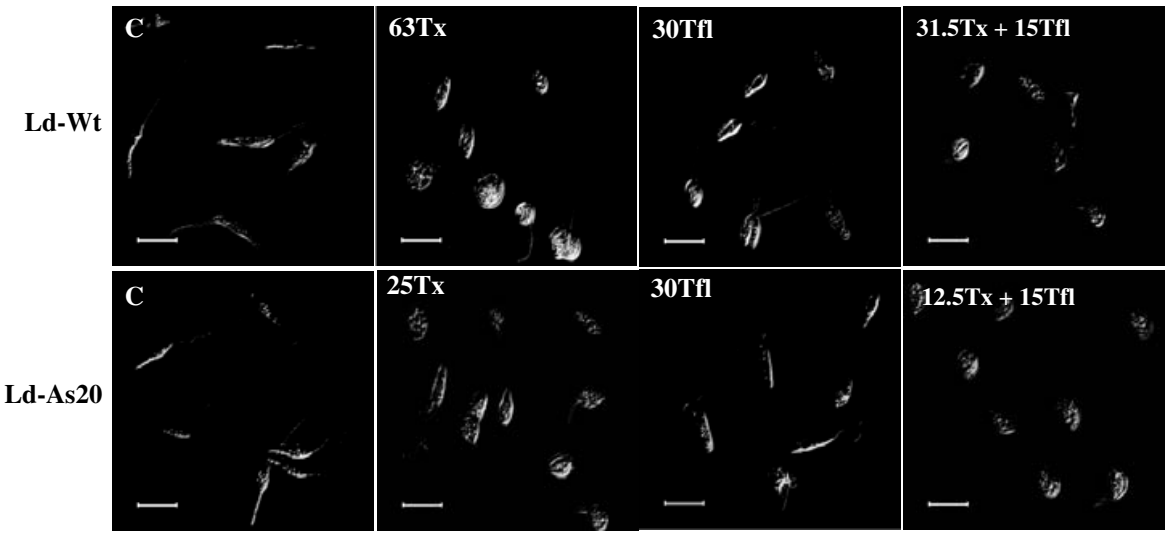

Fig. 2. Effect of paclitaxel, trifluralin and the paclitaxel-trifluralin combination on cellular morphology of Ld-Wt and Ld-As20. The treated and untreated cells were watched under a microscope fitted with DIC module, with 100 × objective. C, untreated cells; 63Tx: Ld-Wt treated with 63·0 μM paclitaxel; 25Tx: Ld-As20 treated with 25·0 μM paclitaxel; 30Tfl: Ld-Wt or Ld-As20 treated with 30·0 μM trifluralin; 31·5Tx + 15Tfl: Ld-Wt treated with 31·5 μM paclitaxel plus 15·0 μM trifluralin; 12·5Tx + 15Tfl: Ld-As20 treated with 12·5 μM paclitaxel plus 15·0 μM trifluralin. Scale bars, 10 μm.

25·0 μM paclitaxel induced motile cells that had altered morphology with the cell body losing the normal elongated shape and attaining a rounded morphology (Fig. 2, 25Tx). More than 90% of the treated Ld-As20 cells had rounded morphology with truncated flagella, multiple flagella or few cells without flagella. Ld-Wt and Ld-As20 exposed to trifluralin at respective IC_{50} concentrations (30·0 μM in Ld-Wt and Ld-As20) also revealed drastic morphological changes (Fig. 2, 30Tfl). Trifluralin treatment to both Ld-Wt and Ld-As20 induced motile cells with a rounded morphology. Both the strains showed cells with truncated flagella and few cells with multiple flagella. The morphology of ~80–90% of both Ld-Wt and Ld-As20 were affected and was consistent with disruption of tubulin function.

When subjected to treatment with a combination of half the IC_{50} concentrations of trifluralin and paclitaxel, all the cells showed an aberrant morphology with few cells having truncated flagella, multiple flagella or no flagella (Fig. 2; 31·5Tx + 15Tfl and 12·5Tx + 15Tfl). The changes in morphology observed were consistent with alterations in the cytoskeleton and specifically, microtubular organization.

Effects on expression of α- β- *and* γ-*tubulins*. To further investigate the cellular events involved in the anti-microtubule activity of paclitaxel, trifluralin and the combination of paclitaxel-trifluralin, the expression profile of the α-, β- and γ-tubulins in Ld-Wt and Ld-As20 were determined. Aliquots of samples obtained from a single treatment group, were simultaneously subjected to Western immunoblotting probed with antibody against α-, β- and γ- isoforms of tubulin. Alterations in the expression of α-, β- and γ-tubulins of Ld-Wt and Ld-As20 in response to paclitaxel have been previously reported from our laboratory (Prasad *et al*. 2000*b*; Jayanarayan & Dey, 2002). Under the current experimental conditions, Ld-Wt and Ld-As20 treated with trifluralin did not show any significant alteration in expression levels of α-, β- or γ-tubulins (data not shown). However, Ld-Wt cells treated with a combination of both trifluralin and paclitaxel showed ~40% increase in the α-tubulin expression in comparison with the untreated cells (Fig. 3A). In Ld-As20, the α-tubulin expression remained unaltered (Fig. 3A). The β-tubulin expression in Ld-Wt cells treated with a combination of half of IC_{50} of both trifluralin and paclitaxel (15·0 μM and 31·5 μM respectively) showed ~40% increase in comparison with the untreated cells (Fig. 3B). The expression of β-tubulin remained unchanged in Ld-As20 in response to similar treatment (Fig. 3B). No significant alteration could be found in the expression of γ-tubulin of both Ld-Wt and Ld-As20 under the tested conditions (Fig. 3C).

Effects on in vivo *tubulin polymerization dynamics*. Paclitaxel has been reported to increase the *in vitro* polymerization of tubulin in *Leishmania* parasites (Kapoor *et al*. 1999). The effect of paclitaxel on the *in vivo* tubulin dynamics of *Leishmania* has not yet been reported either in wild type or drug-resistant Leishmania. Oryzalin, a compound related to trifluralin, and a series of oryzalin analogues have been reported to inhibit tubulin assembly *in vitro* (Bhattacharya, Salem & Werbovetz, 2002). No

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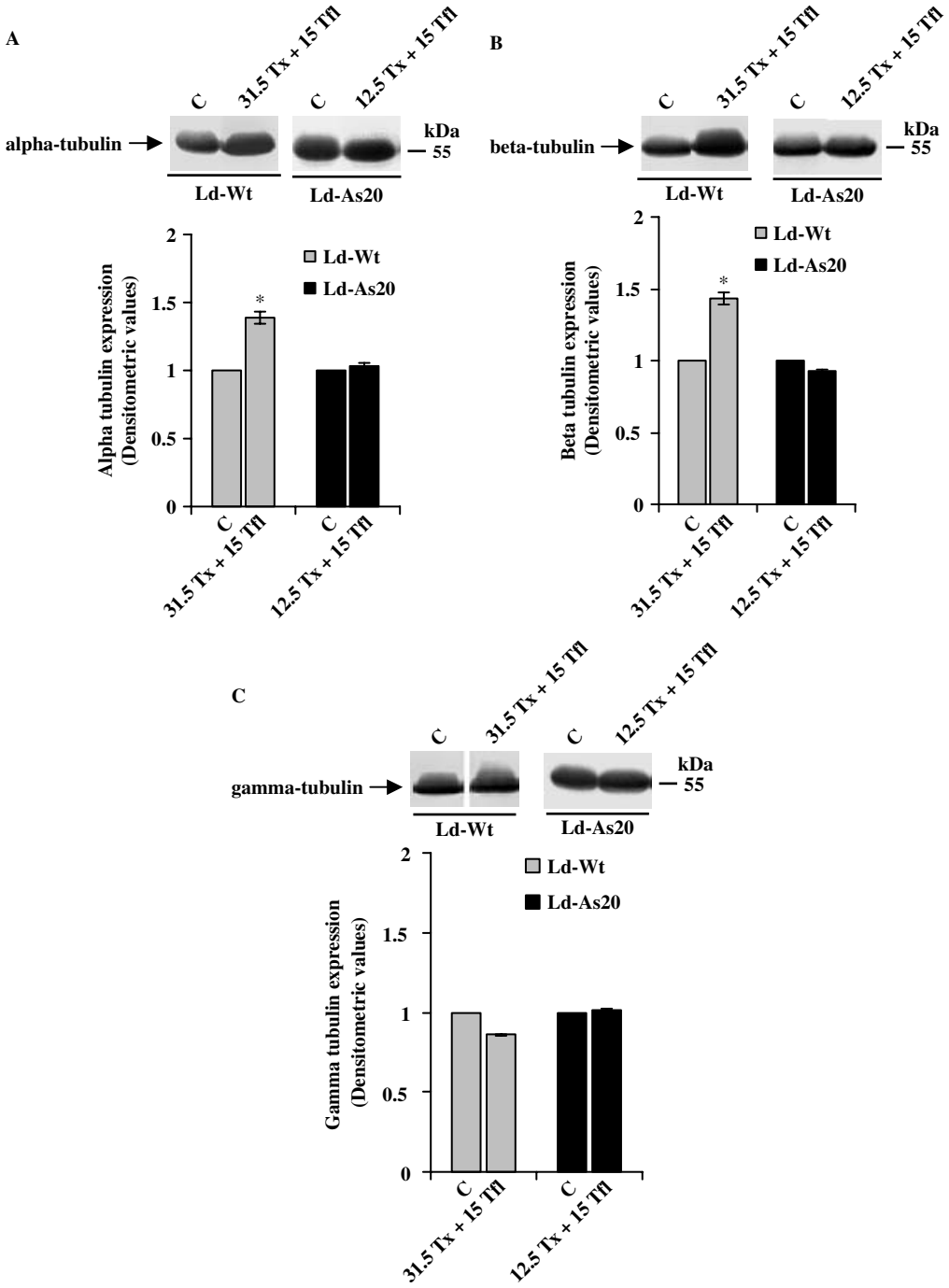

Fig. 3. Effect of a combination of paclitaxel and trifluralin on the α-, β- and γ-tubulin expression in Ld-Wt and Ld-As20. Whole cell lysates of treated and untreated promastigotes were resolved by SDS-PAGE and Western immunoblot using anti-α-, β- or γ-tubulin antibody as described in the Materials and Methods section. (A) Upper panel: Western blots of α-tubulin expression; lower panel: densitometric analysis of Western blots. (B) Upper panel: Western blots of β-tubulin expression; lower panel: densitometric analysis of Western blots. (C) Upper panel: Western blots of γ-tubulin expression; lower panel: densitometric analysis of Western blots. C, untreated Ld-Wt or Ld-As20; 31·5Tx+15Tfl: Ld-Wt treated with 31·5 μM paclitaxel plus 15·0 μM trifluralin; 12·5Tx+15Tfl: Ld-As20 treated with 12·5 μM paclitaxel plus 15·0 μM trifluralin. Results are mean ± S.E.M. of 3 experiments. *$P<0·01$ (all other comparisons to the control were not significant).

reports are available of *in vitro* or *in vivo* tubulin assembly studies of trifluralin in drug-resistant *Leishmania* such as Ld-As20. Hence, we examined the effects of paclitaxel and trifluralin on the dynamics of microtubule formation *in vivo* in wild type and Ld-As20. The treated cells were subjected to separation of the polymerized and unpolymerized tubulins and compared. Fig. 4 shows the Western blot analysis of the unpolymerized and polymerized α-tubulins in Ld-Wt and LdAs-20. The

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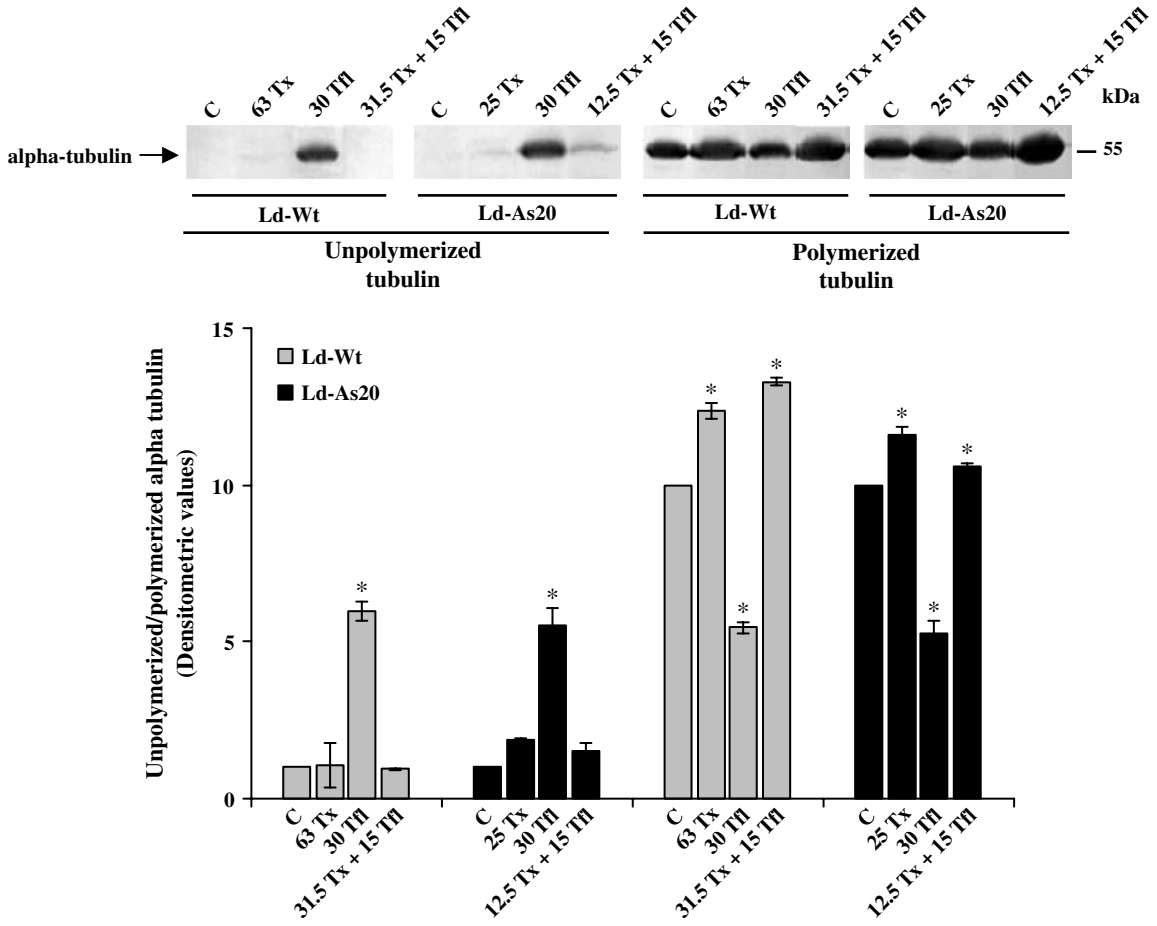

Fig. 4. *In vivo* determination of unpolymerised and polymerized α-tubulin in Ld-Wt and Ld-As20 treated with paclitaxel, trifluralin and a combination of paclitaxel and trifluralin. Upper panel: Western blots of unpolymerized and polymerized α-tubulin; lower panel: densitometric analysis of Western blots. C, untreated cells; 63Tx: Ld-Wt treated with 63·0 μM paclitaxel; 25Tx: Ld-As20 treated with 25·0 μM paclitaxel; 30Tfl: Ld-Wt or Ld-As20 treated with 30·0 μM trifluralin; 31·5Tx+15Tfl: Ld-Wt treated with 31·5 μM paclitaxel plus 15·0 μM trifluralin; 12·5Tx+15Tfl: Ld-As20 treated with 12·5 μM paclitaxel plus 15·0 μM trifluralin. Results are mean $\pm$ S.E.M. of 3 experiments. *$P<0·01$ (all other comparisons to the control were not significant).

unpolymerized tubulin levels in Ld-Wt treated with 63·0 μM paclitaxel and Ld-As20 treated with 25·0 μM paclitaxel did not vary significantly from the untreated cells (Fig. 4). In Ld-Wt treated with 63·0 μM paclitaxel and Ld-As20 treated with 25·0 μM paclitaxel, the polymerized tubulins were $\sim$25% more as compared to the untreated cells (Fig. 4). Results in both Ld-Wt and Ld-As20 show paclitaxel to be favouring microtubule assembly.

The unpolymerized tubulin levels in Ld-Wt and Ld-As20 treated with 30·0 μM trifluralin showed a 5 to 6-fold increase compared to the untreated samples (Fig. 4). Polymerized tubulins of Ld-Wt and Ld-As20 treated with 30·0 μM trifluralin were 40–50% lower as compared to the untreated cells (Fig. 4). Results strongly suggest trifluralin to be favouring microtubule disassembly in both Ld-As20 and Ld-Wt as opposed to paclitaxel, which favours microtubule assembly.

The unpolymerized tubulin pool of both Ld-Wt and Ld-As20 remained unaltered when treated with a combination of paclitaxel and trifluralin (31·5 μM paclitaxel plus 15·0 μM trifluralin in Ld-Wt and 12·5 μM paclitaxel plus 15·0 μM trifluralin in Ld-As20) (Fig. 4). A 45% increase was found in the polymerized tubulin of Ld-Wt and $\sim$35% increase in the polymerized tubulin of Ld-As20, when the cells were treated with a combination of paclitaxel and trifluralin (Fig. 4). The results were similar when the treated samples were probed for β-tubulin (data not shown).

Effects on cellular distribution of α- *and* β-*tubulins*. In addition to the alterations in expression and *in vivo* dynamics of microtubule formation in response to paclitaxel, trifluralin and the combination treatment as described above, the possibility of alterations in cellular distribution of α- and β-tubulins were investigated. The untreated Ld-Wt and Ld-As20 probed for α-tubulin showed orderly distribution of α-tubulins with higher localization at the flagellar and posterior ends (Fig. 5). Ld-Wt and

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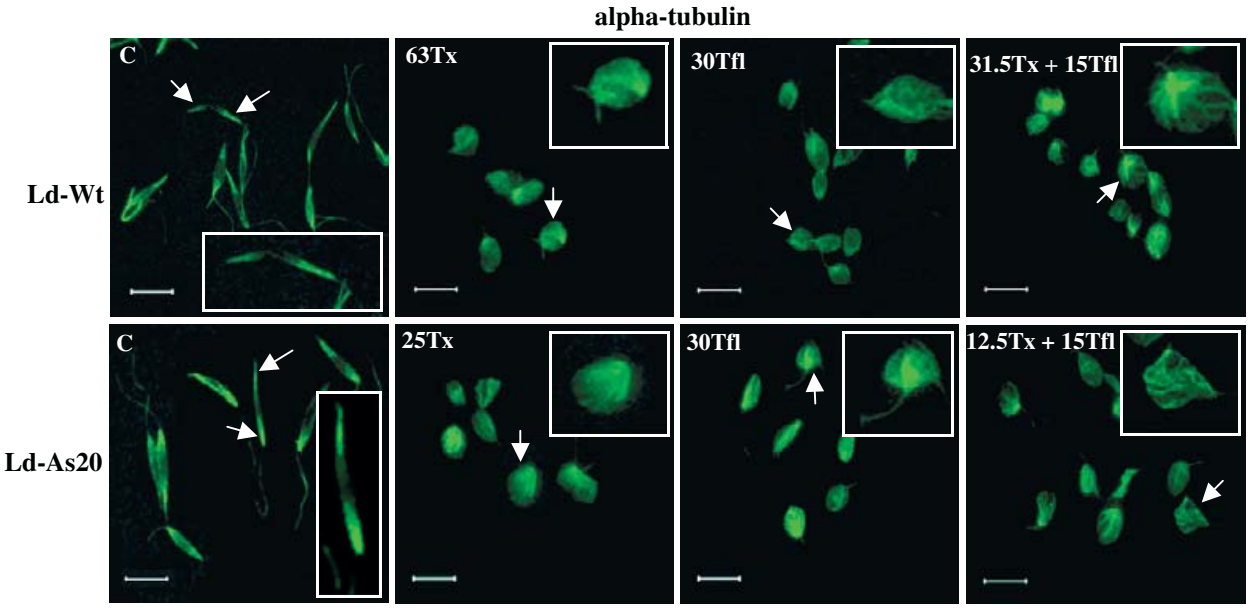

Fig. 5. Effect of paclitaxel, trifluralin and a combination of paclitaxel and trifluralin on cellular distribution of α-tubulin in Ld-Wt and Ld-As20. Treated and untreated promastigotes were probed with anti-α-tubulin antibody and processed as described in the Materials and Methods section. C, untreated cells; 63Tx: Ld-Wt treated with 63·0 μM paclitaxel; 25Tx: Ld-As20 treated with 25·0 μM paclitaxel. 30Tfl: Ld-Wt or Ld-As20 treated with 30·0 μM trifluralin; 31·5Tx+15Tfl: Ld-Wt treated with 31·5 μM paclitaxel plus 15·0 μM trifluralin; 12·5Tx+15Tfl: Ld-As20 treated with 12·5 μM paclitaxel plus 15·0 μM trifluralin. Arrows show a representative cell that is enlarged in the inset. Scale bars, 10 μm.

Ld-As20 treated with paclitaxel at their respective IC_{50} concentrations showed loss of regulation of the orderly distribution of α-tubulins (Fig. 5, 63Tx and 25Tx). A loss of normal cell shape and a distribution of α-tubulin throughout the cell body were also observed. The flagella were not distinctly visible and the cellular distribution of α-tubulin was consistent with the morphological data described above. The treated cells (Fig. 5, 63Tx and 25Tx) also showed areas of high intensity fluorescence randomly spread over the cell body suggesting the presence of microtubular aggregates.

Ld-Wt and Ld-As20 treated with 30·0 μM trifluralin showed deregulation of the distribution of α-tubulins when compared to the untreated cells (Fig. 5, 30Tfl). In both Ld-Wt and Ld-As20 treated with trifluralin, distribution of α-tubulin could be observed throughout the cell body. The flagella were not visible in the treated cells and the cellular distribution of α-tubulin was consistent with the morphological data described above and could also be compared to the effects induced by paclitaxel.

In Ld-Wt and Ld-As20 treated with both paclitaxel and trifluralin, the cells showed a distinct aggregation of α-tubulin at the attachment zone of the flagella with discrete bundles of microtubules radiating through the cell body (Fig. 5; 31·5Tx+15Tfl and 12·5Tx+15Tfl). The distribution patterns of β-tubulins observed were identical to α-tubulin in similar treated samples (data not shown). This is on expected lines since the experimental procedure followed is specific for detergent insoluble cytoskeleton and hence the detected α- and β-tubulins, which are part of the polymerized microtubule and not the free, soluble tubulin isoforms, show similar distribution.

Effects on concentration and cellular distribution of acetylated and tyrosinated tubulins

Acetylated tubulins and tyrosinated tubulins are related to microtubule stability and dynamics (Westermann & Weber, 2003), but not well defined in *Leishmania*. Investigation of the intracellular levels of acetylated tubulins in the wild type and arsenite resistant *L. donovani* revealed that in Ld-As20, the basal level of acetylated tubulin was approximately 1·7-fold higher than in Ld-Wt (Fig. 6A, i). In Ld-Wt, on treatment with 63·0 μM of paclitaxel, the acetylated tubulin levels were ~50% higher and on treatment with 30·0 μM of trifluralin, the acetylated tubulin levels were ~33% higher than the untreated cells (Fig. 6A, ii). Further, a combination of paclitaxel and trifluralin induced an increase in acetylated tubulins by ~50% (Fig. 6A, ii). However, in the Ld-As20 the acetylated tubulin levels did not show any significant change with any of the treatments (Fig. 6A, ii). The basal level of tyrosinated tubulin was found to be similar in Ld-Wt and Ld-As20. The tyrosinated tubulin levels remained unaltered in both Ld-Wt and Ld-As20 in

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response to treatment with paclitaxel, trifluralin or the paclitaxel-trifluralin combination (Fig. 6A, iii).

The untreated Ld-Wt and Ld-As20 probed for cellular distribution of acetylated tubulin showed distinct localization at the flagellar attachment zone and the apex of the cell body (Fig. 6B, i). On treatment of Ld-Wt and Ld-As20 with paclitaxel at their respective IC_{50} concentrations, the acetylated tubulins at the flagellar attachment zone were unaffected in terms of distribution and localization pattern despite the accompanied morphological changes (Fig. 6B, i; 63Tx and 25Tx). However, the localization at the apex of the cell body, as seen in untreated cells, was lost in all the treated cells of both Ld-Wt and Ld-As20. On treatment with trifluralin at their respective IC_{50} concentrations, the localization of the acetylated tubulins at the apex of the cell body, as seen in untreated cells, was lost in all the treated cells of Ld-Wt and Ld-As20 in a similar fashion as seen in response to paclitaxel treatment (Fig. 6B, i; 30Tfl). The acetylated tubulins at the flagellar attachment zone, refractory to trifluralin in terms of distribution and localization pattern, were also comparable to similar observations in paclitaxel-treated cells as described before. When treated with the combination of paclitaxel and trifluralin, the acetylated tubulins at the flagellar attachment zone of both Ld-Wt and Ld-As20 did not show any changes in terms of distribution and localization pattern despite the accompanied morphological changes as compared to the untreated cells (Fig. 6B, i).

The untreated Ld-Wt and Ld-As20 probed for tyrosinated tubulin showed intense labelling along the boundary of the cell body with the flagella being very weakly labelled (Fig. 6B, ii). On treatment of Ld-Wt and Ld-As20 with paclitaxel, trifluralin or a combination of both, the localization pattern was altered and the tyrosinated tubulin was distributed throughout the cell body (Fig. 6B, ii). The flagella were very weakly stained in both Ld-Wt and Ld-As20 treated with trifluralin.

Determination of mode of cell death induced by paclitaxel and trifluralin

The results described above have shown paclitaxel, trifluralin and a combination of paclitaxel and trifluralin to be cytotoxic on both Ld-Wt and Ld-As20. Data also show the accompanying disruption of microtubule function and organization. Many agents that disrupt the microtubule function are reported to induce the apoptotic pathway leading to cell death in many mammalian systems (Ireland & Pittman, 1995). We have recently reported the occurrence of an apoptosis-like death accompanied with alterations in microtubule function in response to sodium arsenite in Ld-As20 (Jayanarayan & Dey, 2004). Therefore, we sought to investigate the possibility of the occurrence of apoptosis-like cell death in Ld-Wt and Ld-As20 in response to paclitaxel, trifluralin or their combination.

DNA fragmentation analysis. DNA fragmentation analysis of Ld-Wt treated with 63·0 μM paclitaxel showed fragmentation of DNA into oligonucleosomal fragments in samples as seen during apoptosis (Fig. 7A). In Ld-As20, DNA ladders were observed in response to treatment with 25·0 μM paclitaxel (Fig. 7B). Trifluralin at 30·0 μM failed to induce DNA laddering in both the strains (Fig. 7A and B). Ld-Wt and Ld-As20 treated with a combination of trifluralin and paclitaxel showed fragmentation of DNA into oligonucleosomal fragments (Fig. 7A and B). The oligonucleosomal fragments were seen with a certain degree of smearing, which is not uncommon in protozoan apoptosis (Das *et al*. 2001; Jayanarayan & Dey, 2004). Data imply an apoptosis-like cell death in Ld-Wt and Ld-As20 promastigotes treated with IC_{50} concentration of paclitaxel and the combination of paclitaxel and trifluralin.

In situ *labelling of DNA fragments*. To further characterize the changes occurring in the DNA during cell death, TUNEL staining was used to detect the free ends of DNA after breakage, which is one of the hallmarks of eukaryotic apoptosis. Ld-Wt treated with 63·0 μM paclitaxel and Ld-As20 treated with 25·0 μM paclitaxel showed TdT-labelled nuclei (~20–25% of total cells), which fluoresced brightly, indicating DNA fragmentation (Fig. 8B and F). Trifluralin treatment did not induce TUNEL-positive cells in either of the strains (Fig. 8C and G). Ld-Wt and Ld-As20 treated with a combination of trifluralin and paclitaxel showed TUNEL-positive cells (~40% of total cells) indicating DNA fragmentation (Fig. 8D and H). Results are consistent with the DNA fragmentation analysis data and provide further evidence for the induction of apoptosis-like cell death in Ld-Wt and Ld-As20 treated by IC_{50} of paclitaxel or a combination of paclitaxel and trifluralin. Data also confirm that trifluralin does not induce apoptosis-like death under the experimental conditions used.

DISCUSSION

In this communication, the effects of two oppositely acting anti-microtubule agents, paclitaxel and trifluralin and a combination of both, on the expression, function and organization of different tubulin isoforms in a sodium arsenite-resistant strain of *L. donovani* have been presented. Further, an apoptosis-like cell death has been demonstrated in both wild type and arsenite-resistant strains in response to treatment with paclitaxel and the combination.

We have shown Ld-As20 to be equally susceptible to trifluralin (IC_{50} = 30·0 μM) as the Ld-Wt. In a study by Chan *et al*. (1995), a vinblastine-resistant

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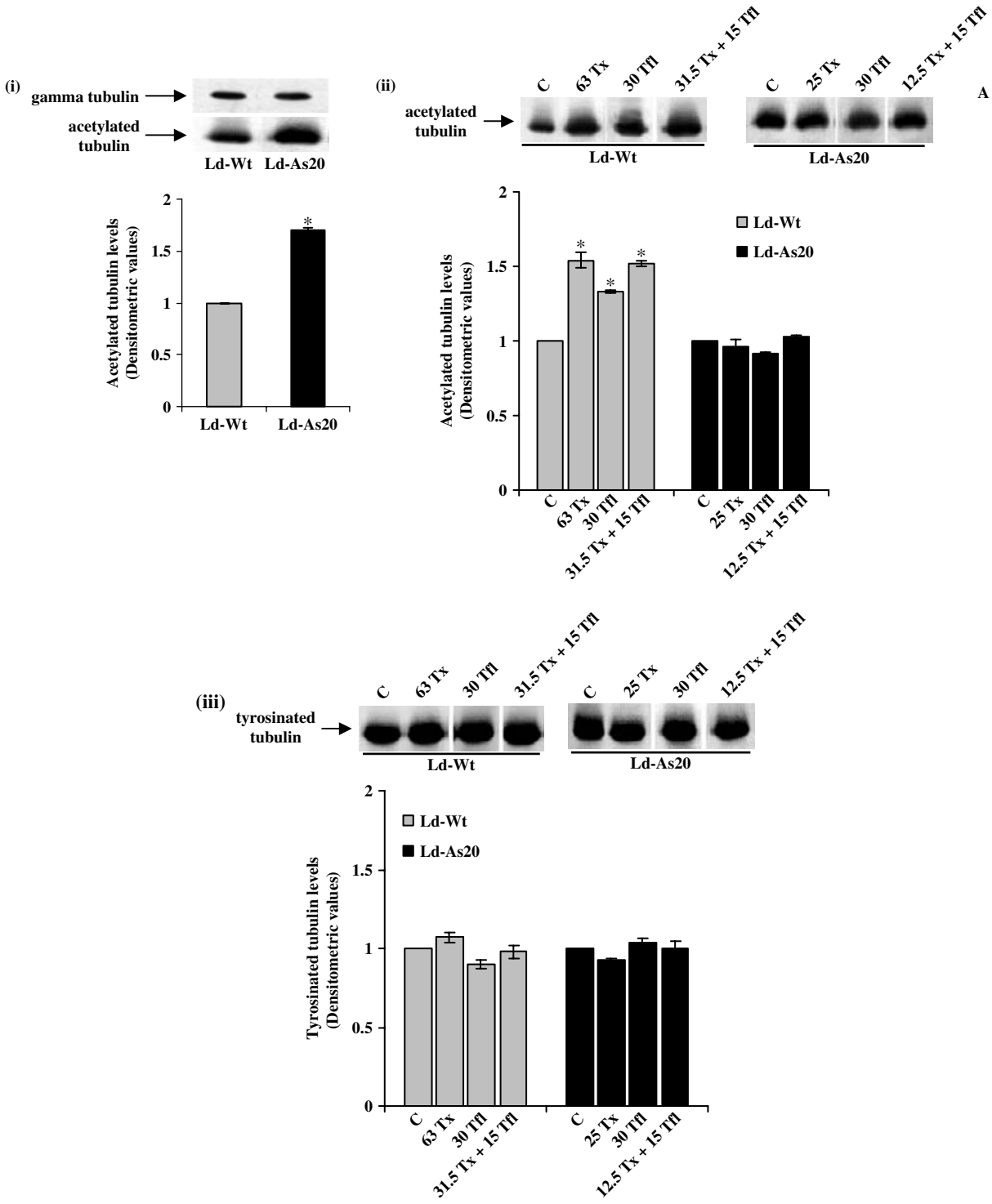

Fig. 6 (*Cont.*). For caption see opposite page.

strain of *L. donovani* having an amplified gene, *ldmdr1*, was shown to have an ED_{50} of trifluralin similar to its parenteral strain. In the same study, Chan *et al.* (1995) investigated the efficacy of trifluralin against P-glycoprotein expressing strains of *Leishmania* and reported them to be equally susceptible to trifluralin as the wild type strain. In a previous communication from our laboratory, the arsenite-resistant *L. donovani* strain, Ld-As20, was reported to overexpress a P-glycoprotein-like protein (Kaur & Dey, 2000). Whether this may correlate with the equal susceptibility of Ld-Wt and Ld-As20 to trifluralin remains to be investigated.

A combination of docetaxel and vinorelbine has been shown to exert a synergistic effect on the growth inhibition of SBC-3 cells (Aoe *et al.* 1999). Empiric combinations of vinca alkaloids with taxanes have been recently used in clinical oncology (Budman *et al.* 2000). The *in vitro* cytotoxicity of paclitaxel and vinorelbine alone, in combination and in sequence,

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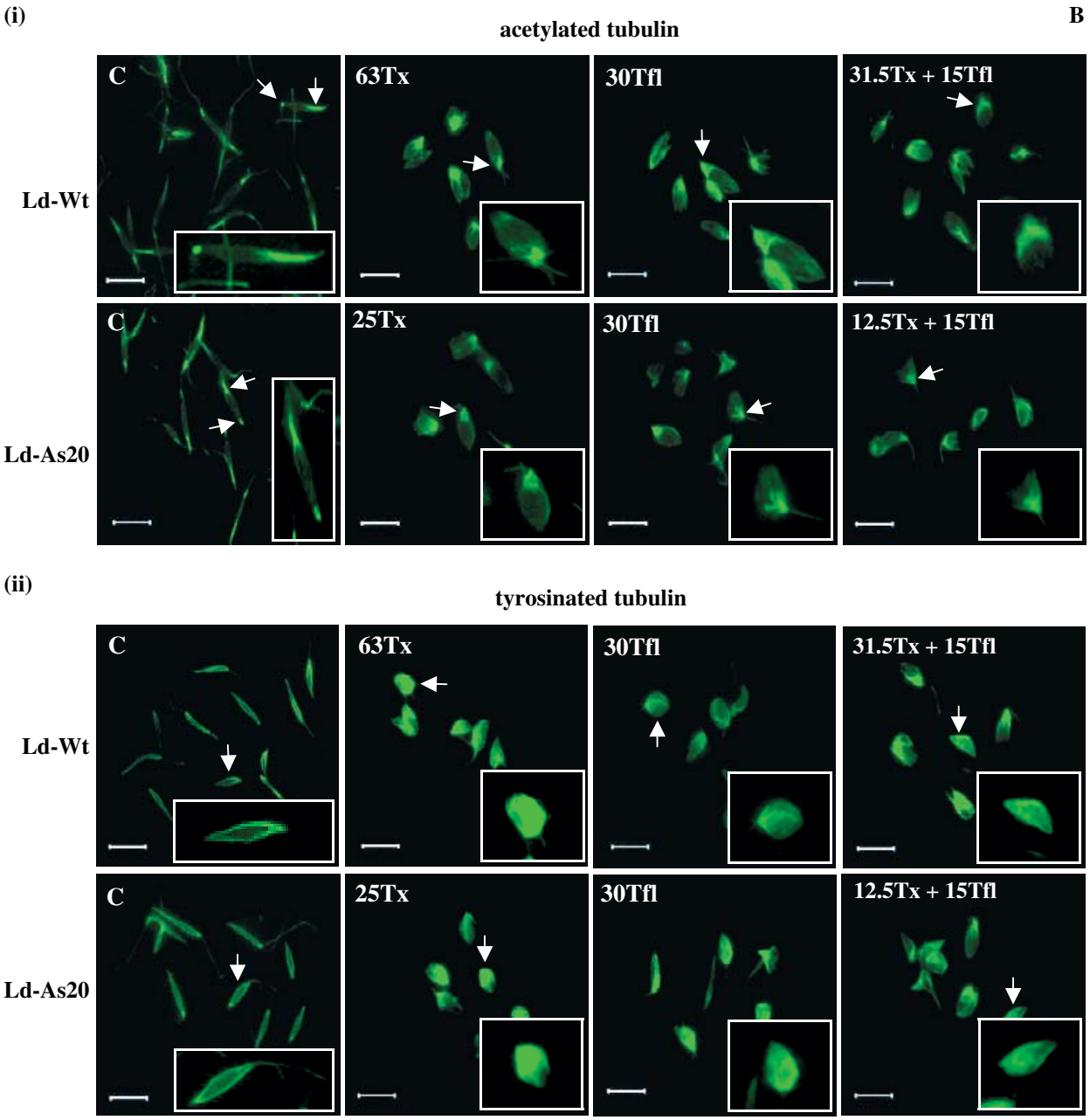

Fig. 6. (Ai) Basal levels of acetylated tubulin in Ld-Wt and Ld-As20. Upper panel: Western blots acetylated tubulin; lower panel: densitometric analysis of Western blots. Gamma tubulin is shown as protein loading control. (Aii) Effect of paclitaxel, trifluralin and a combination of paclitaxel and trifluralin on cellular concentration of acetylated tubulin in Ld-Wt and Ld-As20. Whole cell lysates were resolved by SDS-PAGE and Western immunoblotted using anti-acetylated tubulin. (Upper panel: Western blots of acetylated tubulins; lower panel: densitometric analysis of Western blots.) (Aiii) Effect of paclitaxel, trifluralin and a combination of paclitaxel and trifluralin on cellular concentration of tyrosinated tubulin in Ld-Wt and Ld-As20. Upper panel: Western blots of tyrosinated tubulins; lower panel: densitometric analysis of Western blots. C, untreated cells; 63Tx: Ld-Wt treated with 63·0 μM paclitaxel; 25Tx: Ld-As20 treated with 25·0 μM paclitaxel. 30Tfl: Ld-Wt or Ld-As20 treated with 30·0 μM trifluralin; 31·5Tx+15Tfl: Ld-Wt treated with 31·5 μM paclitaxel plus 15·0 μM trifluralin; 12·5Tx+15Tfl: Ld-As20 treated with 12·5 μM paclitaxel plus 15·0 μM trifluralin. Results are mean ± S.E.M. of 3 experiments. *$P<0·01$ (all other comparisons to the control were not significant). (Bi) Cellular distribution of acetylated tubulins. (Bii) Cellular distribution of tyrosinated tubulins. C, untreated cells; 63Tx: Ld-Wt treated with 63·0 μM paclitaxel; 25Tx: Ld-As20 treated with 25·0 μM paclitaxel; 30Tfl: Ld-Wt or Ld-As20 treated with 30·0 μM trifluralin; 31·5Tx+15Tfl: Ld-Wt treated with 31·5 μM paclitaxel plus 15·0 μM trifluralin; 12·5Tx+15Tfl: Ld-As20 treated with 12·5 μM paclitaxel plus 15·0 μM trifluralin. Arrows show a representative cell that is enlarged in the inset. Scale bars, 10 μm.

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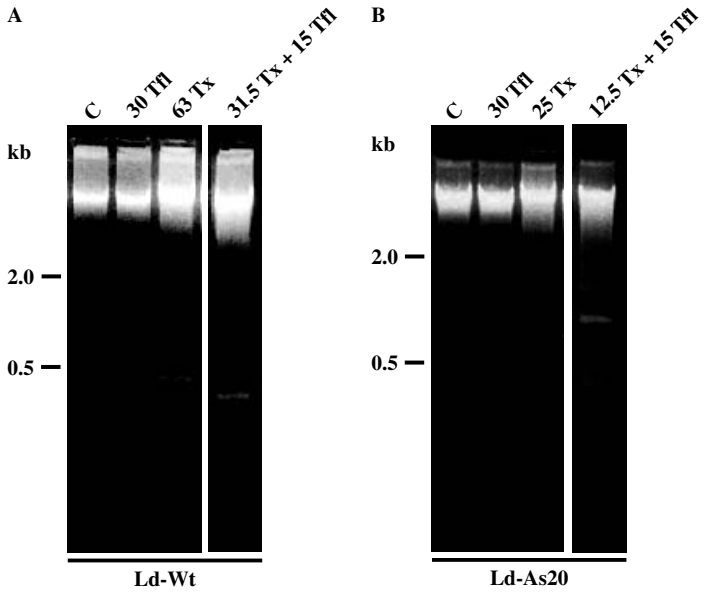

Fig. 7. DNA fragmentation analysis of Ld-Wt and Ld-As20 treated with paclitaxel, trifluralin and their combination. Agarose gel electrophoresis of DNA is shown. (A) Ld-Wt cells. (B) Ld-As20 cells. C, untreated cells; 30Tfl: Ld-Wt or Ld-As20 treated with 30·0 μM trifluralin; 63Tx: Ld-Wt treated with 63·0 μM paclitaxel; 25Tx: Ld-As20 treated with 25·0 μM paclitaxel; 31·5Tx+15Tfl: Ld-Wt treated with 31·5 μM paclitaxel plus 15·0 μM trifluralin; 12·5Tx+15Tfl: Ld-As20 treated with 12·5 μM paclitaxel plus 15·0 μM trifluralin.

has been reported against the human doxorubicin-resistant MCF7 (MCF7-R) breast carcinoma cell line (Culine *et al*. 1999). Combination treatments of docetaxel and vinorelbine have been reported to be beneficial with no substantial increase in associated toxicity in non-small-cell lung carcinoma (Dieras *et al*. 1997). Conversely, in another study, combinations of both paclitaxel and docetaxel with vinorelbine have been reported to cause severe toxicity (Parimoo, Jeffers & Muggia, 1996). Therefore it is evident that any use of a combination of anti-microtubule agents will need extensive validation. The paclitaxel-trifluralin combination induced effects on the MT organization and dynamics were comparable to the effects induced by these drugs individually, although variations in the magnitude were observed under different experimental conditions. The drug-induced effects were consistent with disruption of MT functions, lending further credence to the use of the paclitaxel-trifluralin combination against Ld-Wt and Ld-As20.

In our experiments, paclitaxel, trifluralin and their combination induced altered morphology and abnormal flagella in Ld-Wt and Ld-As20. In trypanosomes, as the cells progress through the cell cycle, alterations in presence and length of the new flagellum occurs along with organelle repositioning (Robinson *et al*. 1995). The images shown in this study represent the natural balance of cells at different stages of cell cycle within the exponentially growing asynchronous culture. Since nearly 90% of the treated Ld-Wt or Ld-As20 show altered morphology and abnormal flagella, the observations are attributed to the drug treatments.

In the *in vivo* tubulin polymerization assays, the combination of paclitaxel and trifluralin increased the polymerized pool of intracellular tubulins in both Ld-Wt and Ld-As20. However, in these assays, a non-specific aggregation of some trifluralin-disrupted MTs with the paclitaxel-stabilized MTs leading to an apparent increase of the polymerized tubulin pool of both the strains cannot be ruled out.

Tubulin post-translational modifications have been extensively investigated in *Trypanosoma* (Schneider *et al*. 1987; Sherwin *et al*. 1987; Sasse & Gull, 1988; Schneider, Plessmann & Weber, 1997; Khol & Gull, 1998). Acetylation is mostly associated with stable microtubules e.g. in trypanosomes, the axoneme and subpellicular microtubules (Sasse & Gull, 1988). The presence of tyrosinated α-tubulin is reported to be a marker for newly formed microtubules in *T. brucei brucei* (Sherwin *et al*. 1987). Anti-acetylated tubulin antibody (clone 6-11B-1) and anti-tyrosinated tubulin antibody (clone TUB-1A2) were used in this study to detect acetylated and tyrosinated tubulins respectively. Werbovetz *et al*. (1999) have used anti-acetylated tubulin antibody (clone 6-11B-1) and anti-tyrosinated tubulin antibody (clone TUB-1A2) for detecting leishmanial acetylated and tyrosinated tubulin by Western immunoblotting. Sasse & Gull (1988) have used the anti-acetylated tubulin antibody (clone 6-11B-1) to detect the acetylated tubulin in *T. brucei* by immunoblotting and immunofluorescence. Therefore, it is very unlikely that the anti-acetylated and anti-tyrosinated tubulin antibodies used in this study would bind non-specifically to any different protein. Paclitaxel, trifluralin and the combination caused an

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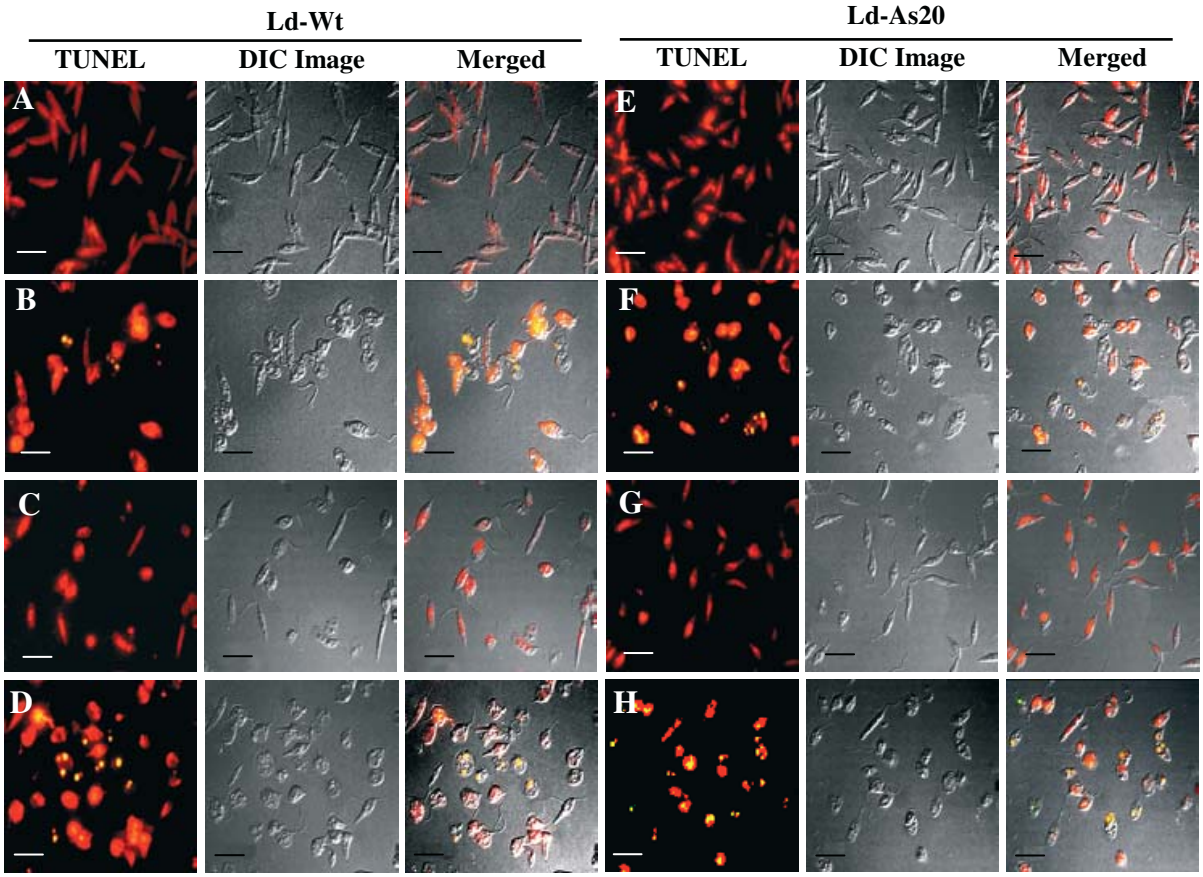

Fig. 8. *In situ* labelling of DNA fragments in Ld-Wt and Ld-As20. The DNA of treated and untreated cells was subjected to terminal deoxyribonucleotide transferase (TdT)-mediated dUTP nick end labelling as described in the Materials and Methods section. Fragmented DNA show intense fluorescence as compared to normal DNA. (A) Untreated Ld-Wt; (B) Ld-Wt treated with 63·0 μM paclitaxel; (C) Ld-Wt treated with 30·0 μM trifluralin; (D) Ld-Wt treated with 31·5 μM paclitaxel plus 15·0 μM trifluralin; (E) untreated Ld-As20; (F) Ld-As20 treated with 25·0 μM paclitaxel; (G) Ld-As20 treated with 30·0 μM trifluralin. (H) Ld-As20 treated with 12·5 μM paclitaxel plus 15·0 μM trifluralin. Scale bars, 10 μm.

increase in the acetylated tubulin levels in Ld-Wt but not in Ld-As20. The possibility of alterations, if any, to the enzyme(s) involved in the post-translational modifications of tubulins by the anti-microtubule agents, leading to an increase in the acetylated tubulin levels of Ld-Wt remains to be investigated. The unaltered localization of acetylated tubulins at the flagellar attachment zone in both Ld-Wt and Ld-As20 on treatment with paclitaxel, trifluralin or both, suggests the presence of a pool of acetylated tubulins refractory to the drug treatment. Such acetylated tubulins non-responsive to microtubule depolymerizing agents have been reported in *T. brucei* (Schneider *et al*. 1987). However, the effect of altered acetylation on the response to paclitaxel has not yet been reported. The treatments also caused loss of localization of tyrosinated tubulins observed along the cell boundary in both the strains suggesting that the deregulation of the localization may have been a general response to the alterations to normal microtubule function/organization and not specific to the type of antimicrotubule agent used. Phosphorylation of γ-tubulin is known to regulate microtubule organization in budding yeast (Vogel *et al*. 2001). Post-translational modification(s) of gamma-tubulins have been predicted to have an important role in the regulation of microtubule nucleation in neuronal cells (Sulimenko *et al*. 2002). Acetylated tubulins are known to regulate the binding of microtubule-associated proteins to microtubules (MacRae, 1997; Westermann & Weber, 2003). Therefore it is possible that the observed alterations in acetylated and tyrosinated tubulins may be related to disruption of microtubule function and organization observed in the affected cells. It was evident from the fluorescent photomicrographs that the flagella of both Ld-Wt and Ld-As20 treated with paclitaxel, trifluralin and the combination were not distinctly stained when probed for α-/β-tubulins or the acetylated and tyrosinated tubulins. However, during morphological observations of treated Ld-Wt and Ld-As20, flagella could be seen in the DIC images suggesting a loss of tubulins from the flagella on treatment with the anti-microtubule agents.

The mode of cell death induced by the anti-parasitic agents can influence the chemotherapeutic

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strategy adopted to limit the parasite growth. Although a common consequence of microtubule disruption in mammalian cells is the induction of apoptosis, there are no reports of paclitaxel, trifluralin or their related analogues causing apoptosis-like death in *Leishmania*. A form of cell death resembling metazoan apoptosis has been reported in parasitic protozoans (Blanco *et al*. 2001; Das *et al*. 2001; Sereno *et al*. 2001; Zangger, Mottram & Fazel, 2002; Debrabant *et al*. 2003). We have recently reported the occurrence of an apoptosis-like death accompanied with alterations in microtubule function in response to sodium arsenite in Ld-Wt and Ld-As20 (Jayanarayan & Dey, 2004). Another recent report form our laboratory has shown apoptosis-like death in miltefosine treated *L. donovani* (Verma & Dey, 2004). Havens *et al*. (2000) have reported the absence of apoptotic indicators in *L. donovani* exposed to antimicrotubule agents such as ansamitocin P3, hemiasterlin and taxol. However, in the current study IC_{50} concentration of paclitaxel in both Ld-Wt and Ld-As20 caused apoptosis-like death whereas trifluralin under similar conditions did not induce apoptosis. The fact that trifluralin did not induce apoptosis-like death under the conditions tested in this study implies that disruption of microtubule function by the anti-microtubule agents may not be the only factor involved. Further investigations are necessary to understand the specific pathways involved in apoptosis-like death mediated by specific anti-microtubule agents in *Leishmania*.

In our experiments, a combination of trifluralin and paclitaxel at respective half of IC_{50} concentrations in Ld-Wt and Ld-As20 showed an additive effect inhibiting ~55% of parasite growth. No changes in the culture medium such as pH shift or precipitation, which could hinder parasite survival, could be observed during the treatment of leishmanial cells with the combination under the stated experimental conditions. By combining trifluralin with paclitaxel, the concentration of paclitaxel required to inhibit the Ld-As20 (by 50%) could be reduced as evidenced in this study. Such an approach could be useful since paclitaxel, although effective *in vitro* against Ld-As20, has considerable toxicity towards mammalian cells (Werbovetz, 2002). We hypothesize that the combination of a drug that specifically binds to parasite tubulin, such as trifluralin, with paclitaxel has resulted in the exploitation of the hypersensitivity of the resistant strain to paclitaxel along with the species specificity of trifluralin. In this study, the cells were simultaneously treated with both paclitaxel and trifluralin. Further investigations *in vivo* are necessary to determine the dependence of these effects on simultaneous or sequential drug addition, dose or duration of treatment.

In conclusion, in this study the arsenite-resistant Ld-As20 has been shown to be susceptible to both paclitaxel and trifluralin. As evidenced by the *in vivo* polymerization assays, trifluralin and paclitaxel have been shown to have opposite anti-microtubule effects with trifluralin favouring microtubule disassembly unlike paclitaxel, which enhanced microtubule assembly. For the first time, a combination of two oppositely acting anti-microtubule drugs have been shown to have anti-leishmanial activity, both in wild type and the resistant strain. The mechanism by which the combination induced cytotoxicity in both Ld-Wt and Ld-As20 has also been established to involve anti-microtubule events that included alteration to tubulin expression, formation dynamics, post-translational modifications and cellular distribution of the α-/β-tubulins. Investigations into the mode of cell death induced by paclitaxel and the combination have revealed drug-specific induction of apoptosis-like death in both Ld-Wt and Ld-As20. Taken together, the above findings provide valuable information for further development of chemotherapeutic strategies based on anti-microtubule agents against drug resistant *Leishmania* parasites. However, the effects of these anti-microtubule agents both *in vivo* and on the amastigote forms of the parasite merits further investigations since, variations in the basal levels of tubulins between the promastigotes and amastigotes have been reported (Prasad *et al*. 2000*b*; Jayanarayan & Dey, 2002). Havens *et al*. (2000) have also reported many-fold increased susceptibility of amastigotes to taxol as compared to promastigotes of *Leishmania*. Since arsenite is a model drug and not used currently for treating leishmaniasis, further extension of these investigations to determine the effects of the anti-microtubule agents on strains resistant to other clinically used drugs may provide valuable information.

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