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ORIGINAL ARTICLE

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Antiproliferative and Antiproteolytic activity of Pentoxifylline in cultures of B16F10 Melanoma cells

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Abstract *Purpose*: Pentoxifylline (PTX), a methyl xanthine derivative is widely used as a haemorheological agent in the treatment of peripheral vascular disease. In the present study, we investigated the in vitro effects of PTX on B16F10 melanoma cell proliferation, adhesion and secretion of Matrix metalloproteinases. *Methods*: The toxic range of PTX was evaluated using MTT test and colony formation assay. The cell cycle study of PTX treated cells was carried out using flow cytometric analysis. Adhesion assay of pretreated melanoma cells was carried out on extracellular matrix (ECM) substrates. The relative levels and activity of matrix metalloprotienase-9 (MMP-9) and MMP-2 were determined by gelatin zymography and western blotting. *Results*: Pentoxifylline significantly inhibited the in vitro proliferation of B16F10 cells in a concentration dependent manner and displayed an IC_{50} of 15.2 mM. Non-cytotoxic concentration of 1–3 mM of PTX for an exposure of 24 h demonstrated significant changes in cell morphology. A significant inhibition in G1-S phase transition was observed on PTX treatment. Pretreated F10 cells showed inhibition in adhesion to ECM components and markedly inhibited the secretion of MMP-9 and MMP-2 gelatinases. *Conclusion*: The results suggest that PTX even at non-toxic pharmacological concentrations acts as an effective antiproliferative agent with significant antiproteolytic and antiadhesive effects.

Keywords Pentoxifylline · B16F10 melanoma · Adhesion · Proliferation · Matrix metalloproteinases

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Introduction

Two hallmarks of cancer are unlimited proliferation and metastasis. While several approaches are being used to target cancer cell growth, relatively few focus specifically on drugs preventing metastasis, which can be safely administered on a long-term basis. Because a majority of cancer patients eventually succumb to metastatic disease, agents that prevent or significantly delay metastasis, without excessive collateral toxicity to other organs, offer a tremendous potential benefit [5].

Pentoxifylline (PTX), a methyl xanthine (Theo bromine) derivative is used as a haemorrheological agent that augments erythrocyte flexibility and decreases blood viscosity resulting in increased microcirculatory blood flow [21]. As an anticancer agent, PTX has been used to enhance tumour sensitivity to both radiation [17] and chemotherapeutic agents. PTX augments the effect of alkylating agents on FSaIIC fibrosarcoma and mammary adenocarcinoma both in vitro as well as in vivo [29, 30]. PTX has been reported to increase tumour perfusion in chronically hypoxic tumours like WiDr tumours thus increasing its radio sensitivity [2].

Pentoxifylline is a well known phosphodiesterase inhibitor that alters membrane fluidity and causes cell deformability in cancer cells which may pass the microcirculation easily and are thus less likely to attach and form metastasis. Moreover it has been shown to be a highly effective inhibitor of platelet aggregation [20]. Based on these properties, researchers have looked into the antimetastatic effects of PTX. Recent studies have showed that PTX has a differential influence on tumour metastasis which might be cell line dependent. PTX facilitates development of murine colon adenocarcinoma but inhibits melanoma derived tumour in the lung [15]. Another study has shown a cytostatic effect of PTX on Neuro 2a mouse neuroblastoma and exerts an antitumour effect on liver metastasis [1].

In our earlier reports, we have demonstrated that PTX inhibits B16F10 experimental metastasis and

growth of solid murine tumours [13] which is mediated via its inhibitory action on cell adhesion, matrix metalloprotienase-9 (MMP-9) secretion [12] and tumour angiogenesis [11]. However, the extent to which the increase in duration of drug exposure facilitates the reduction in the effective concentration thus achieving the pharmacokinetic concentration needs to be evaluated. Taking a step further in this direction, in our present study, we have tried to investigate the effect of non-toxic concentrations of PTX, following an exposure for 24 h, on cell proliferation, cell cycle distribution, cell adhesion and MMP secretion in the B16F10 melanoma model.

Materials and methods

Cell and culture conditions

B16F10, a highly metastatic lung selected subline derived from C57/BL6 murine melanoma [9] was purchased from National Centre for Cell Science, Pune, India. The cell line was maintained as a continuous culture in IMDM (Iscove's Minimum Dulbecco's medium, GIBCO BRL, MD, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO BRL, MD, USA),100 U/ml penicillin and 100 μg/ml streptomycin [12]. Cells were grown in a humified atmosphere of 5% CO_2 and 95% air at 37°C. Media was replenished every third day.

MTT chemosensitivity assay

B16F10 cells were harvested in exponential phase and seeded in 96-well flat bottom tissue culture plate at a concentration of 5×10^3 cells/100 μl/well. The cells were allowed to grow and stabilize for 24 h. Subsequently, the cells were treated with serial concentrations of PTX (Sigma Aldrich) prepared in complete medium. Each treatment was performed in six well replicates. Post-treatment cells were washed with PBS and allowed to grow in complete medium for 24 h. After incubation, the cell viability was determined by MTT colorimetric assay. Briefly 20 μl of MTT reagent (Sigma Aldrich) was added to each well to make a final concentration of 1 mg/ml of media and incubated for 4 h at 37°C. Plates were then centrifuged at 2,000 rpm for 10 min. Medium was aspirated from the wells and 100 μl of dimethyl sulphoxide was added to each. The optical density was measured in an enzyme linked immuno sorbant assay plate reader (Molecular Devices, Spectra Max 190 with Soft max Pro) at 540 nm with a reference wavelength of 690 nm. Cell viability was plotted as a percentage of untreated control. Results are expressed as MEAN ± SEM and are representative of three independent experiments. Inhibitory concentration 50 (IC_{50}) of PTX was determined from the dose effect curve as the drug concentration that decreased the cell viability to 50%.

Colony formation assay

Colony formation assay was carried out as described [4] with minor modifications. Briefly, 30 mm Petri plate was seeded with 500 viable cells in complete medium and allowed to grow for 24 h. The cells were then incubated in the presence of various concentrations of PTX for 24 h. The drug was removed; cells were washed in PBS and incubated for 8–10 days in complete medium. Each treatment was done in duplicate. The colonies obtained were washed with PBS and fixed with methanol for 10 min at room temperature followed by staining with 0.2% crystal violet solution. Colonies with 50 or more number of cells were counted on a Zeiss inverted light microscope with the help of Palm Robo software.

Haematoxilin eosin (H and E) staining of cells to study cytopathic effects.

Pentoxifylline-induced changes in cell and nuclear morphology were observed by H and E staining. In brief, subconfluent culture of B16F10 melanoma grown on coverslips was treated with different concentrations of PTX in complete medium for 24 h. Coverslips were washed in PBS and cells were fixed with methanol. Fixed cells were stained with haematoxilin and eosin, washed and mounted using DPX mountant. The cytoplasmic and nuclear changes were observed under a Phase contrast Microscope and images were captured at 80×250 magnification.

Flow cytometry analysis of cell cycle distribution

Sub confluent B16F10 cells were treated with various concentrations of PTX (0, 1, 2 and 3 mM) for 24 h. Cells were harvested, washed twice with PBS and fixed in chilled 70% ethanol. After centrifugation, the fixed cell pellet was treated with RNAse at a concentration of 0.5 mg/ml (MBI Fermentas) and finally stained with propidium iodide (50 μg/ml) (Sigma Aldrich) for 10 min at room temperature. Ten thousand events were acquired on Becton-Dickinson FACS SCAN and analyzed using Modfit software.

Adhesion assay

Ninety-six-well flat bottom plates was coated with ECM substrates (50 μl/well): Matrigel (10 μg/ml), Fibronectin (2.5 μg/ml), collagen type IV (50 μg/ml) and Laminin (5 μg/ml). Plates were kept overnight at 4°C for polymerization. Unpolymerized substrates were washed with PBS and the plates were blocked with 1% BSA for 2 h at 37°C. Subconfluent B16F10 cultures were treated with 3 mM PTX in medium supplemented with FBS for 24 h. The cells were harvested using saline EDTA, washed and diluted to a final concentration of 3×10^5 cells/ml in IMDM containing 0.1% BSA. One hundred microliter of

the cell suspension was added to each substrate coated well and kept for incubation at 37°C for 1 h. Non-adherent cells were removed by giving two washes with PBS. The adherent cells were quantified using MTT assay and expressed as a relative percentage of the respective total unwashed cells (adherent as well as non-adherent).

Condition media preparation and gelatin zymography

To investigate the effect of PTX on the secretion of matrix metalloproteinases (MMPs), gelatin zymography was performed for gelatinases MMP-2 and MMP-9 as described [25]. Briefly sub confluent cultures treated for 24 h with non-toxic concentrations of PTX (0, 1, 2 mM) in complete medium were washed with PBS and further incubated in serum-free IMDM for 18–20 h. The conditioned media so obtained was centrifuged to remove the cell debris. Media was then dialyzed against PBS and concentrated tenfold by lyophilization. The concentrated conditioned media was normalized with respect to cell count and stored at −80°C until further use. To assess the gelatinase activity, samples were run on 10% SDS-PAGE containing 0.1% gelatin (w/v). The gel was washed in developing buffer (50 mM Tris, 100 mM $CaCl_2$, 1 μM $ZnCl_2$, 1% TritonX 100, 0.02% NaN_3, pH 7.5) for 1 h at room temperature and further incubated in the same buffer at 37°C for 48 h. The gels were stained in Coomassie Brilliant Blue R250 (Sigma Aldrich) and destained in a solution of methanol, water and acetic acid mixture (45:45:10 v/v). Enzymatic activity was visualized as clear zones on a blue background. HT1080 conditioned media was used as a positive control.

Western blotting

The results of the Gelatin Zymography were further confirmed by Western blot analysis. Twenty microliters of the condition media samples were resolved on 10% SDS-PAGE. The proteins were electroblotted to Polyvinyledene diflouride membrane (Amersham, Life Sciences). The membrane was blocked with 5% milk powder in TBST (20 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature. Membranes were further probed with polyclonal antibodies against MMP-2 (Santa Cruz, CA, USA) and MMP-9 (Sigma Aldrich). The blots were washed in TBST and probed with Horseradish Peroxidase conjugated secondary antibody as appropriate. The signal was detected on X-ray film (Kodak) using enhanced chemiluminescent western blotting system according to manufacturer's instructions (Amersham Biosciences). Relative band intensity was calculated using Molecular Analyst 1 software (version 1.4) from Bio-RAD laboratories USA.

Statistical analysis

The results are represented as Mean ± SEM of four or six determinations. For colony formation assay and adhesion assay, the statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparison test. P value < 0.05 was accepted as significant.

Results

In vitro cytotoxicity assay and inhibition in cell proliferation

B16F10 cells treated with PTX for 24 h in a range of 1–20 mM showed concentration-dependent inhibition in cell proliferation as evident from Fig. 1. Hundred percent toxicity was observed at 20 mM. IC_{50} value calculated from the dose effect curve was found out to be 15.2 ± 1.7 mM. A concentration-dependent decrease in the colony formation units was observed with PTX treatment which demonstrates the antiproliferative effects of PTX (Fig. 2). The difference was found to be significant at $P < 0.001$ using one-way ANOVA. The EC50 (concentration that inhibited colony forming units to 50%, which was extrapolated from linear regression analysis of experimental data) was found out to be 7.5 ± 1.9 mM. Based on the results obtained from the cytotoxicity and the proliferation assay, the Therapeutic Index (ratio of IC_{50} to EC_{50}) of PTX for B16F10 cells was calculated to be 2 [4, 28].

PTX induced changes in B16F10 cells morphology

H and E stained cover slips containing F10 cells show significant changes in the morphology of cells. PTX treatment resulted in the formation of a large number of dendrites and cell protrusions even at non-toxic concentrations (Fig. 3). Cells appeared spindle-shaped with some amount of hypertrophy as compared to control cells which were star-shaped and much more spread out.

PTX induced changes in cell cycle distribution of B16F10 cells.

To dissect the mechanism for the antiproliferative effects of PTX, we determined whether the growth inhibitory effects of PTX are associated with specific changes in cell cycle progression. Analysis of DNA content of PI stained B16F10 melanoma cells showed significant dose-dependent inhibition in the G1-S phase transition on PTX treatment, with a maximum inhibition of 78% at 3 mM PTX concentration. This resulted in a clear increase in the percentage of cells in G1 phase as compared to the control cells (Fig. 4).

PTX inhibited B16F10 melanoma adhesion to ECM substrates

Pretreatment of B16F10 cells with a 3 mM PTX concentration for 24 h significantly inhibited their ability to adhere to the reconstituted basement membrane (Matrigel) (20.2% ± 1.8), Collagen type IV (24.1% ± 2.51),

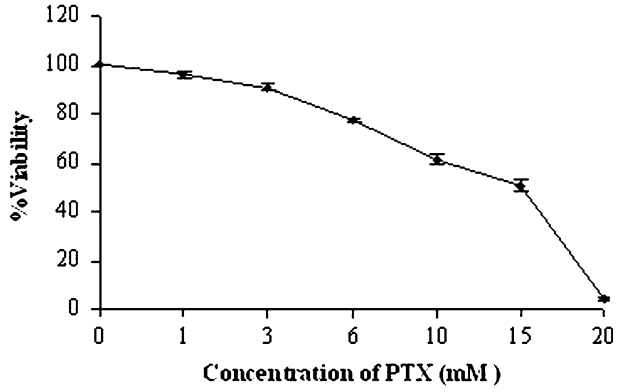

Fig. 1 Chemosensitivity of B16F10 cells towards pentoxifylline (PTX) was studied using MTT colorimetric assay that measures the dehydrogenase activity of the cells. The *dose effect curve* shows concentration-dependent inhibition in the percent viability of cells on PTX treatment (IC50 value = 15.2 ± 1.7 mM), considering untreated control as 100% viable. *Values* depict an average (±SEM) of hex plate measurements. Results are representative of three independent experiments

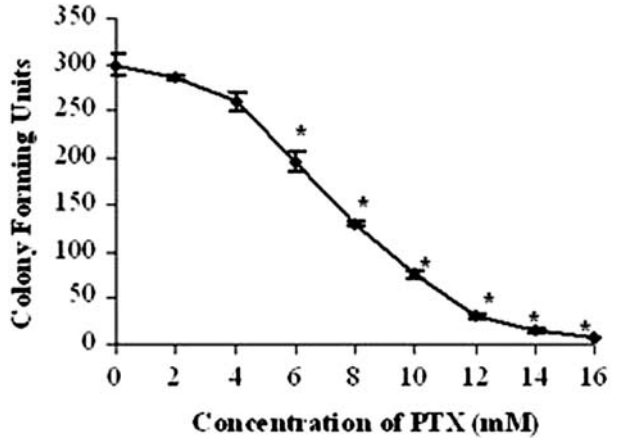

Fig. 2 Pentoxifylline-induced inhibition in cell proliferation was studied using colony formation assay. Twenty-four hour treatment of PTX showed a dose-dependent inhibition in the colony forming units as counted after 10 days incubation. The inhibition in the colony formation units was found to be significant at 6 mM and higher concentrations of PTX with $*P < 0.001$, using one-way ANOVA. The effective concentration 50 (EC50) was found out to be 7.5 ± 1.9 mM which is half of the IC 50 of the drug. The values depict average (± SEM) of duplicate measurements. Results are representative of three independent experiments

laminin (27.1% ± 1.24) fibronectin (16% ± 0.61) as compared to the untreated control (100%) (Fig. 5). The percentage of relative inhibition values were analyzed using one-way ANOVA and were found to be statistically different with a P value < 0.05 using Dunnet Multiple Comparisons Test.

Matrix metalloproteinase secretion was reduced with PTX treatment

Matrix metalloprotienases are key enzymes involved in the process of cancer cell invasion and metastasis. To study the effect of PTX on the secretion and activity of these proteases, gelatin zymography was carried out. As can be seen in Fig. 6 concentrated serum-free media revealed the band of lysis at 72 and 105 kDa that corresponds to the latent forms of the proteins, proMMP-2 and proMMP-9, respectively. A difference of 13 kDa sequence in the mouse and human MMP-9 protein explains the difference in the position of the MMP-9 bands of B16F10 and HT1080. The zymogram also shows the presence of active MMP-9 whereas the active form of MMP-2 in F10 condition media did not show any gelatinolytic activity. Densitometry analysis of the gel showed a significant inhibition in the enzymatic activity of proMMP-9 (52% inhibition), active MMP-9 (70% inhibition) and proMMP-2 (74.2% inhibition) with PTX treatment.

The immunoblot of the condition media with the polyclonal rabbit antibody for MMP-9 recognized both latent and active forms of protein. A concentration dependent inhibition in both the forms of the MMP9 was observed on 24 h PTX treatment and the graphical representation of the relative density of the bands showed 75% inhibition in the MMP-9 protein secreted from F10 cells at 2 mM PTX treatment (Fig. 7a). Presence of latent form (72 kDa) as well as active form (66 kDa) of MMP-2 in the F10 condition media was evident after the Western blot analysis with a polyclonal goat antibody that recognized both the forms of the protein. Both the 72 and 66 kDa forms of the protein were found to be inhibited on drug treatment as evident from the percentage of relative density values in (Fig. 7b).

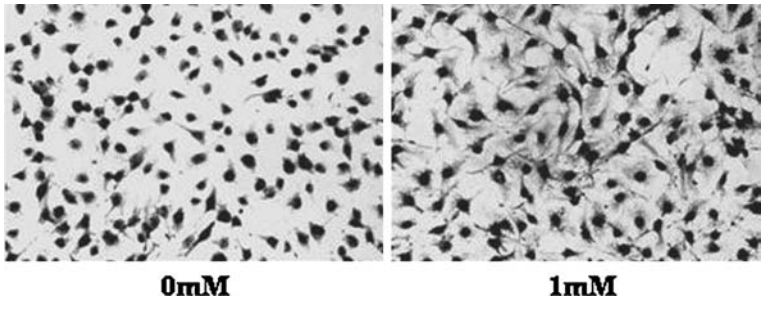

Fig. 3 Haematoxylin and eosin stained micrographs of control and PTX treated B16F10 cells (image at 80×250 magnifications). Significant changes in the cell morphology can be seen even at non-toxic doses of PTX treatment

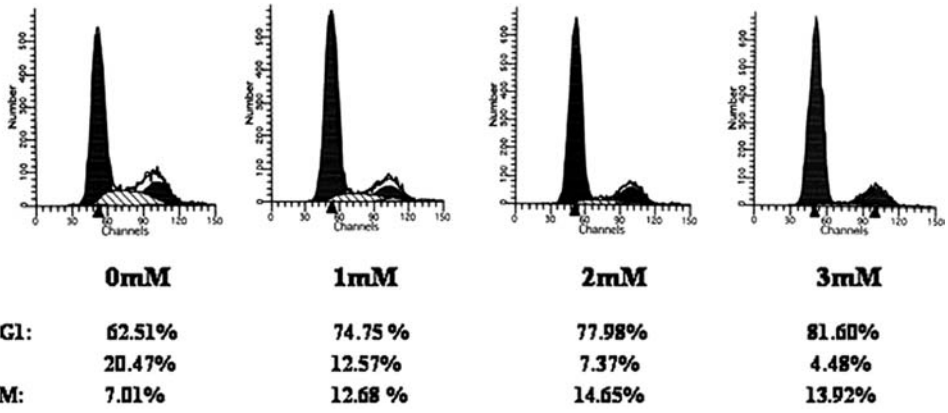

Fig. 4 Cell cycle of PTX-treated B16F10 cells. The DNA content of control as well as untreated cells stained with propidium iodide was analyzed using flow cytometry. The figures show a significant decrease in the percentage of cells in S phase and a subsequent increase in G1 phase with increasing concentration of PTX

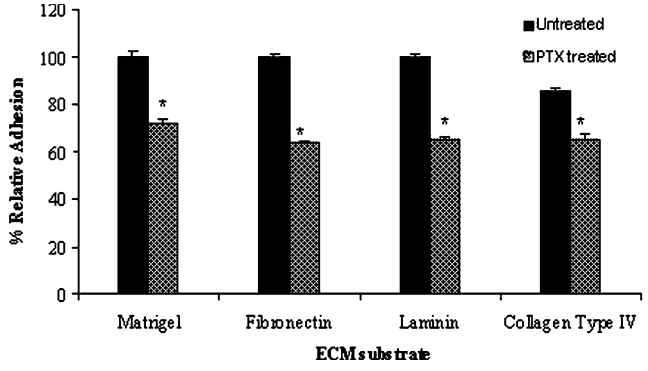

Fig. 5 Adhesion Assay of B16F10 cells pretreated with 3 mM PTX for 24 h showed an inhibition in cell attachment on Matrigel, Collagen Type IV, Fibronectin and Laminin. The number of cells adhered on the substrates are represented as the percentage of relative of the respective unwashed cells (adherent as well as non-adherent). The difference in adhesion of treated and untreated cells was found to be significantly different (P value < 0.005) using one way ANOVA with Dunnett Multiple Comparison Test. The results are representative of three independent experiments

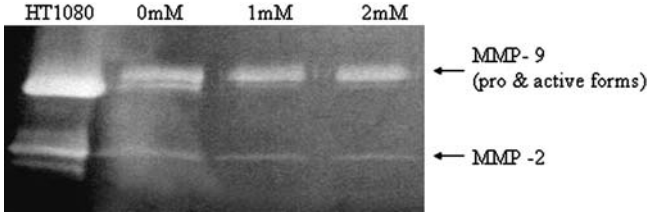

Fig. 6 Gelatin zymography of condition media of B16F10 cells treated with PTX. Condition media from untreated as well as 24 h PTX-treated cells were collected and concentrated. Matrix metalloprotienase-2 (MMP-2) and MMP-9 activity was observed using gelatin zymography. Coomassie stained gel show a dose-dependent inhibition in the latent and active form of MMP-9 and latent form of MMP-2. HT1080 condition media was used as a positive control

Discussion

Cancer metastasis occurs through a multiple series of events that involves the ability of tumour cells to invade the local tissue and cross the tissue barriers. The adhesion of tumour cells to extracellular matrix (ECM) is crucial for metastasis [16, 8]. This is followed by ECM degradation which includes secretion and activation of proteolytic enzymes, such as MMPs [3] that degrade ECM components, such as type IV collagen, glycoproteins and proteoglycans. Proteolytic modifications of the matrix barriers are followed by pseudopodial protrusions and locomotion of the tumour cells finally enabling them to reach to a distant site and form secondary tumour foci.

The reports on the molecular mechanisms involved in the metastatic process led many investigators to look for experimental therapies directed towards inhibiting or regulating one or subsequent steps of the metastatic cascade. One of the possibilities is investigating anti-metastatic activity of the drugs that had already been demonstrated to treat other diseases. Aspirin, a non-steroidal anti-inflammatory drug has been shown to inhibit metastasis [18]. PTX has been widely used in dilated cardiomyopathy, nephropathy [6] and is presently in focus as an anticancer and antimetastatic agent.

There is much evidence to suggest that PTX acts as a non-specific phosphodiesterase inhibitor resulting in up regulation of cAMP [20]. It also inhibits lipopolysaccharide and TPA-induced production of TNFα [26]. Subject to its unique mechanism and based on our earlier studies [13], in the present piece of work we have examined the effect of 24 h exposure of PTX at non toxic doses on B16F10 cell proliferation, cell cycle, adhesion and secretion of proteolytic enzymes.

The 24 h incubation of B16F10 cells in the presence of PTX showed two significant observations. Eighty percent viability was observed at PTX concentration below 6 mM. However above 6 mM PTX concentration, the toxicity was visible and IC 50 was found out to be 15 mM. The IC 50 showed a continuous drop with the increase in the duration of exposure, i.e. 48–72 h (data not shown). Further, PTX inhibited F10 cells proliferation in a concentration-dependent manner with effective concentration being half of the cytotoxic concentration.

Pentoxifylline treatment at a non-toxic concentration of 1 mM showed significant alterations in the cell morphology as evident from H-E staining. Cells showed

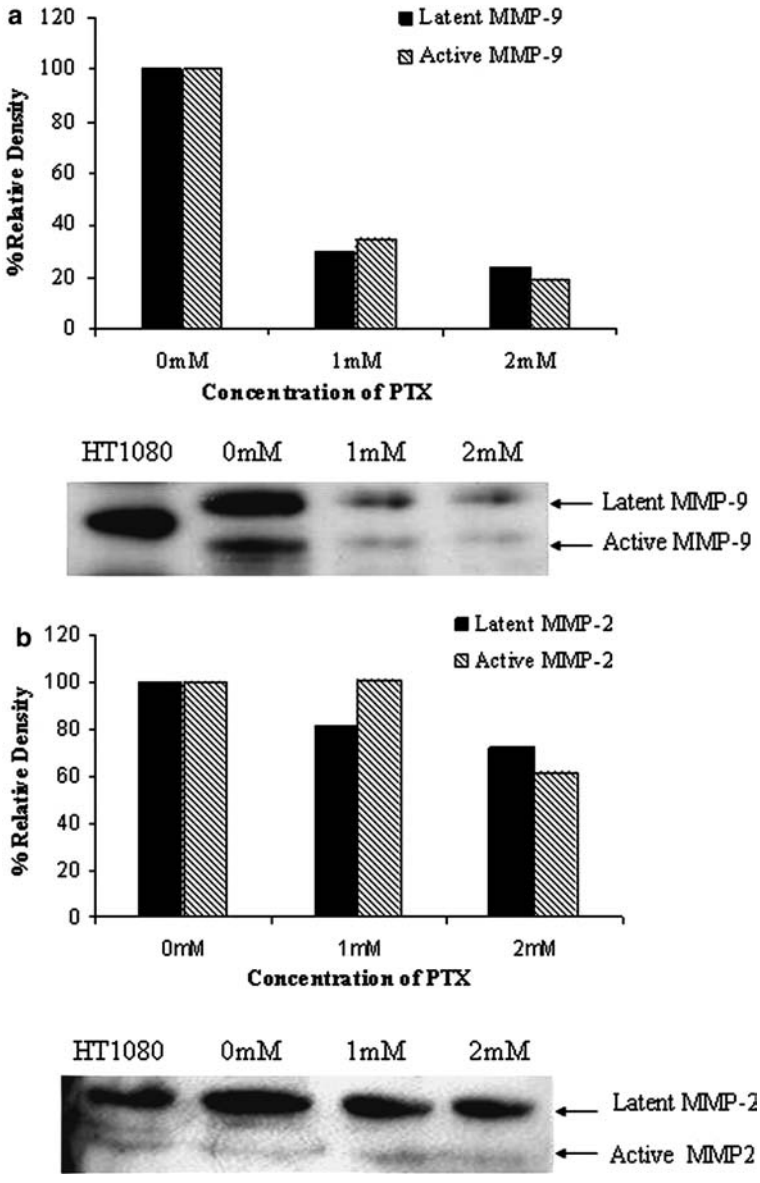

Fig. 7 **a** Western blot analysis of MMP-9 secretions in the condition media of B16F10 cells treated with PTX. Protein bands separated on 8% SDS-PAGE was transferred on PVDF membrane and immunoblotted with polyclonal antibody against mouse MMP-9. The *upper and lower band* corresponds to latent and active forms of MMP-9, respectively. PTX showed a dose-dependent inhibition in both the forms as evident from the densitometric analysis. **b** Western blot analysis of MMP-2 secretions in the condition media of B16F10 cells treated with PTX. Protein bands separated on 10% SDS-PAGE was transferred on PVDF membrane and immunoblotted with a polyclonal antibody for human MMP-2. Densitometric analysis show a dose-dependent inhibition in the latent and active form of the protease

cytoplasmic streaming and dendrite formations which are typical cAMP induced changes in the cell morphology referred to as "cAMP phenotype". Interestingly, PTX treatment also led to the induction in melanin expression which was evident from the color of the cell pellet post treatment (data not shown). Intracellular cAMP elevating agents mimic the action of α-melanocyte stimulating hormone (α-MSH) in inducing murine melanogenesis [7]. Melanin secretion, being a marker of melanoma cell differentiation [23], these observations indicate that PTX can be used to induce differentiation in B16F10 cells. The cell cycle profile of F10 cells treated with PTX show a dose-dependent inhibition in G1-S phase transition, the maximum being 78% at a concentration of 3 mM. This observation accounts for the antiproliferative effects of PTX. Reports on other anticancer agents that are cAMP elevators also show G1 arrest [10]. It suggests that inhibition in cell proliferation and G1-S phase transition in B16F10 melanoma is a result of increase in cAMP and can be attributed to the phosphodiesterase inhibitor property of PTX.

A significant reduction in the attachment of PTX-treated F10 cells to the ECM substrates was observed. Collagen Type IV is a major component of basement membrane whereas Laminin and Fibronectin besides being present in the basement membrane also form a part of the interstitial connective tissue [32]. Adhesion of tumour cells to ECM substrates is an important step of metastatic cascade. Therefore, PTX induced inhibition in this process contributes to the antimetastatic effects of the drug.

Matrix metalloprotienase-2 and MMP-9 expression levels are especially high in lung carcinoma and melanoma cells [33] that play a major role in the facilitation of cancer metastasis [14]. In B16F10 mouse melanoma cells, invasive potential has been related to the activity

and expression of MMP-9 and MMP-2, because of their potential to degrade basement membrane [27]. MMPs are secreted as proenzymes or latent forms and the activation of these enzymes occur via the proteolytic removal of the propeptide domain, thus exposing the active site. In our present study, we have seen a concentration dependent inhibition in both MMP-2 and MMP-9 proteins and their gelatinolytic activitiy. TNFα is a potent transcriptional activator of MMP-9 [22] and PTX have been shown to inhibit TNFα induced MMP-9 secretion in HL60 leukemia cell line [26].We propose a similar mechanism of action for PTX induced inhibition in MMP-9 in B16F10 melanoma.

The PTX mediated inhibition in MMP-2 can be attributed to the phosphodiesterase property of the compound as demonstrated by the inhibitory action of many cAMP analogues on MMP-2 activation [24]. Both the gelatinases show a down regulation in the pro and active forms of the protein on PTX treatment. This indicates that PTX, apart from inhibiting the zymogen forms of the protein, might also affect other molecules that play a role in the activation of these proteins [19, 31].

In conclusion, we provided evidence that PTX, even at non-toxic concentrations can act as a promising antimetastatic drug, by inhibiting B16F10 melanoma cell adhesion to ECM substrates and reducing the secretion and activation of MMP-2 and MMP-9 gelatinases. The antimetastatic properties in addition to its inhibitory effect on B16F10 cell proliferation and role in cell cycle perturbation make PTX an attractive anti tumour agent with significant clinical applications.

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