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ORIGINAL ARTICLE

A new microtubule-targeting compound PBOX-15 inhibits T-cell migration via post-translational modifications of tubulin

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Abstract The ordered, directional migration of T-lymphocytes is a key process during immune surveillance, immune response, and development. A novel series of pyrrolo-1,5-benzoxazepines have been shown to potently induce apoptosis in variety of human chemotherapy resistant cancer cell lines, indicating their potential in the treatment of both solid tumors and tumors derived from the hemopoietic system. Pyrrolobenzoxazepine 4-acetoxy-5-(1-naphtyl)naphtho[2,3-*b*]pyrrolo[1,2-*d*][1,4]-oxazepine (PBOX-15) has been shown to depolymerize tubulin in vitro and in the MCF7 breast cancer cell line. We hypothesized that this may suggest a role for this compound in modulating integrin-induced T-cell migration, which is largely dependent on the microtubule dynamics. Experi-

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ments were performed using human T lymphoma cell line Hut78 and peripheral blood T-lymphocytes isolated from healthy donors. We observed that human T-lymphocytes exposed to PBOX-15 have severely impaired ability to polarize and migrate in response to the triggering stimulus generated via cross-linking of integrin lymphocyte function associated antigen-1 receptor. Here, we show that PBOX-15 can dramatically impair microtubule network via destabilization of tubulin resulting in complete loss of the motile phenotype of T-cells. We demonstrate that PBOX-15 inhibitory mechanisms involve decreased tubulin polymerization and its post-translational modifications. Novel microtubule-targeting effects of PBOX-15 can possibly open new horizons in the treatment of overactive inflammatory conditions as well as cancer and cancer metastatic spreading.

Keywords T-cell migration · PBOX · Tubulin

Introduction

T-lymphocytes are the central regulatory cells of the immune response and require distinct signals for activation. After leaving the vasculature, T-cells acquire a polarized morphology and initiate crawling, probably as a result of chemotactic stimuli, earlier activating stimuli or both [1]. The chemotactic 'hand-mirror' shape of a crawling T-cell consists of a flattened leading edge followed by the nucleus and a handle-like tail termed the uropod [2]. The intrinsic ability of T-lymphocytes to migrate has been observed in vivo within lymph nodes and also in in vitro models of migration [3–6]. T-cell migration from the vascular compartment across tissue barriers and through the extracellular matrix is a key event during inflammation. The capacity to migrate and localize in tissues is of vital importance for the protective function of lymphocytes against infectious agents. However, the capacity of lymphocytes to migrate and infiltrate tissues is also a major contributing factor in the development of autoimmunity, allergy and graft rejection. In addition, tissue specific accumulation is a feature of some neoplastic lymphocytes.

Lymphocyte migration and homing requires a series of ligand-receptor interactions involving adhesion molecules of the integrin family [7]. T-cells make use of the integrin lymphocyte function associated antigen-1 (LFA-1) when migrating in response to chemoattractants either across the vasculature into lymph nodes or across the vessels associated with inflamed tissues [8]. These transmembrane proteins connect the extracellular matrix with the cell interior both physically, being linked to cortical cytoskeleton, and functionally, serving as bi-directional signal transducers. By engagement with intracellular adhesion molecules, LFA-1 provides a strong adhesive force to promote T-cell and antigen presenting cell conjugate formation and generally stabilize this interaction. Locomotory T-cells triggered via LFA-1 in the absence of chemotactic gradient produce movement in a random manner [8]. In addition, LFA-1 has the ability to transduce a variety of transmembrane signals including protein kinase C activation [6] and cytoskeletal rearrangement [9]. However, the exact sequence of integrin-mediated signaling events resulting in cytoskeletal rearrangements and cell locomotion is not fully understood.

Microtubules (MTs) are essential components of the cytoskeleton and are important for many aspects of mammalian cell responses including cell division, growth, migration, and signaling [10, 11]. The MTs play a key role in T-cell locomotion. MT retraction into the cellular uropod is an important step in T-cell motility [8]. MT-targeting agents thus can affect migration of malignant T-cells into endothelial monolayers. Tubulins, the building block of MTs, are subject to specific post-translational modifications including acetylation, detyrosination, and tyrosination [12, 13]. These post-translational modifications of tubulins are thought to modulate the functions and localization of MTs within the cell.

MTs are necessary for directed migration of endothelial and other cells, and there are several possible mechanisms by which MT-disrupting compounds could block cell motility. These include impairment of the repositioning of the microtubule organizing center (MTOC), interfering MT interaction with focal adhesions, inhibition of MT polymerization, and depolymerization cycle, inhibition of intracellular protein trafficking and vesicle transport, and inhibition of MT-mediated integrin clustering and increased avidity. These processes vary in their sensitivity to inhibition by MT-targeting drugs. Therapeutic agents that target cytoskeletal proteins inhibit T-cell migration [14]. This inhibition of T-cell migration could be exploited therapeutically in autoimmune T-cell infiltrative diseases. MT-targeting agents like taxol and nocodazole are being used clinically both as anti-inflammatory and anticancer drugs. It has also been established through various and clinical studies that drug possessing anticancer activity also exhibit anti-inflammatory properties.

Our research center has developed and reported a novel series of pyrrolo-1,5-benzoxazepine (PBOX) compounds which induce apoptosis in a wide variety of human chemotherapy resistant cancer cells including those derived from hematological malignancies, breast carcinomas, and chronic myeloid leukemia cells [15–20]. Furthermore, PBOX compounds demonstrated significant anti-tumor activity in vivo in an aggressive murine model of mammary carcinoma that has been developed in our research center [15]. These suggest the potential of these drugs as novel anticancer therapeutics in the treatment of both solid tumors

and tumors derived from the hematopoietic system. Current on-going investigations are aimed to characterize the molecular mechanisms by which these drugs induce apoptosis of cancer cells. It has recently been demonstrated that some of the PBOX compounds targeted microtubule network in human breast carcinoma-derived MCF7 cells and inhibited the assembly of purified tubulin in vitro [21]. Therefore, the aim of this study was to investigate the effect (s) of a novel microtubule-targeting agent Pyrrolobenzoxazepine 4-acetoxy-5-(1-naphtyl)naphtho[2,3-*b*]pyrrolo[1,2-*d*][1,4]-oxazepine (PBOX-15) on active T-cell migration, which is largely dependent on MT dynamics [8].

Materials and methods

Materials

PBOX-15 was developed by Campiani's research group according to a previously described procedure [22]. (Roswell Park Memorial Institute) RPMI 1640 medium, fetal bovine serum (FBS), and L-glutamine were from Gibco BRL (Grand Island, NY, USA). Antibodies used for induction of T-cell motility as previously described [6] were of the clone SPVL7 (mAb to the α–chain of LFA-1 from Sanbio). Goat anti-mouse IgG was obtained from Dako A/S (Denmark). Human recombinant ICAM-1Fc (rICAM-1) was purchased from R&D Systems (Minneapolis, MN, USA). Mouse monoclonal anti-α-tubulin, anti-acetylated tubulin (clone 6-11B-1) and anti-tyrosinated tubulin (TUB-1A2) antibodies were purchased from Sigma (St Louis, MO, USA). Rabbit polyclonal anti-detyrosinated tubulin (Glu-tubulin) was obtained from Chemicon (Millipore Corporation, Billerica, MA, USA). Alexa fluor 488 conjugated anti-mouse was from Molecular Probes (Invitrogen Corporation, CA, USA). Lymphoprep® was obtained from Axis-Shield PoC AS (Oslo, Norway). Polyvinylidene fluoride (PVDF) membrane was obtained from Pall Gelman Laboratories (Ann arbor, MI, USA). Acrylamide–bisacrylamide solution, Acrylogel (30%) was purchased from BDH (VWR International Ltd., England). Enhanced chemiluminescence (ECL) plus reagent was purchased from Amersham (Arlington Heights, IL, USA). All the reagents unless attributed specifically, were from Sigma (St Louis, MO, USA).

Cell cultures

Human cutaneous T lymphoma cell line Hut78 (American Type Culture Collection (ATCC), Manassas, VA, USA) was used and cultured as described previously [6]. Briefly, they were cultured in RPMI 1640 medium containing 10% (*v*/*v*) heat inactivated FBS, L-glutamine, and antibiotics (penicillin 100 IU/ml, streptomycin 100 μg/ml) in 5% CO_2 at 37°C.

Peripheral blood T-lymphocytes (PBTLs) were isolated from healthy donors using standard techniques. Briefly, peripheral blood mononuclear cells were prepared using Lymphoprep®. T-cells were expanded by culturing in RPMI 1640 medium containing 10% (*v*/*v*) heat inactivated FBS in the presence of 1 μg/ml phytohemagglutin for 3 days. These cells were then maintained in culture medium supplemented with 20 ng/ml IL-2. After 10 days, PBTLs population were >95% positive for CD3.

Induction of cell motility

The ability of T-cells to display integrin-induced locomotory behavior was determined using our well-characterized assay as previously described [6]. Briefly, 6- or 96-well tissue culture plates, depending on the particular assay type were pre-coated with goat anti-mouse IgG or mouse anti-human Fc and subsequently incubated with cross-linking monoclonal motility inducing anti-LFA-1 antibody (clone SPVL-7) or human rICAM-1. Cells were loaded into the anti-LFA-1 coated wells (60×10^4 cells/well in 6-well plate or 10×10^3 cells/well in 96-well plate for Hut78 cells; 30×10^3 cells/well in 96-well plate for PBTLs).

Using previously described procedure, rICAM-1 cross-linking of PBTLs following LFA-1 activation was performed [23] with minor modifications. Briefly, 10 days old cultures were washed twice in wash buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 20 mM (pH 7.4), NaCl 140 mM, glucose 2 mg/ml). Cells were then resuspended in activation buffer (HEPES 20 mM (pH 7.4), NaCl 140 mM, glucose 2 mg/ml, $MgCl_2$ 5 mM, and ethylene glycol tetraacetic acid (EGTA) 1 mM) prior to being loaded into the rICAM-1 coated tissue culture plates.

As a control (resting cells), wells were coated with poly-L lysine (cells adhered to this surface without activation). Cells were pretreated with indicated compounds for 30 min at 37°C before being seeded. PBOX-15 was dissolved in ethanol to the stock concentration of 10 mM and stored at -20°C. Subsequent working dilutions, as indicated in the text, were prepared extemporarily in the culture medium. Control, untreated cells received equivalent volumes of the appropriate solvent.

Deformation Index

In order to study migratory phenotype of T-cells, Deformation Index (DI) was calculated as described previously [24]. DI provides a stronger measure of the degree of cell polarization in comparison to elongation index and circularity alone and, therefore, more accurately reflects the phenotype of polarized locomotory cells. Typically, values of 1–3 are assigned to cells that were non-migratory, i.e., cell shape is nearing circular. Those cells that display a

polarized phenotype (cell body and trailing uropod) and, therefore, a higher degree of deformation had higher DI values (>5) [24]. On average, >40 randomly chosen cells were scored for each set of conditions. Semi-automated analysis was performed using the Scion Image software (Scion Corporation, Fredrick, MC, USA).

Preparation of cellular extracts of Hut78 cells for immunoblotting

The cellular extracts were prepared as described previously [25] with minor modifications. Briefly, cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in lysis buffer (HEPES 50 mM (pH 7.4), NaCl 150 mM, $MgCl_2$ 1.5 mM, EGTA 1 mM, sodium pyrophosphate 10 mM, sodium fluoride 50 mM, β-glycerophosphate 50 mM, Na_3VO_4 1 mM, 1% Triton X-100, phenylmethylsulphonyl fluoride 2 mM, leupeptin 10 μg/ml and aprotinin 10 μg/ml). Lysis was carried out at 4°C for 30 min. Lysates were centrifuged at 16,000×*g* for 15 min at 4°C. The protein content of the supernatant was determined by Bradford assay. Cell lysates were boiled with Laemmli sample buffer (final concentration: Tris-HCl 62.5 mM (pH 6.7), Glycerol 10% (*v*/*v*), sodium dodecyl sulfate 2% (*w*/*v*), bromophenol blue 0.002% (*w*/*v*) containing β-mercaptoethanol 143 mM) for 5 min. Equal amounts of lysates were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The transfer of separated proteins to PVDF membrane was performed by semi-dry blotting. The PVDF membranes were blocked with blocking buffer containing 5% dry milk in PBST (0.1% (*v*/*v*) Tween-20 in PBS) at room temperature for 1 h. Following blocking of the membrane, the immunoblot was incubated with the indicated primary antibody overnight at 4°C, followed by incubation with horseradish peroxidase conjugated secondary antibody for 1 h at room temperature. The immunoblots were visualized using the ECL detection system.

Immunofluorescence microscopy

Confocal microscopy was performed as previously described [6]. Resting or LFA-1 stimulated cells (as described above) on the poly-L lysine or anti-LFA-1-coated Lab-Tek multi-chamber Permanox® slides (Nalge Nunc International, Rochester; NY, USA) were fixed by incubating with 3% paraformaldehyde in PBS for 30 min. Cells were washed twice in PBS for 10 min and were permeabilized in 0.5% Triton X-100 before staining. Cells were incubated with primary antibody for 2 h, washed thrice with PBS, and then incubated with secondary antibodies and Hoechst for 1 h at room temperature. Slides were finally washed with PBS, drained, and mounted with DAKO fluorescent mounting medium (Dako A/S, Denmark). Fluorescent microscopy was performed using 100X objective on a Perkin Elmer LCI confocal workstation with a Kr–Ar laser attached to a Nikon TE2000-U inverted microscope (Nikon, Tokyo, Japan) under Ultraview (Perkin Elmer) image acquisition software. At least 20 different microscopic fields were observed for each sample.

Ex vivo polymerized tubulin assay

The evaluation of tubulin polymerization in live cells (ex vivo assay) was performed as previously described [26]. Briefly, untreated or PBOX-15-treated resting and migrating HUT78 cells (as explained above) were harvested into MT-preserving buffer [Pipes 0.1 M (pH 6.9), glycerol 2 M, $MgCl_2$ 5 mM, EGTA 2 mM, 0.5% Triton X-100, and protease inhibitors) supplemented with 4 μM taxol to maintain stability of assembled MTs during isolation. The supernatant containing unpolymerized tubulin was clarified by centrifugation (20,000 g for 45 min) and separated from the pellet containing sedimented polymerized tubulin. The pellet was washed once in MT-preserving buffer before being denatured in Laemmli sample buffer.

Densitometric analysis

Densitometric analyses of the western blots were performed by using GeneTools software (Syngene, Cambridge, UK). The relative values of the samples were determined by giving an arbitrary value of 1.0 to the respective control samples of each experiment.

Statistical analysis

The data are expressed as mean ± SEM. For comparison of two groups, *p*-values were calculated by two-tailed unpaired student's *t*-test. In all cases $p<0.05$ was considered to be statistically significant.

Results

PBOX-15 inhibits T-cell migration

Activated T-lymphocytes triggered via LFA-1 receptor or ligand interaction rapidly polarize and acquire active motile behavior [6]. They start spreading and subsequently undergo dramatic cytoskeletal changes resulting in a polarized phenotype with long cytoplasmic projections [8]. To test the effect of PBOX-15 on T-cell migration, Hut78 cells were pretreated with different concentrations of PBOX-15 for 30 min and incubated on anti-LFA-1-coated 96-well plates for 4 h. Control untreated cells developed a polarized and elongated morphology (Fig. 1a). Pretreatment

of Hut78 cells with different concentrations of PBOX-15 ranging from 0.2 to 1 μM resulted in the inhibition of LFA-1-induced migration in a dose-dependent manner (Fig. 1b–e). At 1 μM concentration of PBOX-15, complete loss of the locomotion-associated phenotype was observed (Fig. 1e). Quantitation of the observed inhibitory effect of PBOX-15 using DI as a quantitative descriptor of cell morphology also indicated that the development of motile phenotype was inhibited by PBOX-15 in a dose-dependent manner (Fig. 1f).

To test the in vivo efficacy of PBOX-15, we determined whether the inhibitory effect was present in LFA-1-stimulated PBTLs migration. Normal human PBTLs isolated from a healthy donor showed active locomotory behavior when exposed to anti-LFA-1 ligands. (Fig. 2a). Pretreatment of PBTLs with different concentrations of PBOX-15 resulted in the inhibition of migration in a dose-dependent manner (Fig. 2b–f) with complete loss of migration at 1 μM (Fig. 2e), as observed with Hut78 cells. Similar inhibitory effect of PBOX-15 was observed with LFA-1-activated human PBTLs when exposed to immobilized rICAM-1 ligands (data not shown).

To test the potential apoptotic effect of PBOX-15, Hut78 cells were treated with 1 μM concentration of PBOX-15 for 30 min or 24 h. No cell death was observed after 30 min of the treatment (Supplementary Fig. 1). However, treatment of the cells for 24 h induced apoptosis up to 37%, as detected by flow-cytometry after Annexin V and propidium iodide labeling (Supplementary Fig. 1). Therefore, for further experimentation, short pretreatment of the cells with 1 μM concentration of PBOX-15 for 30 min was employed.

Further, to determine if the failure of T-cells to develop a migratory phenotype was also accompanied by the loss of active locomotion, high content analysis experiments utilizing time-lapse video imaging by InCell-1000 Analyzer (GE Healthcare, England) were performed. A slow and apparently random mode of locomotion was observed in control Hut78 cells triggered via cross-linking of LFA-1. Measuring live cell migration over a period of 20 min gave a clear evidence that PBOX-15 treatment significantly decreased LFA-1 stimulated Hut78 cells migration (Supplementary Fig. 2). Average speed of Hut78 cell migration was recorded to be 1.055 μm/min which was decreased by 65.4% when were pretreated with PBOX-15 (0.365 μm/min). Average distance traveled by control cells was 21.1 μm (ranging from 8.3 to 69.5 μm), which was decreased to 7.3 μm (ranging from 0.1 to 42.1 μm) when cells were pretreated with PBOX-15 (Supplementary Fig. 2a vs b).

PBOX-15 targets microtubules of T-cells

Microtubules represent a central cytoskeleton system controlling active locomotion in T-cells. Recent studies from our laboratory provided evidences that PBOX-15 induces apoptosis by affecting tubulin polymerization [21]. To determine whether PBOX-15 exerts similar effects on T-cells microtubules causing inhibition of its migration, intracellular distribution and functional involvement of the microtubule were analyzed. Control untreated resting Hut78 cells showed radial arrays of organized tubulin distribution at the centrosome (Fig. 3a). When the Hut78 cells were stimulated with LFA-1, the characteristic array of MT was

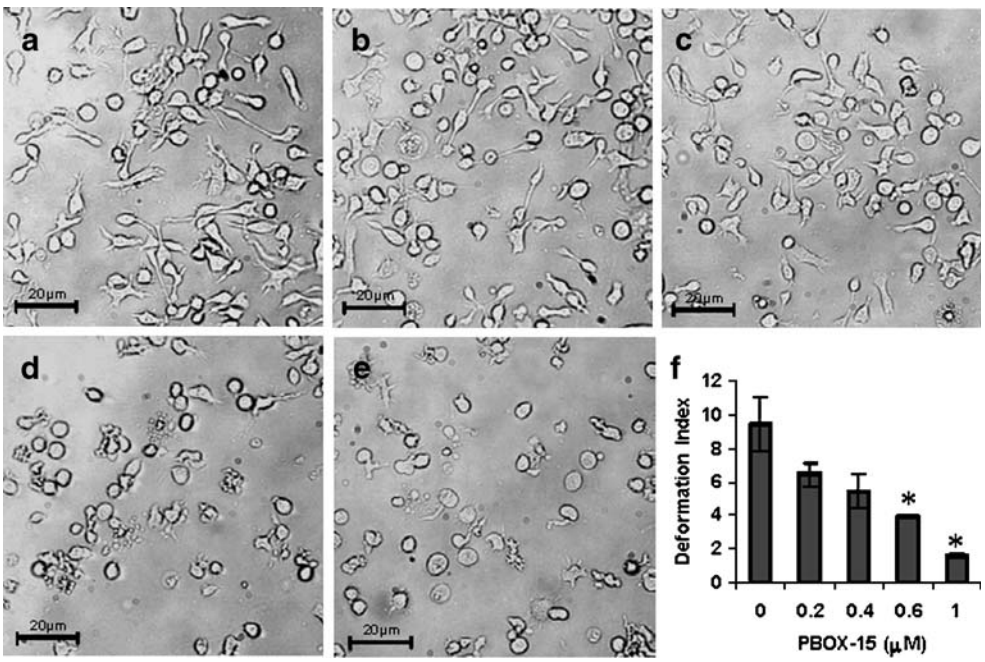

Fig. 1 Effect of PBOX-15 on LFA-1-induced locomotory phenotype of Hut78 cells. Cells were pretreated with either vehicle [0.1% (*v*/*v*) ethanol; control] (**a**), PBOX-15 0.2 μM (**b**), 0.4 μM (**c**), 0.6 μM (**d**), or 1.0 μM (**e**) for 30 min and incubated on anti-LFA-1 coated 96-well plate for 4 h. Experiments were performed in triplicate and repeated three times. At least 20 microscopic fields were photographed, and a representative figure is shown. Dose-response migration inhibition by PBOX-15 in Hut78 cells stimulated via immobilized anti-LFA-1 was determined by measurement of DI (**f**). Data are mean ± SEM of three independent experiments. *$p<0.05$ with respect to control

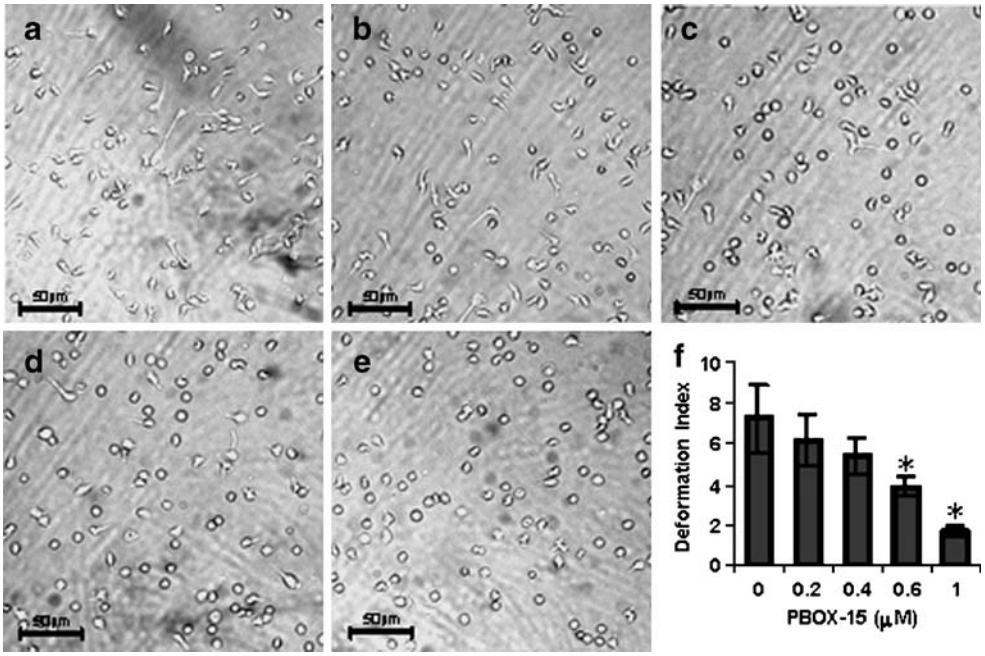

Fig. 2 Effect of PBOX-15 on LFA-1-induced locomotory phenotype of PBTLs. PBTLs isolated from healthy donor were pretreated with either vehicle [0.1% (*v*/*v*) ethanol; control] (**a**), PBOX-15 0.2 μM (**b**), 0.4 μM (**c**), 0.6 μM (**d**) or 1.0 μM (**e**) for 30 min and incubated on anti-LFA-1-coated 96-well plate for 1 h. Experiments were performed in triplicate and repeated three times. At least 20 microscopic fields were photographed, and a representative figure is shown. Dose-response migration inhibition by PBOX-15 in Hut78 cells stimulated via immobilized anti-LFA-1 was determined by measurement of DI (**f**). Data are mean ± SEM of three independent experiments. *$p<0.05$ with respect to control

displayed, in that MTOC was located at the side of the nucleus opposing the direction of cell migration and long MT extended to the uropods from here, reflecting the state of cell polarization (Fig. 3b). In contrast, exposure of Hut78 cells to PBOX-15 dramatically impaired the tubulin network resulting in complete loss of the typical motile phenotype upon LFA-1 ligation. PBOX-15 treated T-cells displayed disorganized tubulin with thin discontinuous filaments and disrupted staining; microtubules were no longer radiating from the centrosome to the plasma membrane (Fig. 3c,d). Similar effects were observed when Hut78 cells were pretreated with microtubule depolymerizing agent nocodazole (Fig. 3e,f). In contrast, stable microtubules in bundles were observed when cells were pretreated with paclitaxel, a microtubule stabilizing drug (Fig. 3g,h).

A similar tubulin-targeting effect of PBOX-15 was observed in resting as well as LFA-1 stimulated PBTLs (data not shown). PBOX-15 did not significantly affect actin cytoskeletal network (Supplementary Fig. 3).

Effects of PBOX-15 on post-translational modifications of tubulin in T-cells

Acetylated tubulins and tyrosinated tubulins are related to microtubule stability and dynamics [27, 28]. To further explore the effect of PBOX-15 on post-translational modification of tubulin in T-cells during LFA-1-induced migration, PBOX-15 treated or untreated resting and LFA-1 stimulated Hut78 cells were immunostained with antibody directed against acetylated α-tubulin (Fig. 4). Untreated control resting cells showed uniform distribution of acetylated tubulin radiating from the centrosome (Fig. 4a); however, this subset of microtubule was dramatically reduced when Hut78 cells were allowed to migrate upon LFA-1 cross-linking (Fig. 4b). Its localization was limited to the area near the MTOC with a characteristic spindle-like appearance (Fig. 4b). When these cells were treated with PBOX-15, the detectable levels of acetylated tubulin were reduced and it was localized as a compact spot near the MTOC in the resting cells (Fig. 4c). Moreover, when PBOX-15-treated cells were cross-linked with LFA-1, tubulin acetylation was further reduced and was only hardly visible around the MTOC (Fig. 4d). As post-translational acetyl modification correlates with increased MT stability [28], these results indicate that the PBOX-15 destabilized long life MTs. Similar effects were observed when Hut78 cells were pretreated with nocodazole (Fig. 4e,f). Paclitaxel treatment stabilized acetylated tubulin and was observed in microtubule bundles in resting as well as LFA-1 cross linked cells (Fig. 4g,h).

Tyrosinated tubulin represents the major form of active microtubules in cells [29]. Therefore, we used an antibody directed against tyrosinated form of tubulin to observe changes in this subset of microtubule in Hut78 cells. Untreated resting as well as LFA-1-stimulated Hut78 cells showed intense labeling for tyrosinated tubulin, which was distributed throughout the cell body radiating from the MTOC (Fig. 5a,b). Strikingly, tyrosinated tubulin network of Hut78 cells after PBOX-15 treatment appeared collapsed with disorganized and punctate staining (Fig. 5c,d), apparently due to the depolymerization of both dynamic and stable microtubules. Similar effects were observed

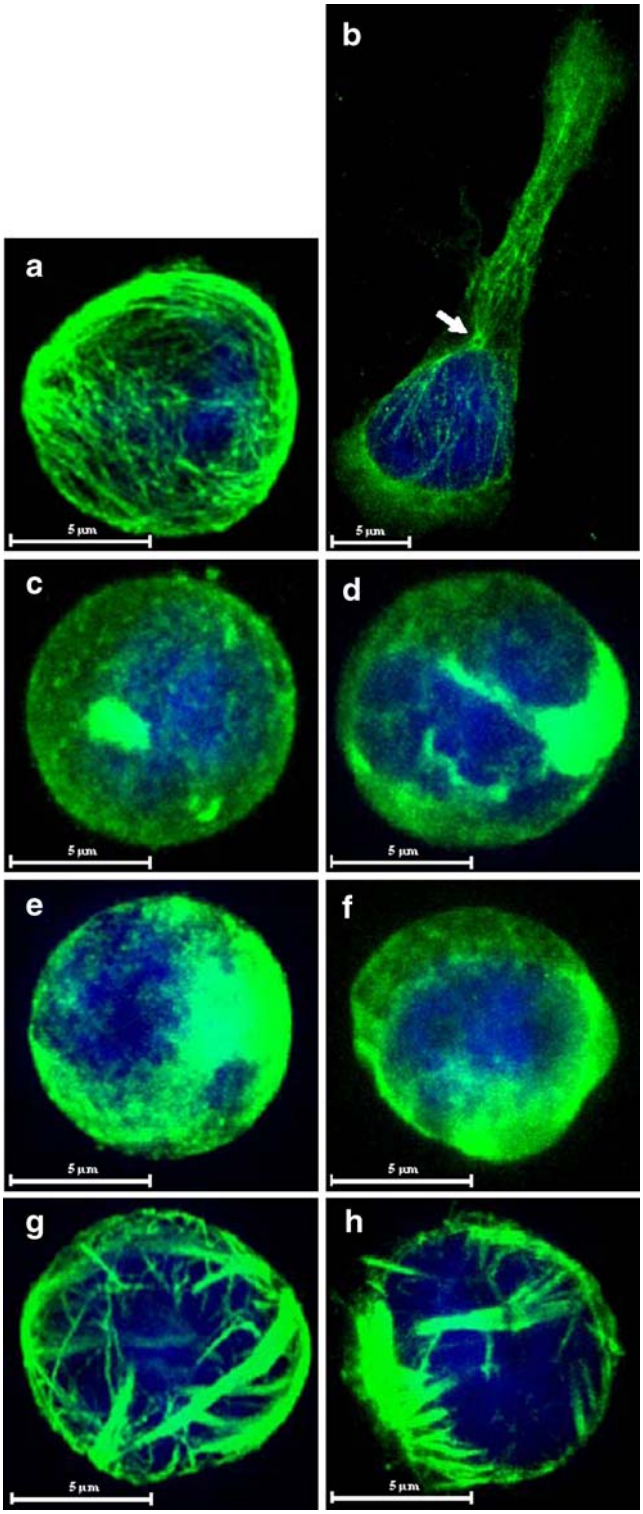

Fig. 3 Effect of PBOX-15 on the organisation of cellular MT network in resting and LFA-1 stimulated T-cells. Hut78 cells were pretreated with either vehicle [0.1% (*v*/*v*) ethanol] (**a, b**), 1.0 μM PBOX-15 (**c, d**), 10.0 μM nocodazole (**e, f**) or 4.0 μM paclitaxel (**g, h**) for 30 min and incubated on poly-L lysine (**a, c, e, g**) or anti-LFA-1 (**b, d, f, h**) coated Permanox® chamber slides for 4 h. After this time, the medium was carefully removed and cells were fixed in 3% PFA. Cells were incubated with mouse monoclonal anti-α-tubulin antibody for 1 h and then with Alexa488 conjugated anti-mouse secondary antibody for further 1 h at room temperature. After washing, nuclei of the cells were stained with Hoechst (*blue*). The organisation of MT network (*green*) was visualized by confocal microscopy using a 100× oil immersion lens. *Arrow* indicates clearly distinguishable MTOC. Results shown are representative of three independent experiments

when Hut78 cells were pretreated with nocodazole (Fig. 5e,f). However, stable microtubules bundles formed due to paclitaxel treatment stained for localized tyrosinated tubulin (Fig. 5g,h).

As carboxy-terminal (C-terminal) tyrosine is encoded for in most α-tubulin by the messenger RNA, the primary modification is its removal by tubulin tyrosine carboxypeptidase [27]. The resulting detyrosinated tubulin exposes a carboxy-terminal glutamic acid and is therefore referred to as Glu-tubulin. Glu-tubulin is abundant in stable MTs of various cell types. Although detyrosination itself does not stabilize MTs, it can be used as a marker for how long an MT has been assembled [27]. Untreated resting cells showed intense staining for Glu-tubulin towards leading edge of cells (Fig. 6a). Glu-tubulin was abundant in LFA-1-stimulated untreated cells toward the leading edge and MTOC (Fig. 6b). Detyrosinated subset of tubulin in Hut78 cells after PBOX-15 treatment appeared collapsed and disorganized (Fig. 6c,d). Similar effects were observed when Hut78 cells were pretreated with nocodazole (Fig. 6e,f). Paclitaxel treatment stabilized the tubulin, making Glu-tubulin appear as bundles (Fig. 6g,h).

To further confirm these results biochemically, we examined the total expression of α-tubulin, acetylated α-tubulin, and tyrosinated tubulin by western blotting. Initially, we examined the total expression of α-tubulin in resting and LFA-1-stimulated Hut78 cells treated with or without PBOX-15 (Fig. 7a). Densitometric quantitation of α-tubulin bands, normalized for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) expression, indicated that there was no significant difference between these samples (not depicted). Acetylated α-tubulin level in LFA-1 stimulated Hut78 cells was 57% lower as compared with resting cells (Fig. 7b; open bars, lanes 2 vs 1). When these cells were pretreated with PBOX-15, acetylated tubulin was reduced to 47% in resting Hut78 cells (Fig. 7b; open bars, lanes 3 vs 1), which was further reduced to 25% after LFA-1 cross-linking as compared to control untreated resting cells (Fig. 7b; open bars, lanes 4 vs 1). Tyrosinated tubulin level in migrating Hut78 cells was increased up to 47% compared to the resting cells (Fig. 7b; closed bars, lanes 2 vs 1). Treatment of these cells with PBOX-15 reduced the level of tyrosinated tubulin by 49% in resting (Fig. 7b; closed bars, lanes 3 vs 1) and by 46% upon LFA-1 cross-linking as compared to control (untreated) cells (Fig. 7b; closed bars, lanes 4 vs 1).

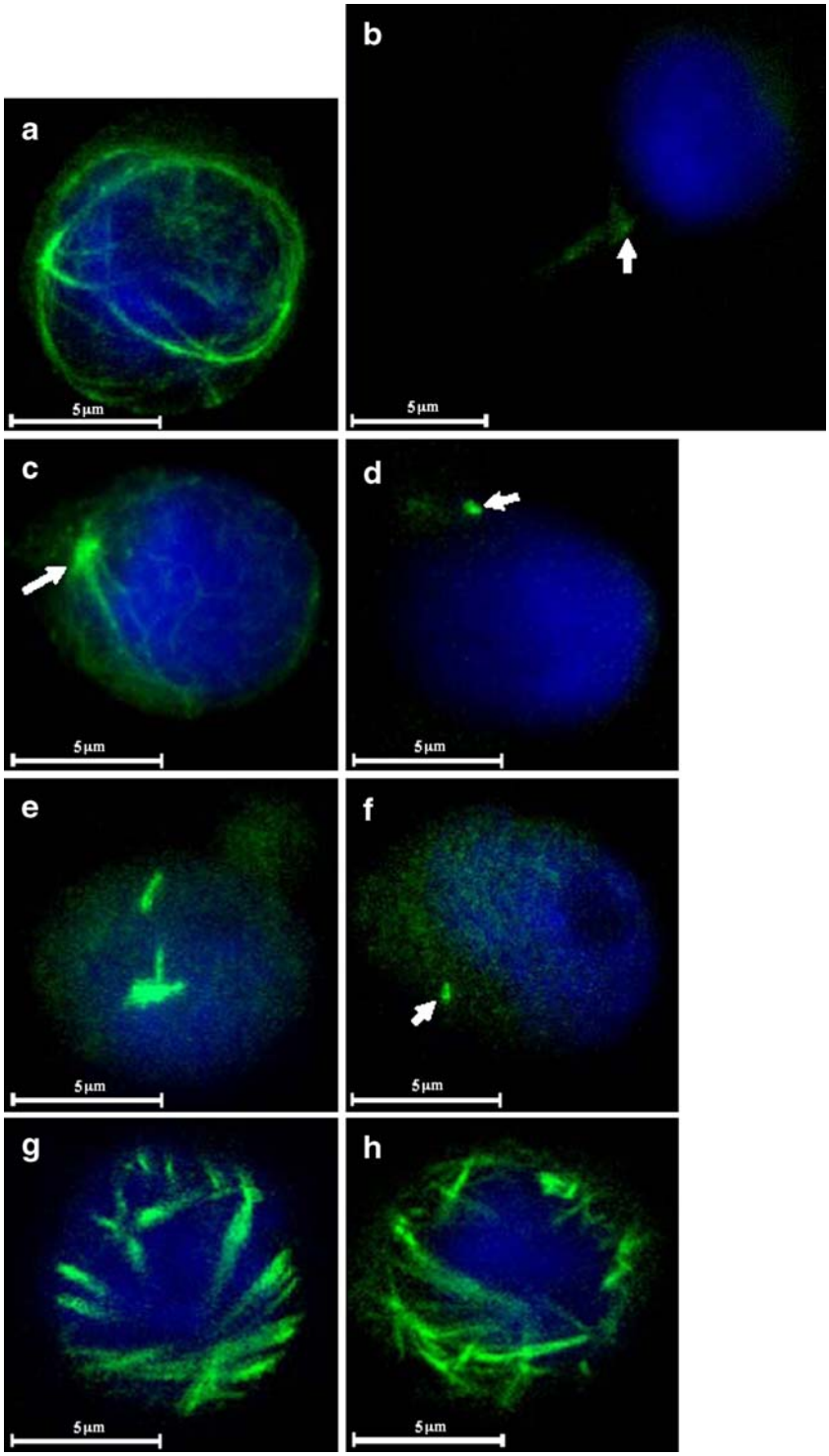

Fig. 4 Effect of PBOX-15 on the acetylation of tubulin in resting and LFA-1 stimulated T-cells. Hut78 cells were pre-treated with either vehicle [0.1% (*v*/*v*) ethanol] (**a, b**), 1.0 μM PBOX-15 (**c, d**), 10.0 μM nocodazole (**e, f**) or 4.0 μM paclitaxel (**g, h**) for 30 min and incubated on poly-L lysine (**a, c, e, g**) or anti-LFA-1 (**b, d, f, h**) coated Permanox® chamber slides for 4 h. After this time, the medium was carefully removed and cells were fixed in 3% PFA. Cells were incubated with mouse monoclonal anti-acetylated tubulin antibody for 1 h and then with Alexa488 conjugated anti-mouse secondary antibody for further 1 h at room temperature. After washing, nuclei of the cells were stained with Hoechst (*blue*). Acetylated tubulin (Ac-tubulin; *green*) was visualized by confocal microscopy using a 100× oil immersion lens. *Arrow* indicates clearly distinguishable MTOC. Results shown are representative of three independent experiments

PBOX-15 induced alterations in tubulin polymerization dynamics

Because PBOX-15 markedly disrupted the cellular microtubule network, we investigated whether it could affect tubulin polymerization in live cells. The effect of PBOX-15 on the live cell tubulin dynamics has not yet been reported. Hence, to study the dynamics of microtubule formation ex vivo, Hut78 cells pretreated with or without PBOX-15 were stimulated by incubating on anti-LFA-1 coated plate for 4 h or left unstimulated and lysed. As MTs could be partially destroyed during the preparation of crude cell lysates, we lysed the cells in MT-preserving buffer and performed differential sedimentation of polymerized tubulin (pellet

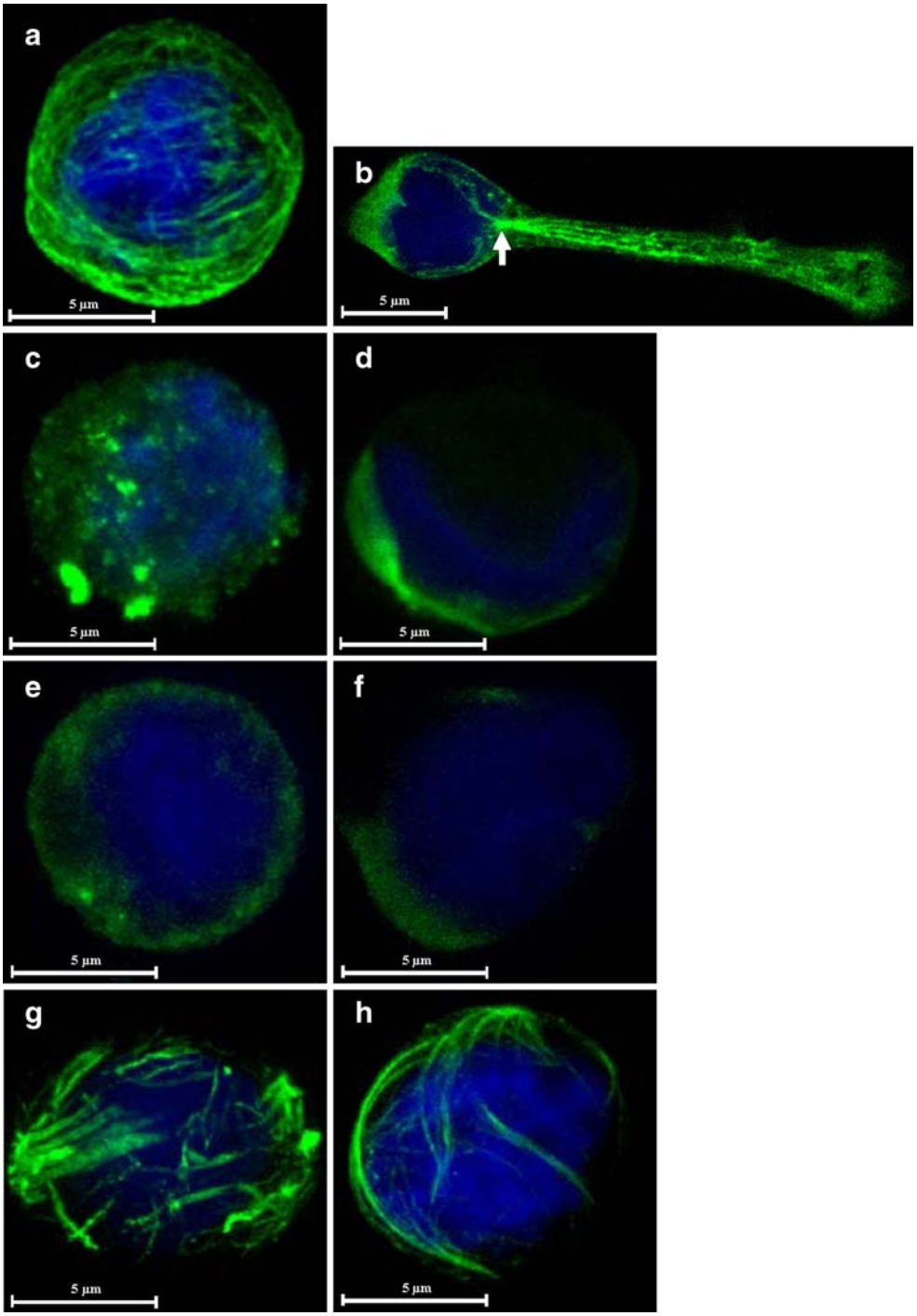

Fig. 5 Effect of PBOX-15 on the tyrosination of tubulin in resting and LFA-1 stimulated T-cells. Hut78 cells were pre-treated with either vehicle [0.1% (*v*/*v*) ethanol] (**a, b**), 1.0 μM PBOX-15 (**c, d**), 10.0 μM nocodazole (**e, f**) or 4.0 μM paclitaxel (**g, h**) for 30 min and incubated on poly-L lysine (**a, c, e, g**) or anti-LFA-1 (**b, d, f, h**) coated Permanox® chamber slides for 4 h. After this time, the medium was carefully removed and cells were fixed in 3% PFA. Cells were incubated with mouse monoclonal anti-tyrosinated tubulin antibody for 1 h and then with Alexa488 conjugated anti-mouse secondary antibody for further 1 h at room temperature. After washing, nuclei of the cells were stained with Hoechst (*blue*). Tyrosinated tubulin (Tyr-tubulin; *green*) was visualized by confocal microscopy using a 100× oil immersion lens. *Arrow* indicates clearly distinguishable MTOC. Results shown are representative of three independent experiments

fraction) from unpolymerized free tubulin (supernatant fraction) pool. Western blot analysis (Fig. 8a) showed that when untreated Hut78 cells were stimulated with LFA-1, the level of unpolymerized tubulin was reduced down to 64% (Fig. 8b; lanes 1 vs 3), with a concomitant increase of polymerized tubulin from 40% to 110% (Fig. 8b; lanes 5 vs 7). In resting Hut78 cells, treatment with PBOX-15 increased the amount of unpolymerized tubulin up to 44% (Fig. 8b; lanes 1 vs 2), with simultaneous decrease of polymerized tubulin level from 40% to 6% (Fig. 8b; lanes 5 vs 6). In a similar manner, when PBOX-15-treated Hut78 cells were stimulated with LFA-1, the level of unpolymerized tubulin was increased from 64% to 139% (Fig. 8b; lanes 3 vs 4), with simultaneous decrease of polymerized tubulin level from 110% to 11% (Fig. 8b; lanes 7 vs 8).

Taken together, these results demonstrate that PBOX-15 inhibits T-cell migration by depolymerizing microtubules. It disrupts the microtubule network, interferes with post-

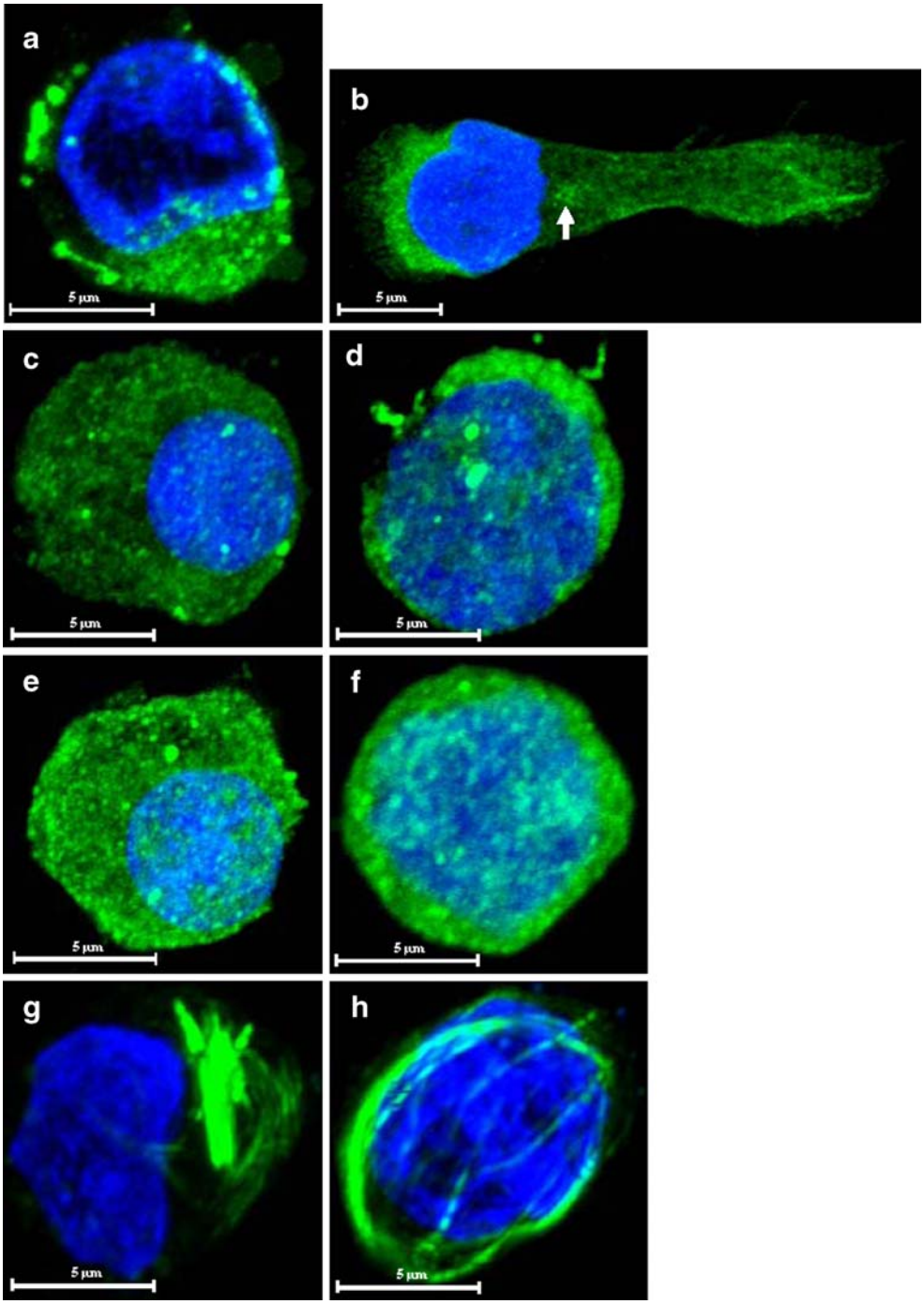

Fig. 6 Effect of PBOX-15 on the detyrosination of tubulin in resting and LFA-1 stimulated T-cells. Hut78 cells were pre-treated with either vehicle [0.1% (*v*/*v*) ethanol] (**a, b**), 1.0 μM PBOX-15 (**c, d**), 10.0 μM nocodazole (**e, f**) or 4.0 μM paclitaxel (**g, h**) for 30 min and incubated on poly-L lysine (**a, c, e, g**) or anti-LFA-1 (**b, d, f, h**) coated Permanox® chamber slides for 4 h. After this time, the medium was carefully removed and cells were fixed in 3% PFA. Cells were incubated with rabbit polyclonal anti-detyrosinated tubulin antibody for 1 h and then with Alexa488 conjugated anti-rabbit secondary antibody for further 1 h at room temperature. After washing, nuclei of the cells were stained with Hoechst (*blue*). Detyrosinated tubulin (Glu-tubulin; *green*) was visualized by confocal microscopy using a 100X oil immersion lens. Arrow indicates clearly distinguishable MTOC. Results shown are representative of three independent experiments

translational modifications of tubulin, and suppresses microtubule dynamics, thus, affecting the function of this cytoskeletal system.

Discussion

PBOX-15 is a potent proapoptotic member of the novel series of PBOX compounds which has previously been shown to induce apoptosis in many human tumor cell lines and to have anticancer properties in various cell culture systems, animal models, and clinical samples [15–19]. The mechanisms by which PBOX-15 exert its antiproliferative effects have been attributed to tubulin depolymerizing activity, thus, resulting in cell death by apoptosis [21]. We demonstrate here that PBOX-15 inhibits LFA-1 integrin-induced migration of human T lymphoma cell line Hut78 as well as PBTLs. The observed effect was dramatic, leading to a complete loss of LFA-1-mediated T-cell polarization and migration.

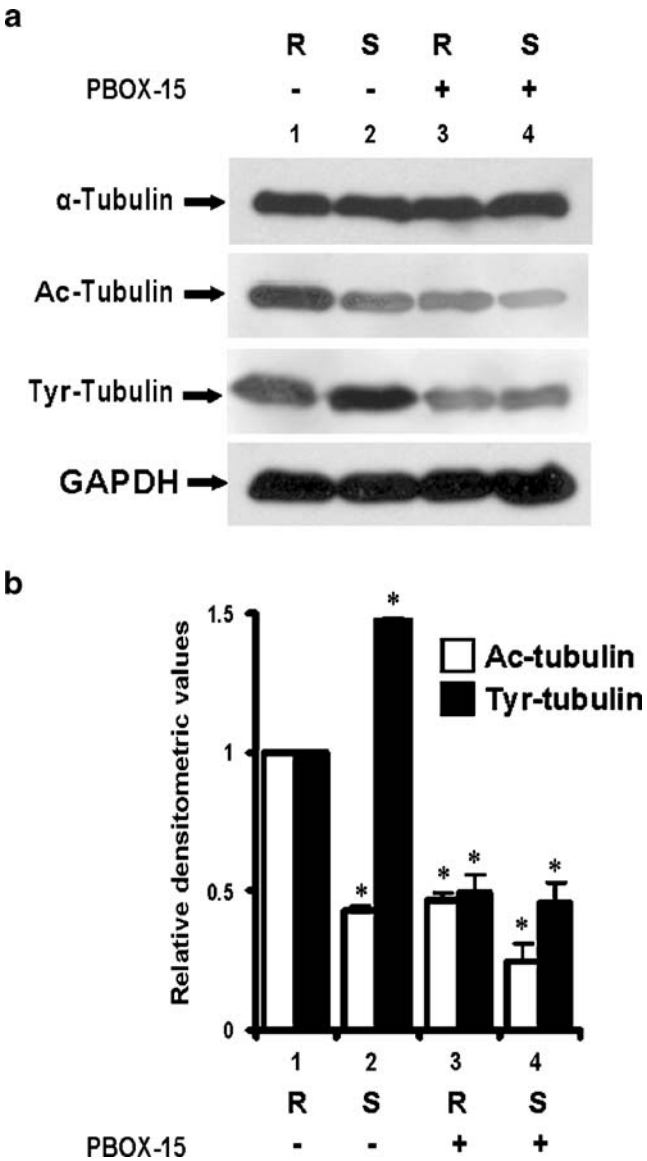

Fig. 7 Effect of PBOX-15 on post-translational modifications of tubulin in resting and LFA-1 stimulated T-cells. Hut78 cells were pretreated with either vehicle [0.1% (*v*/*v*) ethanol] (control) or 1.0 μM PBOX-15 for 30 min, stimulated with (*S*) or without (*R*) anti-LFA-1 for 4 h and lysed. Equal amounts of cell lysates (20 μg each) were separated by SDS-PAGE, and after western blotting probed with anti-α-tubulin, anti-acetylated tubulin (*Ac-tubulin*), anti-tyrosinated tubulin (*Tyr-tubulin*) or GAPDH (as a loading control) antibody (**a**). Relative densitometric analysis of the individual band was performed and presented (**b**). Data are mean ± SEM of three independent experiments. *$p<0.05$ with respect to respective controls

The morphological changes in cells exposed to PBOX-15 were similar to those induced by two other MT-targeting drugs, paclitaxel and nocodazole. However, paclitaxel is known to cause microtubule stabilization, whereas nocodazole causes microtubule depolymerization. It has been revealed in earlier studies from our group that PBOX-15 possesses anti-microtubule activity, as PBOX-15 treatment disrupted microtubule networks [21]. Specific anti-microtubule effect of PBOX-15 has also been proved in vitro using cell-free tubulin polymerization assay [21]. The disruption to the microtubules following treatment with PBOX-15 was similar to that elicited by nocodazole. Increasing concentrations of nocodazole and other known tubulin depolymerizers are known to kinetically 'cap' the actively growing plus end of microtubules, preventing growth and, thus, leading ultimately to disassembly of the microtubules [30]. Both PBOX-15 and nocodazole resulted in a dramatic destruction of the complex MT network of the cell, detected as an intricate mesh of microtubules in the vehicle control cells. The effect of PBOX-15 upon the microtubule network was distinct to that elicited by treatment with paclitaxel. Paclitaxel, a known microtubule polymerizer, causes an increase in microtubule mass and a consequently distinctive bundling of the microtubules. In

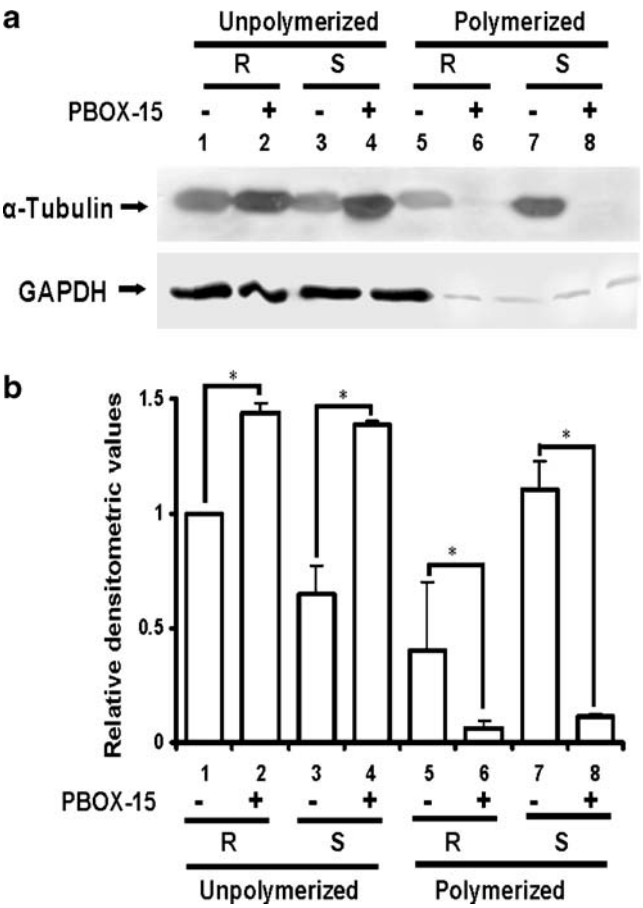

Fig. 8 Ex vivo determination of tubulin polymerization due to PBOX-15 treatment in resting and LFA-1 stimulated T-cells. Hut78 cells were pretreated with either vehicle [0.1% (*v*/*v*) ethanol] (control) or 1.0 μM PBOX-15 for 30 min, stimulated with (*S*) or without (*R*) anti-LFA-1 for 4 h and lysed. Unpolymerized and polymerized protein fractions were separated by centrifugation and collected as supernatant and pellet respectively. Samples were separated by SDS-PAGE, and after western blotting probed with anti-α-tubulin or GAPDH (as a loading control) antibody (**a**). Relative densitometric analysis of the individual band was performed and presented (**b**). Data are mean ± SEM of three independent experiments. * $p<0.05$

contrast, PBOX-15 targets the microtubule network of the cell and causes its disruption via microtubule depolymerization. This strongly indicates that effect of PBOX-15 on depolymerization of tubulin was direct, thus, causing inhibition of subsequent cell migration when stimulated with LFA-1.

In addition to being expressed as multiple gene-encoded isoforms, tubulins undergo a wealth of covalent post-translational modifications that are thought to modulate the functions and localization of MTs within the cell [31]. α-Tubulin undergoes a reversible process by which the C-terminal tyrosine residue is removed and re-added. This cycle of detyrosination and tyrosination is evolutionarily conserved [32]. Whereas the reversible detyrosination–tyrosination cycle of α-tubulin has been studied extensively [27] and implicated in regulating various aspects of cell biology, the precise biological function of this highly specific post-translational modification has remained poorly characterized. Heterogeneity of detyrosinated and tyrosinated forms of α-tubulin in the MTs may be required for coordination and differential interaction of MTs with MT-associated proteins [33] and motor proteins such as kinesin and dynein [34]. Acetylation of α-tubulin is mostly associated with stable microtubular structures, e.g., axonemes, and it occurs after MT assembly [27]. Although the enzyme that is responsible for α-tubulin acetylation 'tubulin acetyltransferase' is not fully characterized, two enzymes that catalyze the opposing reaction, i.e., deacetylation (histone deacetylase-6 and histone deacetylase SIRT2) have been recently described [27]. A role of tubulin acetylation in cell motility has been proposed on the basis of that histone deacetylase-6 overexpression increases the chemotactic movement of NIH-3T3 cells, whereas inhibition of histone deacetylase-6 inhibited cell migration [27]. Whether this reflects a direct effect of acetylation on MT stability and dynamics remains unsolved [27]. Further work is needed to dissect the mechanism by which tubulin acetylation influences motility. Thus, apart from effect of PBOX-15 on tubulin polymerization which plays a leading role in T-cell migration, post-translational modifications of tubulin could potentially represent novel therapeutic targets. Our data clearly indicate that post-transcriptional modifications of tubulin are functionally important for T-cell migration. In this study, we examined the effects of PBOX-15 on the post-translational modification of α-tubulin in T-cells leading to inhibition of their migration. We demonstrated that the tyrosinated form of α-tubulin is reduced after PBOX-15 treatment. Moreover, the acetylated form of tubulin was also reduced by PBOX-15 treatment. Therefore, the data suggest that PBOX-15, in addition to its possible direct tubulin targeting, can also affect post-translation modifications of this crucial cytoskeletal protein. Effect of PBOX-15 on other signaling processes could have additional and discrete consequences for cell migration that were not explored in this study. A series of focused experiments are presently being carried out to characterize the signaling events in T-cells following anti-LFA-1 exposure in detail.

While the ability of PBOX-15 to induce apoptosis of T-cells has been demonstrated, no information was available about the ability of PBOX-15 to regulate other T-cell functions, such as migration. Here, we propose a novel anti-inflammatory effect of PBOX-15 by reducing T-cell migration. We have used our in vitro model for T-cell migration which has been well characterized [3, 6, 8, 24]. The in vivo efficacy of PBOX-15 on the LFA-1 integrin-induced migration was also confirmed using PBTLs isolated from a healthy donor. Therefore, the results obtained might be extrapolated in a broader context.

MT-targeting anticancer drugs (e.g., taxol) also possess anti-inflammatory properties [35]. From this perspective, one can expect direct beneficial effect of the new PBOX-15 compound in leukemic cancers arising from the cells of immune system. However, the precise therapeutic approaches and schedules will certainly have to be optimized by clinicians in order to prevent the possibility of uncontrolled immune suppression and/or resulting infectious complications.

In conclusion, we have demonstrated that PBOX-15 is capable of inhibiting T-cell migration by dramatically altering MT dynamics. Microtubules are important for many aspects of mammalian cell responses including growth, migration, and signaling. The clinical efficacy of anti-microtubule agents is well established, although increasing evidence of resistance to these agents has prompted the search for new agents with a similar mechanism. Thus, the novel MT-targeting compound PBOX-15 may be used therapeutically to downregulate inflammatory reactions, such as those associated with autoimmunity and allergy. The ability of PBOX-15 to inhibit T-cell migration explains its beneficial effects in process where T-cell play an important role and suggests that PBOX-15 may be used therapeutically with chronic inflammatory diseases. Although the various molecular mechanisms involved in disrupting the immunosuppressive network for cancer patients still require clarification, we are approaching the time when it may be possible to use immunotherapy as a primary systemic cancer treatment. Therapeutic agents that target cytoskeletal proteins and are effective in inhibiting cell migration can open new horizons in the treatment of cancer and cancer metastatic spreading.

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