Effects of Methylxanthine on STAT3 mediated regulation of cytokines involved in tumor development and tumor induced angiogenesis in melanoma cells

By

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Recommendations of the Viva Voce Board

As members of the Viva Voce Board, we certify that we have read the dissertation prepared by Md. Zahid Kamran entitled **"Effects of Methylxanthine on STAT3 mediated regulation of cytokines involved in tumor development and tumor induced angiogenesis in melanoma cells"** and recommend that it may be accepted as fulfilling the thesis requirements for the award of Degree of Doctor of Philosophy.

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January 2014

DECLARATION

I, hereby declare that the investigation presented in the thesis titled "Effects of Methylxanthine on STAT3 mediated regulation of cytokines involved in tumor development and tumor induced angiogenesis in melanoma cells" has been carried out by me. This work is original and has not been submitted earlier as a whole or in part for a degree/diploma at this or any other institution or university.

Md. Zahid Kamran

Navi-Mumbai
January 2014

LIST OF PUBLICATIONS

Journal articles

1. Mohammad Zahid Kamran, Rajiv P. Gude. Preclinical evaluation of the antimetastatic efficacy of Pentoxifylline on A375 human melanoma cell line. Biomedicine & Pharmacotherapy, 66 (2012), 617-626.

2. Mohammad Zahid Kamran, Rajiv P. Gude. Pentoxifylline inhibits melanoma tumor growth and angiogenesis by targeting STAT3 signaling pathway. Biomedicine & Pharmacotherapy, 67 (2013), 399-405.

3. Mohammad Zahid Kamran, Prachi Patil, Rajiv P. Gude. Role of STAT3 in Cancer Metastasis and Translational Advances. BioMed Research International, 2013 (2013), 15 pages.

4. Mohammad Zahid Kamran, Prachi Patil, Kavita Shirsath, Rajiv P. Gude. Tyrosine Kinase inhibitor AG490 inhibits the proliferation and migration and disrupts actin organization of cancer cells. Journal of Environmental Pathology, Toxicology and Oncology, 32 (2013), 361-371.

Conference abstracts

1. Mohammad Zahid Kamran, Rajiv P. Gude. Pentoxifylline as an inhibitor of oncogenic signaling pathways: molecular mechanism and clinical implication. Signaling and acquired resistance to targeted cancer therapeutics. 5-7 January, 2014, Robinson College, University of Cambridge, United Kingdom, **(Poster presentation).**

2. Mohammad Zahid Kamran, Rajiv P. Gude. Hemorrheological agent Pentoxifylline modulates oncogenic signaling pathways: an old drug with new perspectives. Current Advances in Immunobiology and Cancer. 28-30 November, 2013, Mumbai, India, **(Poster presentation).**

3. Mohammad Zahid Kamran, Rajiv P. Gude. Novel mechanistic insights for phosphodiesterase inhibitor Pentoxifylline: as a STAT3 inhibitor. 4th International Conference on Stem Cells and Cancer (ICSCC-2013): Proliferation, Differentiation, and Apoptosis. 19 - 22 October, 2013, Mumbai, India, **(oral presentation).**

4. Mohammad Zahid Kamran, Rajiv P. Gude. Pentoxifylline inhibits melanoma tumor growth and angiogenesis by targeting STAT3 signaling pathway: Novel mode of action(s) for an old drug. 81st Annual meeting of the Society of Biological Chemist (I). 8-11 November, 2012, Kolkata, India, **(poster presentation).**

5. Mohammad Zahid Kamran, Rajiv P. Gude. Pentoxifylline inhibits melanoma angiogenesis via STAT3 signaling pathway. AACR International Conference: New Horizons in Cancer Research: Biology to Prevention to Therapy. 13-16 December, 2011, New Delhi, India, **(poster presentation).**

Md. Zahid Kamran

DEDICATED TO MY PARENTS

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Md. Zahid Kamran

CONTENTS

Homi Bhabha National Institute

Ph.D. PROGRAMME

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2. Name of the Constituent Institution: Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre, Kharghar, Navi-Mumbai.

3. Enrolment No.: LIFE09200804014

4. Title of the Thesis: Effects of Methylxanthine on STAT3 mediated regulation of cytokines involved in tumor development and tumor induced angiogenesis in melanoma cells.

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SYNOPSIS

Introduction

Metastasis, the spread of tumor cells from the primary site to distant organs, is the major problem in the management of cancer. Cancer, when detected at an early stage, before it has metastasized, it can often be cured successfully by surgery or by local irradiation. However, when invading tumor cells are metastasized to distant organs treatment is much less successful and leads to the death of a majority of cancer patients. Metastasis is a complex, multistep process in which tumor cells primarily invade surrounding tissue and basement membrane and enter into circulatory or lymphatic system (called intravasation). While surviving during circulation, the tumor cells extravasate and colonize into distant organs and induce angiogenesis to form secondary tumor (1). Each of these steps of metastasis is regulated by the action of numerous genes and signaling pathways whose aberrant activation triggers metastasis.

In recent years, compelling evidence places Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) signaling pathway at a central position in the development and progression of cancer metastasis (2). STAT family proteins are latent cytoplasmic transcription factors that convey the signal from cell surface receptors to the nucleus (3). It is a highly conserved family of proteins and comprises seven members STAT1 to STAT4, STAT5a, STAT5b and STAT6 (3). STATs are generally located in the cytoplasm in their inactive state in resting cells. Phosphorylation of specific tyrosine residue is an essential step for STAT activation. Once activated, STAT dimerises to each other by reciprocal SH2 phospho tyrosine interaction, leading to its translocation into the nucleus and thus its binding to the specific enhancer elements for initiation of transcription (3). Studies from knockout mice revealed that individual

STAT protein is essential for various normal physiological functions such as embryonic development, cell differentiation, immune response and organogenesis (4).

Among seven mammalian STAT proteins, persistent activation of STAT3 is frequently detected in majority of human cancers including melanoma (5). This persistent activation of STAT3 in cancer cells plays an important role in tumorigenesis by enhancing cellular proliferation, cell survival, invasion, angiogenesis and metastasis (5). However, in contrast to this, several lines of evidence have implicated that inhibition of STAT3 with a dominant negative form of STAT3 or other inhibitors attenuates the proliferation and survival of a wide variety of cancers with little or no effect on normal cells (6). Although inactivation of STAT3 leads to embryonic lethality in mice, many normal adult tissues are unaffected by loss of STAT3 (6). As STAT3 plays a central role in tumorigenesis and its function is dispensable in many adult tissues, it is therefore regarded as a well validated target for cancer therapy (7). Methylxanthine, a methylated derivative of xanthine, is a phosphodiesterase inhibitor that can elevate intracellular cAMP level in the cells (8). The members of methylxanthine family are pentoxifylline (PTX), caffeine, theobromine and theophylline. Among these, PTX has emerged as least toxic and thus received much attention for its clinical use (9). PTX is used to improve the effectiveness of microcirculation, increase red blood cell deformability, decrease platelet aggregation and lower plasma viscosity (10). Food and Drug Administration has approved PTX for treatment of peripheral vascular disease (11). Preclinical studies of PTX in animal model demonstrated that it is a well tolerated and non-toxic drug and has potent anti-metastatic activity. Earlier studies from our laboratory have demonstrated that PTX inhibited B16F10 experimental metastasis and growth of murine solid tumors (12), which is mediated via its inhibitory action on cell adhesion, matrix metalloproteinase-9 (MMP9) secretion (13) and tumor angiogenesis

(14). However, none of the studies conducted so far describes the exact molecular mechanism(s) for the anti-metastatic or anti-angiogenic activity of the PTX. Therefore, in the present study, we have tried to evaluate the anti-metastatic or anti-angiogenic activity of PTX with underlying molecular mechanism using A375 human melanoma cell line as a study model.

Aims and objectives

Objective 1: To study the anti-metastatic effect of PTX using A375 human melanoma cell line.

Objective 2: To study the effects of PTX on STAT3 signaling pathway using A375 human melanoma cell line.

Objective 3: To study the anti-tumor and anti-angiogenic potential of PTX in a xenograft model of A375 human melanoma tumor *in vivo*.

Materials and methods

Cell lines and culture conditions

A375 human melanoma, A549 lung carcinoma, U87 glioblastoma, HEK-293 human embryonic kidney, HaCaT human keratinocytes, HT1080 human fibrosarcoma and NIH3T3 mouse fibroblast cell lines were used. All these cell lines were purchased from National Centre for Cell Science, Pune, India. A375 cell was maintained in RPMI-1640 supplemented with 4 mM L-glutamine and 10% fetal bovine serum (FBS) while A549, U87, HEK-293, HaCaT and NIH3T3 were maintained in DMEM containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were grown in a humidified atmosphere of 5% CO_2 and at a temperature of 37 ºC.

Cytotoxicity by MTT assay

Five thousand cells/hundred microliter were seeded in 96 well plate. After 24 h, cells were treated with different concentrations of PTX (0 to 30 mM) for 2, 24, 48 and 72 h.

Thereafter, cells were washed twice with PBS and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added at a concentration of 1mg/ml. After overnight incubation at 37 ºC, the plate was centrifuged at 1500 rpm for 20 minute. DMSO was added to dissolve the formazan crystals and optical density was taken using ELISA reader (Molecular Devices, Spectra Max 190 with Soft max Pro) at a wavelength of 540 nm with a reference of 690 nm.

Colony formation assay

Five hundred cells/ml was seeded in 35 mm culture plate for 24 h. Cells were treated with PTX concentration of 0 to 6 mM for 24 h. Cells were washed with PBS and incubated in 10% serum containing medium for 8-10 days. Colonies formed were fixed with methanol and stained with 1% crystal violet solution. Colonies of 50 or more cells were counted under microscope and photographs were taken.

Cell cycle analysis by Flow cytometry

Sub-confluent cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h. Cells were harvested, washed twice with PBS and fixed in chilled 70% ethanol. The cell pellet was then treated with RNase at a concentration of 0.5 mg/ml and finally stained with propidium iodide (50 µg/ml). Ten thousand events were acquired on Becton-Dickinson FACS calibur and analyzed using Modfit software.

Estimation of apoptosis

A375 cells were seeded in 96-well plates at a concentration of 1.6 $x10^4$ cells/well. After 24 h, cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h. Plates were later centrifuged and ethidium bromide/acridine orange (EB/AO) dye 1:1 mix (16 µl) was added to each well. Cells were viewed and counted using a Nikon eclipse TS100 inverted microscope at 20X magnification. Tests were done in triplicates, counting a minimum of 100 cells.

Adhesion assay

Ninety six well flat bottom plate was coated with extra-cellular matrix (ECM) substrates (50 µl/well): matrigel 10 µg/ml, collagen type IV 50 µg/ml. Plates were kept overnight at 4ºC for polymerization. Unpolymerized substrates were washed with PBS and the plates were blocked with 1% bovine serum albumin (BSA) for 2 h at 37 ºC. Subconfluent A375 cells were treated with sub-toxic doses of PTX (0, 2 and 3mM) for 24 h and then harvested using saline EDTA. Cells were then suspended in media containing 1% BSA at a concentration of $3X10^5$cells/ml. Hundred microliter of the cell suspension was added to each substrate coated well. The plate was incubated for 15, 30, 45, 60 and 90 minute at 37 ºC. The wells were aspirated at the indicated time points i.e. 15, 30, 45, 60 and 90 minute. The plates were then washed twice with PBS to remove non-adherent cells. 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). All assays were performed in triplicate.

Wound healing assay

A375 cells were seeded in 35 mm culture plate. When 70-80% confluency was achieved, cells were treated with mitomycin C (1µg/ml) for 1 h. Cells were washed with PBS and a wound was made using 200µl tip. Cells were incubated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h. Cells were washed with PBS and further incubated for 24 h for wound coverage. At the 0 time point wound widths were measured under the microscope using an ocular grid. Post incubation cells were fixed with 100% methanol and the final wound width was measured. The migration observed was represented as % migration considering migration in untreated control as 100%. Images were taken using AxioVision inverted microscope from Zeiss.

Gelatin zymography

Cells were grown in 90 mm culture plates until 60-70% confluency was achieved. The medium was then replaced with medium containing PTX (0, 1, 2 and 3 mM) for 24 h. Followed by incubation, two PBS washes was given and serum free medium was added. The conditioned medium was collected after 48 h, concentrated and electrophoresed through 10% polyacrylamide gel containing 1mg/ml gelatin. The gel was given two washes with zymograph developing buffer and incubated in developing buffer for 48 h at 37°C. Staining was done in coomassie brilliant blue R250 and destained in a solution of methanol, water and acetic acid mixture (45:45:10 v/v). Enzymatic activities of MMP2 (72 KD) and MMP9 (92 KD) were visualized as clear zones on a blue background.

Actin staining using Phalloidin- Fluorescein isothiocyanate (Phalloidin-FITC)

Cells were grown to a confluency of 60-70% on coverslips and treated with sub-toxic doses of PTX (0, 1, 2 and 3mM) for 24 h. Cells were fixed in 4% paraformaldehyde at 37 °C and permeablized with 0.1% Triton X100. Coverslips were then overlaid with 30 µl of Phalloidin-FITC mixture (50 µg/ml in 0.05% BSA) and incubated at 37°C for 10 minute. Coverslips were washed with PBS and were mounted on a glass slide in the mounting medium and sealed with the help of nail polish. Images were acquired at 63X using the LSM 510 META laser scanning microscope from Carl Zeiss, Germany. Images were analyzed using the software LSM 510.

Immunofluorescence staining

Cells were grown to a confluency of 60-70% on coverslips and treated with PTX (0, 1, 2 and 3mM) for 24 h. cells were fixed in 4% paraformaldehyde and permeablized with chilled methanol. Cells were incubated with the primary antibody (against STAT3 and pSTAT3) overnight at 4 °C followed by incubation with secondary antibody conjugated

to Alexa fluor 568 for 1h at room temperature. Coverslips were washed with PBS and were mounted on a glass slide in the mounting medium and sealed with the help of nail polish. Images were acquired at 63X using the LSM 510 META laser scanning microscope from Carl Zeiss, Germany. Images were analyzed using the software LSM 510.

DNA binding assay

Cells were grown to a confluency of 60-70% and treated with PTX (0, 1, 2 and 3mM) for 24 h. Cells were collected and nuclear fraction was extracted using the TransAM nuclear extraction kit (Active Motif, USA) according to manufacturer's protocol. DNA binding assay was carried out using the TransAM STAT3 activation kit (Active Motif, USA) following the manufacturer's protocol. 10 µg of nuclear extract was added to the oligonucleotide coated 96 well plate. After 1 h primary antibody was added and incubated at 37 °C for 1 h followed by incubation with HRP- conjugated secondary antibody. After extensive washing, developing solution was added and reading was taken using ELISA reader at wavelength of 450 nm with a reference of 655 nm.

Western blotting

Total protein from untreated and PTX treated cell was extracted in lysis buffer and quantified using the bradford reagent. Equal protein (50µg) from all samples was loaded and separated on 8-10% SDS-PAGE. Proteins were transferred to PVDF (polyvinylidene difluoride) membrane. The membrane was blocked with 5% non fat milk in TBST (Tris Buffered Saline with Tween 20) solution for 2 h at room temperature (RT) and incubated with primary antibody (against STAT3, pSTAT3, JAK2, pJAK2, JAK1, pJAK1, Src, pSrc, SHP2, pSHP2, CyclinD1, cMyc, CDK4, CDK6, BclXL, Caspase 3, Caspase 9, VEGF, VEGFR2, HIFα, MMP2, MMP9, p38 MAPK, pp38 MAPK, p44/42 MAPK, pp44/42 MAPK, SAPK/JNK, PI3K, Akt, pAkt,

STAT1 and pSTAT1) overnight at 4 °C and HRP-conjugated secondary antibody for 1 h at RT. The pierce Femto chemiluminescence system was used to detect the signal.

Semiquantitative RT-PCR

Total cellular RNA from untreated cells as well as cells treated with sub-toxic doses of PTX (0, 1, 2 and 3mM) were extracted by Trizol method. cDNA preparation and PCR were performed using kit from Fermentas according to manufacturer's instructions. The following Primers (Sigma Aldrich, USA) were used. GAPDH: Forward, 5'—GAGTCAACGGATTTGGTCGT - 3'; Reverse, 5' —TGTGGTCATGAGTCCTTCCA - 3'; MMP2: Forward, 5' —AGGGCGCTCTGTCTCCTGGG - 3'; Reverse, 5' —CCCACTTGGGCTTGCGAGGG - 3'. MMP9: Forward, 5'—GTGCTCCTGGTGCTGGGCTG - 3'; Reverse, 5'—GCCAGCTGCCTGTCGGTGAG – 3'. VEGF: Forward- 5′-CTACCTCCACCATGCCAAGT-3′; Reverse, 5′-GCAGTAGCTGCGCTGATAGA-3′.

ELISA

Cells were grown to a confluency of 60-70% and treated with PTX (0, 1, 2 and 3 mM) for 24 h. Cells were washed with PBS and serum free medium was added. After 48 h incubation culture supernatant were collected. IL6 and VEGF secretions were measured in culture supernatant using kit (biorbyt, U.K) according to manufacturer's instruction. Hundred microliter of culture supernatant was added to the IL6 and VEGF coated 96 well plate. Primary antibody was added for 1 h followed by HRP-conjugated secondary antibody. After washing, developing solution was added and reading was taken using ELISA reader at a wavelength of 450 nm.

Animal experiments

Animal studies were conducted with the approval of Institutional Animal Ethics Committee (IAEC). Animals were collected from animal house, ACTREC.

Sub-cutaneous xenograft model: A375 cells were grown to 70 to 80% confluency in complete medium and harvested using trypsin-EDTA. One and half millions cells in plane medium were injected subcutaneously into the right flank of 6- to 8-week-old female NOD-SCID mice. The mice were randomised (5 mice/group) and divided into three groups viz, PBS alone, 40 mg/kg PTX and 60 mg/kg PTX. Mice were injected PTX by intra-peritoneal (i.p) route from day 9^{th} when tumors were visible (around 20 to 25 mm^3). Treatment continued from day 9 to day 17 i.e. total 9 days. Animals were sacrificed on 30^{th} day. Tumor size was determined alternate day by vernier caliper and volume was calculated using the formula $0.5ab^2$.

Intra-dermal angiogenesis model: A375 cells were grown to 70 to 80% confluency in complete medium and harvested using trypsin-EDTA. One million cells were injected intra-dermaly into 6- to 8-week-old female NOD-SCID mice. The mice were randomised (5 mice/group) and divided into three groups viz, PBS alone, 40 mg/kg PTX and 60 mg/kg PTX. Mice were injected PTX by i.p route from day 5^{th} when tumors were visible. Treatment continued from day 5 to day 13 i.e. total 9 days. Animals were sacrificed on 18^{th} day. Tumor size was determined alternate day by vernier caliper and volume was calculated using the formula $0.5ab^2$.

Results

***In vitro* cytotoxicity and inhibition in proliferation by PTX**

PTX had dose and time-dependent toxicity against A375 cells. Hundred percent toxicity was observed at 30 mM. IC 50 value calculated from the dose effect curve was found to

be 17, 7, 2.3 and 1.8 mM when exposed to the drug for 2, 24, 48 and 72 h respectively **(Fig 1)**. This inhibition in cellular growth was further confirmed by colony formation assay. A concentration-dependent decreased in the colony forming units was observed with PTX treatment. Interestingly, at sub-toxic doses of 1, 2 and 3 mM PTX 7.8%, 25% and 45% decrease in colony forming units were observed demonstrating anti-proliferative effect of PTX at these sub-toxic doses ($p<0.05$).

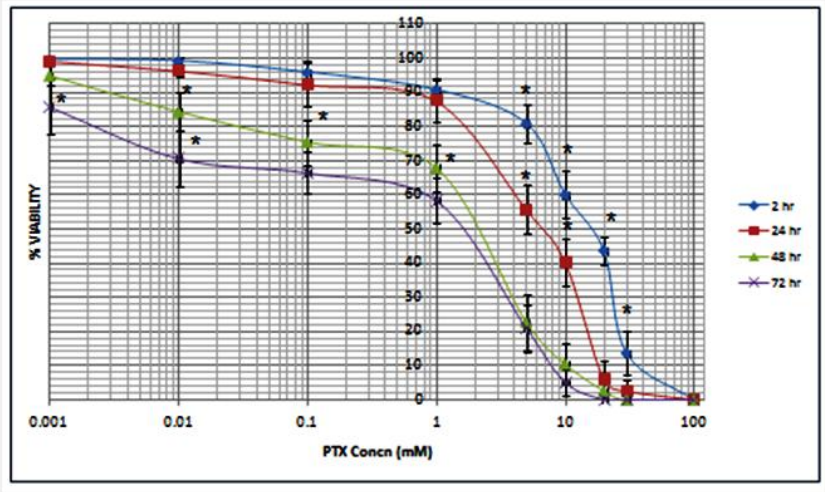

Fig 1: Effect of PTX on the viability of A375 cells. *A375 cells were treated with various concentrations of PTX (0-30 mM) for 2, 24, 48 and 72 h respectively. The numbers of viable cells were determined using MTT assay. There was a dose and time dependent inhibition in the percent viability of cells on PTX treatment. Values are expressed as mean ± SE of three independent experiments. *p < 0.05 compared with untreated control.*

Furthermore, sub-toxic doses (1, 2 and 3 mM) of PTX showed minimal toxicity against normal cells like HaCaT (human keratinocytes) and NIH3T3 (mouse fibroblast). Thus in the following experiments, the sub-toxic doses of 1, 2 and 3 mM with 24 h exposure of PTX was applied in all subsequent experiments unless indicated otherwise.

PTX inhibits cell cycle progression at G1/S phase and also induces apoptosis

The cell cycle profile of A375 cells treated with sub-toxic doses of PTX showed inhibition of G1/S phase transition. The maximum inhibition was 12.2% at a concentration of 2 mM. Also, at 3 mM PTX there was 18.68% increase in sub-G1 fraction, suggesting drug-induced apoptosis. Induction of apoptosis by PTX was further confirmed by ethidium bromide/acridine orange staining assay. PTX significantly induced apoptosis in a dose dependent manner as compared to untreated control ($p<0.05$). At a concentration of 2 mM and 3 mM PTX, 15% and 22% cells were undergoing apoptosis respectively.

PTX inhibits A375 cells adhesion to matrigel and type IV collagen.

Cellular adhesion to ECM is a prerequisite for cellular migration and invasion into the basement membrane. It was observed that pre-treatment of A375 cells with 3 mM PTX showed significant inhibition of adhesion to matrigel (21%, 25.29%, 16.51%, 22.48% and 21.09% at 15, 30, 45, 60 and 90 min incubation respectively) and type IV collagen (38.21%, 19.66%, 15.27%, 14.5% and 15.39 % at 15, 30, 45, 60 and 90 min incubation respectively) as compared to untreated control ($p<0.05$).

PTX inhibits MMP2 and MMP9 secretion

Gelatin zymography was carried out to evaluate the effect of PTX on MMP2 and MMP9 secretion. It was observed that the activity of MMP2 and MMP9 was decreased by PTX treatment in a dose dependent manner as compared to untreated control. Densitometry analysis of the gel showed a significant inhibition in the enzymatic activity of MMP2 (25.27% and 56% inhibition at 2 and 3 mM respectively) and MMP9 (21.8% and 50.19% inhibition at 2 and 3 mM respectively). Further, PTX treatment did not have any effect on the level of MMP2 and MMP9 mRNA as shown by semiquantitative RT-PCR.

PTX inhibits migration of the A375 cells

PTX effectively inhibited migration of cells in a dose dependent manner compared to the untreated control cells ($p<0.05$). This inhibition in cell migration was 19%, 43% and 62% at 1, 2 and 3 mM respectively. Reorganization/polymerization of actin cytoskeleton plays an important role in cell migration. It was observed that PTX disrupted actin organization of the cells in a dose dependent manner.

PTX inhibits constitutive and IL-6 inducible pSTAT3 expression

When A375 cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h and western blot was carried out to evaluate the effect of PTX on STAT3 and pSTAT3 expression, it was observed that PTX inhibited constitutive as well as IL-6 inducible STAT3 activation in A375 melanoma cells in a dose dependent manner **(Fig 2a and 2b)**. Moreover, PTX treatment also inhibited pSTAT3 expression at earlier time points i.e. 30, 60 and 120 minute of PTX treatment. However, total STAT3 protein remained unchanged at all the time points. Interestingly, in normal cells (HEK-293), PTX treatment neither inhibited pSTAT3 nor STAT3 expression on IL-6 stimulation. Additionally, PTX treatment inhibited pSTAT3 expression in other cell lines such as A549 lung carcinoma and U87 glioblastoma.

PTX inhibits kinases and modulates the activity of phosphatase

As PTX inhibited phosphorylation of STAT3, the effect of PTX on various upstream kinases such as JAKs and Src that phosphorylate STAT3 protein was evaluated. Results suggested that PTX treatment inhibited pJAK1 and pJAK2 expression in a dose dependent manner. However total JAK1 and JAK2 protein remained unchanged. Also, PTX did not have any effect on non-receptor tyrosine kinase Src.

Protein tyrosine phosphatases, particularly SHP family phosphatase play an important role in negatively regulating STAT3 signaling pathway. Immunoblotting assay showed

that sodium vanadate (tyrosine phosphatase inhibitor) reversed the effect of PTX, suggesting a contributive role for protein tyrosine phosphatases in PTX-induced inhibition of STAT3 activation. Further, we have shown that PTX enhanced the expression of pSHP2 phosphatase in a dose dependent manner without having any effect on total SHP2 expression.

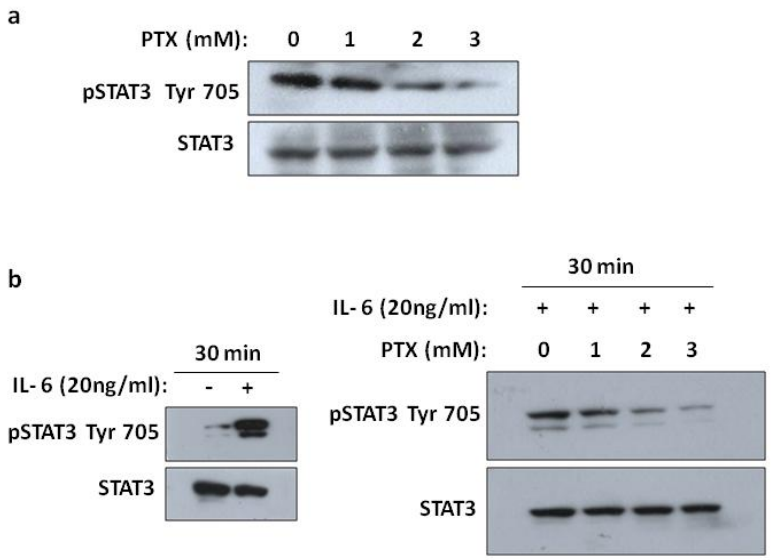

Fig 2: PTX inhibits constitutive as well as IL-6 inducible phospho STAT3 expression in A375 melanoma cells. ***(a)*** *Cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h.* ***(b)*** *Cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h and then stimulated with IL6 (20 ng/ml) for 30 minutes. Whole cell extracts were prepared and subjected to western blotting to determine level of phospho STAT3. The same blots were stripped and reprobed with STAT3 to verify equal protein loading.*

PTX inhibits STAT3 nuclear translocation

After phosphorylation, STAT3 dimerizes and translocates to the nucleus for activation of transcription. Confocal microscopy was carried out to evaluate the effect of PTX on

STAT3 nuclear translocation. Results suggested that PTX treatment inhibited nuclear translocation of STAT3 in a dose dependent manner. Further, PTX treatment consistently inhibited pSTAT3 nuclear expression in a dose dependent manner.

PTX inhibits binding of STAT3 to DNA

In nucleus, binding of STAT3 to the promoter of target genes is essential for initiation of transcription. By using ELISA based DNA binding assay, we have showed that treatment with PTX inhibited DNA binding activity of STAT3 **(Fig 3)**.

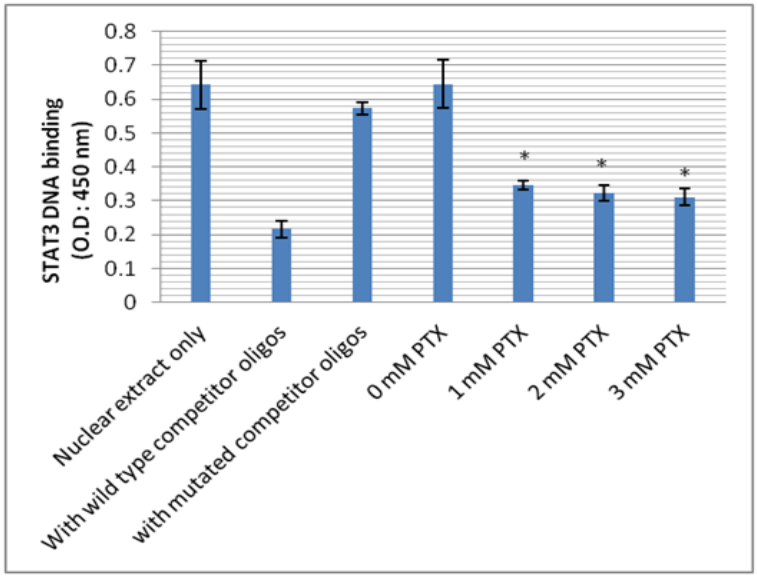

Fig 3: PTX inhibits STAT3-DNA binding in A375 melanoma cells. *Cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h. Nuclear extracts were prepared and DNA binding assay was carried out as described in materials and methods.*

PTX inhibits expression of STAT3 target genes

We studied expression of various genes that are either directly or indirectly regulated by STAT3. PTX significantly decreased the expression of genes involved in (i)

Angiogenesis: VEGF, VEGFR2 and HIFα, (ii) Cellular proliferation: Cyclin D1, CDK6 and cMyc and (iii) Apoptosis: BclXl, caspase 3 and caspase 9.

PTX inhibits IL6 secretion in culture supernatant of A375 melanoma cells

STAT3 activation can occur through the actions of many autocrine and paracrine growth factors including IL-6. Studies have suggested that high level of IL-6 plays key role in tumorigenesis such as anti-apoptotic, pro-invasive, and immune-stimulatory effects. When IL-6 secretion in culture supernatant of melanoma cells was estimated, it was observed that treatment with PTX at sub-toxic doses significantly inhibited IL-6 secretion.

PTX does not inhibit pSTAT1 or STAT1 expression

STAT1 is another member of STAT family proteins that shares 72% sequence homology with STAT3. As, PTX suppressed STAT3 phosphorylation, we evaluated the effect of PTX on STAT1 and pSTAT1 expression. Western blot results suggested that PTX did not affect expression of both pSTAT1 and STAT1.

Effects of PTX on MAP kinase and PI3K/Akt pathways

MAP kinase and PI3K/Akt pathways have also been shown to cross-react with STAT3 signaling, and are frequently activated in a wide range of human cancers. PTX significantly decreased the expression of pp38, SAPK/JNK and pAkt expression without having any effect on expression of pp44/42, p44/42, p38, PI3K and Akt.

Effect of PTX *in vivo*

Since PTX showed significant inhibition in the growth and metastasis of A375 cells *in vitro*, the efficacy of PTX was evaluated against A375 melanoma xenograft. When established xenografts were treated for 9 days i.p with a dosage of 40 mg/kg and 60 mg/kg, the mean volume of tumors treated with PTX was significantly decreased as compared to tumors treated only with PBS. PTX treated tumor tissue had lower

pSTAT3 expression as compared to untreated control as shown by western blot. Furthermore, when established intra-dermal xenograft were treated for 9 days i.p with a dosage of 40mg/kg and 60mg/kg of PTX, there was a significant decreased in tumor induced angiogenesis as compared to untreated control. The PTX was well tolerated and did not show any visible toxicity.

Discussion

Metastasis, rather than the primary tumor is the major cause of mortality in a majority of cancer patients. To cut down this mortality improved ways or novel therapies to treat metastatic disease are needed. PTX, a non-specific phosphodiesterase inhibitor has been shown to have anti-metastatic and anti-angiogenic activity against many human cancers. However, underlying molecular mechanisms are unknown. In the present study we have shown that PTX inhibits A375 human melanoma growth and metastasis *in vitro* as well as *in vivo* by targeting STAT3 signaling pathway.

PTX showed time and dose dependent toxicity against A375 cells. IC 50 value calculated from the dose effect curve was found out to be 17, 7, 2.3 and 1.8 mM when exposed to drug for 2, 24, 48 and 72 h respectively. Further, PTX inhibited cellular proliferation in a dose dependent manner. Interestingly, at sub-toxic doses of PTX (1, 2 and 3mM), 7.8%, 25% and 45% decrease in colony forming units respectively was observed confirming its anti-proliferative effects of PTX at these sub-toxic doses. In addition, the same sub-toxic doses of PTX (1, 2 and 3 mM) had minimal effect on viability of immortalized non-tumorigenic cell lines HaCaT (human keratinocyte) and NIH3T3 (mouse fibroblast). Thus, PTX at sub-toxic doses could be proposed to be a promising candidate for restricting the growth and proliferation of human melanoma cells. Anti-proliferative and growth inhibitory activity of PTX was not only restricted to melanoma cells. It also inhibits cellular growth and proliferation of A549 lung

carcinoma, U87 glioblastoma, MDA-MB 231 breast carcinoma and cervical cancer cell lines HeLa and SiHa (15, 16).

The cell cycle profile of A375 cells treated with PTX showed inhibition in G1/S phase transition and induction of apoptosis. This observation accounts for the anti-proliferative effects of PTX.

Deregulated proliferation and inhibition of apoptosis represents a major causative factor in the development and progression of cancer and provides an obvious target for therapeutic intervention. Our data revealed that at an effective sub-toxic dose of PTX, a significant percentage of cells were observed to undergo apoptosis. This result is consistent with an earlier study showing that PTX induces apoptosis in MDA-MB 231, Hut-78 and MyLa cells (15, 17).

Degradation of basement membranes is a key step in cellular invasion and metastasis. Several lines of evidence strongly implicate that matrix metalloproteinases (MMPs) play a crucial role in these complex multistep processes. Elevated expression of MMPs particularly MMP2 and MMP9 were correlated with migration and invasive potential of tumor cells (18). Thus, the inhibition of MMPs activity may be crucial for the inhibition of cancer metastasis. Our data revealed that PTX decreased the activity of MMP2 and MMP9 in A375 cells. PTX was also found to inhibit MMP2 and MMP9 activity in MDA-MB 231 breast carcinoma (15).

Cellular migration is another property of cancer cells that is essential for the formation of clinically relevant metastasis. For migration, cells require combination of protrusive and contractile forces that are co-ordinately regulated and these driving forces are provided by the dynamic reorganization of the actin cytoskeleton where protrusion acts at the front of the cell and retraction at the rear end. A study showed that depolymerization of actin filament impairs cell migration (19). In this study it was

observed that PTX inhibited migration of A375 cells at sub-toxic doses. Further, PTX disrupted actin organization of the cells, thus suggesting that in some cellular context PTX would inhibit migration of the cells by disrupting actin organization.

The initial invasive action of metastatic cells involves interaction of tumor cells with the ECM through the process of cell matrix adhesion. Once malignant cells are detached from the primary tumor, they bombard the surrounding basement membrane and adhere to its meshwork of ECM substrate. Any alteration of this property would interrupt the metastatic cascade. A significant reduction in the attachment of PTX treated A375 cells to the ECM substrate collagen type IV and matrigel was observed. Therefore, PTX induced inhibition of this process contributes to the anti-metastatic effects of the PTX. Taken together, our data revealed that PTX at sub-toxic doses inhibited multiple steps of metastasis such as cellular proliferation, migration, invasion and cellular adhesion, thus suggesting that PTX has a potential to show anti-metastatic activity against cancer cells.

STAT3 is overactive in almost all cancers, and promotes tumorigenesis by enhancing proliferation, angiogenesis, invasiveness and metastasis. Thus, it is regarded as a druggable target for cancer therapy. Our data revealed that PTX inhibited constitutive as well as IL-6 inducible STAT3 activation in A375 melanoma cells in a dose dependent manner. Also, PTX inhibited phosphorylation of the upstream kinases such as JAK1 and JAK2 and increased the expression of pSHP-2 phosphatase. In contrast to this, PTX did not inhibit pSTAT3 expression in normal cells like HEK-293.

STAT3 phosphorylation at Tyr705 causes it to dimerize and then translocate to the nucleus for binding to specific promoter sequences on its target genes. We found that treatment with PTX not only inhibited nuclear expression of pSTAT3 but also its binding to DNA promoter, thus confirming that PTX had potential to inhibit STAT3

activation. Apart from phosphorylated STAT3, unphosphorylated STAT3 continuously shuttle from cytoplasm to nucleus and may play a role in oncogenesis and transcriptional regulation (20). In our study, treatment with PTX inhibited nuclear translocation of unphosphorylated STAT3 in a dose dependent manner.

Angiogenesis plays an important role in growth and metastasis tumor. A crucial role of vascular endothelial growth factor (VEGF) and its receptors in promoting angiogenesis has been well established. Niu et al reported that constitutive STAT3 activity up-regulates VEGF expression and tumor angiogenesis in melanoma cells (21). In contrast, targeting STAT3 blocks both VEGF and HIFα expression and angiogenesis (22). Consistently, here we found that PTX inhibited VEGF and VEGFR2 expression in a dose dependent manner. STAT3 has also been reported to induce expression of hypoxia-inducible factor-1α (HIF-1α), another key mediator of angiogenesis. We found that PTX treatment inhibited HIF1α expression in a dose dependent manner. Our findings, for the first time, describe the mechanism(s) for anti-angiogenic effect of PTX.

STAT3 phosphorylation plays a critical role in proliferation and survival of tumor cells. We observed that treatment with PTX inhibited the expression of various genes involved in cellular proliferation such as cyclin D1, CDK6 and cMyc. In mammalian cells, cyclin D1 complex with CDK4/CDK6, control G1/S phase transition. Consistent with the result, we have shown that treatment with PTX inhibits melanoma cell proliferation and G1/S phase transition. We have also shown that PTX can induce apoptosis in A375 melanoma cells. Here we observed that treatment with PTX inhibited expression of anti-apoptotic protein BclXL and activates caspase 3 and caspase 9 indicating one of the probable mechanisms by which PTX induces apoptosis.

IL-6, a multifunctional cytokine, is notable for its pleiotropic tumor-promoting activities. High levels of IL-6 have been detected in many types of human epithelial cancers, and correlate with proliferation or survival of multiple myeloma, breast carcinoma, lung adenocarcinoma, prostate cancer, cervical cancer, gastric cancer, and esophageal carcinoma (23). In the present study we observed that treatment with PTX inhibited IL-6 secretion in a dose dependent manner.

STAT1 is the founding member of STAT family transcription factors. In contrast to the growth stimulatory and anti-apoptotic functions of STAT3, STAT1 is generally known to have a tumor suppressor function (24). Thus, the balance between activated STAT1 and STAT3 may play a significant role in directing cells towards proliferation or apoptotic death. Several strategies have been developed to inactivate STAT3. However, so far a very few compounds have been reported that maintain finely tuned equilibrium between STAT1 and STAT3. Here we reported that PTX only inhibited STAT3 signaling without altering STAT1 activation. This fact further strengthens the potential of PTX as a STAT3 inhibitor.

Further, treatment with PTX significantly inhibited solid tumor growth induced by sub-cutaneous route and also inhibited tumor induced angiogenesis in intra-dermal xenograft model *in vivo* without having any visible toxicity.

In conclusion, our data suggest that PTX has potential to inhibit tumor growth, metastasis and angiogenesis *in vitro* as well as *in vivo* by targeting STAT3 signaling pathway. These findings, for the first time, explain the molecular mechanism(s) by which PTX exerts anti-metastatic and anti-angiogenic activity.

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2. Mohammad Zahid Kamran, Rajiv P. Gude. Preclinical evaluation of the antimetastatic efficacy of Pentoxifylline on A375 human melanoma cell line. Biomedicine & Pharmacotherapy, 66 (2012), 617-626.

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List of Abbreviations

ATP	Adenosine tri phosphate
cAMP	cyclic adenosine monophosphate
Concn	Concentration
BSA	Bovine serum albumin
DABCO	1,4-diazabicyclo[2.2.2]octane
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DMEM	Dulbeccos Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
D/W	Distilled water
ECM	Extracellular matrix
FBS	Fetal bovine serum
gm	Gram
h	Hour
JAK	Janus kinase
Kd	Kilo dalton
mg	Milligram
min	Minute
ml	Millilitre
mM	Milli molar
PBS	Phosphate buffered saline
PTX	Pentoxifylline
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell park memorial institute
SDS	Sodium dodecyl sulphate
STAT	Signal transducer and activator of transcription
V	Volts
µg	Microgram
µM	Micro molar
µl	Microlitre

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Cancer

Cancer is one of the leading cause of disease and mortality worldwide (1). It arises in the cells of our body. Normal cells are constantly subjected to signal that dictate whether the cell should divide, differentiate into other cells or die. Cancer cells develop a degree of autonomy from these signals, resulting in uncontrolled growth and proliferation. Although, the biology of cell division and cell differentiation is exceedingly similar in normal and cancer cells, the normal control systems that prevent cell overgrowth and the invasion of other tissues are disabled in cancer cells. As these cells grow they develop new characteristics, including changes in cell structure, decreased cell adhesion, and production of new enzymes etc. These heritable changes allow the cells and its progeny to divide and grow results in abnormal growth and cancer.

Although the processes that occur during the development of cancer are only incompletely understood, it is clear that the successive accumulation of mutations in key genes is the force that drives cancer development (2). These genes can be broadly classified into three groups. The first group, called proto-oncogenes, produces protein products that normally enhance cell division or inhibit normal cell death. The mutated forms of these genes are called oncogenes e.g. bcr-abl, ras, myc etc. The oncogenes activate the signaling cascade continuously, resulting in an increased production of factors that stimulate cellular growth. A activating somatic mutation in one allele of an oncogene is generally sufficient to confer a selective growth advantage on the cells. Thus, oncogenes are dominant mutations.

The second group, called tumor suppressor genes, makes proteins that normally prevent cell division or cause cell death. Upon mutation these gene lose their ability to control

cell cycle progression e.g. Rb, p53, APC etc. Each cell in the body except for germ cells has two copies of a particular gene. The mutation in the tumor suppressor genes are recessive in nature i.e. mutation in one copy of the gene is not sufficient (haploinsufficiency) to overcome its ability to control its function; hence it requires mutation in both the copy of the gene.

Proto-oncogenes and tumor suppressor genes work much like the accelerator and brakes of a car, respectively. The normal speed of a car can be maintained by controlled use of both the accelerator and the brake. Similarly, controlled cell growth is maintained by regulation of proto-oncogenes, which accelerate growth, and tumor suppressor genes, which slow cell growth. Mutations that produce oncogenes accelerate growth while those that affect tumor suppressors prevent the normal inhibition of growth. In either case, uncontrolled cell growth occurs.

A third type of gene associated with cancer is the group involved in DNA repair and maintenance of chromosome structure known as stability genes or caretakers genes. This class include the mismatch repair (MMR), nucleotide-excision repair (NER) and base-excision repair (BER) genes responsible for repairing subtle mistake made during normal DNA replication or induced by environmental exposure. When a DNA repair gene is mutated its product is no longer made, preventing DNA repair and allowing further mutations to accumulate in the cells.

Any qualitative or quantitative alterations in these three classes of genes lead to breakdown of their normal functioning. These genetic alterations are known to occur by point mutation, deletion, gene amplification, chromosomal translocation or other mechanisms (3). Besides, qualitative or quantitative changes in the genes, structural alterations in gene can also occur by epigenetic mechanisms. Methylation of cytosine bases in DNA or modification in the expression of genes by changes in histone profile

are the common epigenetic changes that also play a vital role in tumor progression. For instance, there is change in global DNA methylation pattern during cancer progression leading to genomic instability (4). Deregulated expression of microRNAs (miRNAs) is another emerging epigenetic mechanism involved in carcinogenesis. Several of miRNAs are found to be upregulated or downregulated in numerous cancers (5). Apart from genetic and epigenetic alterations, several environmental factors affect one's probability of acquiring cancer. These factors are considered carcinogenic agents when there is a consistent correlation between exposure to an agent and the occurrence of a specific type of cancer. Some of these carcinogenic agents include X-rays, UV light, viruses, tobacco products, pollutants, and many other chemicals.

1.2 Hallmarks of cancer

In spite of their diversity, human cancers share six fundamental properties such as sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis **(Figure 1.1)**. These six characteristics were proposed as hallmarks of cancer in 2000 by Hanahan and Weinberg (6). Different cancers display each of these to different extents. Moreover, these properties may be acquired step by step and become evident at various stages during the progression of a cancer. The six hallmarks of cancer are:

1.2.1 Self-sufficiency in growth signals: Cells require growth factors for their growth and proliferation. Normal tissues carefully control the production and release of growth promoting factors and signaling that instruct entry into and progression through the cell division cycle, thereby ensuring a homeostasis of cell number. However, cancer cells, by deregulating these signals, become masters of their own destinies. Cancer cells are

able to synthesize their own growth factors or they amplify the growth factor signaling. This autonomy from growth factor signaling leads to unregulated growth.

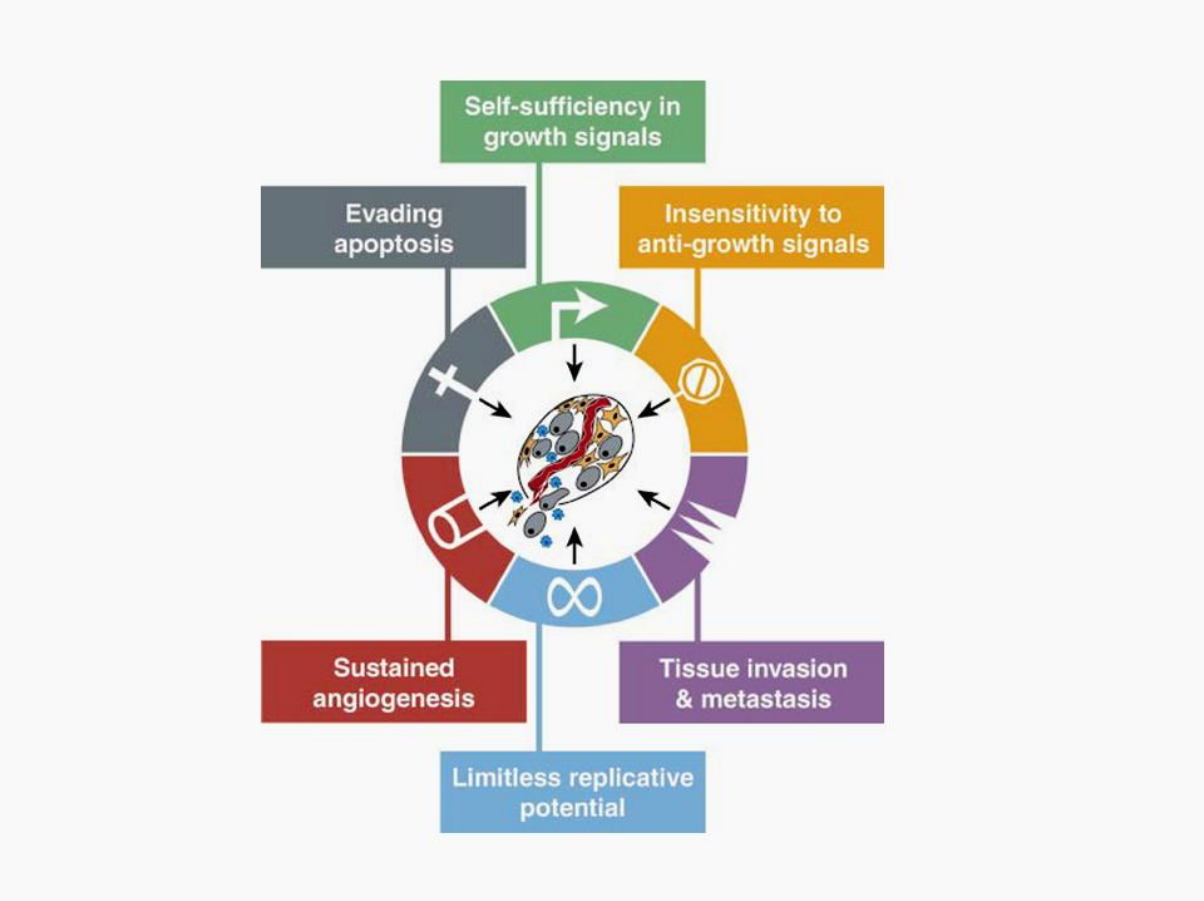


Figure 1.1: Acquired capabilities of cancer [adapted from (6)]

1.2.2 Insensitive to anti-growth signals: In order to maintain cellular quiescence and tissue homeostasis many anti-proliferative or anti-growth signals operate within the normal tissue. Cancer cells, on the other hand, bypass or evade these anti-growth signals to enable their own growth and proliferation. For instance, TGFβ signalling is an important anti-proliferative signal, to avoid such signalling tumor cells downregulate TGFβ receptors (7) or they expresses mutant or dysfunctional receptors (8).

1.2.3 Evading apoptosis: The ability of cancer cells to expand in number is determined not only by the rate of cell proliferation but also by the rate of cell death. Programmed

cell death or apoptosis represents a major source of this attrition. There is increasing evidence to suggest that resistance to apoptosis is a major hallmark of most and perhaps all types of cancer. Cancer cells bypass apoptosis in many ways. The most common method involves mutation of the p53 tumor suppressor gene resulting in the loss of proapoptotic regulators. More than 50% of all human cancers (and 80% of squamous cell carcinomas) show inactivation of the p53 protein (9).

1.2.4 Limitless replicative potential: Normal cells undergo finite number of divisions before they stop dividing completely (senescence), this is called Hayflick effect. After every round of cell division, telomere lengths (50-100 bp DNA from ends of each chromosome) get progressively shorter, until it provokes the cell to stop dividing and enter senescence. Cancer cells prevent telomere shortening by producing the enzyme, telomerase, which keeps extending telomeres, thus preventing senescence (10).

1.2.5 Sustained angiogenesis: Angiogenesis, a multistep process of sprouting new blood vessels from pre-existing ones, is essential for patho-physiological processes such as wound healing, chronic inflammation and embryogenesis. However, compelling evidence suggests that angiogenesis has a critical role in the progression of cancer (11). Under normal physiological condition there is presence of finely tuned equilibria between pro-angiogenic and anti-angiogenic factors. However, this equilibrium is disturbed under malignant conditions and shifted towards angiogenic switch. Continuous expression or up-regulation of various pro-angiogenic factors such as VEGF, bFGF, angiopoietin etc have been reported in tumor cells and their microenvironment. As a result, blood vessels grow around tumor cells that help in maintaining their continued growth, proliferation and metastasis.

1.2.6 Tissue invasion and metastasis: During the development of human cancer, cancer cells invade the surrounding normal tissue, breach the organ basement membrane and disseminate to distant organ sites via lymphatics or blood circulation and form tumors at these sites. This property of dissemination is referred to as metastasis (12). Metastasis enables cancer cells to survive and grow in new environments where there are no restrictions of space or nutrients. More than 90% of the patients die because of the invasive and metastatic cancers rather than the primary tumors. Although important, this is the least understood aspect of cancer biology because of the complexity and multistep nature of this process.

To the above six initial hallmarks of cancer, conceptual progress in the last decade has added two emerging hallmarks to this list - reprogramming of energy metabolism and evading immune destruction (13). Cancer cells are highly proliferating cells and they have increase demand of energy for the generation of new cells. Normal cells depend on glycolysis, krebs cycle and mitochondrial oxidative phosphorylation pathway for glucose metabolism. However, cancer cells can reprogram the glucose metabolism mainly to glycolysis even in the presence of oxygen (Warburg effect). Additionally, expression and activity of lipid biosynthesis enzymes like fatty acid synthase and hydroxymethylglutaryl-coenzyme A reductase are increased in cancer cells. A second enabling characteristic allows cancer cells to evade immunological destruction, in particular by T and B lymphocytes, macrophages, and natural killer cells. According to this logic, cancer cells that do appear have somehow managed to avoid detection by the various arms of the immune system or have been able to limit the extent of immunological killing, thereby evading eradication.

1.3 Metastasis

Metastasis, the spread of tumor cells from the primary site to distant organs, is the major cause of mortality in a majority of cancer patients (12). Advances in understanding the molecular mechanisms involved in metastasis have lagged behind other developments in cancer field, because of complexity of this multistep process. The process of cancer metastasis consists of a long series of sequential, interrelated steps each of which is rate limiting **(Figure 1.2)**. The first step is the detachment of cancer cells from the primary site, which involves alterations in the cell adhesion profile. To gain access to the blood vessels for further transportation, the tumor cells must invade basement membrane and surrounding tissues and enter into the circulation, a process called intravasation. During circulation, not all tumor cells are equipped to survive the high shear forces in circulation and the host immune defences. However, many tumor cells have evolved mechanisms to survive in circulation. The next step is the extravasation and colonization into new organs, where they proliferate and vascularised to form secondary tumor (12). Several tumors metastasize only to the organs in the anatomic vicinity like the regional lymph nodes or the organs receiving the afferent blood vessel from the primary. However, many tumors bypass several organs in the blood flow path and very specifically colonize distinct organ sites (14).

To understand the process of metastasis more precisely, various *in vitro* as well as *in vivo* experimental models were developed (15). This not only helped in confirming the involvement of steps in metastasis but also resulted in identification of large number of host and tumor derived molecules that participate in these steps of metastasis. In these assays, cancer cells are injected into experimental animals, either orthotropically or directly into the circulation to model the later phases of metastatic process. These assays are called spontaneous or experimental metastasis assay respectively, and the

end result of both the assays is the formation of visible metastases at the secondary site (16). In addition to above models some *in vitro* and *in vivo* assays have been developed to study the complex biological processes like cell motility, invasion, and angiogenesis, which are otherwise difficult to study in animals. The classical *in vivo* models used to study invasion and angiogenesis include the chick embryo chorioallantoic membrane (CAM) assays, corneal neovascularisation assays, the rabbit ear chamber, and hamster cheek pouch assays. The invasive potential of cancer cells is also studied using *in vitro* matrigel invasion assay. Motility and adhesion assay are used to study cell motility and adhesive properties respectively. All these assays have led to the identifications of many molecular alterations in cancer cells that can contribute to their ability to metastasize.

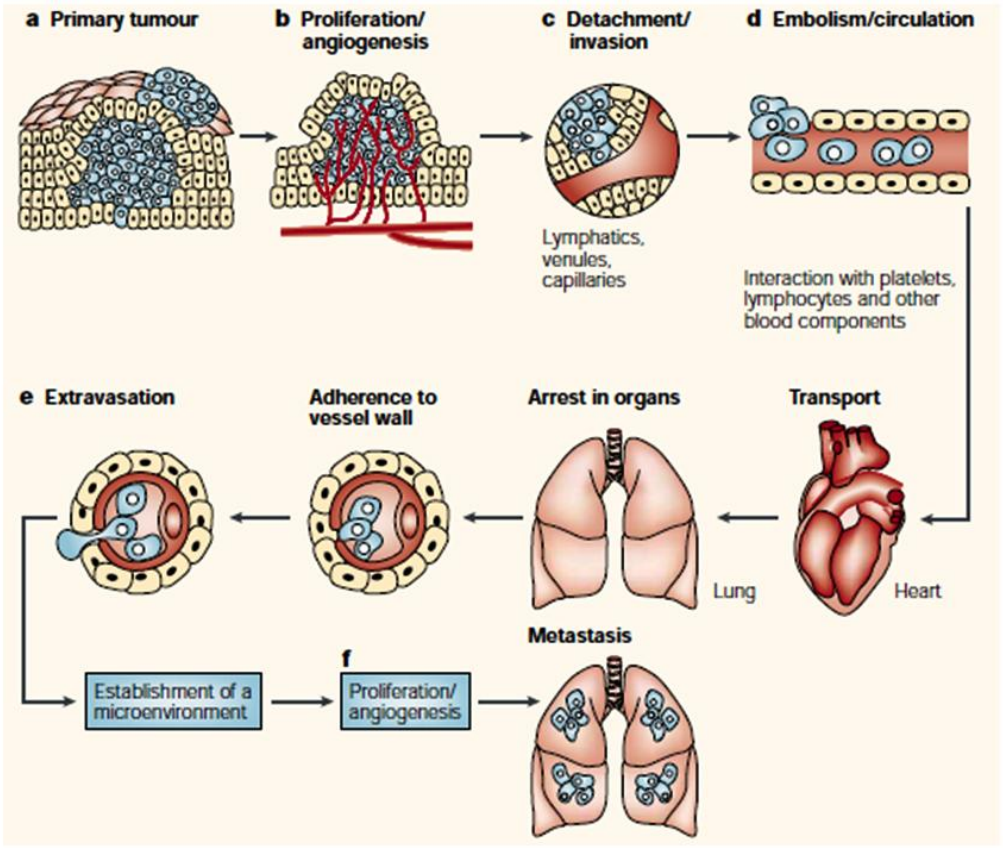


Figure 1.2: Different steps of metastasis [adapted from (17)]

1.4 Different steps of metastasis.

1.4.1 Detachment of tumor cells from primary tumor mass

The first step in the process of metastasis is the detachment of tumor cells from the primary site. For this, cells needs to overcome the mechanisms that hold the cells in place i.e. cell-cell and cell-extracellular matrix (ECM) interactions. Though, adhesion mechanism must be disrupted for tumor cells to metastasize, the reattachment of malignant cells to metastatic sites requires an increase in cellular adhesive capacity (18). This concept of 'disordered adhesion' was first put forward in 1944, when Coman recognized that adhesion between malignant cells must be downregulated as a prerequisite for invasive behaviour (19). Since then our knowledge of the molecular basis for these alterations in adhesive capacity has increased considerably, and it is now well recognized that cell-cell and cell-ECM adhesion mediated by specific cell surface molecules, play a critical role in tumor cell metastasis. In particular tumor invasion and metastasis, both hallmarks of tumor malignancy, frequently coincide with the loss of E-cadherin mediated cell-cell adhesion. Expression of E-cadherin, the most abundant adhesion molecule in adherence junctions of epithelia, is downregulated in most, if not all, epithelial cancers (20). This observation, led to hypothesis that the loss of intracellular contact would generate permissive conditions for tumor cell migration. Indeed, several research groups have shown that reconstitution of a functional E-cadherin adhesion complex suppresses the invasive phenotype of many different tumor cell types (20).

As well as cell-cell communication, interactions between tumor cells and the ECM occur during the initial invasive action of metastatic cells and following their extravasation. These interactions are mediated mainly through integrins, a family of

transmembrane glycoprotein, heterodimer of α and ß subunits (21). To date, comprising 8β and 18α subunits, so far known to assemble into 24 distinct integrins **(Figure 1.3)** (21)**.** These are the receptors that bind to a variety of ECM components including laminin, fibronectin, vitronectin, and collages. Altered expression or expression of newer set of integrin receptors appear to aid cancer cells in not only breaking cell-ECM interaction but also in their motility (22). Studies on cell lines have shown that integrin expression changes with malignant transformation. Comparison between melanocytes and melanoma cells in culture revealed that the neoplastic cells tend to over-express α2ß1 and α4ß1 integrins relative to their benign counterparts (23)

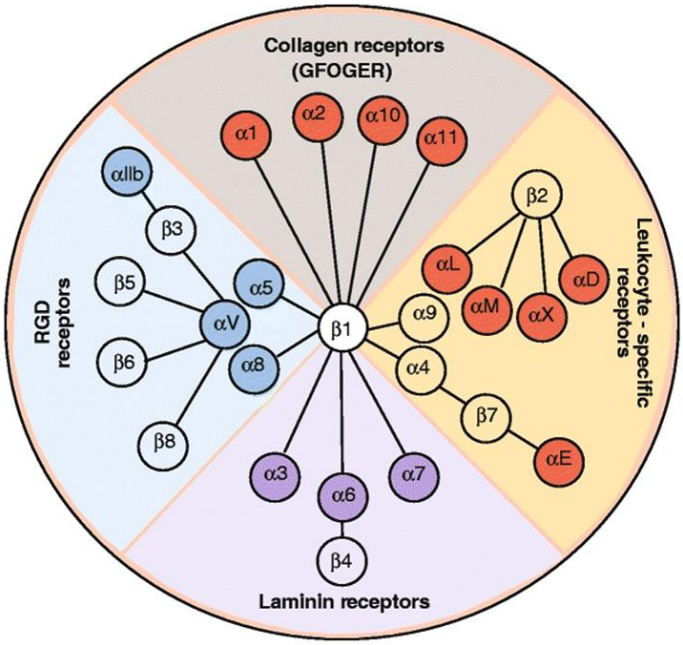


Figure 1.3: Representation of the integrin family [adapted from (21)].

1.4.2 Proteolytic degradation

Degradation of basement membrane and invasion of the surrounding tissue is a very important step in metastatic cascade. The degree of fragmentation of the basement membrane, whose main components include type IV collagen, laminin and heparan

sulphate proteoglycan, has been shown to correlate with tumor invasion and metastasis (24). A number of proteolyitc enzymes are involved in this process and can be classified into four major categories, depending on nature of the active sites. These are:

1. Matrix metalloproteinases
2. Serine proteinases
3. Cysteine proteinases
4. Aspartyl proteinases

1.4.2.1 Matrix metalloproteinases

The Matrix metalloproteinases (MMPs) are endopeptidases that can cleave virtually any component of the ECM. It is precisely regulated under normal physiological conditions such as embryogenesis, normal tissue remodelling, wound healing and angiogenesis but when deregulated it becomes a cause of many diseases such as arthritis, nephritis, cancer, encephalomyelitis and chronic ulcers (25). There are more than 21 human MMPs and the homologues from other species **(Table 1.1)** (26). On the basis of the specificity of MMPs for ECM components, they are divided into collagenases, gelatinases, stromelysins and matrilysins (**Figure 1.4)** (27). The common names of the MMPs mirror this classification. Out of eight distinct structural classe s of MMPs: five are secreted and three are membrane-type MMPs (MT-MMPs)**.** The MMPs are synthesized as inactive zymogens (pro-MMPs). They are kept inactive by an interaction between a cysteine-sulphydryl group in the propeptide domain and the zinc ion bound to the catalytic domain, activation requires proteolytic removal of the propeptide prodomain (27). Most of the MMPs are activated outside the cell by other activated MMPs or serine proteinases. Direct evidence for a role of MMPs in tumor progression comes from xenograft experiments using cancer cells with decreased and increased expression levels of MMPs, and from carcinogenesis experiments with mice that either

lack a specific MMP or have organ-specific MMP overexpression. MMPs are upregulated in almost every type of human cancer, and their expression is often associated with poor survival (28). Whereas some of the MMPs (for example, MMP7) are expressed by the cancer cells, other MMPs (for example, MMP2 and MMP9) are synthesized by the tumor stromal cells, including fibroblasts, myofibroblasts, inflammatory cells and endothelial cells. MMPs promote tumor progression not only through ECM degradation, as originally thought, but also through signalling functions. MMPs counter apoptosis, orchestrate angiogenesis, regulate innate immunity, and promote metastasis and tumor growth (26). As MMPs seemed to be attractive cancer targets, several second-generation small-molecule and peptidomimic-based MMP-inhibitor (MMPI) drugs advanced to phase III clinical trials in patients with advanced cancer (**Table 1.2)** (29).

Recent studies show that members of the MMP family exert different roles at different stages during cancer progression. In particular, they may promote or inhibit cancer development depending among other factors on the tumor stage, tumor site (primary, metastasis), enzyme localization (tumor cells, stroma) and substrate profile (28). For example, MMP8 provides a protective effect in the metastatic process, decreasing the metastatic potential of breast cancer cells when it is overexpressed (30). Similarly, MMP8 expression in squamous cell carcinoma of the tongue is correlated with improved survival of patients and it is proposed that this protective action is probably correlated with the role of estrogen in the growth of tongue squamous cell carcinomas (31). On the other hand, MMP9 might function as tumor promoter in the process of carcinogenesis as well as an anticancer enzyme at later stages of the disease in some specific situations. This dual role is based on the findings in animal models, where it observed that MMP9 knockdown in mouse models exhibited decreased incidence of

carcinogenesis, whereas tumors formed in MMP9 deficient mice were significantly more aggressive (28).

MMP designation*	Structural class	Common name(s)
MMP-1	Simple hemopexin domain	Collagenase-1, interstitial collagenase, fibroblast collagenase, tissue collagenase
MMP-2	Gelatin-binding	Gelatinase A, 72-kDa gelatinase, 72-kDa type IV collagenase, neutrophil gelatinase
MMP-3	Simple hemopexin domain	Stromelysin-1, transin-1, proteoglycanase, procollagenase-activating protein
MMP-7	Minimal domain	Matrilysin, matrin, PUMP1, small uterine metalloproteinase
MMP-8	Simple hemopexin domain	Collagenase-2, neutrophil collagenase, PMN collagenase, granulocyte collagenase
MMP-9	Gelatin-binding	Gelatinase B, 92-kDa gelatinase, 92-kDa type IV collagenase
MMP-10	Simple hemopexin domain	Stromelysin-2, transin-2
MMP-11	Furin-activated and secreted	Stromelysin-3
MMP-12	Simple hemopexin domain	Metalloelastase, macrophage elastase, macrophage metalloelastase
MMP-13	Simple hemopexin domain	Collagenase-3
MMP-14	Transmembrane	MT1-MMP, MT-MMP1
MMP-15	Transmembrane	MT2-MMP, MT-MMP2
MMP-16	Transmembrane	MT3-MMP, MT-MMP3
MMP-17	GPI-linked	MT4-MMP, MT-MMP4
MMP-18	Simple hemopexin domain	Collagenase-4 (*Xenopus*; no human homologue known)
MMP-19	Simple hemopexin domain	RASI-1, MMP-18[†]
MMP-20	Simple hemopexin domain	Enamelysin
MMP-21[§]	Vitronectin-like insert	Homologue of *Xenopus* XMMP
MMP-22	Simple hemopexin domain	CMMP (chicken; no human homologue known)
MMP-23	Type II transmembrane[¶]	Cysteine array MMP (CA-MMP), femalysin, MIFR, MMP-21/MMP-22[†]
MMP-24	Transmembrane	MT5-MMP, MT-MMP5
MMP-25	GPI-linked	MT6-MMP, MT-MMP6, leukolysin
MMP-26	Minimal domain	Endometase, matrilysin-2
MMP-27[#]	Simple hemopexin domain	
MMP-28	Furin-activated and secreted	Epilysin
No designation	Simple hemopexin domain	Mcol-A (Mouse)
No designation	Simple hemopexin domain	Mcol-B (Mouse)
No designation	Gelatin-binding	75-kDa gelatinase (chicken)

Table 1.1: The matrix metalloproteinase family [adapted from (26)].

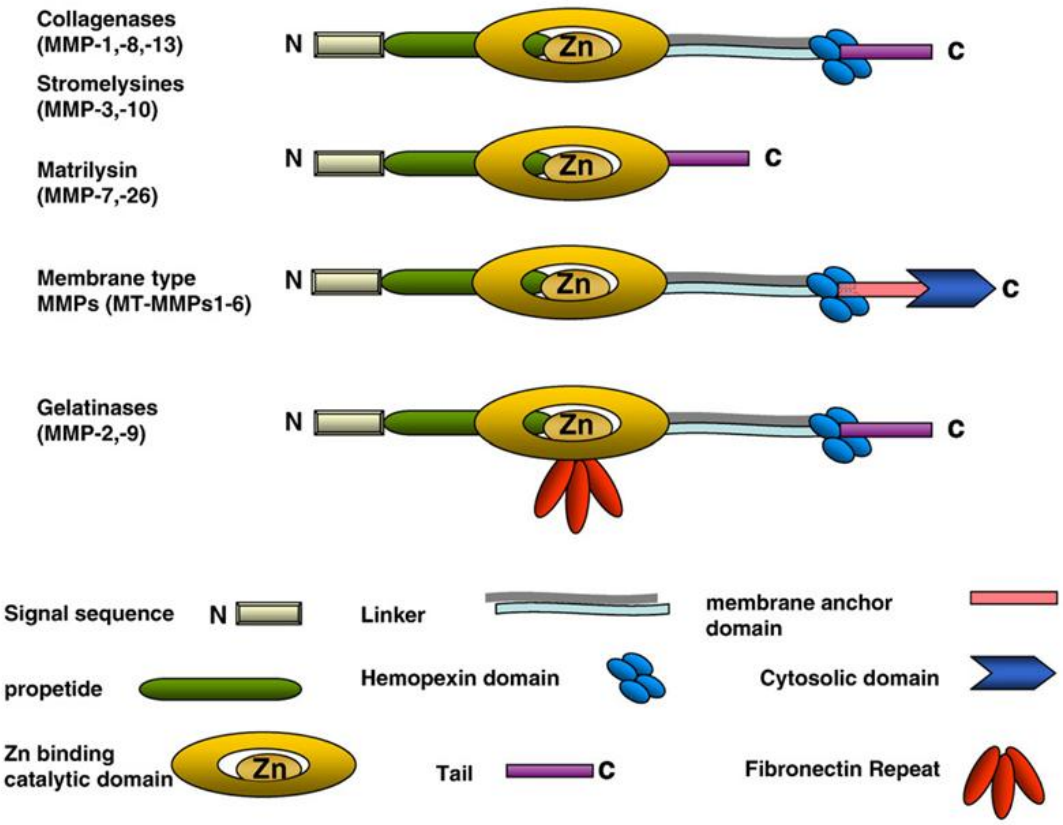


Figure 1.4: Domain organization of MMPs. *All MMPs share similar domain organization. The N-terminal domains of MMPs contain a signal peptide, a pro-peptide, and a catalytic domain with a conserved zinc binding motif. The conserved cysteine switch residue resides on the pro-peptide and binds the catalytic zinc ion in the zymogen form. The gelatinases contain three fibronectin repeats for the binding of gelatin. A linker domain of various lengths connects the N-terminal to the C-terminal hemopexin domain (absent in MMP-7). The MT-MMPs are bound to the cell surface through a C-terminal membrane anchor domain* [adapted from (27)].

Inhibitor	Structure	Specificity	Comments
Marimastat (BB-2516)	Peptido mimetic	Broad spectrum	Survival benefit in a subset of gastric cancer patients. Survival benefit in glioblastoma multiforme patients in combination with temozolomide.
Tanomastat (BAY 12-9566)	Non-peptido mimetic	MMP-2, 3, 9	Development halted because treated patients showed poorer survival than control.
Prinomastat (AG3340)	Non-peptido mimetic	Broad spectrum	No survival benefits in NSCL cancer patients. No difference in progression of prostate carcinomas.
Metastat (COL-3)	Tetracycline derivative	Gelatinases	Multiple mechanisms of action against MMPs. Currently recruiting Kaposi's sarcoma patients.
Neovastat (AE-941)	Shark cartilage extract	Broad spectrum	Multiple mechanisms of action on MMPs. Currently recruiting renal-cell carcinoma, multiple myeloma and NSCL cancer patients.
BMS-275291	Non-peptido mimetic	Broad spectrum	Currently recruiting NSCL cancer patients.
MMI-270	Non-peptido mimetic	Broad spectrum	Anti-angiogenic and anti-metastatic effects in animal models. Phase I studies in patients with advanced malignancies.

Table 1.2: Matrix metalloproteinase inhibitors in clinical development for cancer therapy [modified and adapted from (29).

Tissue inhibitors of metalloproteinases (TIMPs) are natural endogenous inhibitors of MMPs. Four homologous TIMPs (TIMP1, 2, 3 and 4) have been identified to date. All of them share a conserved structure divided into an N and a C-terminal domain and containing three conserved disulfide bonds (32) . TIMPs regulate MMPs activity by

binding to either pro MMPs or MMPs. The net balance between protease and inhibitor activity determines the proteolytic potential of tumors, and decreases in TIMP levels generally correlates with tumorigenesis (33). Promoter hypermethylation and lost expression of TIMP2 gene have been reported in prostate cancer cells and tumor samples (34). Overexpression of TIMPs reduced experimental metastasis of melanoma (35). Transgenic studies reveal that mouse 3T3 cells became tumorigenic after antisense depletion of TIMP1 (36). TIMP1 overproduction slowed chemical carcinogenesis in skin and liver carcinogenesis in transgenic mice (37). In addition, TIMPs have shown apoptosis-inducing properties (38).

The recent experimental data on TIMPs in tumorigenesis indicate a new paradigm for TIMPs in cancer. TIMPs block the activity of MMPs, which is important in inhibiting tumorigenesis and subsequent malignant progression. On the other hand, TIMP may also promote tumorigenesis (33). Increased expression of TIMP1 is associated with poor prognosis in colorectal cancer (39), breast cancer, gastric carcinoma, lymphoma, prostate, and lung cancer (26). Systemic delivery of TIMP4 by intramuscular administration of naked TIMP4-DNA significantly stimulated mammary tumorigenesis *in vivo* (40). TIMP4 upregulates Bcl-2 and Bcl-XL protein and protects breast cancer cells from apoptosis both *in vitro* and in nude mice (40). Therefore, the net effect of TIMPs on tumorigenesis may depend on bioavailability of local amount of TIMPs in tumor microenvironment, the time when the TIMP is presented to the tumor cells, and the presence of putative TIMP receptor on tumor cells.

1.4.2.2 Serine proteases

Members of this class of endopeptidases are characterised by a serine residue at the active site and are produced in an inactive pro-form. Important members of the family

include trypsin, thrombin, plasmin, cathepsin G and plasminogen activator (uPA). There are two types of plasminogen activators: the urokinase-type (uPA) and the tissue type (tPA). These activators are coded by two different genes. However, they are quite similar in structure. Both are capable of catalyzing the conversion of inactive zymogen plasminogen to active proteinase plasmin. There is evidence that the primary role of tPA is to generate plasmin for thrombolysis, while it is the uPA that generates plasmin in events involving degradation of ECM. As a consequence uPA participates in cancer invasion and metastasis (41). uPA expression has been correlated to the ability of blue nevi to transform, as gene ablation in mice inhibited melanoma formation (42). uPA could be detected in both, the tumor and the surrounding stroma of basal cell carcinomas (43). Similarly, increased expression of uPA, tPA, and PAI-1 was observed in squamous cell carcinoma (44).

1.4.2.3 Cysteine proteinases

The family of cathepsins is characterised by a cysteine residue at their active site and include cathepsins B, L, H, C, S, F, K, O, V, W and X (45). These are lysosomal proteases that can degrade a variety of ECM components at a neutral pH, but whose optimal activity is at an acid pH. Among these, cathepsin B H, and L have major implications in cancer metastasis. It has been found that cathepsin B mRNA, protein level and activity in tumors and cancer cell lines, especially with high metastatic potential, are increased (45). It can affect extra-cellular connective matrix directly, causing its proteolytic degradation or indirectly via activation or amplification of other ECM degrading proteases. Cathepsin B, importantly, can activate certain MMPs and also receptor bound uPA (46). It displays preferential binding to cell membranes in malignant tumors (47). Tumorigenicity and the metastatic phenotype of human melanoma cells correlate with the expression of cathepsin L (48). Procathepsin L

degrades both fibronectin and laminin while cathepsin L degrades types I and IV collagens, fibronectin and laminin (48). Cathepsin H is easily distinguished from other endosomal cysteine proteases by its unique aminopeptidase activity (49). There is growing evidence that the expression of cathepsin H increases in disease states including breast carcinoma, melanoma, glioma, and lung, colorectal, prostate and other carcinomas (50). A possible function of cathepsin H proteolytic degradation is its ability to degrade fibrinogen and fibronectin, suggesting that along with other proteases, cathepsin H may be involved in the destruction of ECM components leading to cancer proliferation, migration and metastasis.

1.4.2.4 Aspartyl proteinases

Cathepsin D is an aspartyl protease that appears to cleave a variety of substrates such as fibronectin and laminin. Cancer cells are characterized by overexpression of cathepsin D at mRNA and at protein levels (51). Cathepsin D is synthesized in an inactive form as procathepsin D, which has no proteolytic activity. It is auto-activated in an acidic environment (pH<5) in acidic intracellular vesicles or activated during the action of other proteases (52). Marked expression of cathepsin D takes place in carcinomas of uterus, ovary, lung, intestines and many other organs (51). Cathepsin D, as an endopeptidase, degrades many intracellular and endocytosed proteins, ECM proteins and proteins of the basal epithelium (53).

1.4.3 Intravasation

The entry of tumor cells into the circulation occurs by a process termed as intravasation. The blood vessels are usually impermeant to the passive traversal of cells because they are lined by the basement membrane, whose composition is very similar to organ BM. Metastatic cells gain entry into circulation by degrading basement membrane, by the

production of various degradative enzymes (24). Moreover, the intravasation of metastatic cells is further facilitated by angiogenesis in tumors. Tumor often induce the growth of new blood vessel in and around them by a process a termed as angiogenesis (11). Tumor angiogenesis, in fact, an obligatory step for tumors to grow beyond a size of 2 mm (11). Since, newly formed blood vessels are usually immature with a leaky basement membrane, tumor cells from highly angiogenic tumors have a greater inclination to invade, and an easier access to the circulation. Strikingly, it has been demonstrated recently that there is a major difference in intravasation between metastatic cells and non-metastatic cells. Although both the metastatic variants of an adenocarcinoma cell line were equally motile in the primary tumor, the metastatic cells were polarized towards the blood vessels, while the non-metastatic cells get 'fragmented' during intravasation (54).

1.4.4 Survival in circulation

As metastatic-tumor cells enter the blood vessels, they are subjected to various non-specific forces such as mechanical stress, hemodynamic turbulence, loss of adhesion-induced cell death and cell mediated cytotoxicity (55). As a result, a very low percentage of tumor cells survive during circulation which further establishes micrometastasis in distant organs. This was termed as 'metastatic inefficiency' (56). STAT3 signaling has a major role in protecting the tumor cells from body's immune surveillance during their transit through circulation. Nguyen and co-workers observed that activation of STAT3 signaling in tumor cells or in inflammatory immune cells modulate secretion of various inflammatory factors such as IL-6 and TNFα that act as an immunosuppressors and increase the probability of survival of tumor cells (57). Moreover, STAT3 activation in tumor microenvironment also reduces the activity of

Natural Killer (NK) cells thereby protecting tumor cells during circulation (58). Additionally, tumor cells make association with platelets which protect them from the stresses of shear flow (59). Thus, STAT3 activation increases the number of surviving tumor cells that invade distant potent organs to form secondary tumor.

1.4.5 Extravasation and organ colonization

Once in circulation tumor cells are able to reach almost all organ sites. However, some tumors metastasize only regionally either to the lymph nodes or to the organs in the anatomic vicinity, while several others metastasize to very specific distant organ sites. It has long been recognized that different cancer types show an organ-specific pattern of metastasis. For instance, prostate cancer preferentially forms metastases in the bones and metastases from breast cancer are often detected in the brain, lungs, bone and liver. Already in 1889, Stephen Paget published a paper that described the seed and soil theory (60). He had noticed that certain types of tumor cells (the seed) had a high propensity to form metastases in specific organs (the soil) and he proposed that this was due to the compatibility between the seed and the soil. Forty years later, Paget's theory was challenged by James Ewing, who proposed that metastasis was determined by the anatomy of the vascular and lymphatic channels that drain the primary tumor i.e. the patterns of blood flow were the primary reason for organ-specific metastasis (61). His view then prevailed until seminal studies by Isaiah Josh Fidler conclusively demonstrated that while tumor cells reached the vasculature of all organs, the development of metastases occurred selectively in certain organs but not others (17). Today we know that all these models are valid. Autopsy studies and experimental animal studies support the concept that both blood flow and compatibility factors contribute to metastatic spread to various organs (62). Together, these studies show that

the blood flow determine the initial fate of the tumor cells and decide in which organ the tumor cells will end up after they have left the primary tumor. However, after the cancer cells have arrested in an organ, their ability to grow there is dependent on molecular interactions between the cancer cells and the new environment. In addition, the new organ must be able to support the cancer cells with the proper growth factors. Because chemokines and their receptors are involved in the homing of lymphocytes and hematopoietic cells to specific organs, it could be reasoned that also cancer cells use chemokines to home to specific organs. In an elegant study by Muller and co-workers, it was shown that breast cancer cells express the chemokine receptors that match the chemokines that are expressed in the organs where these cells end up as metastases (63).

1.4.6 Angiogenesis

One of the key factors in the growth of tumors in the host organ is neovascularisation, usually by the process of angiogenesis (11). Angiogenesis is the formation of new blood vessels from pre-existing ones. Angiogenesis is orchestrated by a variety of activators and inhibitors. Activators of endothelial cell proliferation and migration are mainly tyrosine kinase ligands, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and the angiopoietins. The receptors for VEGF and angiopoietins are exclusively present on endothelial cells. The remodelling of ECM that occurs during angiogenesis generates several fragments of ECM components. Some of them are potent angiogenic inhibitors, for example thrombospondin-1, angiostatin (a fragment of plasminogen that binds ATP synthase and annexin II), endostatin, tumstatin and canstatin (fragment of collagen that binds to integrins). In general, the levels of activators and inhibitors dictate whether an endothelial cell will be in a quiescent or an angiogenic state.

Neovascularisation not only provides blood supply to the tumor mass but also provides an escape route for the tumor cells to form distant metastasis (64). The newly formed vessels often lack the basement membrane and the endothelial lining is discontinuous, and thus provides an easy escape route for cancer cells into the circulation (64).

1.4.7 Growth of tumor cells in the target organ

Following adhesion and extravasation, efficient survival and proliferation of tumor cells is required for successful metastatic growth, and these processes require a receptive microenvironment at the destination site (65). Tumor cell survival and proliferation may be influenced by cell-cell and cell-matrix interactions in the metastatic niche. In recent years, evidence has emerged that specific interactions between tumor cells and molecular components of the metastatic niche such as fibronectin may be important in the evasion of cell death within the foreign soil (65). The metastatic niche would also constitute a rich source of growth factors and cytokines, many of which (including VEGF) may directly regulate tumor cell proliferation in addition to survival. The small proliferations of tumor cells at metastatic niches constitute micrometastases. Subsequently, the assembly of a functional vasculature is required to enable further cellular expansion and progression to macrometastases, a process for which activation of the angiogenic switch is required (64). Recent studies exploring the cellular and molecular pathways that mediate the micro- to macro- metastatic switch identified bone marrow derived endothelial progenitor cells (EPCs) as critical regulators of this process (66). In addition to EPCs, haematopoietic and mesenchymal cells aid in macrometastatic progression. Tumor-associated macrophages potentiate the angiogenic stimulus by expression of VEGF and angiopoietins, accelerate recruitment of other inflammatory cells, and secrete proteases for further matrix remodelling (65).

1.5 Methylxanthines

Xanthine is a purine base that can be found in a majority of body tissue and fluid. It can also be found in some plants which are used for medicinal purposes. Methylxanthines are methylated derivative of xanthine. It is an integral part of everyday food and drink consumption. Three important classes of methylxanthine are caffeine, theobromine and theophylline. Caffeine is a widely consumed compound because it is present in many common beverages such as tea, coffee and soft drinks. These classes of compound acts as both:

1. Competitive nonselective phosphodiesterase inhibitors (67), which raise intracellular cyclic adenosine monophosphate (cAMP), activate protein kinase A (PKA), inhibit TNF-alpha and leukotriene synthesis, and reduce inflammation (68, 69).
2. Nonselective adenosine receptor antagonists which inhibit sleepiness-inducing adenosine (70).

Methylxanthines are bronchodilators and represent a unique class of drugs for the treatment of asthma and chronic obstructive pulmonary disease (71). It also serves as a smooth muscle relaxant and immunomodulator (72). The exact mechanism of action of methylxanthines is not well understood, but it appears that increase in the concentration of cyclic AMP level in cells by methylxanthines is responsible for these effects.

1.6 Pentoxifylline

Pentoxifylline (PTX) is a derivative of theobromine, a synthetic methylated xanthine. The chemical name of PTX is 1-(5-oxohexyl)-3, 7-dimethylxanthine. It is a FDA approved drug for treatment of peripheral vascular disease (73).

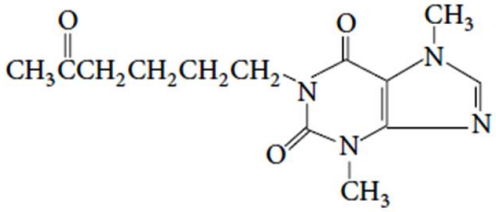


Figure 1.5: Structural formula of Pentoxifylline

Like other methylxanthine derivatives, PTX is an inhibitor of phosphodiesterase, resulting in an elevated intracellular pool of the second messenger cAMP (74). It is more soluble in water (77mg/ml) than other methylxanthine derivatives and has emerged as least toxic compound, thus received much attention for its clinical use (75). PTX is used to improve the effectiveness of microcirculation, increase red blood cell (RBC) deformability, decrease platelet aggregation and lower plasma viscosity (76). PTX has also been shown to modify the immune system. For instance, this drug improves leukocyte deformability and chemotaxis, depresses neutrophil degranulation, decreases endothelial leukocyte adhesion and lowers the sensitivity of leukocytes to cytokines (76). Furthermore, it has been reported that PTX can inhibit the production of inflammatory cytokines, and, thus, reduces neutrophil adhesiveness to endothelial cells, enhances chemotaxis and lowers the production of free radicals (77). In other studies, PTX has been shown to augment the production of prostacyclins and a vasodilator, eicosanoid (78). Furthermore, PTX has been reported to promote the oxygenation of ischemic areas and lower the amount of metabolic derangements associated with ischemia-reperfusion injury (79). PTX downregulates human immunodeficiency virus (HIV-1) long terminal repeat (LTR) directed gene expression in the human monocytic cell line U38 (80). Studies on T lymphocytes have shown that anti CD3 induced

expression of c-Rel was blocked by PTX, whereas the induction of other NF-κB family members was not affected (81).

PTX has been shown to have an effect also on growth factors and signal transduction pathways, affecting growth and proliferation. PTX has been shown to inhibits IEC18 intestinal epithelial cell proliferation via a differential modulation of TGFα and TGFβ2 expression (82) and also been shown to inhibit growth factor induced PI3K activation in smooth muscle cells (83). PTX has also been shown to inhibit proteins involved in cell cycle regulation including cyclin D1, the downregulation of which causes a G1 arrest (83).

Pentoxifylline and cancer

The first clue regarding anticancer or antimetastatic activity of PTX came from the study of Gastpar and co-workers. In 1974, Gastpar and co-workers showed that PTX can inhibit the cancer cell "stickiness" and thus increase the circulating time of tumor cells (84). This finding prompted further studies on the antimetastatic action of PTX. Since then various studies suggested anticancer or antimetastatic activity of PTX in various types of cancer cells. For instance, Ambrus and co-workers reported on the ability of PTX to reduce spontaneous metastasis (85) and studies from Futakuchi and co-workers has shown that PTX can strongly inhibit lung metastasis from rat HCCs *in vivo* (86). Recent studies showed that PTX has a differential influence on tumor metastasis which might be cell line dependent. PTX facilitates development of murine colon adenocarcinoma but inhibits melanoma derived tumor in the lung (87). Another study has shown that PTX significantly reduces liver metastasis in the mouse Neuro2a neuroblastoma model (88).

As an anticancer agent, PTX has been used to enhance tumor sensitivity to both radiation and chemotherapeutic agents (89, 90). PTX induces tumor perfusion therefore

better oxygenation. This enhances radiosensitivity as well as facilitates delivery of anticancerous drugs. PTX also interferes with cytokine-mediated inflammatory reactions, which occur in the acute phase after irradiation, thus reduces side effects post radiation exposure. Further, it abrogates G2M phase block created by treatment with cytotoxic agents and radiations, leading to increased apoptosis (91). PTX has been reported to increase radiosensitivity in chronically hypoxic WiDr tumors, head and neck cancer and hepatoma cell lines (91, 92). PTX could prevent early and late normal lung tissue damage due to radiotherapy in a double blind, randomized trial (93).

Recently Hernandez-Flores and co-workers suggested that PTX sensitizes cervical cancer cells to cisplatin-induced apoptosis and decreases its senescence via inhibition of NFκβ signaling pathway (94). PTX inhibited tissue factor expression and VEGF release under hypoxic as well as normal conditions in A375 melanoma, MCF7 breast carcinoma and A549 lung carcinoma (95). PTX has also been shown to induce the sensitization of multidrug-resistant cells to vincristine via downregulation of P-glycoprotein, stimulation of apoptosis and reduction of MMPs released from drug-resistant L1210/VCR cells (96). Another report showed that PTX in combination with proteasome inhibitor MG132 induces apoptosis in leukemic cells (97).

Pharmacokinetics of Pentoxifylline

Pentoxifylline is well absorbed from the gastrointestinal tract after oral administration. The initial peak concentration appeared 2 or 3 hours after ingestion, indicating that the drug was readily released (98). This has also been documented by *in vitro* dissolution studies. The peak concentration of PTX was about 100 ng/ml and reached a plateau at about 60 ng/ml 4-8 hours after administration (99). Oral preparations have a bioavailability of only 20% to 30%, because of a high first-pass clearance (100). In

healthy volunteers PTX is eliminated from the circulation with a short mean elimination half-life of about 0.9 hr after oral and about 1 to 2 hours after intra-venous administration (101). Seven metabolites of PTX are known **(Table 1.3)**. Hinze and co-workers reported that among the seven metabolites detected in urine after oral administration of PTX, 80% is represented by metabolite V, 12% by a combination of metabolites II and Ill, 8% by metabolite IV and less than 1% by metabolites I, VI and VII (99). The unchanged form appears in the urine only in traces, indicating that the drug is almost completely metabolized before excretion by the kidneys. Metabolite's plasma concentration was always higher than that of PTX: a 300 ng/ml peak and 200 ng/ml plateau for the 5- hydroxyhexyl metabolite (metabolite I) (98). A sustained-release 400 mg tablet is currently available commercially, which allows a patient to reduce dosing frequency while maintaining therapeutic plasma concentrations.

Metabolites of Pentoxifylline

Chemical Substitution No. 1 Position	Site of Formation	Urinary Excretion
Pentoxifylline $CH_2CH_2CH_2CH_2CO\text{-}CH_3$	(Ingested)	Traces
Metabolite I $CH_2CH_2CH_2CH_2CH(OH)CH_3$	Erythrocytes	< 1%
Metabolite II $CH_2CH_2CH_2CH_2CH(OH)CH_2OH$	Liver	12% (including III)
Metabolite III $CH_2CH_2CH_2CH(OH)CH(OH)\text{-}CH_3$	Liver	12% (including II)
Metabolite IV $CH_2CH_2CH_2CH_2\text{-}COOH$	Liver	8%
Metabolite V $CH_2CH_2CH_2COOH$	Liver	80%
Metabolite VI[b] $CH_2CH_2CH_2CH_2CO\text{-}CH_3$	Liver	< 1%
Metabolite VII[b] $CH_2CH_2CH_2CH_2CH(OH)CH_3$	Liver	< 1%

Table 1.3: Metabolites of Pentoxifylline [adapted from (98)].

1.7 Rationale of the study

More than 90% of cancer deaths are the consequence of metastatic spread (102). In spite of this, not only current drugs but also most of the investigational drugs aim at eradicating or controlling the primary tumor and no specific antimetastatic drug is being marketed at the moment. That's why cancer researchers vigorously search for drugs that can prevent metastases. During the past decade, new technologies such as structure-based drug discovery have been created, research expenditure by the US National Institutes of Health (NIH) has increased by more than two-fold, and pharmaceutical industries have doubled their R&D spending (103). This investment, however, has not resulted in proportionate quantities of novel and effective anticancer or antimetastatic drugs. Only one of every 5000–10,000 prospective anticancer agents receives FDA approval and only 5% of oncology drugs entering Phase I clinical trials are ultimately approved (103). These failure rates underscore the need for alternative efforts for drug development.

Researchers and clinicians have adopted numerous strategies to reduce the cost and time involved in cancer drug development. One such strategy is to evaluate established non-cancer drugs that have already been FDA approved for noncancerous diseases. This approach, alternatively called 'new uses for old drugs', 'drug repositioning', 'drug repurposing', 'drug re-profiling' or 'therapeutic switching'(103). By using this approach, specifically, drug development risk is reduced because repositioning candidates have been through several stages of clinical development and therefore have well known safety and pharmacokinetic profiles. Shorter routes to the clinic are also possible because *in vitro* and *in vivo* screening, chemical optimization, toxicology, formulation development and even clinical development have already been completed and can therefore be bypassed. In sum, these factors enable several years, and

substantial risks and costs, to be removed from the pathway to the market **(Figure 1.6)**. With a similar kind of approach, our laboratory is working on a drug known as Pentoxifylline (PTX). As stated earlier, PTX is a FDA approved drug for the treatment of peripheral vascular disease. Preclinical studies of PTX demonstrated that it is well tolerated, safe, non-toxic drug and has potent antimetastatic and antiangiogenic activity against many human cancers *in vitro* as well as *in vivo*. Some of the non-cancerous drugs that have anticancerous property are shown in (**Table 1.4)**.

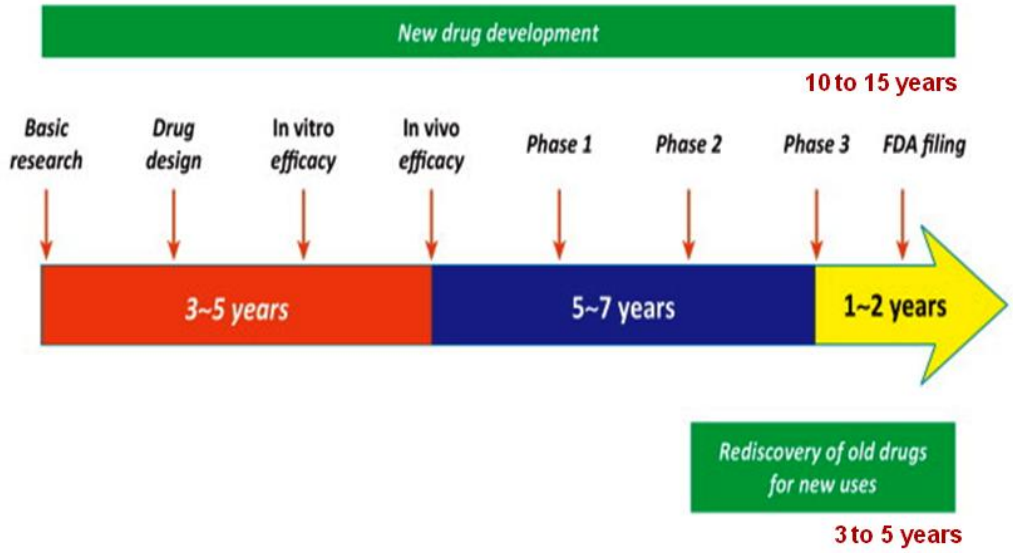


Figure 1.6: A comparison of traditional new drug discovery versus drug repositioning. *Major steps and estimated time involved in the conventional drug development process, which involves basic research, drug design, testing of safety and efficacy with preclinical and clinical studies, and finally filing for FDA approval takes around 10 to 15 years. The estimated time of drug development can be significantly reduced to 3 to 5 years by repurposing old drugs.[modified and adopted from (103)]*

Drug	Original indication (mechanism)	New anticancer indication (mechanism)
Thalidomide	Antiemetic in pregnancy (TNF-α ↓)	Multiple myeloma (NF-κB ↓, STAT3 ↓)
Aspirin	Analgesic, antipyretic (COX-1 ↓, COX-2 ↓)	Colorectal cancer (COX-2 ↓, NF-κB ↓, AP-1 ↓)
Valproic acid	Antiepileptic (GABA ↑)	Leukemia, solid tumors (HDACI ↓, HDACII ↓, NF-κB ↓, IL-6 ↓)
Celecoxib	Osteoarthritis, rheumatoid arthritis (COX-2 ↓)	Colorectal cancer, lung cancer (COX-2 ↓, NF-κB ↓)
Statins	Myocardial infarction (HMG-CoA reductase ↓)	Prostate cancer, leukemia (NF-κB ↓, HMG-CoA reductase ↓)
Metformin	Diabetes mellitus (AMPK ↑[a])	Breast, adenocarcinoma, prostate, colorectal (AMPK ↑[a], NF-κB ↓, TNF ↓, MCP-1 ↓)
Rapamycin	Immunosuppressant (mTOR ↓)	Colorectal cancer, lymphoma, leukemia (NF-κB ↓, IL-6 ↓, IKK ↓)
Methotrexate	Acute leukemia (DHFR ↓)	Osteosarcoma, breast cancer, Hodgkin lymphoma (NF-κB ↓, TNF-α ↓)
Zoledronic acid	Anti-bone resorption (osteoclast ↓)	Multiple myeloma, prostate cancer, breast cancer (CXCR-4 ↓, MMPs ↓, IL-6 ↓, Bcl-2 ↓, Bax ↑, FOXO3a ↑[a])
Leflunomide	Rheumatoid arthritis (DHODH ↓)	Prostate cancer (PDGFR ↓, EGFR ↓, FGFR ↓, NF-κB ↓)
Wortmannin	Antifungal	Leukemia (NF-κB ↓, AP-1 ↓)
Minocycline	Acne	Ovarian cancer, glioma (MMPs ↓)
Vesnarinone	Cardioprotective	Oral cancer, leukemia, lymphoma (NF-κB ↓, IL-8 ↓, VEGF ↓, AP-1 ↓)
Thiocolchicoside	Muscle relaxant (GABA ↓)	Leukemia, multiple myeloma (NF-κB ↓)
Nitroxoline	Antibiotic	Bladder, breast cancer (MetAP-2 ↓)
Noscapine	Antitussive, antimalarial, analgesic (bradykinin ↓)	Multiple cancer types (NF-κB ↓, HIF-1α ↓, Bcl-2 ↓, p21 ↑, p53 ↑, AIF ↑)

Table 1.4: Non-cancer drugs and their mechanism of action for cancer activities. *[Adapted from (103)]*.

Earlier studies from our laboratory

Earlier reports from our laboratory demonstrated that PTX inhibited B16F10 murine melanoma growth and metastasis in both *in vitro* as well as *in vivo* (104). Treatment with PTX inhibited B16F10 experimental metastasis and growth of murine solid tumors (105), which is mediated via its inhibitory action on cell adhesion, MMP9 secretions (106) and tumor angiogenesis (107). It was observed that PTX even at sub-toxic pharmacological doses acts as an effective antiproliferative agent with significant antiproteolytic and antiadhesive effects in culture of B16F10 melanoma cells (108). PTX has also demonstrated novel combination with suramin and has synergistic antitumor and antimetastatic activity in B16F10 melanoma (109). Our report also confirmed the antimetastatic activity of PTX against MDA-MB 231 breast carcinoma (110). However, none of the studies conducted so far describes the exact molecular mechanism(s) for the antimetastatic or antiangiogenic activity of the PTX. Therefore, in the present study, we have tried to evaluate the antimetastatic or antiangiogenic activity

of PTX with underlying molecular mechanism using A375 human melanoma cell line as a study model.

Details of the proposed research

Cellular proliferation, adhesion, invasion, migration and angiogenesis are the key steps in the metastasis of cancer. Any drugs that have antimetastatic activity should interfere in either of these processes. We therefore divided our study into two parts. In first part, we proposed to evaluate the effect of PTX on different key steps of metastasis such as cellular proliferation, invasion, migration, cellular adhesion and angiogenesis, using combination of *in vitro* and *in vivo* assays standardized in our laboratory. In the next part, we planned to investigate the underlying molecular mechanisms by which PTX has antimetastatic or antiangiogenic activity.

1.8 Aims and objectives of the study:

Objective 1: To study the antimetastatic effect of Pentoxifylline using A375 human melanoma cell line as study model.

Objective 2: To elucidate the mechanism(s) by which Pentoxifylline has antimetastatic and antiangiogenic activity.

Objective 3: To study the antitumor and antiangiogenic potential of Pentoxifylline in a xenograft model of A375 human melanoma tumor *in vivo*.

CHAPTER 2

To study the antimetastatic effect of Pentoxifylline.

2.1 Introduction

Metastasis, rather than the primary tumor, is the major cause of mortality in the majority of cancer patients (102). To reduce this mortality, improved ways or novel therapies to treat the metastatic disease are needed. Metastasis is complex, multistep process involving cellular proliferation, invasion into surrounding tissues, intravasation, migration through blood or lymphatic system, extravasation and cellular adhesion into new organ and angiogenesis (102). If any of the steps, such as proliferation, invasion, migration or adhesion are prevented by small molecule or drug, then this molecule or drug would be more clinically promising and, it is hoped, would reduce cancer mortality.

Earlier studies from our laboratory have demonstrated that PTX even at sub-toxic pharmacological doses acts as an effective antiproliferative agent with significant antiproteolytic and antiadhesive effects in culture of B16F10 melanoma cells (108). No studies have been undertaken to assess the antimetastatic effect of PTX in human melanoma cell line. Therefore, taking a step further in this direction, in our present objective, we have evaluated the effect of sub-toxic doses of PTX on different steps of metastasis such as cellular proliferation, invasion, migration and cellular adhesion using A375 human melanoma cell line as a study model.

2.2: MATERIALS AND METHODS

2.2 MATERIALS

2.2.1 Fine Chemicals

Name	Source
Acrylamide	Amersham Bios., UK
Bovine Serum Albumin Fraction V	Sigma Aldrich, USA
Bradford reagent	Sigma Aldrich, USA
Collagen Type IV	Sigma Aldrich, USA
1,4-diazabicyclo[2.2.2]octane (DABCO)	Sigma Aldrich, USA
Dimethyl Sulphoxide (tissue culture grade)	Sigma Aldrich, USA
Ethylene Diamine Tetra acetic acid	Amersham Bios., UK
IL-6 lyophilized powder	Sigma Aldrich, USA
Matrigel	Sigma Aldrich, USA
Methyl tetrazolium bromide	Sigma Aldrich, USA
Mitomycin C	Sigma Aldrich, USA
N,N'-Methylene-bis-acrylamide	Amersham Bios., UK
Pentoxifylline	Sigma Aldrich, USA
Poncue S	Sigma Aldrich, USA
Polyoxyehylene Sorbitan Monolaurate (Tween 20)	Sigma Aldrich, USA
Paraformaldehyde	Sigma Aldrich, USA
Sodium Orthovnadate	Sigma Aldrich, USA
Sodium Lauryl Sulphate	Sigma Aldrich, USA
Sodium Citrate	Sigma Aldrich, USA
Sodium deoxycholate	Sigma Aldrich, USA
TritonX100	Sigma Aldrich, USA
Tris	Sigma Aldrich, USA

NNN'N'-Tetramethylethylenediamine	Amersham Bios. USA

2.2.2 Tissue culture reagents and plastic ware

Name	Source
Benzyl penicillin	Alembic Ltd. India
DMEM	Gibco, USA
Fetal Bovine Serum US origin	Gibco, USA
RPMI 1640	Gibco, USA
Streptomycin	Nicholas Piramel, India
35mm culture dishes	B.D.Falcon, USA
60mm culture dishes	B.D.Falcon, USA
90mm culture dishes	B.D.Falcon, USA
96 well flat bottom plates with lid	B.D.Falcon, USA
24 well plate with lid	B.D.Falcon, USA
Cell scrapers	Nunc Nalgene Int., USA
0.2 micrometre Syringe driven filters	Millipore, USA
30 KDa Centrifugal cut-of filters	Amicon Millipore, USA

2.2.3 General chemicals

Name	Source
Agarose	Sigma Aldrich, USA
Ammonium persulphate	Sisco Laboratories, India
Calcium Chloride	S.D fine Ltd, India
Coommasie Brilliant Blue R	Sisco Laboratories, India
Magnesium Chloride	S.D. fine Ltd, India
Potassium Chloride	SD Fine Ltd, India
Phenol Red	SD Fine Ltd, India
Skimmed milk powder	Marvel, UK

Sodium azide	Spectrochem Pvt. Ltd., India
Sodium bi carbonate	S.D. Fine Ltd, India
Sodium hydroxide	Sisco Laboratories, India
Disodium hydrogen phosphate	Qualigens, India
Dihydrogen sodium phosphate	S.D.Fine Ltd, India
Trypsin	Himedia Pvt Ltd, India

2.2.4 Antibodies

Name	**Source**
Primary antibodies	
Mouse monoclonal STAT3	Santacruz Biotech., USA
Rabbit monoclonal pSTAT3	Cell Signaling, USA
Rabbit monoclonal STAT1	Cell Signaling, USA
Rabbit monoclonal pSTAT1	Cell Signaling, USA
Rabbit monoclonal JAK1	Cell Signaling, USA
Mouse monoclonal pJAK1	Cell Signaling, USA
Mouse monoclonal JAK2	Santacruz Biotech., USA
Rabbit monoclonal pJAK2	Cell Signaling, USA
Rabbit monoclonal Src	Cell Signaling, USA
Rabbit monoclonal pSrc	Cell Signaling, USA
Mouse monoclonal VEGF	Santacruz Biotech., USA
Rabbit monoclonal VEGFR2	Cell Signaling, USA
Mouse monoclonal HIF-1α	Santacruz Biotech., USA
Rabbit monoclonal p38	Cell Signaling, USA
Rabbit monoclonal pp38	Cell Signaling, USA
Rabbit monoclonal p44/42	Cell Signaling, USA
Rabbit monoclonal pp44/42	Cell Signaling, USA

Rabbit monoclonal SAPK/JNK	Cell Signaling, USA
Rabbit monoclonal PI3K	Cell Signaling, USA
Rabbit monoclonal Akt	Cell Signaling, USA
Rabbit monoclonal pAkt	Cell Signaling, USA
Rabbit monoclonal cyclin D1	Cell Signaling, USA
Rabbit monoclonal CDK4	Cell Signaling, USA
Rabbit monoclonal CDK6	Cell Signaling, USA
Rabbit monoclonal cMyc	Cell Signaling, USA
Rabbit monoclonal BclXl	Cell Signaling, USA
Rabbit monoclonal caspase 3	Cell Signaling, USA
Rabbit monoclonal cleaved caspase 3	Cell Signaling, USA
Rabbit monoclonal caspase 9	Cell Signaling, USA
Rabbit monoclonal cleaved caspase 9	Cell Signaling, USA
Mouse monoclonal MMP2	Santacruz Biotech., USA
Mouse monoclonal MMP9	Santacruz Biotech., USA
Mouse monoclonal β-Tubulin	Sigma Aldrich, USA
Secondary antibodies	
Anti-mouse Horseradish Peroxidase (HRP)	Sigma Aldrich, USA
Anti-rabbit Horseradish Peroxidase (HRP)	Sigma Aldrich, USA
Anti-mouse FITC (Fluoresceine-isothiocyanate)	Invitrogen, USA
Anti-rabbit Alexa 568	Invitrogen, USA

2.2.5 Kits and bioactive molecules.

Name	**Source**
Trans AM Nuclear extraction kit	Active Motif, USA
Trans AM STAT3 transcription factor assay kit	Active Motif, USA
VEGF ELISA kit	Biorbyt Ltd, USA

IL-6 ELISA kit	Biorbyt Ltd, USA
TNFα ELISA kit	Biorbyt Ltd, USA
cDNA synthesis kit	MBI Fermentas, USA
PCR amplification kit	MBI Fermentas, USA
DNA ladder 1KB	MBI FErmentas, USA
Protein ladder prestained	MBI Fermentas, USA
Phalloidin, FITC labelled	Sigma Aldrich, USA
Primers	Sigma Aldrich, USA
Protease Inhibitor Cocktail	Sigma Aldrich, USA
Protein ladder prestained	MBI Fermentas, USA
Ribonuclease A	MBI Fermentas, USA
Supersignal West Femto Detection kit	Thermo Scientific, USA

2.2.6 Solvents and acids

Name	Source
Acetic Acid	Qualigens, India
Dimethyl Sulphoxide	Qualigens, India
Ethyl Alcohol	S.D. Fine Ltd., India
Methanol	S.D. Fine Ltd., India
Sulphuric acid	Qualigens, India
37-41% Formaldehyde	Merck, India
2-Mercapto ethanol	Fluka AG, Germany
Hydrochloric Acid	Qualigens, India
Glycerol	Qualigens, India
Chloroform	Qualigens, India
Isopropanol	Sigma Aldrich, USA
Ethanol	Sigma Aldrich, USA

2.2.7 Instruments and machines

Name	Source
ELISA Plate Reader (Spectra Max 190)	Molecular Devices, USA
Centrifuge RC 5C plus	Sorvall, USA
Table Top Ultracentrifuge (TL100)	Beckman, USA
Spectrophotometer (UV 160A)	Shimadzu, Japan
Stereomicroscope	Zeiss , Germany
UV illuminator	UVP Bioimaging Sys., USA
Upright Light Microscope	Zeiss, Germany
Laser Confocal Microscope (510 Meta)	Zeiss, Germany
Rota 4R Centrifuge	Plastocrafts Ind. Pvt. Ltd.
Rocker	Neolab, India
Rotary Shaker	Remi Equipments, India
Transblot cell	Biorad, USA
Power pack Electragel100	Techno Source, India
Horizontal gel apparatus Monokin	Techno Source, India
Water bath	Neolab, India

2.2.8 Cell lines and animals

A375 human melanoma cell line	NCCS, Pune, India
A549 lung carcinoma cell line	NCCS, Pune, India
U87 glioblastoma cell line	NCCS, Pune, India
HEK-293 human embryonic kidney cell line	NCCS, Pune, India
HaCaT human keratinocytes cell line	NCCS, Pune, India
HT1080 human fibrosarcoma cell line	NCCS, Pune, India
NIH3T3 mouse fibroblast cell line	NCCS, Pune, India
Female NOD SCID mice	Animal house, ACTREC

2.3 METHODS

2.3.1 Maintenance of cell lines

Reagents

RPMI 1640 (Roswell Park Memorial Institute medium): 16.2 gm of media powder was dissolved in 1 litre of fresh D/W. 3.1 gm of NaHCO3 was added to the dissolved media. Media was sterilized by filtration through 0.2 micrometre filter and stored in aliquots at 4°C.

DMEM (Dulbecco's Modified Eagles medium): 13.5 gm of media powder was dissolved in 1 litre of fresh D/W. 3.7 gm of NaHCO3 was added to the dissolved media. Media was sterilized by filtration through 0.2 micrometre filter and stored in aliquots at 4°C.

FBS (Fetal Bovine Serum): Aliquoted and stored at -20°C.

Penicillin: A 5,00,000 unit vial was dissolved in 25ml of PBS and filter sterilized. Aliquots were made and kept at-20°C.

Streptomycin: 1gm of powder was dissolved in 32ml of PBS; filter sterilized and stored at 20°C in aliquots.

Phosphate buffered saline (PBS):

NaCl: 8.0 gm

KCl: 0.2 gm

Na_2HPO_4: 1.44 gm

KH_2PO_4: 0.24 gm

The salts were dissolved in D/W, pH was adjusted to 7.2 and the volume was made up to 1 litre. Sterilization was done by autoclaving.

Trypsin EDTA: 0.25% Trypsin and 0.2% EDTA in PBS; sterilized by filtration; stored at 4°C.

Saline EDTA: 0.02% EDTA in PBS; sterilized by filtration; stored at 4°C.

Trypan Blue: 0.4% trypan blue powder was dissolved in PBS; filtered using Whatman filter paper and kept at 4°C.

Protocol

A375 cell was maintained in RPMI-1640 supplemented with 4mM L-glutamine and 10% FBS while A549, U87, HEK-293, HaCaT and NIH3T3 were maintained in DMEM containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were grown in a humidified atmosphere of 5% CO2 and at a temperature of 37 ºC. Cells were passaged on reaching 80-100% confluency. Confluent monolayers were rinsed in PBS and harvested with Trypsin EDTA. Subsequently, to this dissociated cell suspension, complete media was added to inactivate trypsin. Cells were centrifuged at 2000 rpm for 5 minute and the pellet was resuspended in 1ml of complete medium. Cell viability was determined using trypan blue dye exclusion test. Cell suspension was diluted in trypan blue and counted in a haemocytometer. The viable cells excluded the dye while the dead cells retained it and appeared blue colored under the microscope. Cell count was determined using the formula:

No of cells /ml= no of cells x 10^4 x dilution factor.

It was ensured that the cells were at least 95% viable for all the *in vitro* and *in vivo* assays. Routine cryopreservation of the cell lines were done in which cells were harvested and to it chilled freezing mixture (RPMI 1640/DMEM with 30%FBS and 10% DMSO) was added drop wise. One million cells were added in each cryo vial and kept at -20°C for an hour. The vial was then transferred to -80°C, kept overnight and then stored in liquid nitrogen. The revival of the frozen cells was carried out by taking the cryo vial out of liquid nitrogen and immediately thawing it in a 37°C water bath. To this cell suspension, complete media was added to dilute the DMSO. Cells were

centrifuged and given a PBS wash. The cells were then seeded in a culture dish and grown to confluency.

2.3.2 MTT cytotoxicity assay

Reagents

Pentoxifylline: Trental vial containing 300 mg of Pentoxifylline in 15ml saline (73.86 mM).

MTT reagent: MTT powder was dissolved in sterile PBS at a concentration of 5mg/ml and stored at 4 °C.

DMSO (dimethyl sulphoxide).

Protocol

Cells were harvested in exponential phase and a cell suspension was made in complete medium at a concentration of $5x10^4$ cells /ml. From this cell suspension 100 µl was seeded in each well of 96 well flat bottom tissue culture plates except for blank. The cells were allowed to grow and stabilize for 24 h. Subsequently the media from the wells was removed and replaced with complete media containing the different concentration of the PTX (0 to 30 mM) and incubated for 2, 24, 48 and 72 h. Each treatment was performed in six wells replicates. Post incubation cells were washed twice with PBS and MTT was added at a concentration of 1mg/ml. After overnight incubation at 37 ºC, the plate was centrifuged at 1500 rpm for 20 minute. Medium from the plate was removed by gently tapping the plate against a pad of blotting paper. Formazon crystals were dissolved in 100 µl of DMSO. The optical density was measured in ELISA plate reader at 540 nm with a reference wavelength of 690 nm. Absorbance of blank was subtracted from each. Cell viability was plotted as percentage of untreated control and dose effect plot was generated. Inhibitory concentration 50

(IC50) of the PTX was determined from the dose effect curve as the drug concentration that decreased the cell viability to 50 %.

2.3.3 Colony formation assay

Reagents

1% Crystal violet stain.

Protocol

Briefly 500 cells/ml was seeded in 35 mm culture plate. After 24 h, cells were treated with different concentration of PTX (0 to 6 mM) for 24 h. Cells were washed with PBS and further incubated in 10% serum containing medium for 8-10 days till in the untreated control the colonies attain a size of more than 50 cells. The cells were fixed with 100% methanol and stained with crystal violet. Colonies of 50 or more cells were counted and photographs were taken.

2.3.4 Cell cycle analysis by Flow cytometry

Reagents

RNase: 10ug/ul solution

Propidium iodide: 1mg/ml solution in PBS

Protocol

Sub confluent A375 cells were treated with the sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h. Cells were harvested, washed twice with PBS and fixed in chilled 70% ethanol. The fixed cell pellet was washed with PBS and resuspended in 200µl of PBS. Cells were treated with10 µl RNase solution (0.5 mg/ml) for 15 minute at 37°C.The volume was made upto 500 µl and 50 µl propidium iodide (50µg/ml) was added to it. Cells were incubated for 10 minutes at room temperature. Ten thousand events were acquired on FACS Calibre and analyzed using Modfit software.

2.3.5 Ethidium bromide/acridine orange apoptosis staining assay

Reagents

Acridine orange 100 µg/ml and ethidium bromide 100 µg/ml in PBS.

Protocol

A375 cells were seeded in 96 well plates at a concentration of 1.6 $x10^4$ cells/well. After 24 h, cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h. Plates were later centrifuged at 1500 rpm for 15 minute and ethidium bromide/acridine orange (EB/AO) dye 1:1 mix (16 µl) was added to each well. Cells were viewed and counted using a Nikon eclipse TS100 inverted microscope at 20X magnification. Tests were done in triplicates, counting a minimum of 100 cells.

2.3.6 Adhesion assay

Reagents

Matrigel: 10µg/ml working solution made in PBS

Collagen type IV: 50 µg/ml working solution in PBS.

Blocking solution: RPMI 1640 containing 1% BSA.

Protocol

Ninety six well flat bottom plates were coated with ECM substrates: matrigel and collagen type IV (50µl/well). Plates were kept overnight at 4°C for polymerization. Unpolymerized substrates were washed with PBS and the plates were blocked with 1% BSA containing media for 2 h at 37°C. Sub confluent A375 cultures were treated with sub-toxic doses of PTX (0, 2 and 3 mM) for 24 h. The cells were harvested using saline EDTA, washed and diluted to a final concentration of $3x10^5$ cells /ml in medium containing 0.1% BSA. 100µl of the cell suspension was added to each substrate coated well and kept for incubation at 37°C for 15, 30, 45, 60 and 90 minute. Non adherent cells were removed by giving two washes with PBS. Wells containing unwashed cells

were kept as control. The adherent cells were quantified using MTT assay and expressed as relative % of the respective total unwashed cells (adherent as well as non adherent). Experiments were carried out in triplicates. All assays were performed in triplicate.

2.3.7 Gelatin zymography

Reagents

Non reducing lamelli buffer 4X without ß mercapto ethanol.

2% gelatin in sterile milli Q.

Developing and washing Buffer: 50 mM Tris chloride containing 100 mM $CaCl_2$, 1 uM $ZnCl_2$, 1% Triton X 100 and 0.02% NaN3, pH 7.5. Filter sterilized and store at 4 °C.

Staining solution: 0.5% coomassie brilliant blue R250 powder in a destaining solution.

Destaining solution: methanol, water and acetic acid mixture (45:45:10 v/v).

Other reagents include SDS-PAGE reagents.

Protocol

FBS-free culture supernatant of A375 cells, untreated or pretreated with sub-toxic doses of PTX (1, 2 and 3 mM) for 24 h were collected, normalised as per the cell number and concentrated using centricon YM-30 tubes. Equal volumes of samples were incubated in non-reducing lammeli buffer for 30 minute at room temperature and run on 10% SDS-PAGE containing 0.1% gelatin (w/v). 25 µg of HT1080 condition media was loaded as a positive control. The gel was then washed twice in developing buffer and further incubated in same buffer at 37 ºC for 48 h. Staining was done in coomassie brilliant blue R250 and destained in a solution of methanol, water and acetic acid mixture (45:45:10 v/v). Enzymatic activity of MMP2 (72 KDa) and MMP9 (92KDa) was visualized as clear zones on a blue background. Gel image was taken in the gel documentation machine and densitometric analysis of the band intensity was done.

2.3.8 Wound healing assay

Cells were seeded in 35 mm culture plate. Cells were then treated with mitomycin C at a concentration of 1 µg/ml for 2 h. Plates were washed twice with PBS and sub-toxic doses of PTX (0, 1, 2 and 3 mM) was added for 24 h. Post treatment wounds were made using a yellow tip. The peeled off cells were removed completely with thrice PBS washes. Initial wound widths (at the 0 time point) were measured using ocular grid. Cells were further incubated for 24 h in media for wound coverage. Post incubation, cells were fixed with methanol and the final wound width was measured. The migration observed was represented as % migration considering migration in untreated control as 100%. Images were taken using Axio Vision inverted microscope from Zeiss. Experiments were carried out in triplicates. Cell migration was calculated using the formula:

% Cell Migration = 100 X (0 time wound width- Final wound width)/(0 time wound width).

2.3.9 Phalloidin-FITC staining

Reagents

4% Paraformaldehyde (PFA): 4 gm PFA was dissolved in PBS and the temperature was brought to 37°C.

Phalloidin staining solution: A 50 µg/ml solution of phalloidin-FITC was made in 0.05% BSA.

Protocol

Cells were grown to a confluency of 60-70% on cover-slips and treated with sub-toxic doses of PTX (0, 1, 2 and 3mM) for 24 h. Cells were washed with PBS and fixed by incubating them for 10 minute at 37°C with freshly prepared 4% PFA. Cover-slips were washed with PBS and permeablized using 0.1% Triton X100 at room temperature for

10 minute. Coverslips were then overlaid with 30µl of Phalloidin mixture (50 µg/ml in 0.05% BSA) and incubated at 37°C for 10 minute. Coverslips were washed with PBS and were mounted on a clean glass slide in the mounting media and sealed with the help of nail polish. Images were acquired at 63X using the LSM 510 META laser scanning microscope from Carl Zeiss, Germany. Images were analyzed using the software LSM 510.

2.3.10 ELISA

Reagent

IL6, VEGF and TNFα ELISA kit.

Protocol

Cells were grown to a confluency of 60-70% and treated with PTX (0, 1, 2 and 3 mM) for 24 h. Cells were washed with PBS and serum free medium was added. After 48 h incubation, culture supernatant was collected. From culture supernatant ELISA was carried out using kit according to manufacturer's instruction. Hundred microliter of culture supernatant was added to the IL6, VEGF and TNFα coated 96 well plates for 90 minute and incubated at 37 °C. Primary antibody was added for 1 h at room temperature. Plate was given three washes with PBS and HRP-conjugated secondary antibody was added. After 1 h plate was washed thrice with PBS and developing solution was added. When dark blue color appeared in positive control well, stop solution was added and reading was taken using ELISA reader at a wavelength of 450 nm.

2.3.11 Semiquantitative RT-PCR

Sample preparation

Reagents

Trizol (TRI^{TM}) reagent

Chloroform

Isopropanol

75% ethanol

DEPC treated water: 1ml of DEPC was added to 1 litre of fresh D/W. It was mixed thoroughly for 30 minute, incubated overnight at room temperature and autoclaved the next day.

Protocol

Cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h. Cells were harvested, pelleted and TRI^{TM} reagent was added to the cell pellet. The cell pellet was passed through a pipette several times to form a homogenous lysate. The samples were allowed to stand for 5 minute on ice. Chloroform (200 µl) was added for every 1ml of TRI^{TM} reagent used. The sample was covered tightly, shaken vigorously for 15 second and was allowed to stand for 2-5 minute at room temperature. The resulting mixture was centrifuged at 12,000 rpm for 30 minute at 4°C. Centrifugation separated the mixture into 3 phases: a red organic phase, containing proteins, an interphase containing DNA and a colorless upper aqueous phase containing RNA. The aqueous phase was transferred to a fresh tube and an equal volume of isopropanol was added to it and mixed. The mixture was allowed to stand at -20°C for 2 h followed by centrifugation at 12,000 rpm for 30 minute at 4°C. The RNA precipitate formed a pellet at the bottom of the tube. The supernatant was removed and the RNA pellet was washed with 1 ml of 75% ethanol. The sample was centrifuged at 12,000 rpm for 20 minute at 4°C. The RNA pellet obtained after centrifugation was dried for 5-10 minute by air drying. The pellet was resuspended in nuclease free water. The purity and quantification of the RNA sample was performed using a nano-drop biophotometer.

1st strand cDNA synthesis

Reagents

First strand cDNA synthesis kit

DNaseI and DnaseI buffer

Nuclease free water

Dry Bath

Protocol

Total RNA was converted into cDNA using M-MuLV reverse transcriptase. To a nuclease free tube, the following were added:

RNA	2 µg
10X DNaseI reaction buffer	1 µl
DNaseI	1 µl
Ribonuclease inhibitor	1 µl
Nuclease free water	Up to 11 µl

This was incubated at 37°C for 30 minute. The reaction was terminated by adding 1 µl of EDTA and heating at 65°C. For synthesizing the cDNA the following were added to the same tube:

5X reaction buffer	4 µl
10mM dNTP mix	2 µl
Reverse Transcriptase	1 µl
Oligo dT	1 µl

The mixture was incubated at 42°C for 60 minute. The reaction was terminated by heating at 70°C for 5 minute.

Polymerase Chain Reaction (PCR)

Reagents

PCR kit

Nuclease free water

TE buffer: 10 mM Tris pH 8.0, 1 mM EDTA

Thermal cycler

Protocol

All primers were dissolved in TE buffer according to manufacturer's instructions. The PCR reaction mixture for 1 reaction (20 µl) was made as follows:

10X PCR buffer	2.0 µl
10mM dNTP mix	0.4 µl
Primer forward	0.08 µl
Primer reverse	0.08 µl
Taq DNA polymerase	0.1 µl
25mM MgCl2	0.4 µl
Template cDNA	0.8 µl
Nuclease free water	Up to 20 µl

To demonstrate the linearity of PCR conditions GAPDH (house keeping gene) transcripts were also amplified.

The PCR conditions for the thermal cycler were as follows:

1) Initial denaturing was carried out at 94°C for 3 minute.

2) Denaturing was carried out at 94°C for 35 second.

3) The annealing temperature for MMP2, MMP9 and VEGF primers were 66.8°C, 53.2°C and 55.3°C respectively.

4) Annealing time was 35 second.

5) Extension was carried out at 72°C for 35 second.

6) Final extention was carried out at 72°C for 10 minute.

Step 2-5 was repeated for 35 cycles

The primer sequences were as follows:

MMP2:

Forward, 5’ -AGGGCGCTCTGTCTCCTGGG - 3’

Reverse, 5’ - CCCACTTGGGCTTGCGAGGG - 3’

MMP9:

Forward, 5’-TGCTCCTGGTGCTGGGCTG - 3’

Reverse, 5’-GCCAGCTGCCTGTCGGTGAG – 3’

VEGF

Forward, 5’-CTACCTCCACCATGCCAAGT-3’

Reverse, 5’-GCAGTAGCTGCGCTGATAGA-3’

GAPDH:

Forward, 5’-GAGTCAACGGATTTGGTCGT - 3’

Reverse, 5’ -TGTGGTCATGAGTCCTTCCA - 3’

2.3.12 Agarose Gel Electrophoresis

Reagents

1 % Agarose: 1 gm agarose was melted at 100°C in 100 ml TBE buffer

TBE buffer

Tris Base	121gm
Boric acid	61.7 gm
EDTA	7.44gm

Dissolved in 1 litre autoclaved distilled water.

Loading dye (MBI Fermentas)

Protocol

Agarose was melted to a concentration of 1% in TBE buffer and EtBr (1mg/ml) was added to the melted agarose. The gel was poured into the apparatus and cooled. 10 µl of PCR product was mixed along with 2 µl loading dye and loaded onto the agarose gel along with molecular weight markers. The gel was run at a constant voltage of 80 Volts. The DNA bands in resolved gel were visualized and documented on a UVP gel doc system.

2.3.13 Preparation of total cell lysate and protein estimation

Reagents

Lysis Buffer: Triton X-100 (1%), NaCl (150 mM), EDTA (5mM), EGTA (2 mM), Sodium

fluoride (50mM), Sodium orthovanadate (1mM) and protease inhibitor cocktail

(1µl/100µl)

Protocol

Cells were harvested using Trypsin EDTA and washed with PBS. The cells were pelleted and incubated on ice for 40 minute in the presence of lysis buffer. Lysate was clarified by centrifugaton at 10,000 rpm for 20 minute and stored at -80ºC until further use.

Protein Estimation

Reagents

0.5 mg/ml Bovine serum albumin (BSA) in D/W, Bradford‘s reagent

Protocol

In a 96 well plate 1.25, 2.5, 3.75 and 5µg of BSA i.e. 2.5µl, 5µl, 7.5µl and 10µlof 0.5mg/ml of BSA was taken. Volume was made up to 10µl with distilled water. 10µl of sample was taken in the rest of the wells. Water was kept as blank. To it 100µl of

Bradford's reagent was added. Absorbance was taken at 595 nM using SoftMax Pro software. The estimation was done in triplicates. Sample concentration was automatically given by the software, using the BSA standard curve.

Sample Buffer (6X)

Reagents

Glycerol	6ml
1M Tris pH6.8	3ml
SDS	1.2 gm
β ME (2-mercaptoethanol)	600 µl
Bromophenol blue 0.05%	0.005 gm

Protocol

50µg of cell lysate was mixed with 6X sample buffer in a ratio of 5:1. The volume of the samples was normalized with D/W and the samples were boiled for 5 minute in a boiling water bath for denaturation and then resolved on SDS PAGE.

2.3.14 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Reagents

30% Acrylamide mixture (29.2% Acrylamide + 0.8% Bisacrylamide) solution was prepared in D/W, filtered through Whatmann filter # 3 and stored at 4ºC in an amber coloured bottle.

1.5 M TrisHCl pH8.8 (resolving buffer).

1 M TrisHCl pH6.8 (stacking buffer).

10% SDS solution: 10gm of SDS was added to 100ml of water, heated to 70ºC for SDS to dissolve completely.

10% Ammonium persulphate (APS) solution.

N,N,N',N'-Tetramethylethylenediamine (TEMED).

Pre stained molecular protein marker.

Tank Buffer: 196 mM Glycine , 50 mM Tris HCl and 0.1%SDS. pH adjusted to 8.3

Agarose 1%

Mini gel dual assembly

Power Pack

8% resolving gel recipe 30 ml volume (for 2 mini gels):

D/W	13.9 ml
Acrylamide mixture	8 ml
1.5M Tris pH8.8	7.5 ml
10% SDS	300 µl
10% APS	300 µl
TEMED	18 µl

Stacking gel recipe 10 ml volume (for 2 mini gels):

D/W	6.8ml
Acrylamide mixture	1.7 ml
1M Tris pH6.8	1.25 ml
10% SDS	100 µl
10% APS	100 µl
TEMED	10 µl

Protocol

The glass plates were wiped with 70% alcohol and assembled in running gel apparatus. The bottom and the sides of the glass plates were sealed using 1% molten agarose. When solidified the resolving gel mixture was poured gently leaving some space for the stacking gel. The gel mixture was overlaid with methanol to give a uniform gel front and kept for polymerization for 20-30 minute. When polymerization was over the

methanol and the unpolymerised gel mixture was removed, washed with D/W. The stacking gel mixture was poured on top of the resolving gel and a comb was inserted. Stacking gel was allowed to polymerize, following which the wells were washed with D/W and marked. The assembly cathodic and anodic chamber was filled with tank buffer and the wells were loaded with the denatured protein sample. A molecular weight marker was loaded in one well. Electrophoresis was carried out at 25mA for the stacking and then the further run was carried out at 30mA. Run was stopped when the dye front reached 1mm above the gel end.

2.3.15 Protein transfer and western blotting

Protein transfer

Reagents

Wet Transfer buffer: 25 mM Tris, 192 mM glycine, 30% v/v methanol, pH8.3.

Ponceau S solution: 0.1% Poncue powder in 5% acetic acid.

Polyvinylidene difluoride (PVDF) membrane

Protocol

The PVDF membrane was cut according to the size of the gel and pre-wetted with methanol for 5 minute before equilibration. The resolved gel and the membrane both were equilibrated with the transfer buffer for 20 minute. Four Whatmann filter #3 sheets were cut according to the size of membrane. After equilibration, the membrane was kept over the gel and this was sandwiched between the folds of filter paper, two on either side. Care was taken to avoid any bubbles being trapped. The sandwich was then kept between the electrode plates of the transfer assembly (gel towards the cathode). The transfer assembly was filled with the transfer buffer. Transfer was carried out for 16 h at constant 10 V or for 1 h at 100 V in a cold room. The membrane was stained

with Ponceau solution till the pink bands appeared. The side containing the bands were marked and then destained by rinsing in D/W.

Western Blotting

Reagents

TBS: 50 mM Tris pH 7.5, 0.85% NaCl; pH was adjusted to 7.6; solution was stored at 4°C.

TBST: To 1 litre of TBS 1ml of Tween 20 was added, stirred well and used.

Blocking solution: 5% non-fat skimmed milk powder in TBST; kept at 37°C for 15 minute to dissolve completely.

Primary and secondary antibody dilutions: The primary antibodies were diluted in 2.5% non-fat skimmed milk or 5% BSA in TBST. The secondary antibodies were diluted in 2.5% non-fat skimmed milk in TBST.

Super signal west femto kit.

Developing cassette.

Plastic boxes.

X-ray films.

Protocol

The membrane was blocked with the blocking solution for 2 h. The blots were then washed vigorously with TBST for 5 minute. The blot was taken in a plastic bag, overlaid with primary antibody and kept on a rocker for the mentioned time. After incubation, blots were washed with TBST three times, for 10 minute each. Blots were then incubated for 1 h with HRP conjugated secondary antibody. The nonspecific binding was removed by three TBST washes, 15 minute each. The table below provides the details of the western blotting carried out.

Protein	Molecular weight	Blocking	Primary antibody concentration	Primary antibody incubation	Secondary antibody concentration
STAT3	91 Kd	Overnight	1:500	1 h	1:4000
pSTAT3	91 Kd	2 h	1:2000	Overnight	1:4000
JAK1	130 Kd	2 h	1:1000	Overnight	1:4000
pJAK1	130 Kd	2 h	1:1500	Overnight	1:4000
JAK2	125 Kd	2 h	1:500	1 h	1:4000
pJAK2	125 Kd	2 h	1:1500	Overnight	1:4000
Src	60 Kd	2 h	1:2000	Overnight	1:4000
pSrc	60 Kd	2 h	1:2000	Overnight	1:4000
SHP2	72 Kd	2 h	1:1000	Overnight	1:4000
pSHP2	72 Kd	2 h	1:1000	Overnight	1:4000
VEGF	42 Kd	2 h	1:500	1 h	1:4000
VEGFR2	150 Kd	2 h	1:1000	Overnight	1:4000
HIF-1α	132 Kd	2 h	1:500	1 h	1:4000
P38 MAPK	38 Kd	2 h	1:1500	Overnight	1:4000
pP38 MAPK	38 Kd	2 h	1:1500	Overnight	1:4000
P44/42 MAPK	44/42 Kd	2 h	1:2000	Overnight	1:4000
pP44/42MAPK	44/442 Kd	2 h	1:2000	Overnight	1:4000
SAPK/JNK	46, 54 Kd	2 h	1:1500	Overnight	1:4000
Cyclin D1	36 Kd	2 h	1:1000	Overnight	1:4000
cMyc	65 Kd	2 h	1:2000	Overnight	1:4000
CDK4	30 Kd	2 h	1:1000	Overnight	1:4000
CDK6	36 Kd	2 h	1:1000	Overnight	1:4000

BclXl	30 Kd	2 h	1:1000	Overnight	1:4000
Caspase 3	35 Kd	2 h	1:1500	Overnight	1:4000
Cleaved Caspase3	19 Kd	2 h	1:1000	Overnight	1:4000
Caspase 9	47 Kd	2 h	1:1000	Overnight	1:4000
Cleaved Caspase 9	37 Kd	2 h	1:1000	Overnight	1:4000
STAT1	91 Kd	2 h	1:1500	Overnight	1:4000
pSTAT1	91 Kd	2 h	1:1000	Overnight	1:4000
PI3K	102 Kd	2 h	1:500	1 h	1:4000
Akt	60 Kd	2 h	1:1000	1 h	1:4000
pAkt	60 Kd	2 h	1:1500	Overnight	1:4000
MMP2	72 Kd	2 h	1:500	1 h	1:4000
MMP9	92 Kd	2 h	1:500	1 h	1:4000
β Tubulin	50 Kd	1 h	1:3000	1 h	1:4000

The blots were then taken to the dark room for visualization of the signal. Super signal west femto developing reagent was made as per the manufacturer's instructions. The blot was kept on a clean glass plate and on the probed side super signal west femto mixture was added and incubated for 5 minute. After incubation excess reagent was drained on the filter paper and the blot was kept in the developing cassette in between two transparent plastic sheets. On to the protein side X-ray sheet was kept and exposed for various times depending upon the signal intensity. The exposed films were developed in the developing machine. Equal loading was checked by studying ß-tubulin expression.

Statistical analysis

All of the experiments were replicated thrice. Statistical analysis was done with the help of the software SPSS 15.0. Data are expressed as means ± SEM. The significance of the differences between the treatments and the corresponding controls was analyzed by one way ANOVA. A probability value of $p < 0.05$ was considered to be statistically significant.

2.4 RESULTS

2.4.1 *In vitro* cytotoxicity and inhibition in proliferation by PTX

To evaluate the effect of PTX on the growth kinetics, A375 cells were treated with different concentrations of PTX in the range of 0 to 30 mM and were grown for 2, 24, 48 and 72 h. It was observed that PTX had dose and time dependent toxicity against A375 cells **(Figure 2.1)**. Hundred percent toxicity was observed at 30 mM when treated for 48 and 72 h. IC 50 value calculated from the dose effect curve was found out to be 17, 7, 2.3 and 1.8 mM when exposed to drug for 2, 24, 48 and 72 h respectively. This inhibition in cellular growth was further confirmed by colony formation assay. A concentration dependent decreased in the colony forming units was observed with PTX treatment **(Figure 2.2a and 2.2b)**. Interestingly, at sub-toxic doses of 1, 2 and 3 mM of PTX 7.8%, 25% and 45% inhibition in colony forming units were observed demonstrating antiproliferative effects of PTX at these sub-toxic doses. In contraposition, PTX at same sub-toxic doses (1, 2 and 3 mM) had minimal effects on the viability of immortalized non-tumorigenic cell line HaCaT (human keratinocyte) and NIH3T3 (mouse fibroblast) **(Table 2.1)**. Thus in the following experiments, the sub-toxic doses of 1, 2 and 3 mM with 24 h exposure of PTX applied in all subsequent experiments unless indicated otherwise. Further, we have showed that treatment with PTX also inhibited cellular growth and proliferation of A549 lung carcinoma and U87 glioblastoma **(Figure 2.3)**.

2.4.2 PTX inhibits cell cycle progression and induces apoptosis.

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 profile or induction of apoptosis. A375 cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h, and then underwent propidium iodide

staining/FACS analysis. It was observed that PTX inhibited cell cycle progression from G1 to S phase **(Figure 2.4)**. The maximum inhibition was 12.2% at a concentration of 2 mM. Also, at 3 mM PTX there was 18.68% increased in sub-G1 fraction, suggesting drug-induced apoptosis. Induction of apoptosis by PTX was further confirmed by ethidium bromide/acridine orange staining assay. It was observed that PTX significantly induced apoptosis in a dose dependent manner as compared to untreated control **(Figure 2.5)**. At a concentration of 2 and 3 mM PTX, 15% and 22% cells were undergoing apoptosis respectively.

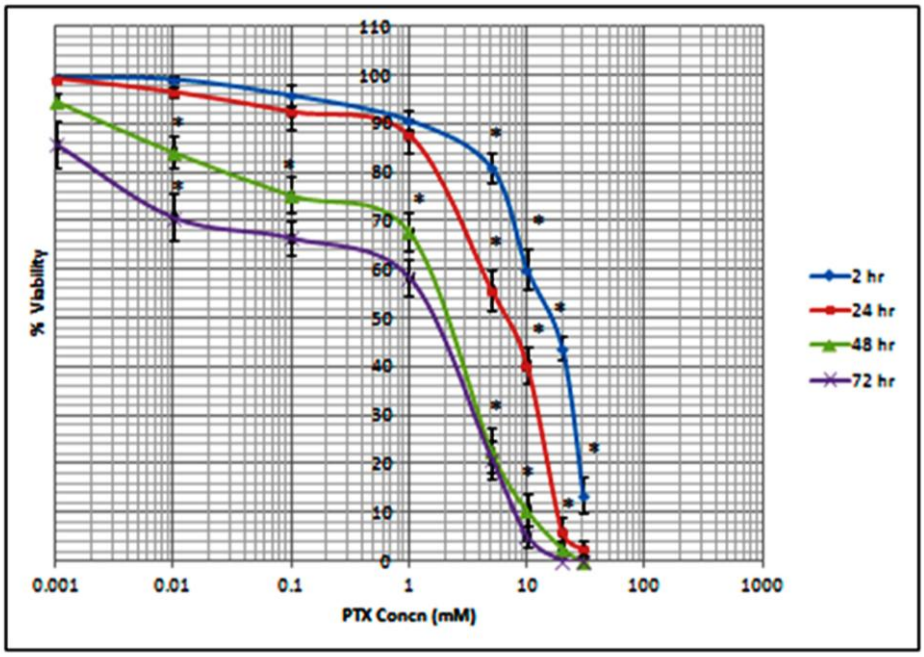
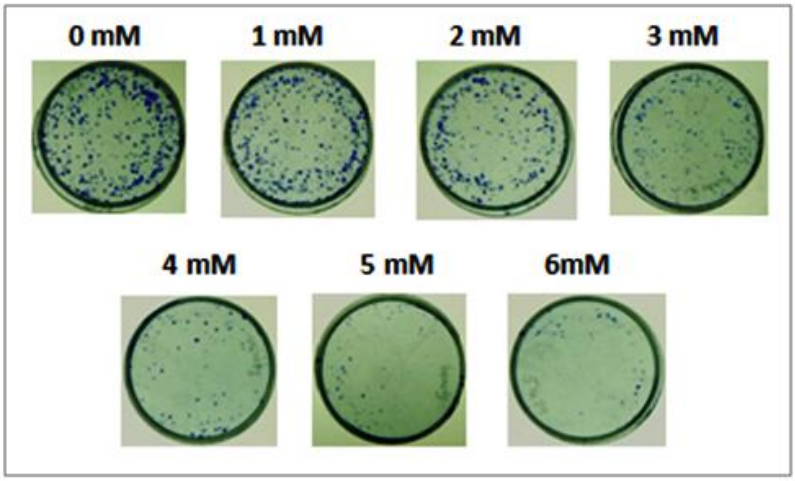


Figure 2.1: Effect of PTX on the viability of A375 cells. *A375 cells were treated with various concentrations of PTX (0-30 mM) for 2, 24, 48 and 72 h respectively. The numbers of viable cells were determined using MTT assay. There was a dose and time dependent inhibition in the percent viability of cells on PTX treatment. Values are expressed as mean ± SE of three independent experiments. *p < 0.05 compared with untreated control.*

a.



b.

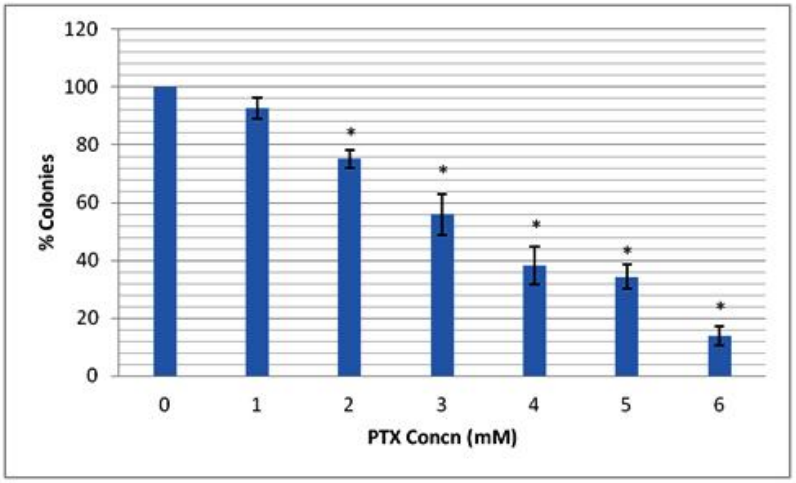


Figure 2.2: PTX inhibits cellular proliferation of A375 cells. *A375 cells were treated with various concentrations of PTX (0-6 mM) for 24 h and allowed to grow for 10 days. The colonies obtained were stained with crystal violet and counted.* ***(a)*** *Representative image of the plates.* ***(b)*** *Quantitative representation. Values are expressed as mean ± SE of three independent experiments. *p < 0.05 compared with untreated control.*

Cell line percentage of cellular viability ± SE		
PTX dose	**HaCaT (% viability)**	**NIH3T3 (% viability)**
1 mM	89.37±4.57	90.28±1.78
2 mM	88.0±5.68	82.0±1.15
3 mM	87.33±6.17	77.35±2.4

Table 2.1: Effect of PTX on viability of normal cells. *Culture cells were treated with PTX for 24 h and cells viability was determined using MTT assay. The results are reported in percentage of cell viability as compared to untreated control groups respectively considered as 100%. Values are expressed as mean ± SE of three independent experiments.*

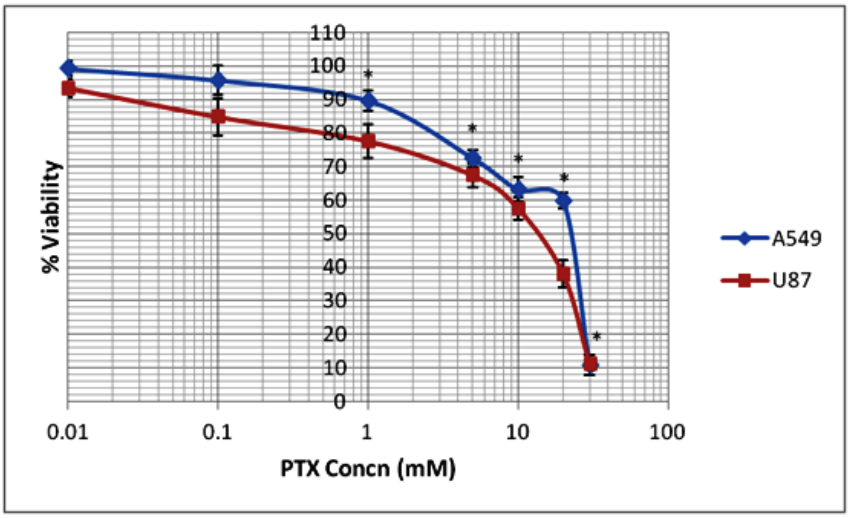


Figure 2.3: PTX inhibits viability of A549 lung carcinoma and U87 glioblastoma. *A549 and U87 cells were treated with various concentrations of PTX (0-30 mM) for 24 h. The numbers of viable cells were determined using MTT assay. There was a dose dependent inhibition in the percent viability of cells on PTX treatment. Values are expressed as mean ± SE of three independent experiments. *p < 0.05 compared with untreated control.*

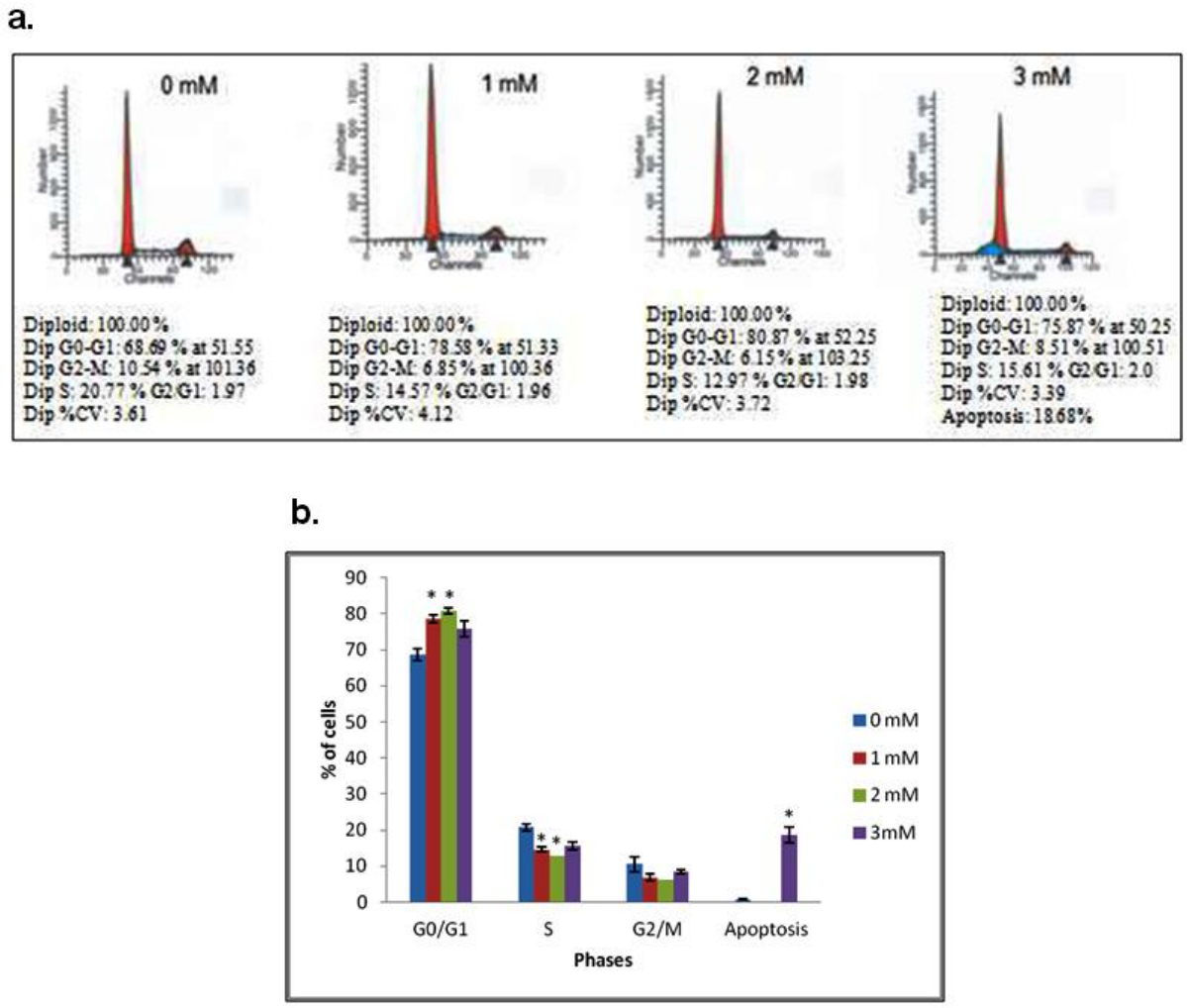
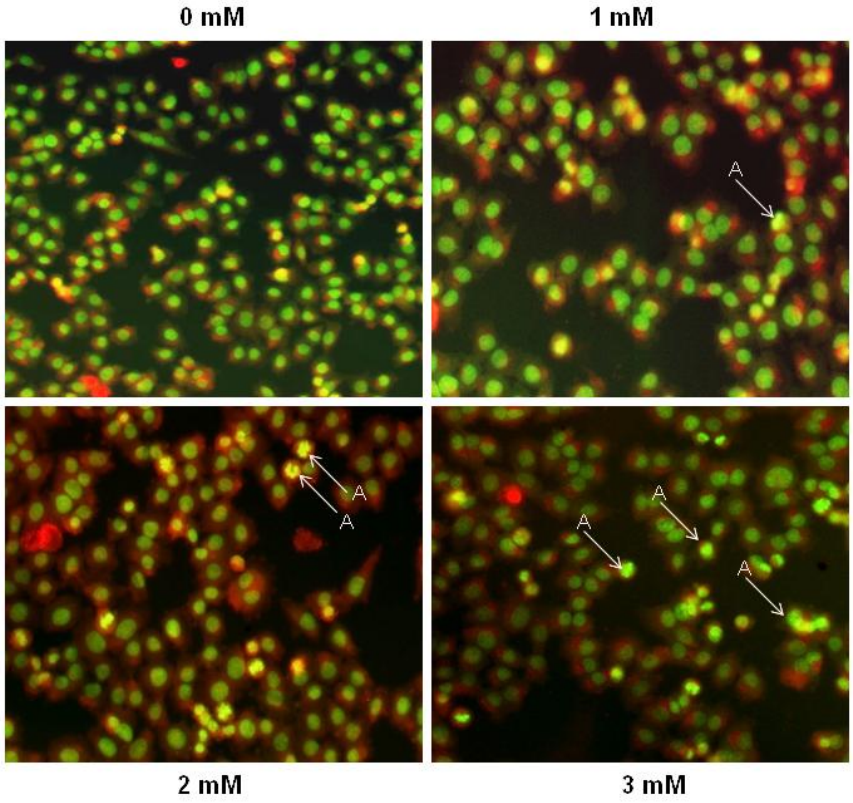


Figure 2.4: PTX induces G0/G1 cell cycle arrest, S phase depletion and apoptosis in A375 cells. *A375 cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h, stained with propidium iodide and analyzed using flow cytometry.* ***(a)*** *A significant G0/G1 phase arrest and reduction in S phase cells was observed. Additionally, an increased in sub G1 population was observed at 3 mM PTX concentration suggesting the induction of apoptosis.* ***(b)*** *Quantitative representation of which is given in the graph. *p < 0.05 compared with untreated control.*

a.



b.

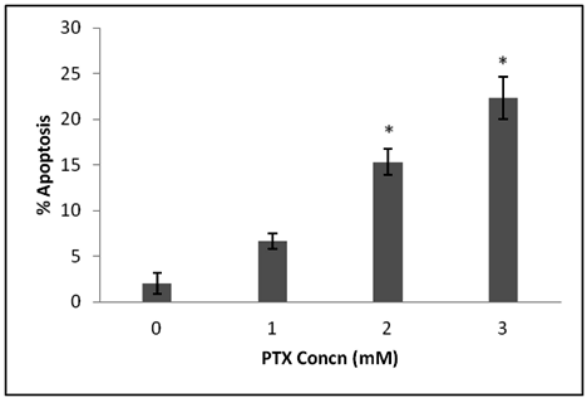


Figure 2.5: PTX induces apoptosis. *A375 cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h. Cells were then stained with ethidium bromide/acridine orange for apoptosis measurement.* ***(a)*** *Images of the cells. Arrow next to "A" indicates apoptotic cells.* ***(b)*** *Quantitative representation of which is given in the graph. Values are expressed as mean ± SE of three independent experiments.*p < 0.05 compared with untreated control.*

2.4.3 PTX inhibits A375 cells adhesion to matrigel and type IV collagen.

The adhesion of tumor cells and interaction with different components of the ECM is a prerequisite for cellular migration and invasion into the basement membrane. We investigated the effect of PTX on the adhesion of A375 cells to matrigel and type IV collagen. It was observer that pre-treatment of A375 cells with 3 mM PTX showed significant inhibition to matrigel (21%, 25.29%, 16.51%, 22.48% and 21.09% at 15, 30, 45, 60 and 90 min incubation respectively) and type IV collagen (38.21%, 19.66%, 15.27%, 14.5% and 15.39 % at 15, 30, 45, 60 and 90 min incubation respectively) as compared to untreated control **(Figure 2.6)**.

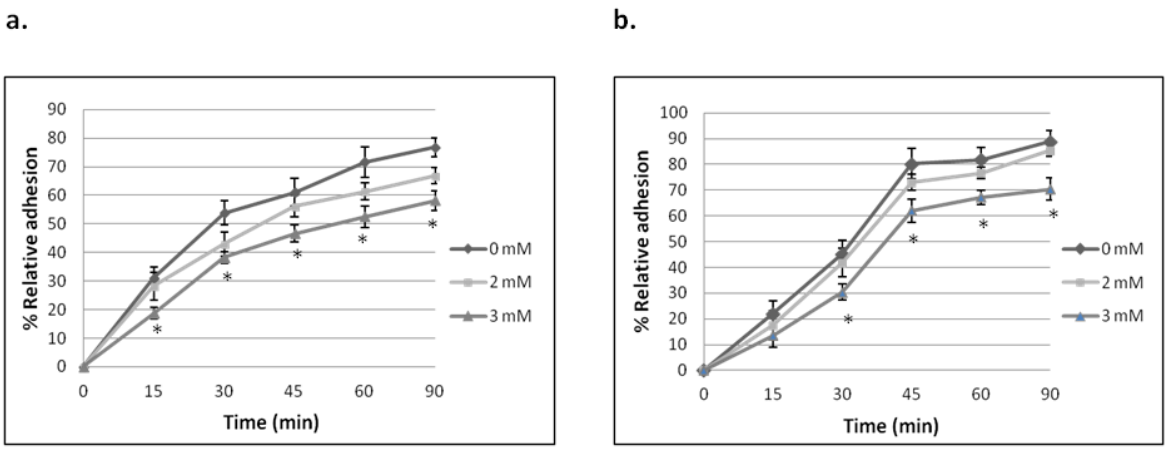


Figure 2.6: PTX inhibits A375 cell adhesion to matrigel and type IV collagen. (a) *Untreated and PTX treated (24 h: 2 and 3 mM) A375 cells were allowed to adhere to plates coated with matrigel (10 µg/ml) for 15, 30, 45, 60 and 90 minute.* ***(b)*** *Untreated and PTX treated (24 h: 2 and 3 mM) were allowed to adhere to plates coated with type IV collagen (50 µg/ml) for 15, 30, 45, 60 and 90 minute. Cell adhesion was then assayed using MTT. The graph represents percentage of adherent cells in comparison to control (untreated cells) taken as 100%. Values are representative of three independent experiments ± SE. *p < 0.05 compared with untreated control.*

2.4.4 Effect of PTX on MMP2 and MMP9 expression and activity

Matrix metalloproteinases (MMPs) are the enzymes critical for the process of invasion, migration and metastasis (28). To determine whether PTX has effect on these MMPs i.e. MMP2 and MMP9, we examined the expression of MMP2 and MMP9 mRNAs by RT-PCR. It was observed that PTX treatment has no effect on MMP2 and MMP9 mRNAs **(Figure 2.7a)**. Next, gelatin zymography was carried out to study the effects of PTX on MMP2 and MMP9 activity. It was observed that the activity of MMP2 and MMP9 in concentrated serum-free media of A375 cells was decreased by PTX treatment in a dose dependent manner **(Figure 2.7b)**. Densitometry analysis of the gel showed a significant inhibition in the enzymatic activity of MMP2 (25.27% and 56% inhibition at 2 and 3 mM respectively) and MMP 9 (21.8% and 50.19% inhibition at 2 and 3 mM respectively) **(Figure 2.7c)**. The results of zymography were confirmed by western blot analysis. Data suggested that PTX inhibited MMP2 expression in a dose dependent manner. However, level of MMP9 expression remained unchanged **(Figure 2.8)**.

2.4.5 PTX inhibits TNFα secretion

TNFα is a vital cytokine involved in inflammation, immunity, and cellular organisation. Expression studies have confirmed abnormally high concentrations of TNFα in tumors, implicating the cytokine in specific preneoplastic lesions and in advanced malignant disease (111). Several studies suggest that TNFα promotes cancer growth, invasion, and metastasis. A375 cells treated with PTX showed decrease in TNFα secretion in culture supernatant as compare to untreated cells **(Figure 2.9)**.

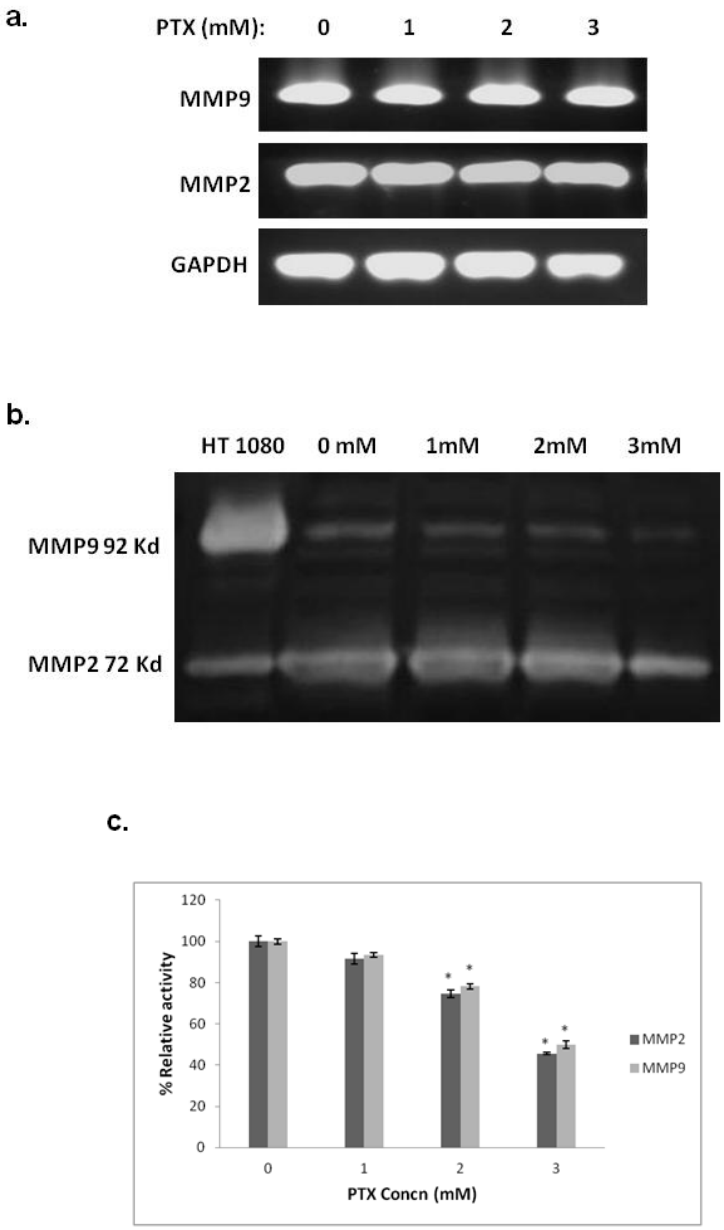


Figure 2.7: Effects of PTX on MMP2 and MMP9 mRNA expression and activity. *A375 cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h.* ***(a)*** *MMP2 and MMP9 mRNA expression was checked using RT-PCR.* ***(b)*** *The activity of MMP2 and MMP9 in conditioned medium was checked using gelatin zymography. HT1080 condition medium was used as a positive control.* ***(c)*** *MMP2 and MMP9 activity were quantified by densitometric analysis of gel. The densitometric data were expressed as mean ± SE of three independent experiments. *p < 0.05 compared with the untreated control.*

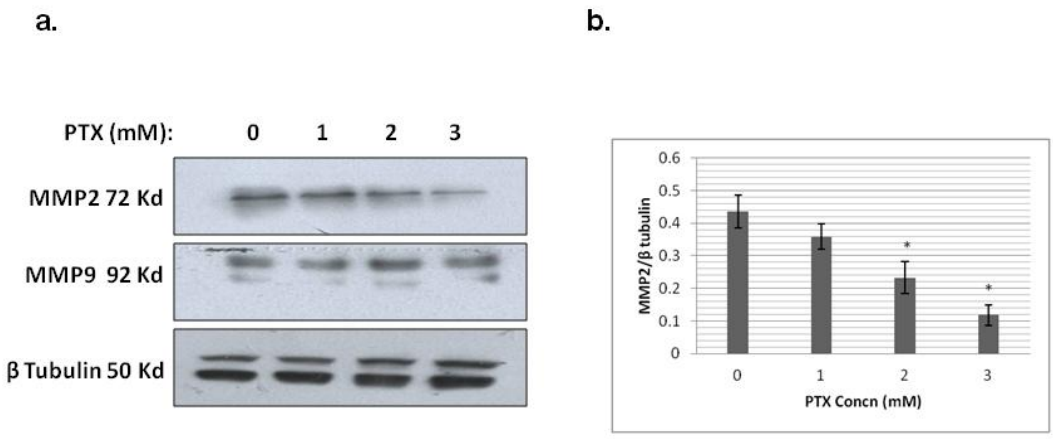


Figure 2.8: Effects of PTX on MMP2 and MMP9 expression. *A375 cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h.* ***(a)*** *Whole cell extracts were prepared and subjected to western blotting to determine the level of MMP2 and MMP9. β tubulin was used as loading control.* ***(b)*** *Densitomeric analysis of gel. The densitomeric data were expressed as mean ± SE of three independent experiments. *p < 0.05 compared with the untreated control.*

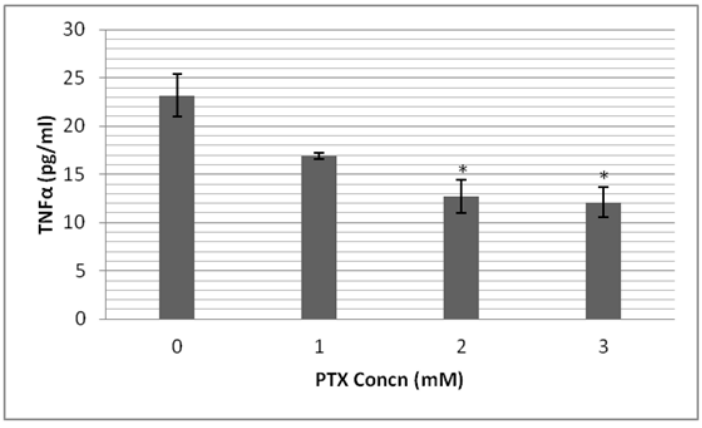
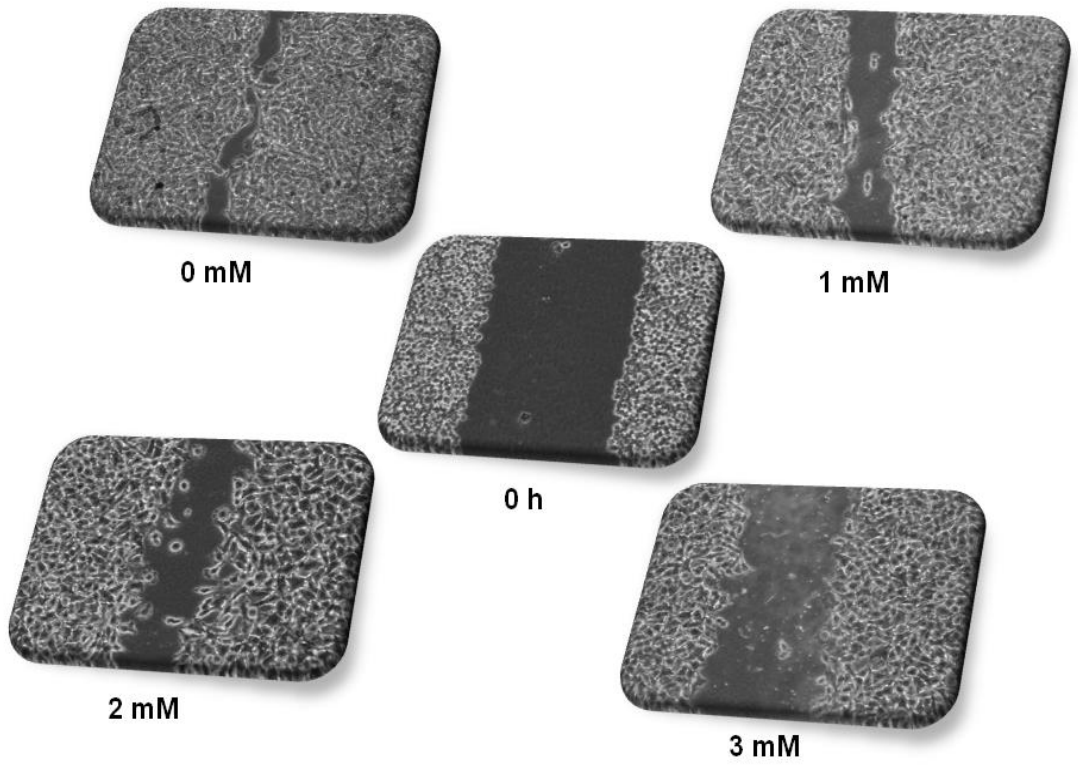


Figure 2.9: PTX inhibits TNFα secretion. *A375 cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h and TNFα secretion was measured by ELISA in culture supernatant after 24 h. Values are representative of two independent experiments ± SE. *p < 0.05 compared with untreated control.*

2.4.6 PTX inhibits migration of the A375 cells

Cellular migration is a critical process of invasion allowing primary tumors to metastasize. To evaluate the effect of PTX on cellular migration, wound healing assay was carried out. The wound healing capacity of cells was measured against a reference plate fixed at zero time after making a wound. It was observed that PTX inhibited cellular migration in a dose dependent manner **(Figure 2.10a)**. This inhibition in cellular migration was found to be 19%, 43% and 62% at 1, 2 and 3 mM respectively **(Figure 2.10b)**.

a.



b.

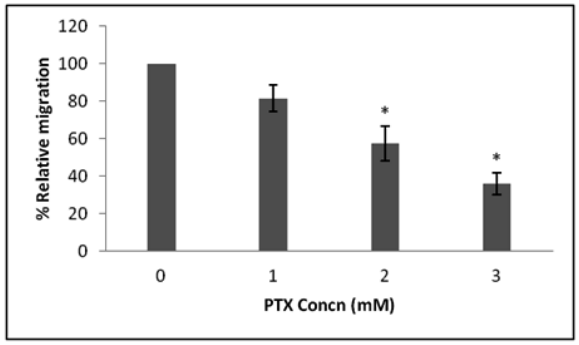


Figure 2.10: PTX inhibits A375 cells migration. *A375 cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h. Wound coverage was observed in PTX pretreated cells. Initial wound widths were measured and a representative picture is shown as '0 time point'.* ***(a)*** *Wound coverage in untreated control and PTX treated cells are shown.* ***(b)*** *Quantitative representation of which is given in the graph. Values are representative of three independent experiments ± SE. *p < 0.05 compared with untreated control.*

2.4.7 PTX disrupts actin organization of the A375 cells

Reorganization/polymerization of actin cytoskeleton plays an important role in cell migration. As PTX inhibited cellular migration, we hypothesized that disruption of F-actin may account for this phenomenon. To evaluate this, cells were treated with sub-toxic doses of PTX for 24 h and F-actin staining was carried out. It was observed that, in PTX treated cells there was huge loss of actin stress fibers as compare to control cells **(Figure 2.11)**.

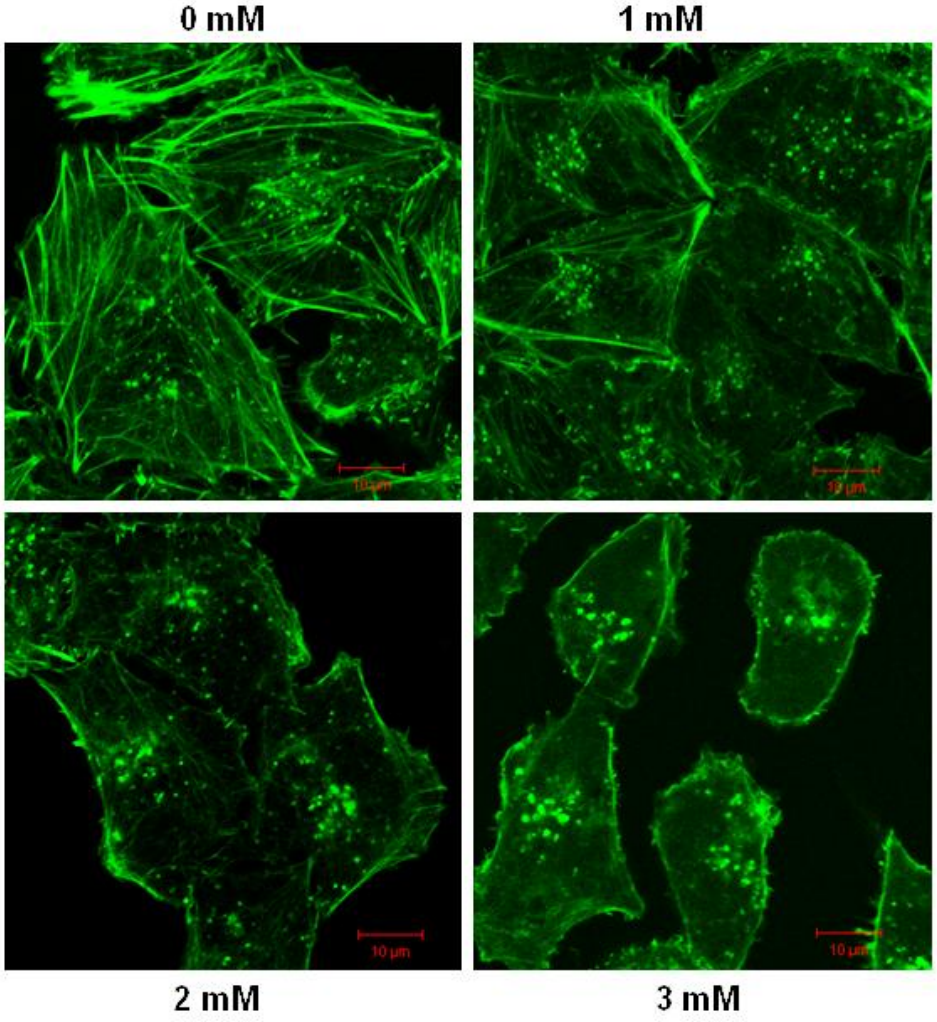


Figure 2.11: PTX disrupts actin organization of A375 cells. *A375 cells were treated with sub-toxic doses of PTX for 24 h and then stained with Phalloidin-FITC (an actin staining agent). Original magnification X63 with 2X zoom.*

2.5 DISCUSSION

Discussion

Metastasis, rather than the primary tumor is the major cause of mortality in majority of cancer patients. It is a complex multistep process involving cellular proliferation, invasion into surrounding tissues, migration, adhesion and angiogenesis. So, any of the steps such as cellular proliferation, invasion or migration if prevented by small molecule or drug then this molecule would be clinically more promising and hopefully reduce cancer mortality.

As, the high degree of cytotoxicity is one of the most common causes of failure of a number of potential drugs, a very viable and promising approach is to investigate antimetastatic activity of the drugs that had already been demonstrated to treat other diseases. This facilitates an easy translation of the compound in a clinical set up as the pharmacokinetics and side effects of the drug are known. PTX, a phosphodiesterase inhibitor has been widely used in dilated cardiomyopathy and nephropathy (76). In the present study we have identified the process or processes, which are being affected by PTX that imparts antimetastatic characteristic to this drug, using A375 human melanoma model.

PTX had dose and time dependent toxicity against A375 cells. IC50 value calculated from dose effect curve was found to be 7 mM when exposed to drug for 24 h. Further, PTX inhibited cellular proliferation in a dose dependent manner. Interestingly, at sub-toxic doses of PTX (1, 2 and 3 mM), 7.8%, 25% and 45% decreased in colony forming units respectively was observed confirming its antiproliferative effects of PTX at these sub-toxic doses. In addition, the same sub-toxic doses of PTX (1, 2 and 3 mM) had minimal effect on viability of immortalized non-tumorigenic cell line HaCaT (human keratinocyte) and NIH3T3 (mouse fibroblast). Thus, PTX at sub-toxic doses could be

proposed to be a promising candidate for restricting the growth and proliferation of human melanoma cells. Anti-proliferative and growth inhibitory activity of PTX was not only restricted to melanoma cells. It also inhibited cellular growth and proliferation of A549 lung carcinoma, U87 glioblastoma, MDA-MB 231 breast carcinoma and cervical cancer cell lines HeLa and SiHa (94, 110).

The cell cycle profile of A375 cells treated with PTX showed inhibition in G1/S phase transition and induction of apoptosis. This observation accounts for the antiproliferative effects of PTX. Induction of apoptosis by PTX was further confirmed by ethidium bromide/acridine orange staining assay. Significant percentage of cells was observed to undergo apoptosis upon PTX treatment. This result is consistent with an earlier study showing that PTX induces apoptosis in MDA-MB 231, Hut-78 and MyLa cells (110, 112).

To be invasive, tumor cells require an optimum level of cellular adhesion to ECM. Once malignant cells are detached from the primary tumor, they bombard the surrounding basement membrane and adhere to its meshwork of ECM substrate. Any alteration of this property would interrupt the metastatic cascade. A significant reduction in the attachment of PTX treated A375 cells to the ECM substrate collagen type IV and matrigel was observed. Integrin may play a critical role in the attachment of tumor cells to the ECM (22). A separate study was being done by our group focuses MDA-MB 231 cell adhesion and effect of PTX on it. It was observed that treatment with PTX significantly inhibited integrin α5 and β1 expression (113).

Degradation of basement membranes and extracellular matrix is a key step in tumor cell migration, invasion and metastasis. Tumor cells regulate indiscriminate degradation of matrix by secreting the MMPs in zymogenic form. In cultured human melanoma cells

elevated expression of MMP1, MMP2 and MMP9 has been shown to be correlated with migration and invasion (114). In human melanocytic lesions a positive correlation between tumor progression and MMP2 expression level was demonstrated (115). Increased expression of MMP9, on the other hand, was found mainly in radial growth phase of primary melanoma, indicating that MMP9 expression correlates with early invasion of melanoma (116). Thus, the inhibition of MMPs activity may be crucial for the inhibition of cancer metastasis. Our data revealed that activities of MMP2 and MMP9 in condition medium of A375 cells were decreased by PTX treatment in a dose dependent manner. However, the levels MMP2 and MMP9 mRNAs were remained unchanged. Western blotting of MMP2 and MMP9 proteins from whole cell lysate obtained from pretreated cells showed that PTX inhibited MMP2 expression in a dose dependent manner without altering MMP9 expression. This discrepancy in result may be attributed to the differential regulation of expression and activity of MMP2 and MMP9. TNFα greatly induced the MMP9 activity in human melanoma cell lines (117) and we have found that PTX inhibited TNFα production in A375 cells. PTX have also been shown to inhibit TNFα induced MMP9 secretion in HL60 leukemia cell line (118). Thus, we suggest that the PTX induced inhibition in MMP9 activity in A375 cell line is through decreased expression of TNFα. The mechanisms accounting for the upregulation of MMP2 activity in metastatic cells are largely unknown. Increasing evidence suggests that constitutively activated STAT3 protein in melanoma directly binds to the promoter of MMP2 gene and upregulates its expression (119). We have demonstrated that PTX significantly inhibited the expression of activated STAT3 in A375 melanoma cell line (next section). Thus, PTX mediated inhibition in MMP2 can be attributed at least in part through its inhibitory effects on STAT3 signaling pathway. Taken together, our finding implies that the antimetastatic effect of PTX was related to

the inhibition of enzymatically degradative processes of tumor metastasis. We have also shown that PTX inhibited MMP2 and MMP9 activity in MDA-MB 231 cells (110).

Cellular migration is another property of cancer cells that is required for migration from the primary site to distant organ and finally proliferates to produce clinically relevant metastasis. Any alteration of this property would interrupt the metastatic cascade. To test whether PTX could inhibit migration in A375 cells, we carried out migration studies. At effective sub-toxic doses of PTX, significant inhibitions in cell migration were observed compared to the control cells. The effect of PTX on cell motility varies with cell type. It is a known pharmacological inducer of human sperm motility (120). However, it inhibits migration in human neutrophils, leukocytes and MDA-MB 231 cells (110, 121, 122). Similar to PTX, other methylxanthine derivative like caffeine has also been shown to inhibit cellular migration of HT29 cells (123). Cell motility is a very dynamic process that continuously requires modifications in cell shape and formation of cell protrusions. This is achieved by highly regulated organization of actin cytoskeleton at the protruding edge and the rear edge (124). A study showed that depolymerization of actin filament impairs cell migration (125). In this study we observed that PTX disrupted actin organization of the cells, thus suggesting that in some cellular context PTX would inhibit migration of the cells by disrupting actin organization. Taken together, our data revealed that PTX at sub-toxic doses inhibited metastasis at multiple steps such as cellular proliferation, migration, invasion and cellular adhesion, thus suggesting that PTX has the potential to show antimetastatic activity against cancer cells.

CHAPTER 3

To study the mechanism(s) by which PTX has antimetastatic and antiangiogenic activity.

3.1 INTRODUCTION AND REVIEW OF LITERATURE

3.1 Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway

The story of the JAK/STAT pathway begins with interferons (IFNs). IFN was the founding member of the cytokine family and was first described by Isaacs and Lindenmann more than 50 years ago (126). Over the subsequent 25 years, characterization of how IFNs mediate their biological response led to identification of the JAK/STAT signaling cascade, where JAKs are receptor-associated kinases and STATs the transcription factors they activate (127). Today, 4 JAKs (JAK1, JAK2, JAK3 and TYK2) and 7 STATs (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6) are known (127). STATs are considered to have a dual function, acting as signal transducers (transmitting signals from the membrane to the nucleus), and as transcription factors (activating the expression of genes once they bind to their promoters). JAK/STAT pathway is one of the principal signalling cascades mediating cytokine receptor derived signals in mammals (128). It is conserved in all vertebrates and even found in some metazoas. Originally implicated in the regulation of survival, proliferation and differentiation of haematopoietic cells, the JAK/STAT pathway has later on also been linked to developmental processes, growth control and maintenance of homeostasis in a variety of other cells and tissues (129, 130). Importantly, accumulating evidence indicates that dysregulation of JAK/STAT signalling is causally related to a variety of human diseases, including cancer (131). STATs are activated by various receptor and non-receptor tyrosine kinases. Upon activation, STATs dimerize, translocate to the nucleus where they activate transcription of specific target genes that lead to altered cellular phenotypes (127). Under normal physiological conditions, STATs activation particularly activation of STAT3 is tightly regulated. However,

compelling evidence suggests that STAT3 is constitutively activated in many human cancers and plays a pivotal role in tumor growth and metastasis. It regulates cellular proliferation, invasion, migration, and angiogenesis that are critical for cancer metastasis **(Figure 3.1)**. In contrast to this, blocking STAT3 signaling in tumor cells inhibits tumor growth, angiogenesis, and metastasis without affecting normal cells, thus confirming STAT3 as a potential target for cancer therapy (131).

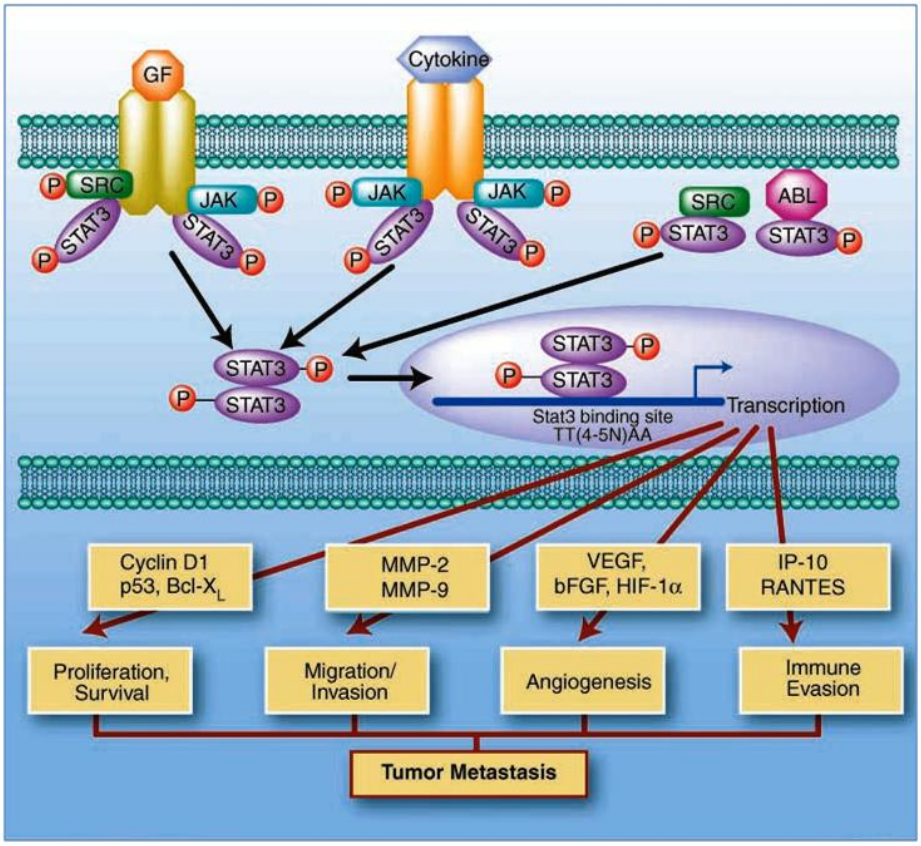


Figure 3.1: Role of STAT3 signaling in cancer metastasis *[adapted from(132)].*

3.1.1 Structure of STATs

All seven STAT family members are ranging in size from 750 to 900 amino acids (130), and are located on three chromosomal locations **(Table 3.1)**. Structural,

biochemical and genetic studies have revealed that all STAT family proteins have seven structurally and functionally conserved domains including the amino-terminal domain (NH2), coiled-coil domain, DNA-binding domain (DBD), linker domain (Lk), SH2 domain, tyrosine activation domain (Y) and transcriptional activation domains (TAD) **(Figure 3.2)**. The amino-terminal domain (~125 residues) is a structurally independent moiety. It is important for protein-protein interactions and for dimer-dimer interactions to form tetrameric STAT molecules (133). Tetramer formation is necessary for strong STAT-DNA binding. Amino-terminal domain has also been implicated in STATs nuclear import and export (134). The coiled-coil domain is span from residues ~135 to 315 and consists of four α- helix bundle. This domain provides large hydrophilic surface to interact with various regulatory proteins and other transcription factors, for example CBP/p300 (130, 135).

Family member	Chromosomal location	
	Human	Murine
STAT 1	2q12-33	1
STAT 4	2q12-33	1
STAT 2	12q13-14.1	10
STAT 6	12q13-14.1	10
STAT 3	17q11.2-22	11
STAT 5a & 5b	17q11.2-22	11

Table 3.1: Chromosomal location of human and murine STAT family members *[modified and adapted from (136)].*

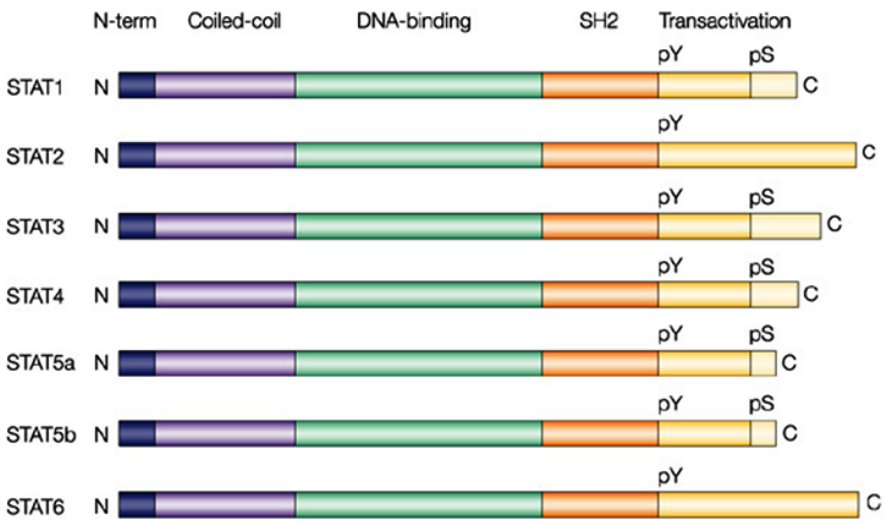


Figure 3.2*: *Structure of STAT family members *[adapted from (127)].*

The DNA binding domain, between residues ~ 320 to 480, contains several β-sheets and determines DNA sequence specificity of individual STATs. The adjacent linker domain, between residues ~ 480 to 580, maintains an appropriate conformation between the dimerization domain and DNA-binding domain. Next, the SH2 domain, a highly conserved domain, spans from residues ~ 575–680. It is involved in receptor recruitment and the formation of active STAT dimer. The tyrosine activation domain is positioned directly adjacent to the SH2 domain, consists of a conserved tyrosine residue usually around 700 position. Phosphorylation of this tyrosine residue is essential for STAT activation. The remaining carboxyl-terminal transactivation domain, a highly variable domain in context of length and sequence among STAT family members, regulates transcriptional activation of target genes through interaction with other transcriptional regulators. Conserved serine phosphorylation site is present in many transactivation domains. Phosphorylation of this serine residue is required for maximal transcriptional activity (137, 138).

3.1.2 Activation of STATs

STATs are activated by phosphorylation of single tyrosine residue located around 700 position. Various kinases that catalyzes this phosphorylation include numerous cytokine receptors (lack intrinsic tyrosine kinase activity but to which JAKs are non-covalently associated), receptors with intrinsic tyrosine kinase activity: such as epidermal growth factor (EGFR), vascular endothelial growth factor receptor (VEGFR), platelet derived growth factor receptor (PDGFR), and colony stimulating factor 1 (139, 140), and various non-receptor tyrosine kinases such as Src and Abl (141, 142). After binding of ligand to the respective receptor, receptor dimerizes or oligomerizes that result in reciprocal tyrosine phosphorylation, and consequent activation, of the intrinsic (EGFR/VEGFR) or the attached kinase (JAKs). Phosphorylation of the kinase is the first of three tyrosine phosphorylation culminating in STAT activation. The activated JAKs phosphorylate tyrosine sites on the cytoplasmic tail of the receptor that serve as docking sites for the SH2 domains that occur in all the STATs. The receptor-bound STAT is then phosphorylated on tyrosine. Once phosphorylated, STAT homodimerizes or heterodimerizes through reciprocal interaction between SH2 domains and phosphotyrosines. STAT1, STAT3, STAT4, STAT5a and STAT5b form homodimers. STAT1 and STAT2 and STAT1 and STAT3 can also form heterodimers. As a result, STAT dimer translocate to the nucleus where they bind to specific STAT DNA-binding elements in the promoter of target genes and activate transcription **(Figure 3.3)**. Individual STAT member recognizes a slightly different palindromic sequence, and can thereby induce a unique gene expression profile. The best-studied pathway for STAT activation is through the JAKs therefore known as the JAK/STAT pathway.

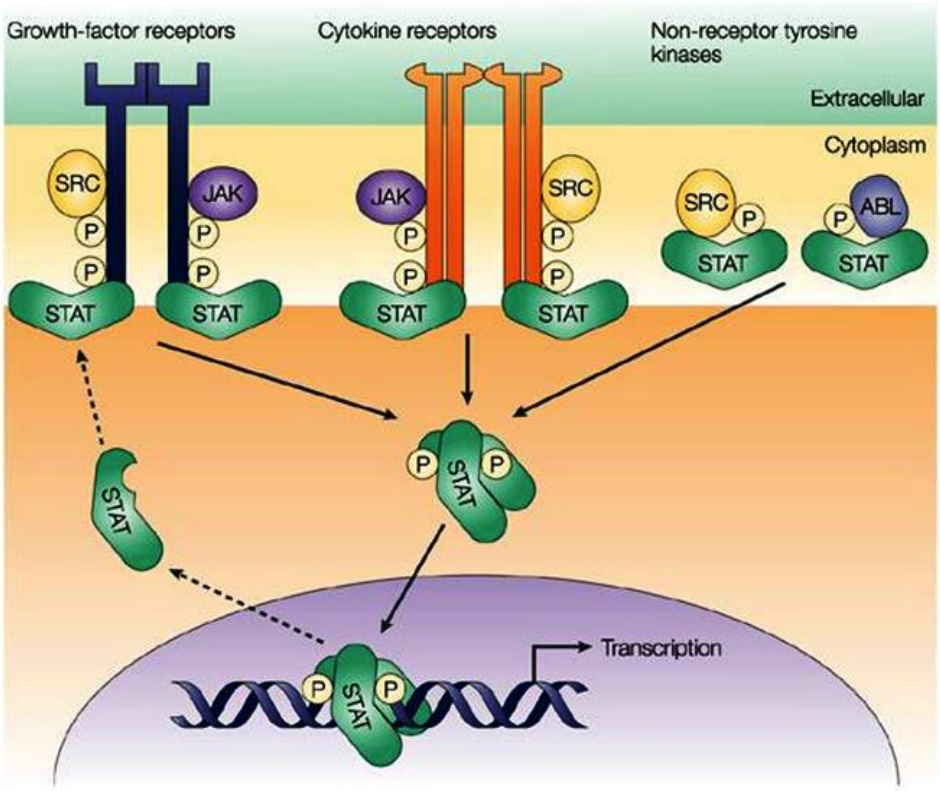


Figure 3.3: Activation of STAT signaling *[adapted from (127)].*

3.1.3 JAK family kinases

The four mammalian JAK family members, JAK1, JAK2, JAK3 and TYK2, are protein tyrosine kinases that are non-covalently attached to cytokine receptors. They range in size from 120 Kd to 140 Kd and are located on three different chromosomes (143). The genes coding for JAK1 and JAK2 are located at chromosome 1p31.3 and 9p24 respectively. The gene coding for JAK3 is located at 19p13.1 while TYK2 is located at chromosome 19p13.2. Expression studies indicate that JAK1, JAK2 and TYK2 are ubiquitously expressed in most tissues while JAK3 is primarily expressed in hematopoietic cells (144). The JAKs are structurally unique in having a C-terminal kinase domain that is preceded by a pseudokinase domain (143). It is this feature that

gives the Janus kinases their name — just like the Roman god, they are two-faced with respect to these domains.

3.1.4 Structure and function of JAKs

JAKs are relatively large proteins containing more than 1,000 amino acids. Seven distinct JAK homology regions (JH) have been identified (JH1 to JH7), and these form the putative structural domains of the JAK family members (143) **(Figure 3.4)**.

Figure 3.4: Schematic structure of JAKs: *The JAK family comprises four structurally related kinases: Jak1, Jak2, Jak3, and Tyk2. Seven JAK homology regions (JH) containing the catalytically active kinase domain (JH1), the enzymatically inactive pseudokinase domain (JH2), the SH2 domain (JH3, JH4), and a FERM domain (JH6, JH7) form the JAK protein. The FERM domain mediates JAK binding to the transmembrane cytokine receptor and regulates kinase activity [adapted from (144)].*

The carboxyl terminus contains the kinase and pseudokinase domains, denoted JH1 and JH2, respectively. The JH1 domain contains all the features typical of a catalytic tyrosine kinase, including tyrosine residues in the activation loop region, the canonical GXGXXG motif in the nucleotide-binding loop and a conserved aspartic acid residue involved in the phosphotransfer reaction in the catalytic loop (145). The sequence of the JH2 pseudokinase domain, also called kinase like domain, is highly homologous to the kinase domain, but lacks characteristic residues of active tyrosine kinases, making it catalytically inactive (146). It has been suggested that the exon–intron structure of the

nucleotide sequence coding for the JAK3 pseudokinase domain resembles that of genes coding for Src kinases, while no homology with other known genes was detected for the kinase domain (147). Thus, it seems unlikely that the pseudokinase domain emerged from a simple duplication of genes coding for the kinase domain. The primary structure of JH3 and JH4 shares homology with Src-homology-2 (SH2) domains (148). However, mutation of a critical residue that is required for the function of the SH2 domain has no effect on cytokine receptor signaling, suggesting a scaffold rather than a signaling role for the SH2-like domain (148). Rounding off the N-terminus, the JH5-JH7 domains contain a predicted FERM (Band-4.1, ezrin, radixin and moesin)-like motif, which plays a role in appending JAKs to their cytokine receptors (149).

The link between JAKs and cytokine signalling was first made when it was shown that TYK2 was required for IFN signalling through the analysis of TYK2-mutant cell lines (150). Shortly thereafter, it was discovered that other JAKs are linked to other cytokine receptors, and subsequent studies using knockout mice have confirmed the essential and specific function of JAKs (151). Although specific JAKs are activated through each cytokine receptor and may partially contribute to specificity, the JAK kinases by themselves are not an absolute determinant of the specificity in cytokine signaling, since many different cytokines activate the same JAKs. Nevertheless, JAK gene targeting studies have identified characteristic signaling defects **(Table 3. 2)** (151). JAK1 knockout mice die perinatally due to a failure to nurse (ascribed to a LIF defect). Their tissues are defective in response to cytokines from the IL-2, IL-6, IFN and IL-10 families. The JAK2 knockout mice exhibit embryonic lethality (i.e., day 12.5), due to the absence of definitive erythropoiesis, additional immunological impairments caused by impaired cytokine signaling are observed. JAK3 expression is limited to lymphoid

tissues (144) . Biochemical and genetic studies have genetically and physically linked JAK3 to the common gamma chain (γC), which is associated with members of the lymphoid predominant IL-2 family of cytokine receptors (e.g., receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21). Consistent with this, JAK3 and γC knockout mice both exhibit severe combined immunodeficiency (SCID)-like defects, highlighting JAK3 as an appealing therapeutic target (152). TYK2 deficiency has been associated with distinct phenotypes in humans and mice. Whereas TYK2 knockout mice feature modest cytokine defects and a proclivity towards type 2 (i.e., allergic) T-cell responses (153), TYK2 deficient humans exhibit a severe allergic phenotype that has been attributed to an impaired antimicrobial response (154). In mice, TYK2 may play a more important role in integrating the response to multiple cytokines (155).

Targeted gene	**Phenotype**
JAK1	Viable but perinatal lethality due to neurological defects, SCID.
JAK2	Embryonic lethality, due to failure in erythropoiesis, impaired cytokine signaling.
JAK3	Viable and fertile, SCID.
TYK2	Viable and fertile, impaired IFN and IL-12 response, increased pathogen susceptibility

Table 3.2: Phenotype of JAK knockout mice. *[Adapted from (151)].*

The JAK family of non-receptor protein tyrosine kinases are essential for receptor cytokine signaling during differentiation of immune responses and also mediates signals for growth, proliferation, and formation of hematopoietic cells and other tissues. However, its aberrant activity has also been implicated in various human malignancies,

such as leukemia (156). For example, the fusion protein TEL-JAK2 results from a fusion between the oligomerization domain of TEL and the JH1 kinase domain of JAK2 exhibits constitutive tyrosine kinase activity. TEL-JAK2 fusion proteins have been found in lymphoid and myeloid leukemia (144). Ectopical overexpression of TEL-JAK2 in hematopoietic cell lines leads to constitutive activation of STAT1, STAT3 and STAT5 and induces growth factor independent proliferation (157). Another oncogenic fusion protein involving JAK2 is the PCM1-JAK2 fusion protein that was identified in several cases of chronic and acute leukemia, such as atypical chronic myeloid leukemia (CML), acute erythroid leukemia and B-cell lymphoma (158-160).

3.1.5 Biological function of STATs

Gene-targeting studies indicate that some STAT proteins are highly specific in their function and that they are responsible for mediating the immune response in mammals **(Table 3.3)** (161).

Targeted gene	Phenotype
STAT1	Compromised innate response to microbial pathogens and viruses.
STAT2	Increased susceptibility to viral infection and a loss of biological response to type I IFN.
STAT3	Early embryonic lethal.
STAT4	Impaired natural killer cell cytotoxicity and Th1 cell response.
STAT5a and 5b	No mammary gland development or lactogenesis.
STAT6	No Th2 cells development.

Table 3.3: Phenotype of STATs knockout mice *[adapted from (161)].*

3.1.5.1 STAT1

STAT1 was the first member of the STATs family transcription factor discovered as a principal target of both type I and type II interferon (IFN) activation. Mice lacking STAT1 are viable and fertile but lost responsiveness to both type I and type II IFNs (162). However, defects in the biological response to other STAT1 activating ligands (e.g., IL-6 and EGF) are considerably more modest. STAT1 null mice are highly susceptible to bacterial and viral infections (162). In humans, defects in IFNγ-STAT1 axis have been intimately linked with increased susceptibility to mycobacterial infection (163). In addition to their role in directing an effective innate response to intracellular bacteria, STAT1 target genes have been associated with suppression of cellular proliferation (154). This contrasts the pro-proliferative and anti-inflammatory activities linked to STAT3 (describe below), and raises the possibility that STAT1 and STAT3 serve to functionally antagonize each other. Interestingly, STAT1 deficient mice also showed sensitivity for both spontaneous tumor development and tumor development after methylcholanthrene treatment (164-166). Thus strengthen the pro-apoptotic function of STAT1.

3.1.5.2 STAT2

STAT2 is an exceptional member of STATs family protein, since it neither homodimerize nor bind DNA directly. Instead, upon activation with type I and type III IFNs, STAT2 forms an active heterodimer with either STAT1α or STAT1β. Therefore, STAT2 knockout exhibits similarities with the STAT1 knockout and exhibit profound defects in their biological response to type I IFNs and likely type III IFNs (167). More detailed analysis of IFN I response in $STAT2^{[-/-]}$ tissues has revealed a loss in IFN I autocrine activity, abnormal DC maturation and a loss in SOCS1 expression.

3.1.5.3 STAT3

STAT3 was first identified as a DNA-binding factor that selectively binds to the interleukin-6 (IL-6)–responsive element in the promoter of acute-phase genes from IL-6–stimulated hepatocytes (168). STAT3 was also independently identified as a DNA-binding protein, in response to epidermal growth factor (169). Consistent with a broad range in activity, STAT3 plays a crucial role in the regulation of cell proliferation, survival, apoptosis, and differentiation. However, despite the roles of STAT3 in a wide variety of physiologic processes, it has been shown that altered STAT3 activation can contribute to oncogenesis (170). STAT3 is the only STAT family member whose knockout leads to embryonic lethal phenotype (171). STAT3 deficient mice develop into the egg cylinder stage but show a rapid degeneration between embryonic days 6.5 and 7.5. This is probably due to nutritional insufficiency, since STAT3 is expressed at day 7.5 in the embryonic visceral endoderm, which is important for nutrient exchange between the maternal and embryonic environment (171). Tissue specific STAT3 knockouts have been associated with an increased inflammatory response, altered energy homeostasis, developmental defects and a decreased oncogenic potential (154). Cardiomyocyte-specific conditional STAT3 knockout mice showed enhanced susceptibility to cardiac injury caused by myocardial ischemia, systemic inflammation, or drug toxicity (172). STAT3-deficient T cells showed a loss of proliferative response due to a defect in IL-6 mediated prevention of apoptosis (173). Mice with STAT3 deficient epidermal and follicular keratinocytes were viable and did not have any defects in the development of epidermis and hair follicles. However, the mice had sparse hair, wound-healing processes were severely impaired and they spontaneously developed ulcers with age (174). Targeted STAT3 knockout in macrophages and neutrophils results in mutant mice that are highly susceptible to endotoxin shock and

develop chronic enterocolitis with age, due to abolished inhibitory effects of IL-10 on inflammatory cytokine production (175). STAT3 deficient hepatocytes severely impaired IL-6 induced acute-phase response in the liver during inflammation (176). Considering all knockout data together it is surprising that STAT3 has such mild phenotypes in the conditional knockout mice compared to the early embryonic lethality in the full knockout. The data suggests that the STAT3 activators in early development are different from the known activators in the adult and it will be of interest to identify them.

3.1.5.4 STAT4

STAT4 is only expressed in myeloid cells, thymus and testis. It is mainly activated by cytokine IL-12, resulting in its tyrosine phosphorylation, in both human and mouse (145). STAT4 can also be phosphorylated in response to IFN-γ stimulation through activation of JAK1 and TYK2, but this has only been observed in human cells (177). Upon encountering antigen, $CD4^+$ T helper lymphocytes differentiate into three distinct subsets, Th1, Th2 or Th17 cells that are distinguished by their cytokine expression profiles. STAT4 specifically plays an important role in Th1 cell development or function, as mice lacking STAT4 unable to generate Th1 cells (178). Further, lymphocytes from STAT4 deficient mice were unable to make high levels of IFNγ, characteristic of Th1 cells, after IL-12 priming (179). STAT4 also plays an important role in the IL-23-dependent polarization of naïve $CD4^+$ T-cells into Th17 cells (180). Additionally, NK cell cytolytic functions were severely impaired in STAT4 knockout mice (181). Recent data indicate that STAT4 deficient mice show a resistance to autoimmune diseases like rheumatoid arthritis, diabetes and experimental, allergic

encephalomyelitis (EAE) (182). Taken together, these data highlight the importance of STAT4 and Th1 responses in pathogenesis of autoimmune diseases.

3.1.5.5 STAT5

STAT5 was first observed in sheep and rat mammary tissue during the lactation period and it was named as MPBF (milk protein binding factor) or MGF (mammary gland factor) respectively (183). It is exist as two closely related genes, STAT5a and STAT5b, which share 95 % identity at the N-terminus and some variability at the C-terminus. Both genes are expressed in all tissues and are known to be activated by a wide variety of cytokines. Despite their higher degree of conservation, STAT5a and STAT5b exert redundant yet distinct functions, as revealed by single and double knockout mice studies (184, 185). STAT5a deficient mice showed incomplete mammopoiesis and failure of lactogenesis based on defective signaling to prolactin stimulation (186), and decreased proliferation of splenocytes to interleukin IL-2 stimulation. On the other hand, STAT5b deficient mice showed a phenotype similar to that of Laron-type dwarfism, which is a human growth hormone (GH)-resistance disease generally associated with a defective GH receptor, and STAT5b was suggested to play a major role in mediating the sexually dimorphic effects of GH pulses in the liver (187). Additionally, STAT5a and -b doubly disrupted mice showed mild lymphocytopenia, profound deficiency in peripheral T cell proliferation, and a detectable reduction of colony forming efficiency of bone marrow cells in response to IL-3, IL-5, IL-7 and granulocyte/macrophage colony-stimulating factor (184).

3.1.5.6 STAT6

STAT6 is ubiquitously expressed and is mainly activated by IL-4 and IL-13 (154). IL-4 is expressed by activated T and B cells and regulates differentiation of $CD4^+$ T cells to

Th2 cells and class switching of B cells resulting in secretion of IgE. Lymphocytes from STAT6-deficient mice were unable to upregulate MHC Class II, CD23, and IL-4Rα expression in response to IL-4, supporting the importance of STAT6 in the activation of IL-4 inducible genes (188) STAT6 deficient B lymphocytes were also unable to switch to the immunoglobulin isotypes IgG1 and IgE in response to IL-4 (189). STAT6 deficient T helper cells were also unable to differentiate into Th2 cells (190).

3.1.6 Regulation of STATs

In normal cells, the duration of STAT activation is transient, usually lasting from a few minutes to several hours. Thus, suggesting that nature has given very sophisticated regulatory mechanisms to attenuate these processes. These includes dephosphorylation by protein tyrosine phosphatases (PTPases), suppressors of cytokine signaling family of inhibitors (SOCS), proteins that inhibit activated STAT family of inhibitors (PIAS), degradation by ubiquitin-proteasome systems, receptor mediated endocytosis and degradation in lysosome and dominant negative STATs.

3.1.6.1 Protein tyrosine phosphatases (PTPases)

Since tyrosine kinases play an important role in STAT3 activation, it is not surprising that tyrosine phosphatases are likely to play a role in STAT3 deactivation. These include classical protein tyrosine phosphatases (PTPs), dual-specificity phosphatases and low molecular weight phosphatases (191). All these protein tyrosine phosphatases even though shares little sequence similarity, exhibit similar tertiary structure and are characterized by presence of signature motif VHCSXGXGR[T/S]G (192). The classical PTPs are divided into 2 groups, the transmembrane tyrosine phosphatase CD45 and

nontransmembrane PTPs, including SH2-domain-containing SHP1 and SHP2, phospho-tyrosine phosphatase 1B (PTP1B) and T cell–protein tyrosine phosphatase (TC-PTP). SH2-domain-containing phosphatases that include SHP1 (previously named PTP1C) and SHP2 (previously named PTP1D) were the first cytoplasmic tyrosine phosphatases found to be involved in inhibiting JAK/STAT activity. These phosphatases are mainly cytoplasmic and are characterized by the presence of two SH2 domains preceding a phosphatase domain. Their SH2 domains allow association with phospho-tyrosine present on activated receptors or on signaling molecules as well as on activated JAKs. This association triggers activation of the phosphatase domain and the subsequent dephosphorylation of the substrate. In case of a receptor, STATs will no longer be recruited to it and thus will no longer be phosphorylated and activated.

SHP1 is predominantly expressed in hematopoietic cells but it is also present in epithelial or smooth muscle cells. It binds to tyrosine-phosphorylated erythropoietin (EPO) receptor and to dephosphorylates JAK2, whereas there are other examples in which SHP1 has bound directly to JAK2 via an SH2-independent mechanism (193). Overexpression of an inactive SHP1 mutant leads to delayed JAK2 and STAT3 dephosphorylation in the cells. SHP1 also downregulates IL-2 induced tyrosine phosphorylation of JAK1 and JAK3 (194).

SHP2 is relatively ubiquitously expressed and deletion of this gene is embryonic lethal for mice, thus revealing a major role for SHP2 in cell growth and development. SHP2 specifically interacts with tyrosine phosphorylated STAT5a through its C-terminal SH2 domain and dephosphorylates STAT5a (195). It also acts as a dual-specificity protein phosphatase, interact with and dephosphorylate STAT1 at both Tyr701 and Ser727

(196). SHP2 also dephosphorylates JAK2 and contributes to the JAK2 stability by decreasing its association with SOCS-1 (197).

PTP1B is a cytosolic PTP and expressed in many tissues. It dephosphorylates prolactin-activated STAT5a and STAT5b. PTP1B also dephosphorylates TYK2 and JAK2, which contain the (E/D)-pY-pY-(R/K) consensus, but not JAK1, which does not have this motif (198)

TC-PTP is mainly expressed in hematopoietic cells and alternative splicing of its gene allows expression of two distinct proteins, a p45 nuclear form called TC45 or TC-PTPa, and a p48 cytoplasmic form called TC48 or TC-PTP1b. TC-PTP interacts with the D/E-pYpY-T/V sequence present in JAK1 and JAK3 and dephosphorylate them (199) . In addition, the nuclear TC45 is responsible for deactivation of nuclear STAT1 and STAT3 (200).

CD45 is the most studied transmembrane PTP involved in negatively regulating JAK/STAT signaling (191). It is expressed in hematopoietic cells and composed of two phosphatase domains in its intracellular part, but only one seems to be active. CD45 is a JAK phosphatase and mice deficient for CD45 show hyperactivation of JAK1 and JAK3 (201).

Dual -specificity phosphatases dephosphorylate both phosphotyrosine and phosphotherionine. STAT3 is phosphorylated in response to angiotensin II at tyrosine as well as serine in vascular smooth muscle cells (202). PP2B, a dual-specificity phosphatase dephosphorylates STAT3 tyrosine phosphorylation while protein phosphatase 2A (PP2A) dephosphorylates STAT3 serine phosphorylation (202).

Higher expression of low molecular weight phosphatase was generally observed in megakaryoblastic cells. In DAM1 megakaryocytic cells, low molecular weight phosphatase dephosphorylates STAT5 by interacting with their C-terminal domain (203).

3.1.6.2 SOCS family of inhibitors

SOCS family proteins are inducible inhibitors of cytokine signaling and comprises eight members CIS along with SOCS1 to SOCS7 (204). These proteins were independently discovered by 3 groups using distinct approaches and named as a JAK binding protein (JAB), as a suppressor of IL-6 signalling (SOCS1), and based on sequence homology with the STAT3-SH2 domain (STAT-induced STAT inhibitor: SSI) (204). All SOCS proteins are characterized by a central SH2 domain flanked by an N-terminal domain of variable length and a C-terminal SOCS box. The SH2 domain is required for association with other proteins, such as the phosphorylated tyrosine residue in the activation loop of JAK2 in the case of SOCS1, and is essential for the inhibitory functions of SOCS proteins (204).

SOCS proteins inhibit cytokine signaling using a number of different mechanisms. SOCS1 binds directly via its SH2 domain to tyrosine phosphorylated JAKs, as a consequence JAK activity is directly inhibited (191). SOCS3 uses a different mechanism for inhibition of JAKs, it binds to the activated receptor directly (191). Unlike SOCS1 and SOCS3, CIS inhibits STAT activation by competing with STATs for binding to receptor docking sites (204). CIS is able to prevent STAT5 from associating with cytokine receptors, blocking STAT5 activation (205). In addition, SOCS proteins also induce proteasomal degradation pathway through SOCS box motif located at the C terminus of all SOCS proteins (206). Thus, SOCS protein-negative

regulation might involve a two-step mechanism- the first being the inhibition of signaling by binding of SOCS protein to an active complex and the second being disposal of those signaling components via proteasomal degradation. Recent work by Ram and Waxman supports this hypothesis, with results indicating that complete inhibition of GH signaling by CIS requires an active proteasomal degradation pathway (207). An interesting feature of SOCS proteins is that they do not affect the activation of the JAK-STAT pathway but rather the deactivation. As a result, prolonged STAT activation induced by cytokines is observed in the absence of SOCS proteins.

CIS was the first of the SOCS family proteins described and has redundant roles, as evidenced in the gene-targeted mice, which lack any phenotypic changes. However, transgenic mice overexpressing CIS demonstrate a phenotype similar to that seen in STAT5a $^{[-/-]}$ and STAT5b$^{[-/-]}$ mice, supporting the importance of CIS in the regulation of STAT5 (205).

SOCS1 gene-targeted mice are healthy at birth but die before weaning because of fatty degeneration of the liver, monocytic infiltration of the organs, reduced thymus size and severe loss of T and B lymphocytes (208).

The SOCS2 knockout mice revealed a critical role for SOCS2 in GH and insulin-like growth factor signaling. SOCS2-deficient mice are significantly larger than their wild-type counterparts because of loss of SOCS2-dependent attenuation of GH signaling (208). Interestingly, mice overexpressing SOCS2 are also significantly larger than wild-type mice, suggesting that SOCS2 might have a dual role (209).

Mice deficient in SOCS3 exhibited embryonic lethality, with erythrocytosis and placental defects (191). Conditional SOCS3 gene targeting studies have demonstrated an important role for SOCS3 in the negative regulation of IL-6 (210).

The roles of the other SOCS family members, including SOCS4, SOCS5, SOCS6, and SOCS7, have not been as well defined.

3.1.6.3 PIAS family of inhibitors

The PIAS family proteins are constitutively expressed and identified as a negative regulator of STATs signaling. It consists of five members namely PIAS1, PIAS3, PIASy, PIASxα, and PIASxβ. The all PIAS proteins are evolutionary conserved and are characterized by conserved LXXLL signature motif at the amino-terminal region (211). Other conserved elements of PIAS proteins include zinc binding domain, acidic domain and serine/therionine rich region, however, PIASy lacks serine/threonine rich domain. PIAS mediated gene regulation involves direct blocking of DNA-binding activity of transcription factors, recruiting transcriptional co-repressors or co-activators, and promoting protein sumoylation. It has been proposed that PIAS1, PIAS3 and PIASx interact with STAT1, STAT3 and STAT4 respectively (212). PIASy also interact with STAT1. Coprecipitation studies have demonstrated that PIAS1 interacts with STAT1 dimer and blocks STAT1 DNA-binding and STAT1-dependent gene induction (213). Similarly, PIAS3 binds to STAT3 homodimer and STAT1-STAT3 heterodimer and blocks DNA-binding activity (213). In contrast, PIASx and PIASy repress the transcriptional activity of STAT1 and STAT4, by recruiting corepressor molecules such as histone deacetylases (HDACs) (211, 214).

Another characteristic of PIAS proteins is that they exhibit E3-small ubiquitin-related modifier (SUMO) ligase activity (215). Sumoylation, the covalent attachment of SUMO to its substrate, is a reversible posttranslational modification involving formation of an isopeptide bond between the C-terminus of SUMO with an ε-amino of a lysine residue in the target protein. Sumoylation has been implicated in the regulation of protein-

protein interaction, protein stability, protein localization, and protein activity. Recently, it was shown that STAT1 can by sumoylated on Lys703 and that this is promoted by PIAS1, PIAS3, and PIASx (216).

3.1.6.4 Degradation by ubiquitin-proteasome systems

Ubiquitin-proteosomal degradation is important for downregulation of the JAK/STAT signaling pathway. It has been observed that proteasome inhibitors prolonged the activation of the JAK/STAT pathway in response to IL-2, IL-3, growth hormone, ciliary neurotrophic factor, IFN, and erythropoietin stimulations (217). The tyrosine phosphorylated forms of STAT4, STAT5, and STAT6, but not other STATs, have been shown to be stabilized by proteasome inhibitors (218). JAK1, JAK2 and JAK3 are also target for ubiquitin-proteasome degradation (217).

3.1.6.5 Receptor mediated endocytosis and degradation in lysosome

Endocytosis is the process by which cells internalise extracellular and plasma membrane bound entities into the cell interior. During this process molecules that are to be internalised including ligand-receptor complexes are enclosed in vesicles that bud from the interior face of the plasma membrane into the cell. Then, cellular trafficking machinery transports this vesicle and its cargo to the appropriate cellular locations through a network of specialized organelles, including early endosome, recycling endosome, late endosome, multi-vesicular bodies and lysosomes.

An important function of endocytosis in cell signaling is to downregulate signal responses by internalizing receptors either constitutively or upon ligand binding. This provides an excellent mechanism to inhibit many signaling pathways by constitutively internalizing a pool of receptors from the plasma membrane and via switching off signaling after ligand binding. However, emerging view also considers endocytosis as

an integral part of signal propagation and processing. Evidence of receptor mediated endocytosis as a negative regulation of STAT signaling originate from studies that indicated that the IL-6R is downregulated by its ligand (219). Recent data demonstrate that the common signal transducer gp130 carries a di-leucine internalization motif within the intracellular domain (220). In addition, mutation of serine 139 to an alanine of gp130 reduces the internalization rate by 50 % suggesting that serine phosphorylation upon IL-6 stimulation leads to a conformation change and exposure of the internalization signal to the endocytotic machinery (220).

3.1.7 Dominant negative STATs

STAT1β, STAT3β and STAT5β are the naturally occurring splice variant of STATs protein. They lack the C-terminal transactivation domain and the serine phosphorylation site and have a competitive dominant negative effect on gene induction, mediated by the STAT pathway (221) . They can be generated either by alternative mRNA splicing or proteolytic processing. The physiological roles of splice product are not completely understood but they are known to be negative regulators of STAT signaling. The splice variant gets tyrosine phosphorylated, dimerize and bind DNA efficiently but do not activate genes transcription as its lack transactivation domain (221). In biological assays STATβ splice variants are often used as inhibitory tool and also to study the role of STAT proteins.

3.2 STATs and cancer

STAT proteins particularly STAT1, STAT3 and STAT5 are frequently overactivated in wide variety of human solid tumors and blood malignancies **(Table 3.4)**. In general, continuous stimulation by cytokines and growth factors, overactivation of upstream

tyrosine kinases or silencing of the negative regulators of STAT activation may be involved in this persistent STATs activation in different tumor tissues.

Tumor type	Activated STAT protein
Solid tumors	
Breast cancer	STAT1, STAT3, STAT5
Head and neck cancer	STAT1, STAT3, STAT5
Melanoma	STAT3
Lung cancer	STAT3
Ovarian cancer	STAT3
Pancreatic cancer	STAT3
Prostate cancer	STAT3
Blood tumors	
Multiple myeloma	STAT1, STAT3
HTLV-1-dependent leukemia	STAT3, STAT5
Acute myelogenous leukemia	STAT1, STAT3, STAT5
Chronic myelogenous leukemia	STAT5
Large-granular-lymphocyte leukemia	STAT3
Acute lymphoblastic leukemia	STAT5
Lymphoma	
EBV-related and Burkitt's lymphoma	STAT3
Cutaneous T-cell lymphoma	STAT3
B-cell non-Hodgkin's lymphoma	STAT3
Anaplastic large-cell lymphoma	STAT3

Table 3.4: Activated STATs protein in human cancers *[modified and adapted from (127)].*

As discussed earlier STAT1 is considered a pro-apoptotic and tumor suppressor, as $STAT1^{[-/-]}$ mice has shown to be tumor prone. One mechanism by which STAT1 suppresses tumor formation is its key role as a transcription factor downstream of type I and type II interferon. IFNα is widely used for the treatment of metastatic melanoma and for certain forms of leukemia, where it can induce disease regression. IFNγ is a key component of tumor surveillance and protects the host against spontaneously arising tumors. In contrast, emerging data revealed that in certain cellular contexts the IFN/STAT1 pathway may mediate tumor cell growth. Kovacic and co-workers showed that presence of STAT1 accelerates leukemia progression *in vivo* (222). One of the possible explanations is that $STAT1^{[-/-]}$ tumor cells acquire increased MHC class I expression upon leukemia progression that allows for the escape of hematopoietic malignancies from immune surveillance. In addition, Hix and co-workers reported that tumor STAT1 activity enhances breast tumor growth and immune suppression mediated by myeloid-derived suppressor cells (223). Thus, STAT1 genes products may serve both as tumor suppressor as well as tumor promoters. Further investigation will be needed to completely unravel the role of STAT1 in cancer.

STAT5 protein is important for the development and functions of the mammary gland (183). However, persistent activation of STAT5 has been seen in many human cancers particularly in haematopoietic malignancies. Most frequently, this aberrant STAT5 activation is due to hyperactivation of tyrosine kinases such as JAK2 (224). JAK2 activates STAT5 and activated STAT5 translocates to the nucleus and regulates gene expression involved proliferation and cell survival. On the other hand, STAT5 can inhibit tumor progression like in the liver, and it is a tumor suppressor in fibroblasts

(224). STAT5 protein are able to regulate cell differentiation and senescence, activating the tumor suppressors SOCS1, p53 and PML (224).

3.3 STAT3 and cancer

STAT3 can be considered as an oncogene, and oncogenic role was revealed for the first time by the finding that overexpression of the constitutively active mutant i.e. STAT3C, can transform cultured cells and induced tumor in immunodeficient mice (225). Further work showed that overexpression of STAT3C enhances malignant progression of skin tumor, triggers the onset of lung adenocarcinoma and sufficient to transform mouse embryonic fibroblast (226). Thus, the activated STAT3 molecule by itself can mediate cellular transformation and initiates tumor growth and progression. Compared with normal cells and tissues in which STAT3 activation is transient and tightly regulated, constitutive activation of STAT3 has been detected in a wide variety of human cancer cell lines and tumor tissues **(Table 3.4)**. In tumor cells, constitutive activation of STAT3 is linked to persistent activity of tyrosine kinases, including Src, EGFR, JAKs, Bcr-Abl, and many others. After activation, STAT3 modulates the transcription of responsive genes involved in the regulation of a variety of critical functions, including cell proliferation, differentiation, apoptosis, angiogenesis, metastasis, and immune responses (156). For many cancers, elevated levels of activated STAT3 have been associated with a poor prognosis (227). However, in contrast to well-characterised oncogenic activity of STAT3 in many cancers, there are also examples were STAT3 activation has pro-apoptotic and growth inhibitory functions. Bromberg and colleagues found that, in clinical thyroid cancer samples, levels of phosphorylated STAT3 negatively correlated with tumor size and metastasis (228). Furthermore, loss of STAT3 expression increased thyroid cell tumorigenicity *in vivo* in both xenograft and

orthotopic mouse models (228). This study highlights a possible tumor suppressor role for STAT3 in thyroid cancer. Similarly, STAT3 behaves as a tumor suppressor in phosphatase and tensin homolog-deficient glioblastomas (229). Moreover, STAT3 deficiency in the adenomatosis polyposis coli (APCMin) model of intestinal cancer led to enhanced tumor progression, characterized by the development of invasive, more proliferative carcinomas (230). Study also shows a correlation with STAT3 activity and better survival. For example, node-negative breast cancer patients with positive phospho-STAT3 nuclear expression have a significantly improved survival at both short (5 year) and long-term (20 year) (231). In conclusion, as noted above, STAT3 is constitutively activated in many cancers and multiple lines of evidence places STAT3 at a central node in the development, progression, and maintenance of various malignancies. Hence, even though not being a *bona fide* oncogene, but because of its direct activation by other oncogenic factors and its role in tumor growth and metastasis, STAT3 can still be considered an appropriate target for cancer therapy.

3.4 STAT3 as a target for cancer therapy

Transcription factors can be the ideal target for the cancer therapy since large numbers of signaling pathways converge on a limited set of nuclear transcription factors. These transcription factors are the final switch that changes the gene expression pattern of the cells, leading to malignancies. Additionally, targeting a single transcription factor can blocks the multitude of upstream genetic aberrations that leading to cancer. Thus, strengthen the concept that transcription factor as a logical choice for cancer therapy (232). However, to be an ideal target for cancer therapy, a transcription factor needs to fulfil certain criteria: First, it must be overactive in large percentage of cells in different tumor types. Second, good therapeutic targets must be susceptible to specific inhibition

by small-molecule inhibitors or drugs. Third, tumor cells should be more dependent on the activity of target than normal cells. On the basis of these criteria, STAT family transcription factors particularly STAT3 emerged as a promising molecular target for cancer therapy (233).

Compelling evidences suggest that STAT3 is overactive in many cancers and it is alone sufficient to induce transformation of the cells and transformed cells forms tumors *in vivo* (225). In contrast, blocking aberrant STAT3 signaling decreased susceptibility to malignant transformation of a number of cell types (234).

Importantly, electroinjection of dominant-negative STAT3 variant into pre-existing B16 tumors caused inhibition of tumor growth as well as tumor regression *in vivo* (235), making the compelling argument that targeting STAT3 can block tumor growth. Additionally, antitumor bystander effects have also been observed in tumors treated with STAT3 dominant-negative gene therapy i.e., adjacent tumor cells also underwent apoptosis without having undergone gene therapy. These results provide *in vivo* proof of principle that STAT3 is a valid molecular target for cancer therapy. Since the discovery of STAT3 as an oncogene, more than 2,500 articles have been published demonstrating the importance of this pathway in virtually all malignancies and confirmed that STAT3 inhibition prevent tumor growth and metastasis (236).

STAT3 signaling is obligatory for growth and survival of various human tumor cells. Interruption of constitutive STAT3 signaling inhibits expression of the anti-apoptotic Bcl family members, Bcl-xL, Bcl-2 and Mcl-1, increases the expression of the pro-apoptotic Bax protein and sensitizes tumor cells to chemotherapy-induced apoptosis (131). Further, suppression of STAT3 activation inhibits invasion (by inhibiting MMP2 and MMP9), migration (by microtubule depolymerisation) and angiogenesis (by inhibiting VEGF and HIF1α) in tumor cells (161).

Finally, tissue-specific ablation of STAT3 has shown that non-oncogenic cells or normal cells lacking STAT3 proliferate and survive well *in vitro* and *in vivo* (237). Furthermore, inhibition of STAT3 in mouse fibroblasts does not have deleterious effects in normal cell growth (237). This selective response to STAT3 inhibitors in tumor versus normal cells might reflect that tumor cell show oncogenic addiction to the STAT3 for growth and survival, whereas normal cells do not or use alternative pathways (238). Overall, STAT3 seems to be the most promising candidate, not only due to its activation in many tumors, but also because of its immunosuppressive activity around tumor microenvironment (239). Its inhibition could slow down the proliferation of oncogenic cells, as well as stimulate antitumor immunity, which should therefore result in a potent reduction of tumor growth.

3.5 Strategies to inhibit STAT3 signaling

As stated above, STAT3 is a valid target for cancer therapy. Large efforts are currently underway to develop STAT3 inhibitors that efficiently inhibit STAT3 signaling. However, this approach faces several challenges because STAT3 does not have enzymatic activity and, unlike the other receptors (e.g. estrogen receptor), it does not naturally bind to small ligands. Nevertheless, the multiple steps involved in STAT3 activation from cell surface receptors to the transcriptional activity in the nucleus affords several possible strategies to inhibit STAT3 signaling. Some of these strategies include:

i) Development of receptor/ligand antagonists and receptor-neutralizing antibodies

As receptor overexpression is frequently associated with many types of cancers, use of receptor antagonists could in principle have promising results. Receptor or ligand antagonists include molecules that are structurally related to but lack the intrinsic

activating property of the physiological ligand and possess higher affinity for receptor. Sant7, an IL-6 superantagonist inhibits constitutive STAT3 activation and tumor growth in human lung fibroblast (240). Similar to ligand antagonists, use of receptor neutralizing antibody, for example, anti-EGF-R antibodies, and anti-HER2 antibody seems appealing as therapeutic approach to block aberrant signaling. Anti-EGFR antibodies, cetuximab and panitumumab and anti-HER2 antibody herceptin/ trastuzumab are FDA approved drugs for treatment of various malignancies (241). Other monoclonal antibodies in clinical development are zalutumumab, nimotuzumab, and matuzumab.

ii) Tyrosine or serine kinase inhibitors

As STAT3 is activated by various receptor or non-receptor tyrosine kinases, inhibiting these kinases represent one of the most exploited approach for therapeutic intervention. Inhibition of receptor tyrosine kinase activity (such as JAKs activity) inhibits growth of various cancer cells and it correlates with inhibition of constitutive STAT3 activity in cultured tumor cells (242). Similarly, inhibition of non-receptor tyrosine kinases such as Src or Bcr-Abl inhibits tumor growth and induces apoptosis by targeting STAT3 signaling. Tyrphostin, selective protein tyrosine kinase inhibitors have antitumor activity both *in vitro* as well as *in vivo*. Ni and co-workers reported that Janus kinase inhibitor, tyrphostin AG490, inhibited the constitutive activation of STAT3 and suppressed the growth of human prostate cancer cells (243). AG490 also inhibited STAT3 signaling in multiple myeloma and Hodgkin lymphoma (244). Ruxolitinib, an oral JAK1 and JAK2 inhibitor is the first JAK inhibitor that has been approved by the FDA for the treatment of myelofibrosis (245). Currently, ruxolitinib is also in phase II trials for the treatment of haematological malignancies and prostate cancer. Another JAK inhibitor SAR302503 is currently in phase I clinical trial for the treatment of solid

tumors. Pacritinib, a JAK2 inhibitor is in a phase III trial to treat myelofibrosis and in several phase I/II trials in other hematologic malignancies (245). Another JAK2 inhibitor AZD1480 potently blocks STAT3 signaling and currently in phase I trial in treatment of solid tumors (245). Other JAK inhibitors such as BMS-911543, AC-430, CEP-33779 are in either in preclinical stages or in early stages of development **(Table 3.5)**.

STAT3 serine phosphorylation is required for its maximal transcriptional activity. So, inhibition of this post-translational modification would be sufficient to impair STAT3 activity. It is observed that pharmacological inhibitor of p38 MAP kinase activity inhibits STAT3 serine phosphorylation and cell transformation induced by v-Src (246).

Inhibitor	Targeted JAK	Indication
BMS-911543	JAK2	Myelofibrosis
AC-430	JAK2	Rheumatoid arthritis, Lymphoma
CEP-33779	JAK2	Rheumatoid arthritis
R723	JAK2	Myeloproliferative neoplasias

Table 3.5: Selected JAK inhibitors in pre-clinical stage *[modified and adapted from(245)].*

iii) Alteration of STAT3 interacting proteins and modulation of phosphatases

Physiological negative regulators of JAK/STAT signaling comprise various endogenous proteins that can be explored for targeting STAT3 pathway. These include protein inhibitors of activated STAT (PIAS) and cytokine-inducible SH2- containing protein (CIS). The PIAS family of protein directly interacts with activated STATs, interfering

with their DNA-binding activity, and inhibiting gene transcription (154). On the other hand, CIS family regulates STATs signaling either by inhibiting the activity of JAKs, or by competition with STATs for phosphorylated docking sites on the receptors, or by targeting bound signaling proteins to the ubiquitin proteasome pathway (206). Thus, designing of small molecule pharmacological inhibitors that mimics PIAS or CIS would have potential therapeutic application. StIP1, a newly identified STAT3 interacting protein acts a scaffold protein that regulates ligand-dependent STAT3 activation (247).

Protein phosphorylation, mediated by kinases plays an important role in regulating almost all fundamental cellular processes. Various diseases including cancer are known to be associated with aberrant kinase signaling. Thus, the activity of protein phosphatases, enzymes that oppose protein kinases represent an essential regulatory mechanism that can be exploited for therapeutic benefits. SHP family phosphatase particularly SHP1 and SHP2 as well as protein serine /threonine phosphatase are involved in modulating STATs functions. SHP1 phosphatase has been found to function as tumor suppressor and its expression is diminished in most leukemia and lymphoma cell lines and tissues, and in some non-hematopoietic cancer cell lines, such as breast cancer cell lines and some colorectal cancer cell lines (248). Recently, Bard-Chapeau and co-workers showed that decrease in SHP2 expression enhances JAK/STAT signaling in hepatocellular carcinoma (249). Also, loss of SHP1 enhances JAK3 and STAT3 phosphorylation (250). Specific strategies will be required to design small molecules that will selectively promote protein phosphatase activity as well as capable of downregulating STAT3 signaling.

iv) Development of small molecule inhibitors that interfere with STATs dimerization

Dimerization of STATs molecule via reciprocal phosphotyrosine-SH2 interactions is a key event in the activation of STAT signaling. STAT3 dimerization is essential for their nuclear translocation and gene transcriptional activity. Disrupting STAT3 dimerization therefore appears to represent an excellent conceptual framework to selectively block the aberrant activity and functional effects of this protein. However, targeting protein functions/dimerization has remained a challenging task. With respect to chemical design, disrupters of STAT dimerization could be SH2-like peptides with high affinity for the phospho-tyrosine region of STATs or small peptide mimetics that are specific for the SH2 sequence of STATs. Ideally these peptides should possess certain characteristics, including a stronger affinity for STAT monomer and cohesive interaction that generates a hetero-complex of STAT-inhibitor over STAT-STAT dimers. Structure-based design led to the identification of a phosphopeptide inhibitor derived from the STAT3 SH2 domain-binding peptide sequence, PY*LKTK (where Y * represents pTyr). PY*LKTK disrupted STAT3:STAT3 dimers and STAT3-DNA binding activity *in vitro* (251). This study provided a validation of the concept to disrupt STAT3:STAT3 dimerization and activity using a small-molecule peptide.

v) Inhibitors of STATs nuclear translocation

Like other transcription factors, the STATs protein need to gain access to the nucleus to produce their effects, and therefore nuclear trafficking has an integral role in their function. However, the detail mechanisms how STATs protein translocates to the nucleus are not clear. Earlier studies suggested that nuclear localization signal present at N terminal region of STAT protein is required for but does not guarantee nuclear translocation. Tyr phosphorylation of STATs is also not essential for nuclear translocation. In addition, certain STATs have been found to interact physically with

non-STAT proteins and this interaction might influence nuclear localization. Thus, understanding the details of translocation mechanisms of STATs, including specificity of interactions with other proteins would provide the necessary information for design of selective inhibitors to target STAT nuclear trafficking for clinical interventions.

vi) Direct blocking of STAT DNA-binding and transcriptional activity

STATs are DNA-binding transcription factors, and thus, their functions depend upon their binding to the specific DNA promoter. Structural studies revealed that the transcriptional activity of STAT3 requires the physical interaction of its DNA-binding domain with the consensus DNA-binding sequence in the promoter regions of responsive genes. Idea about this conserved amino acids pattern could provide the basis for the development of artificial competitors of STAT-DNA binding activity. A class of platinum (IV) compounds has been reported that potentially block the DNA-binding activity of STAT3. Mechanistic studies revealed that these compounds interact with STAT3 DNA-binding domain, thus disrupting the binding to the STAT3-responsive DNA sequences (252). Galiellalactone, a tetrahydro- isobenzofuranone derivative isolated from ascomycete was reported to block STAT3 DNA-binding activity (251). Galiellalactone selectively inhibited IL-6-induced STAT3 DNA-binding and transcriptional activity without affecting STAT3 phosphorylation in HepG2 hepatoma cells (251). Recently, 20-mer peptide aptamers have also been identified to specifically interact with the DNA-binding domain of STAT3 and in turn inhibit STAT3 DNA-binding and transcriptional activity (253).

Apart from DNA-binding domain, other domains such as coiled-coil domain and C-terminal domain of STATs participate in co-operative binding with transcriptional coactivators such as CBP/p300, Sp1 or with nuclear adaptor proteins that are also

essential for their transcriptional activity. These requirements for transcriptional activity suggest it is possible to modulate the transcriptional activity and biological functions of some STATs by using small molecule mimics of coactivators or pseudo-coactivators that would interfere with the cooperative interactions.

vii) Oligonucleotide approaches to inhibit STATs signaling

Oligonucleotide technology including antisense RNA, small interfering RNA (siRNA), and decoy oligodeoxynucleotide (ODN) were developed to inhibit target gene expression. The application of antisense technology is already forging ahead in the number of existing clinical trials. The knockdown of the STAT3 protein by antisense RNA or siRNA approaches has been demonstrated in studies, which showed the induction of tumor cell apoptosis and tumor regression.

Decoy ODN is a double-stranded oligonucleotide, when introduced into cells, compete for the binding of the targeted transcription factor with the endogenous *cis*-elements, resulting in the downregulation of the transcription factor mediated gene transcription. STAT3 decoy comprises of a 15-bp double-stranded oligonucleotide, which corresponds closely to the STAT3 response element in the c-fos promoter and binds competitively to STAT3 (254). STAT3 decoy inhibited proliferation and STAT3 mediated gene expression in squamous cell carcinoma of the head and neck *in vitro* as well as in a xenograft model *in vivo* (254). Sen and co-workers conducted first phase 0 clinical trial using STAT3 decoy in head and neck cancer and reported that intratumoral administration of STAT3 decoy oligonucleotide abrogated target gene expression in patients with head and neck squamous cell carcinoma (255). STAT3 decoy has also been shown to inhibit the growth of human lung cancer and glioma (254). Recently, a new class of ODN inhibitors against STAT3 was developed, designated as G-quartet

ODN. These are G-rich ODN with the ability to form inter- and intra-molecular four-stranded G-quartet structures. Studies suggest that G-quartet ODN disrupts STAT3:STAT3 dimers, inhibits the binding to DNA, and hence selectively blocks STAT3 activity, without affecting the other members of the STAT family (254). **Table 3.6** summarizes the STAT3 inhibitors that are currently in clinical development according to www.clinicaltrails.gov (131).

Inhibitor	Phase	Condition	STAT3 targeting strategy	Status
STAT3 decoy	0	HNSC	DBD oligonucleotide decoy	Complete
ISIS-STAT3$_{RX}$	1/2	Advanced cancers, lymphoma	Antisense oligonucleotide	Recruiting
AZD 9150	1	Advanced hepatocellular carcinoma	Antisense oligonucleotide	Not yet recruiting
OPB-31121	1	Advanced solid tumors	Small molecule	Complete

Table 3.6: STAT3 inhibitors currently in clinical development *[adapted from (131)].*

3.6 STAT1 and cancer

STAT1, the founding member of STATs family protein is a central mediator of both Type I (alpha and beta) and Type II (gamma) IFNs. Most, if not all, normal functions of STAT1 are closely related to the biological effects of IFNs since its utilization is notably specific to IFN ligands. Similarly, as described above, STAT1-deficient mice have lost responsiveness to both types of IFNs and acquired enhanced susceptibility to bacterial and viral pathogens. Studies that showed that STAT1 exert growth inhibition of cultured cells has led to the idea that deficiency of STAT1 might result in altered cell growth and disturbed immune functions, i.e. disorders that are pertinent to malignancy

(154) . Inappropriate STAT1 activation or loss of its expression has been observed in different histological types of tumors such as breast cancer, head and neck cancer, melanoma, leukaemia, and lymphoma. In many types of tumors STAT1 induces anti-proliferative and pro-apoptotic genes that directly hamper tumor growth (256). In particular, STAT1 activates the transcription of genes encoding for caspases, death receptors and their ligands, and inducible nitric oxide synthase (iNOS) (256). STAT1 can also negatively regulate the expression of prosurvival genes such as Bcl-xL and Bcl-2. Moreover, STAT1 was also shown to favour apoptosis by directly interacting either with TNFR1 and TRADD or with p53 in DNA damage-induced apoptosis (256). In addition to pro-apoptotic role, STAT1 was also known to regulate the transcription of genes involved in cell cycle control, either inducing the cyclin dependent kinase (CDK) inhibitors p21waf/cip1 or p27Kip1 or repressing the expression of c-Myc and many cyclins (256). All these studies sustained the idea that STAT1 might function as a tumor suppressor. However, some studies suggest that, under specific conditions STAT1 can instead favour carcinogenesis and tumor survival, confirming the complexity of this biological system (223).

In striking contrast with STAT1, STAT3 is considered an oncogene, since its overexpression of a constitutively active form could itself be responsible for cellular transformation (225). In addition to being involved in cellular transformation, STAT3 also participates in cellular proliferation and cell survival. It upregulates the expression of cyclin D1, cMyc and growth promoting gene pim-1. While on other hand, it suppresses apoptosis by modulating the expression of anti-apoptotic genes such as Bcl2, BclxL, Mcl1, survivin and cIAP2 (257). Moreover, STAT3 was shown to negatively regulate the expression of p53, which is considered to be the most common inhibitor of cellular proliferation as well as inducer of apoptosis (258).

3.7 STAT1:STAT3 cross regulation

As described above, STAT1 and STAT3 often play opposing roles in proliferation, apoptosis and anti-tumor immune responses. One important observation is that they share many activating stimuli. Cells are normally exposed to a complex cytokine milieu, and modulation of their responses depends upon relative abundance and activation level of STAT1:STAT3 (259). Indeed, several studies on STAT-deficient cells have revealed the existence of reciprocal STAT1:STAT3 regulatory mechanisms. For example, in STAT3-deficient murine embryonic fibroblast (MEFs), IL-6 triggers prolonged activation of STAT1 correlating with an IFNγ-like response, including up-regulation of multiple IFNγ-inducible genes. Accordingly, increased and prolonged phosphorylation of STAT1 in response to gp130 cytokines occurs in several systems upon STAT3 gene inactivation (260). These data suggest that in normal cells one of the functions of STAT3 in response to IL-6 is to downregulate STAT1 activity, thus preventing IFNγ-like responses and allowing IL-6-specific signaling. Taken together, these observations suggest that the relative abundance of STAT3 or STAT1 may play a role in determining their relative activation levels and biological effects in response to activating stimuli. In turn, this may be relevant for the development and growth of tumors in the presence of specific tumor microenvironments, where different cytokine/growth factor combinations can modulate the relative levels of STAT1 and STAT3, resulting in their differential activation. Under normal physiological condition, there are very finely tuned equilibria between STAT1:STAT3 **(Figure 3.5)**. However, during tumorigenesis this balance (STAT1:STAT3) is altered. Alterations of the balanced expression and/or activation of STAT1 and STAT3 may lead to unexpected results and directing cells towards tumorigenesis (257). Thus, care should be taken in

the design of therapeutic interventions using compounds that could unbalance the finely tuned equilibrium that lies at the base of STAT1:STAT3 signaling (261).

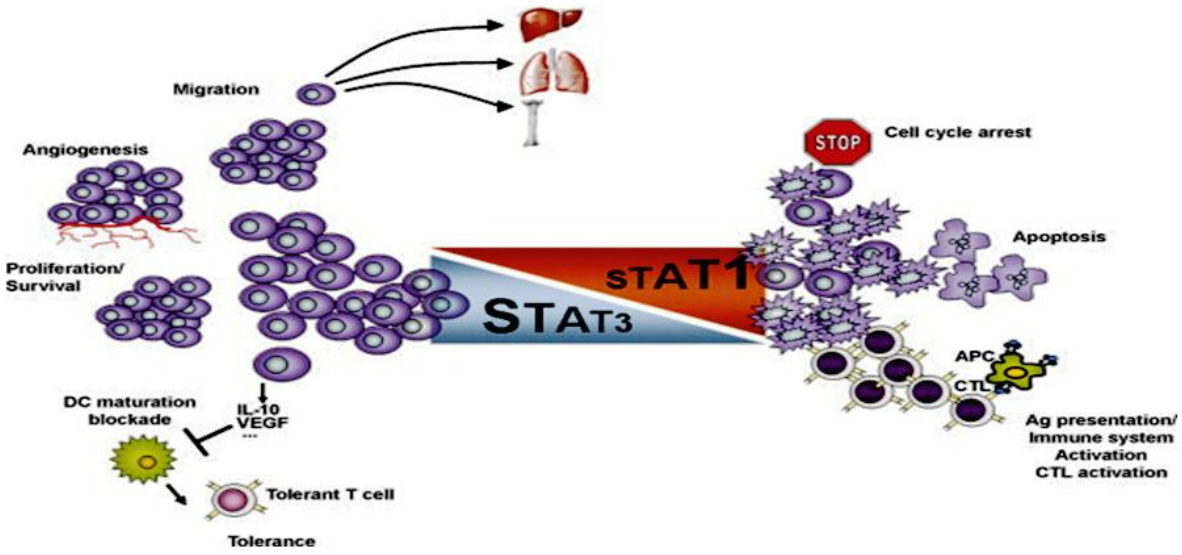


Figure 3.5: STAT1:STAT3 cross regulation and balanced expression *[adapted from (256)]*.

3.8 MATERIALS AND METHODS

3.8.1 Immunofluorescence staining

Reagent

Fixative: 4% paraformaldehyde (PFA).

Permeabilizing solution: Chilled methanol at -20°C.

Blocking solution: 1% BSA made in PBS.

Primary antibodies against STAT3 and pSTAT3: Diluted in PBS.

Secondary antibody conjugated with Alexa 568: Diluted in PBS.

Nuclear counter stain DAPI: 5mg/ml.

Mountant: 1% DABCO in 90% of glycerol PBS.

Transparent nail polish.

Protocol

Cells were grown to a confluency of 60-70% on coverslips and treated with PTX (0, 1, 2 and 3 mM) for 24 h. Cells were fixed in 4% PFA for 3 minute. They were permeabilized with chilled methanol for 10 minute at -20°C and were blocked in 1% BSA for half an hour. The coverslips were overlaid with primary antibody (1:30) and incubated in a humidifying chamber for one hour. After incubation, the coverslips were given three washes with PBS and overlaid with secondary antibody conjugated with the dye Alexa 568 (1:200). After one hour, three washes were given with PBS and the coverslips were then overlaid with DAPI for nuclear staining. Subsequently, three washes were given with PBS and coverslips were mounted on glass slides in 4% DABCO glycerol. Cells incubated with the fluorochrome conjugated secondary antibody alone (no primary antibody) were used as negative controls. Images were acquired at 63X using the LSM 510 META laser scanning microscope from Carl Zeiss, Germany. Images were analyzed using the software LSM 510.

3.8.2 DNA binding assay

Reagent

TransAM Nuclear fraction extraction kit.

TransAM STAT3 activation kit.

Protocol

Cells were grown to a confluency of 60-70% and treated with PTX (0, 1, 2 and 3mM) for 24 h. Cells were harvested and nuclear fraction was extracted using TransAM nuclear fraction extraction kit according to manufacturer's protocol. DNA binding assay was carried out using the TransAM STAT3 activation kit following the manufacturer's protocol. 10 µg of nuclear extract was added to the oligonucleotide coated 96 well plate. After 1 h primary antibody was added and incubated at 37 °C for 1 h. After 3 washes with washing buffer HRP- conjugated secondary antibody was added and incubated for 1 h at room temperature. Plates were given 4 washes with washing buffer and developing solution was added. Plates were monitored for blue color development in the positive control and sample well. When dark blue color appeared in positive control well, stop solution was added. Reading was taken using ELISA reader at wavelength of 450 nm with a reference of 655 nm.

3.8.3 ELISA, western blotting and semiquantitative RT-PCR: as described in chapter 2

3.9 RESULTS

3.9.1 PTX inhibits constitutive as well as IL-6 inducible STAT3 phosphorylation

A375 cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h, whole-cell extracts were prepared and western blot was carried out to evaluate the effect of PTX on STAT3 and pSTAT3 expression. An anti-phospho STAT3 (Tyr 705) antibody was used which detects endogenous levels of pSTAT3 only when phosphorylated at tyrosine 705. Data suggested that PTX inhibited pSTAT3 expression in a dose dependent manner without affecting total STAT3 protein **(Figure 3.6a)**. Additionally, it was also observed that treatment with PTX not only inhibited pSTAT3 expression at 24 h treatment but also at earlier time points i.e. ½ h, 1 h and 2 h of PTX treatment **(Figure 3.6b, c and d)**. However, total STAT3 protein remained unchanged at all the time points.

Because IL-6 induces STAT3 phosphorylation, we evaluated whether PTX could inhibit IL-6-inducible pSTAT3 expression. A375 cells were treated with sub-toxic doses of PTX for 24 h and then stimulated with IL-6 (20ng/ml) for 30 minute. Western blot data suggested that PTX treatment inhibited IL-6-inducible pSTAT3 expression in a dose dependent manner **(Figure 3.6e)**. However, total STAT3 protein remained unchanged. Furthermore, PTX treatment inhibited pSTAT3 expression in other cell lines such as A549 lung carcinoma and U87 glioblastoma **(Figure 3.7)**.

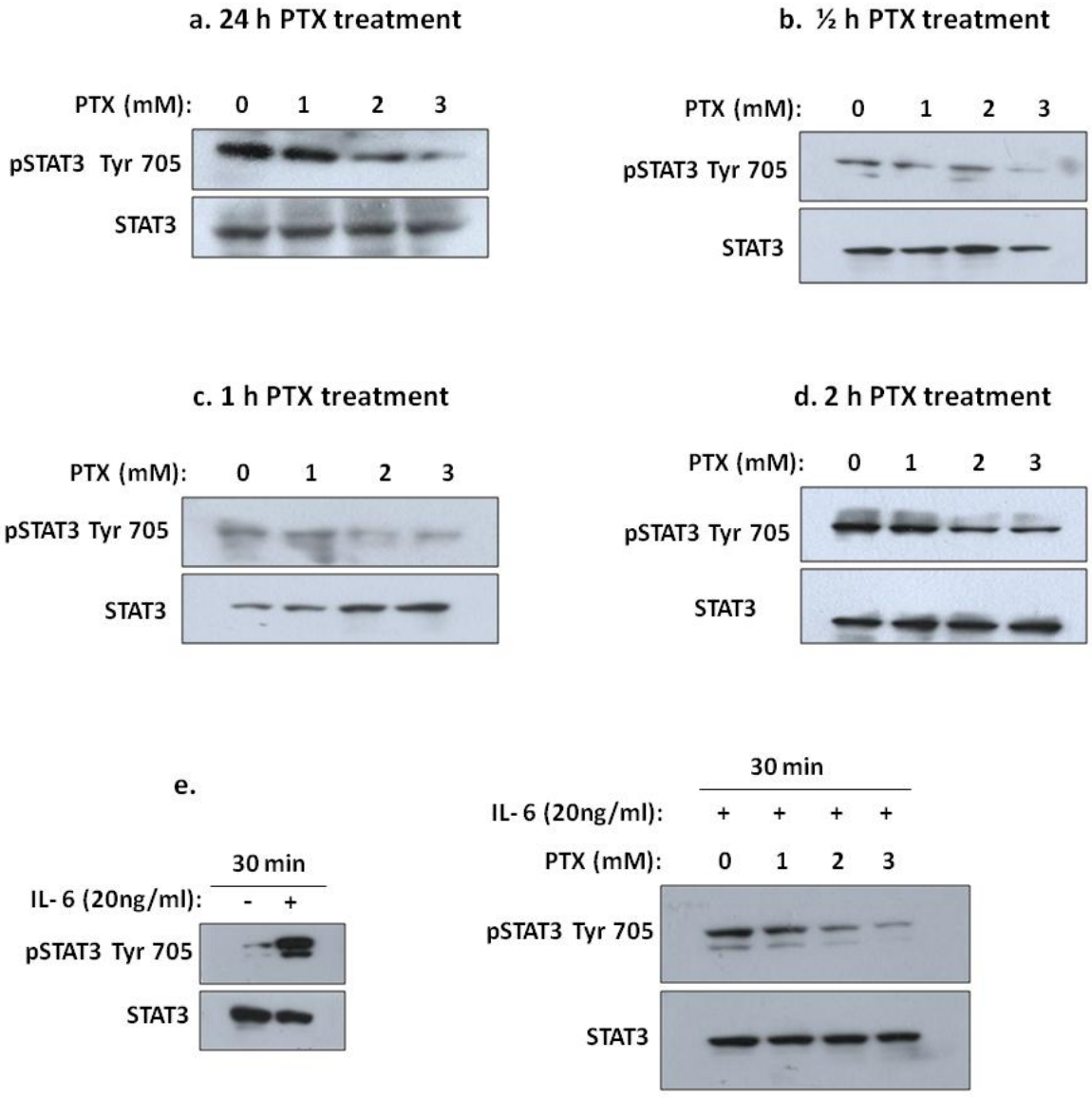


Figure 3.6: PTX inhibits constitutive and IL-6 inducible STAT3 phosphorylation in A375 melanoma cells. *Cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for* ***(a)*** *24 h,* ***(b)*** *½ h,* ***(c)*** *1 h,* ***(d)*** *2 h and* ***(e)*** *cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h and then stimulated with IL-6 (20 ng/ml) for 30 minute. Whole cell extracts were prepared and subjected to western blotting to determine level of phospho STAT3. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading.*

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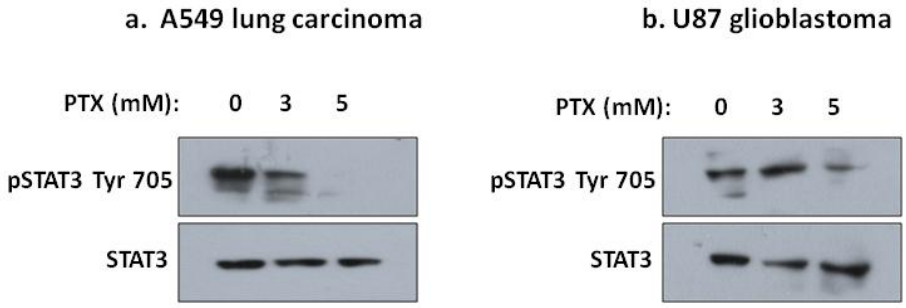

Figure 3.7: PTX inhibits STAT3 phosphorylation in (a) A549 lung carcinoma and (b) U87 glioblastoma. *Cells were treated with sub-toxic doses of PTX (0, 3 and 5 mM) for 24h. Whole cell extracts were prepared and subjected to western blotting to determine level of phospho STAT3. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading.*

3.9.2 PTX does not inhibit phospho STAT3 expression in normal cells.

For this study we used normal cell HEK-293 (Human Embryonic Kidney). HEK-293 cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h and then stimulated with IL-6 (30 ng/ml) for 30 minute. Whole cell extracts were prepared and subjected to western blotting to determine level of pSTAT3 and STAT3. Results suggested that, (a) in un-stimulated cells there was no pSTAT3 expression, but it was induced by IL-6 treatment (30ng/ml) for 30 minute. (b) PTX treatment neither inhibited pSTAT3 nor STAT3 expression in IL-6 stimulated cells **(Figure 3.8)**.

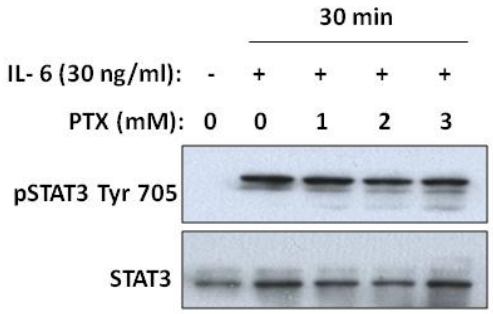

Figure 3.8: PTX does not inhibit phospho STAT3 expression in normal cells. *HEK-293 cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24h followed by stimulation with IL-6 (30 ng/ml) for additional 30 minute. Whole cell extracts were prepared and subjected to western blotting to determine level of phospho STAT3. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading.*

3.9.3 PTX inhibits pJAK1, pJAK2 and VEGFR2 expression

Since PTX inhibited STAT3 phosphorylation, we evaluated the expression of upstream kinases that phosphorylate STAT3 protein. Various kinases that catalyze STAT3 phosphorylation include cytokine receptors (JAK family kinases), receptors with intrinsic tyrosine kinase activity (EGFR or VEGFR) and non-receptor tyrosine kinases (Src or Abl). It was observed that PTX inhibited pJAK1, pJAK2 and VEGFR2 expression in a dose dependent manner **(Figure 3.9)**. Total JAK1 and JAK2 levels were unchanged. PTX treatment neither inhibited pSrc nor Src expression **(Figure 3.9)**. PTX thus appears to regulate the STAT3 phosphorylation at least in part by inhibiting upstream kinases such as pJAK1, pJAK2 and VEGFR2.

3.9.4 PTX induces the expression of pSHP2 phosphatase

One of the other possibility by which PTX may regulates STAT3 phosphorylation is by modulating the expression of tyrosine phosphatases. To confirm this, A375 cells were treated with different concentrations of sodium orthovanadate (broad acting tyrosine phosphatase inhibitor) for 25 minute before 3 mM PTX treatment for an additional 2 h. After that, whole cell extracts were prepared and western blot was carried out. It was observed that phosphatase inhibitor reversed the effect of PTX, suggesting a contributive role for protein tyrosine phosphatase in STAT3 dephosphorylation induced

by PTX **(Figure 3.10a)**. SHP2 is a ubiquitously expressed non-receptor protein tyrosine phosphatase and has been reported to play an important role in negatively regulating of JAK/STAT3 signaling (262). We therefore evaluated the effect of PTX on SHP2 phosphatase. Western blot data suggested that PTX treatment increased the expression of pSHP2 phosphatase in a dose dependent manner without having any effect on expression of total SHP2 protein **(Figure 3.10b)**.

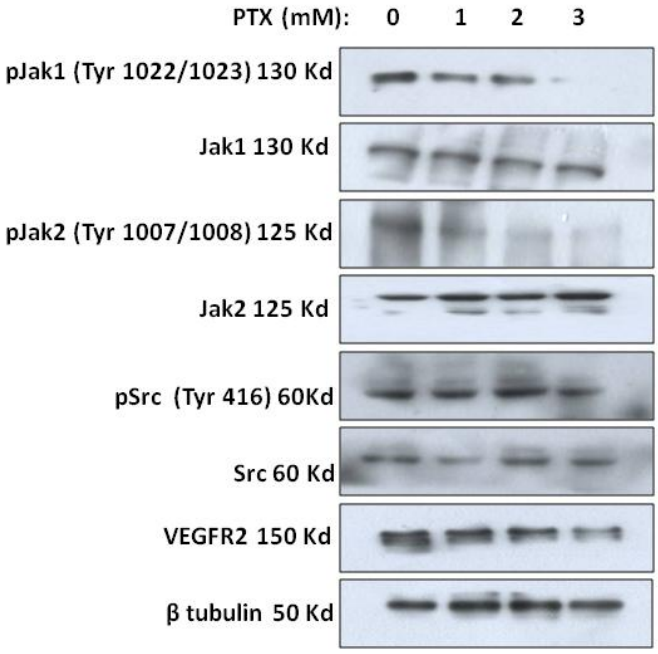

Figure 3.9: PTX inhibits phospho JAK1, phospho JAK2 and VEGFR2 expression in A375 melanoma cells. *Cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h. Whole cell extracts were prepared and subjected to western blotting to determine level of pJAK1, pJAK2, pSrc and VEGFR2. The same blots were stripped and reprobed with JAK1, JAK2, Src and β tubulin antibody to verify equal protein loading.*

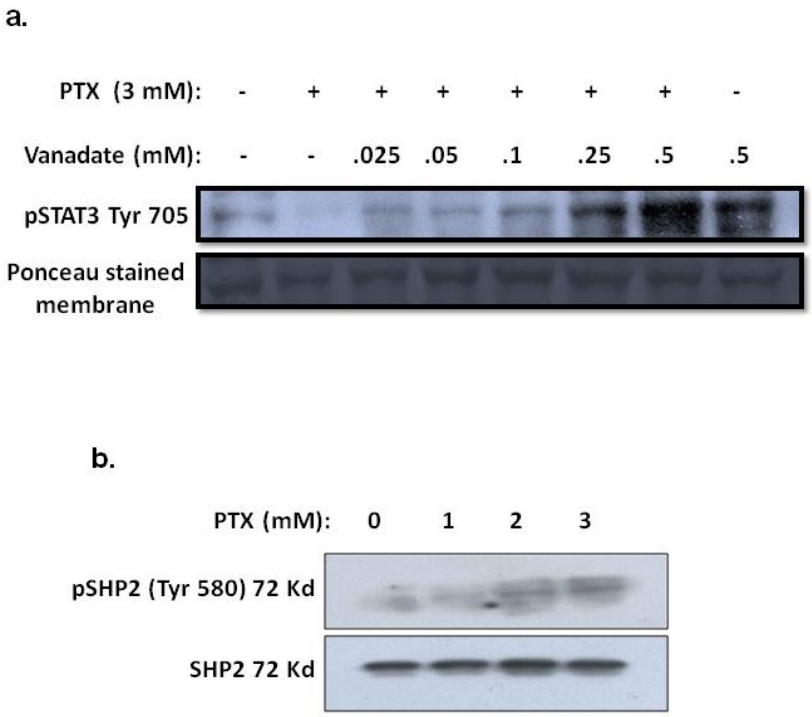

Figure 3.10: PTX modulates the tyrosine phosphatase expression. ***(a)*** *Vanadate reversed the inhibitory effect of PTX. A375 cells were treated with the indicated concentrations of vanadate for 25 minute followed by PTX treatment (3 mM) for additional 2h. Whole cell extracts were prepared and subjected to western blotting to determine level of phospho STAT3. The same ponceau stained membrane was used to verify equal protein loading.* ***(b)*** *PTX increased the pSHP2 expression. Cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h. Whole cell extracts were prepared and subjected to western blotting to determine level of pSHP2. The same blots were stripped and reprobed with SHP2 antibody to verify equal protein loading.*

3.9.5 PTX inhibits STAT3 nuclear translocation

Following activation, STAT3 dimerizes to each other and translocates to the nucleus for activation of transcription. Confocal microscopy study was carried out to evaluate the effect of PTX on STAT3 nuclear translocation. Results suggested that PTX treatment

inhibited STAT3 nuclear translocation in a dose dependent manner **(Figure 3.11)**. Further, we also evaluated the effect of PTX on pSTAT3 expression by confocal microscopy. Consistent with the immunoblottingt results shown in Figure 3.6, immunostaining results suggested that PTX treatment inhibited pSTAT3 expression in a dose dependent manner **(Figure 3.12)**.

3.9.6 PTX inhibits STAT3-DNA binding

In nucleus, binding of STAT3 to the promoter of target genes is essential for initiation of transcription. We evaluated the effect of PTX on STAT3-DNA binding activity using ELISA-based DNA binding assay. First, we standardized this technique in our laboratory using 10μg of nuclear extract. Wild type competitive oligonucleotide reduced STAT3 binding around 75%, while incubation with mutant STAT3 oligonucleotide has a limited effect on STAT3 binding to DNA. This method is upto 10-fold more sensitive and 40-fold faster than manual EMSA (electrophoretic mobility shift assay). A375 cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h and nuclear extracts were prepared. 10μg of nuclear extract were used to access the effect of PTX on STAT3-DNA binding. Results suggested that PTX treatment inhibited STAT3- DNA binding in a dose dependent manner **(Figure 3.13).** In addition, we have also showed that PTX inhibited STAT3-DNA binding in A549 lung carcinoma **(Figure 3.14)**.

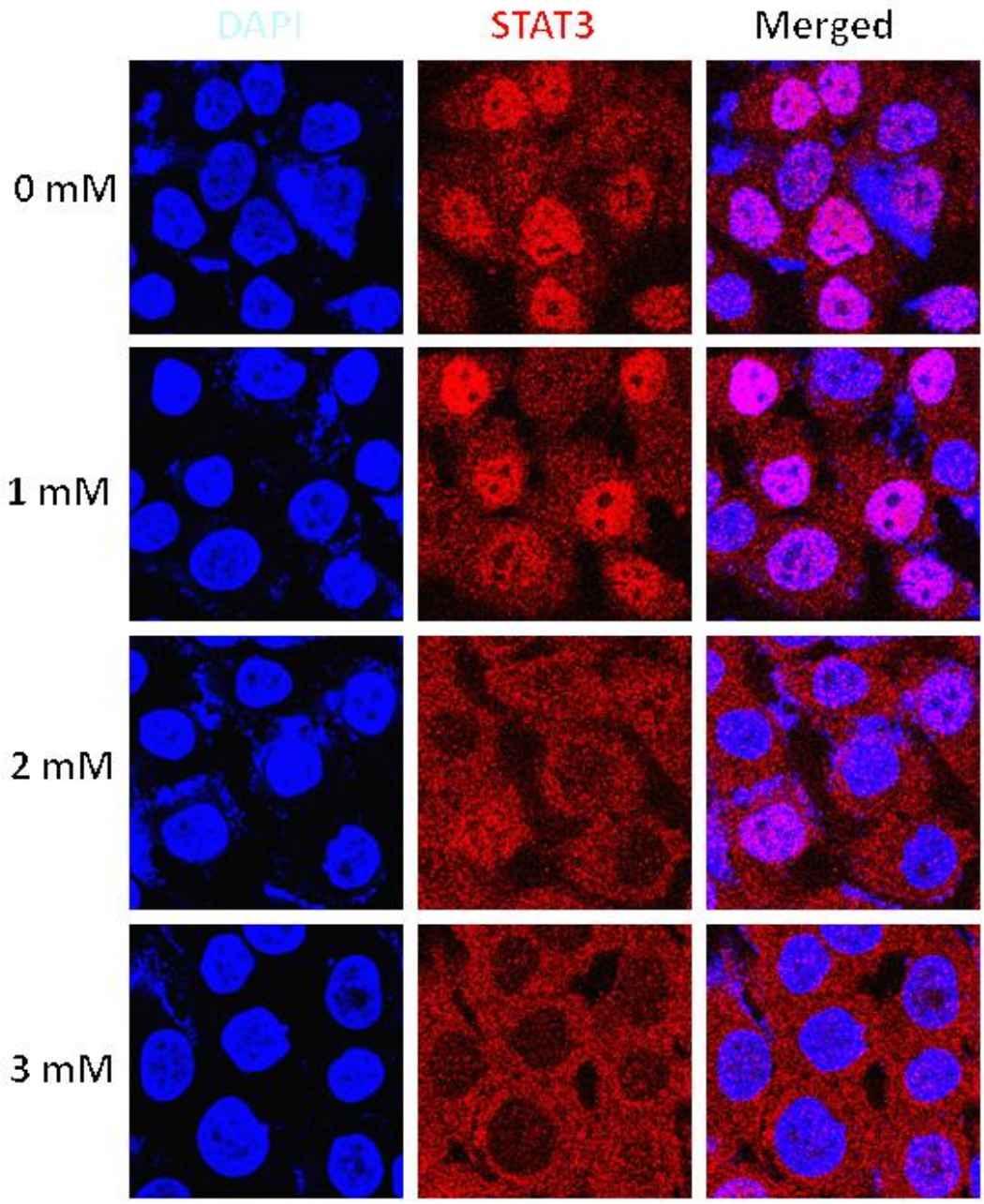

Figure 3.11: PTX inhibits STAT3 nuclear translocation in A375 melanoma cells. *A375 cells were treated for sub-toxic doses of PTX (0, 1, 2 and 3mM) for 24 h, fixed, permeablized and incubated with antibody against STAT3 followed by incubation with fluorescently conjugated secondary antibody. They were then analysed for the STAT3 distribution by confocal microscopy. Original magnification X63 with 2X zoom.*

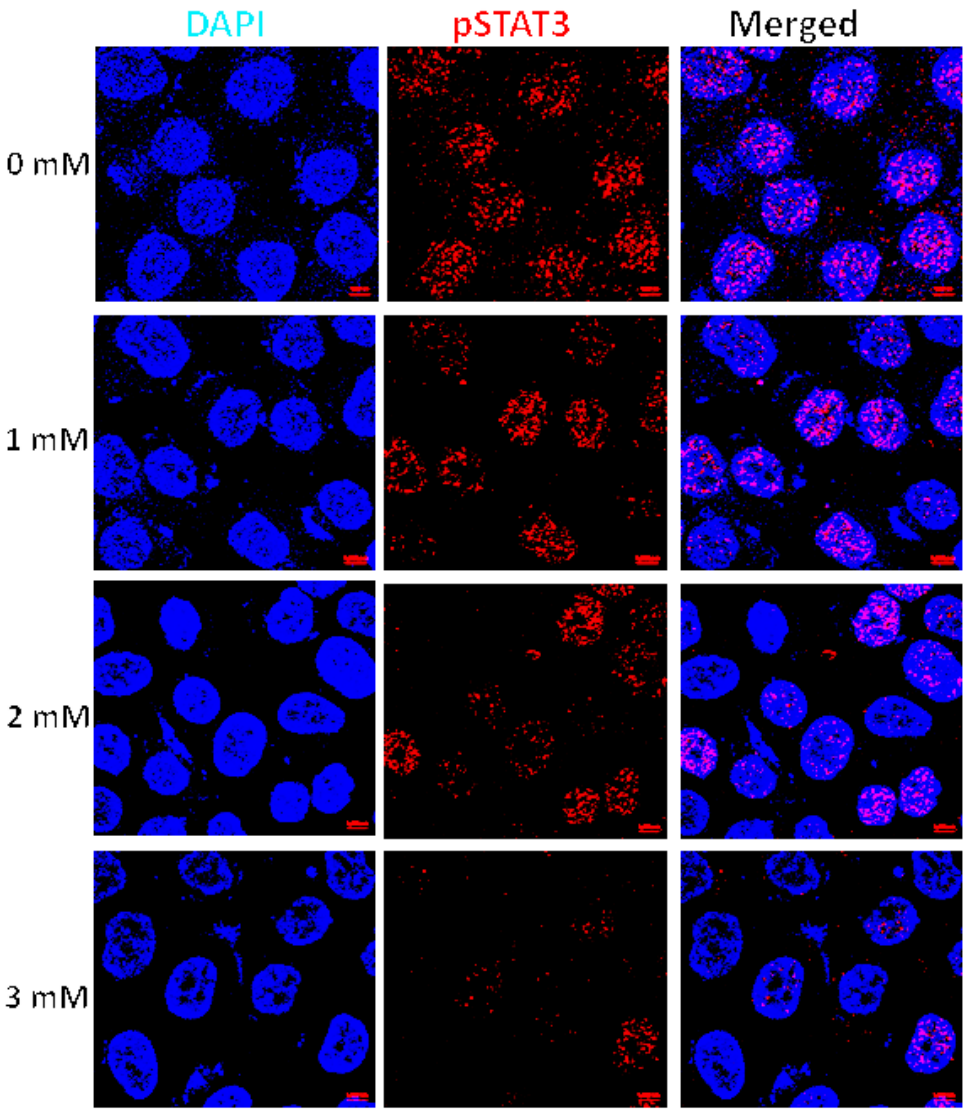

Figure 3.12: PTX inhibits pSTAT3 nuclear expression in A375 melanoma cells. *A375 cells were treated for sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h, fixed, permeablized and incubated with antibody against pSTAT3 followed by incubation with fluorescently conjugated secondary antibody. They were then analysed for the pSTAT3 expression by confocal microscopy. Original magnification X63 with 2X zoom. Scale bar 5µm.*

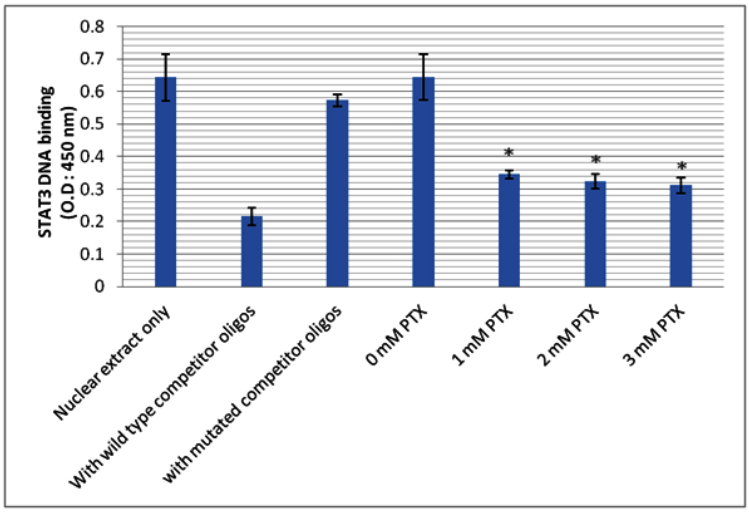

Figure 3.13: PTX inhibits STAT3 DNA binding in A375 melanoma cells. *A375 Cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h. Nuclear extracts were prepared and DNA binding assay was carried out as described in materials and methods.*

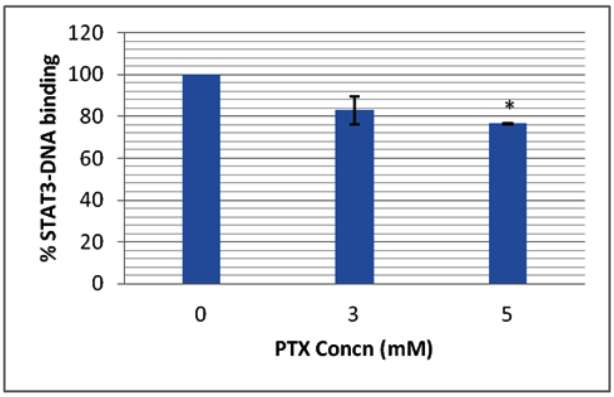

Figure 3.14: PTX inhibits STAT3 DNA binding in A549 lung carcinoma. *A549 cells were treated with sub-toxic doses of PTX (0, 3 and 5 mM) for 24 h. Nuclear extracts were prepared and DNA binding assay was carried out as described in materials and methods.*

3.9.7 PTX inhibits expression of proangiogenic molecules VEGF and HIF1α

VEGF is the major inducer of angiogenesis whose expression is regulated by STAT3. Reports suggest that STAT3 is function as a transcriptional activator of VEGF gene (263). RT-PCR was carried out to evaluate the effect of PTX on VEGF mRNA. It was observed that PTX inhibited VEGF mRNA at sub-toxic doses **(Figure 3.15a)**. In order to evaluate the effect of PTX on VEGF at translational level, western blot was carried out. Results suggested that PTX inhibited VEGF expression in a dose dependent manner **(Figure 3.15b)**. Further, by using ELISA from culture supernatant of melanoma cells, we showed that PTX inhibited VEGF secretion in a dose dependent manner **(Figure 3.16)**.

As a confirmation that PTX inhibited VEGF expression by targeting STAT3 signaling, A375 cells were treated with STAT3 specific inhibitor WP1066 and western blot was carried out. Results suggested that WP1066 inhibited pSTAT3 expression as well as VEGF expression **(Figure 3.17a)**. Additionally, we also used JAK2 inhibitor AG490 and showed that AG490 inhibited pJAK2, pSTAT3 and VEGF expression **(Figure 3.17b)**. Taken together, all these observations confirmed that PTX inhibited VEGF expression by targeting STAT3 signaling.

STAT3 has also been reported to induce expression of hypoxia-inducible factor-1α (HIF-1α), another key mediator of angiogenesis (127). In hypoxic conditions both STAT3 and HIF1α bind simultaneously to the VEGF promoter leading to its maximum transcriptional activation and angiogenesis (264). Our data showed that PTX inhibited HIF-1α expression in a dose dependent manner **(Figure 3.18)**.

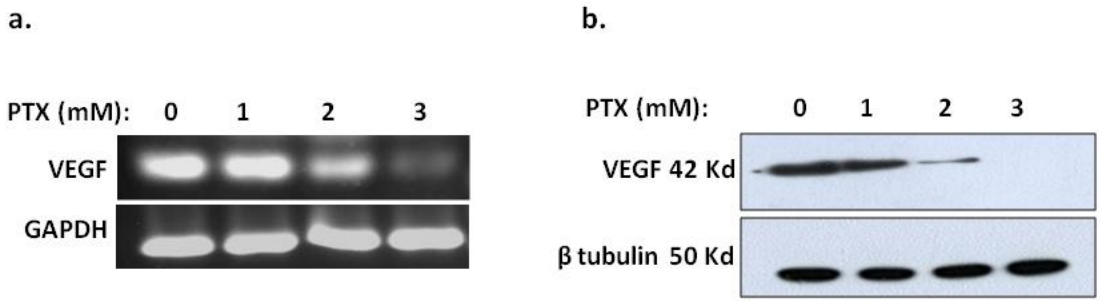

Figure 3.15: PTX inhibits VEGF expression in A375 melanoma cells. *A375 cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h and checked for expression VEGF.* ***(a)*** *RT-PCR results.* ***(b)*** *Western blot results. GAPDH and β tubulin were used as loading control.*

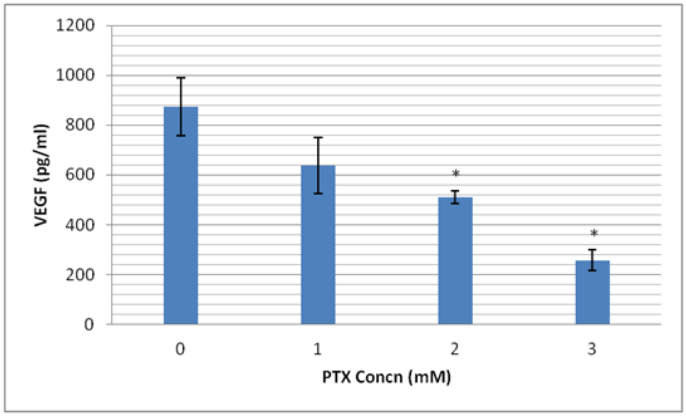

Figure 3.16: PTX inhibits VEGF secretion in A375 melanoma cells. *A375 cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h and VEGF secretion was measured by ELISA in culture supernatant after 24 h.*

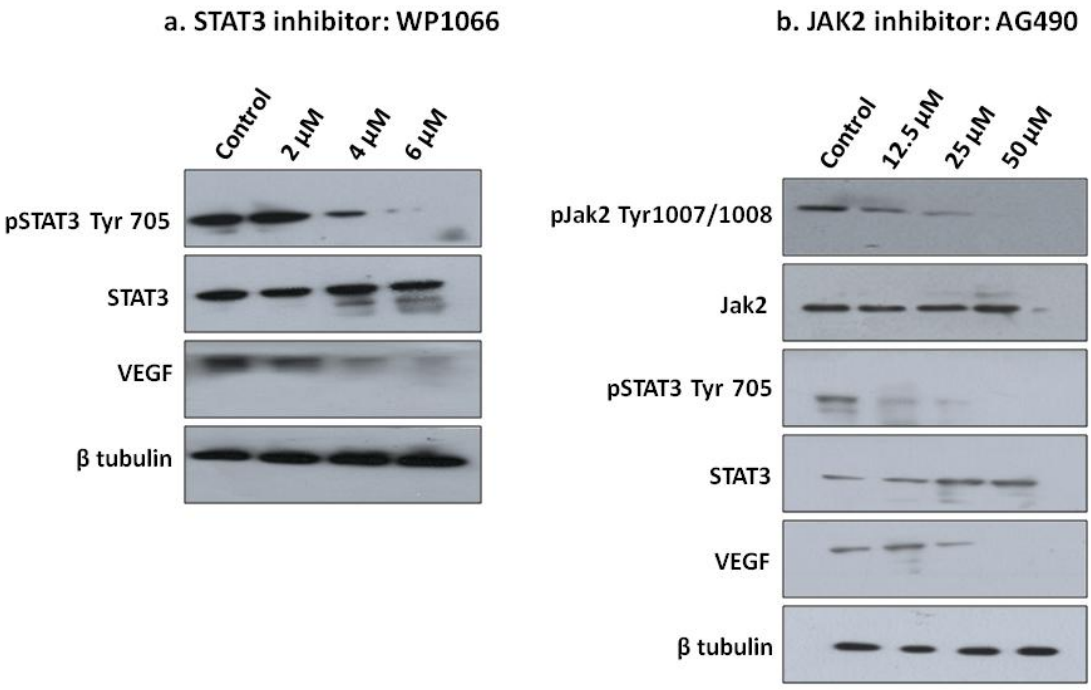

Figure 3.17: PTX inhibits VEGF expression by targeting STAT3 signaling*. A375 cells were treated with indicated concentration of* ***(a)*** *STAT3 inhibitor WP1066 and* ***(b)*** *JAK2 inhibitor AG490 for 24 h. Whole cell extracts were prepared and subjected to western blotting to determine level of pJAK2, pSTAT3 and VEGF. The same blots were stripped and reprobed with JAK2, STAT3 and β tubulin antibody to verify equal protein loading.*

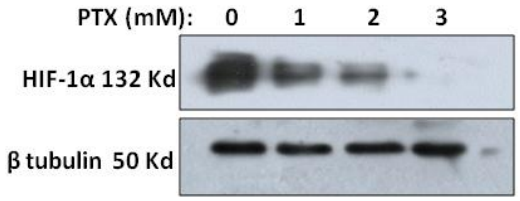

Figure 3.18: PTX inhibits HIF-1α expression. *A375 cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h. Whole cell extracts were prepared and subjected to western blotting to determine level of HIF-1α. β tubulin was used as loading control.*

3.9.8 PTX inhibits expression of genes involved in cellular proliferation and survival

In addition to being involved in angiogenesis, STAT3 also participates in cell growth and survival. Constitutive activation of STAT3 is associated with upregulation of genes involved in cellular proliferation such as cyclin D1 and cMyc. STAT3 has also been shown to upregulates the expression of anti-apoptotic protein BclXL. Western blot was carried out to evaluate the effect of PTX on these genes. Results suggested that PTX inhibited the expression of cyclin D1, CDK6, cMyc, BclXL and activated the caspase 3 and capase 9 activity **(Figure 3.19a and 3.19b).**

3.9.9 PTX inhibits IL-6 secretion in culture supernatant

In addition to upregulating numerous genes involved in cellular proliferation, survival, and angiogenesis, STAT3 induces the expression of many cytokines and chemokines such as IL-6 and other mediators, which are associated with cancer promoting inflammation (239)**.** IL-6 is shown to be elevated in serum level of patients with advanced prostate cancer (265). Increased IL-6 expression is also observed in human colorectal cancer (266). ELISA was carried out to evaluate the effect of PTX on IL-6 secretion. Results suggested that PTX inhibited IL-6 secretion in a dose dependent manner **(Figure 3.20)**.

3.9.10 PTX has no effect on phospho STAT1 expression

STAT1 is a founder member of STATs family transcription factors and shares 70% sequence homology with STAT3 (259). In contrast to STAT3, STAT1 is regarded as tumor suppressor. As PTX inhibited STAT3 phosphorylation, we have checked whether pSTAT1 or STAT1 expression is also affected by PTX in order to test the specificity of PTX towards STAT3. Western blot data suggested that PTX did not exhibit any effect

on both STAT1 and pSTAT1 expression, indicating that it is specific for STAT3 **(Figure 3.21)**.

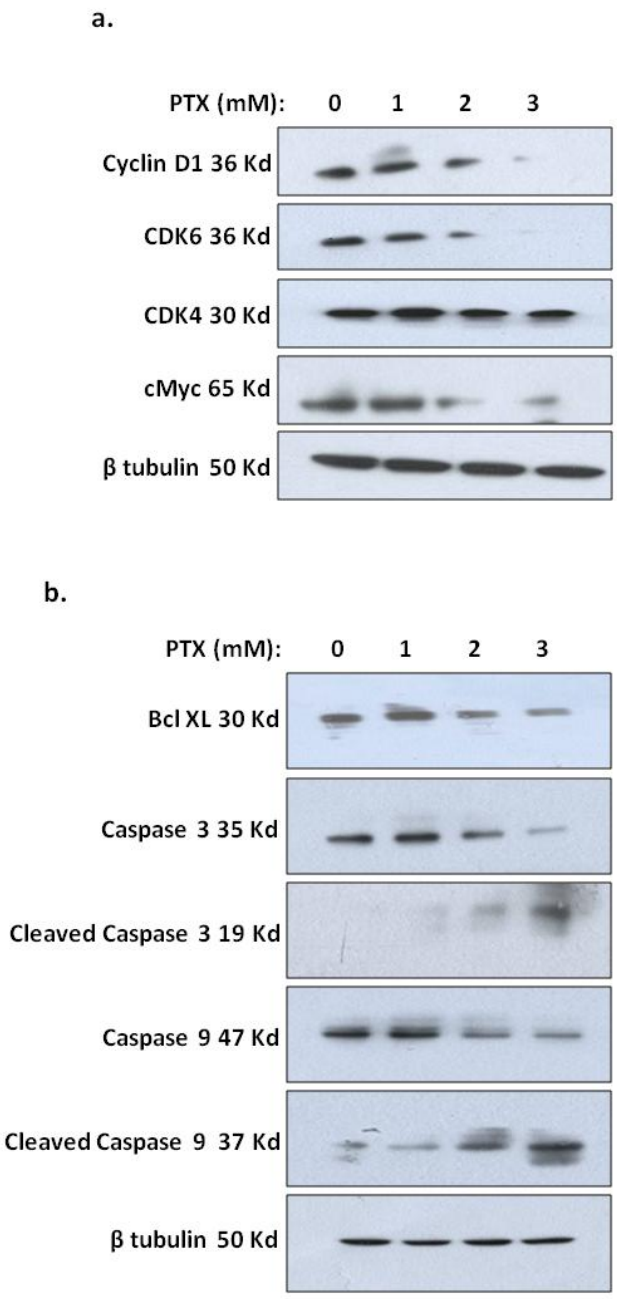

Figure 3.19: PTX inhibits expression of STAT3-regulated gene products involved in proliferation and survival. *A375 cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h. Whole cell extracts were prepared and subjected to western blotting to determine level of* ***(a)*** *Cyclin D1, CDK6, CDK4 and cMYC and* ***(b)*** *BclXL,*

caspase 3, cleaved caspase 3, caspase 9 and cleaved caspase 9. β tubulin was used as loading control.

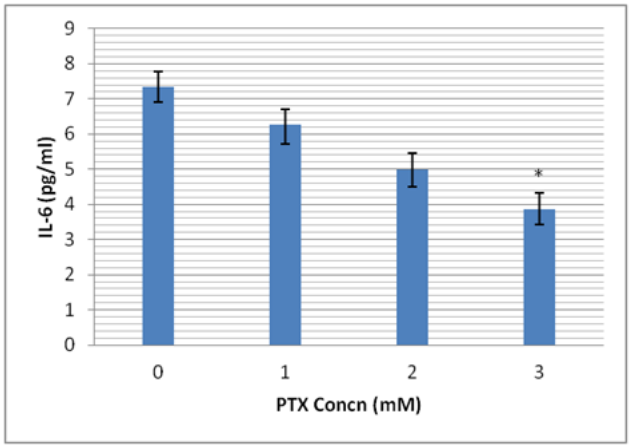

Figure 3.20: PTX inhibits IL-6 secretion. *A375 cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h and IL-6 secretion was measured by ELISA in culture supernatant after 24 h.*

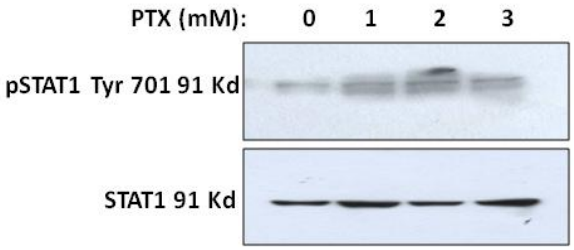

Figure 3.21: PTX does not inhibit pSTAT1 expression. *A375 cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h. Whole cell extracts were prepared and subjected to western blotting to determine level of pSTAT1. The same blots were stripped and reprobed with STAT1 antibody to verify equal protein loading.*

3.9.11 PTX inhibits pp38, SAPK/JNK and pAkt expression

MAP kinase and PI3K/Akt pathway has also been shown to cross-react with STAT3 signaling pathway and are frequently activated in a wide range of human cancers. Therefore, we evaluated the effect of PTX on these pathways. PTX decreased the expression of pp38, SAPK/JNK and pAkt expression without having any effect on pp44/42, p44/42, p38, PI3K and Akt expression **(Figure 3.22).**

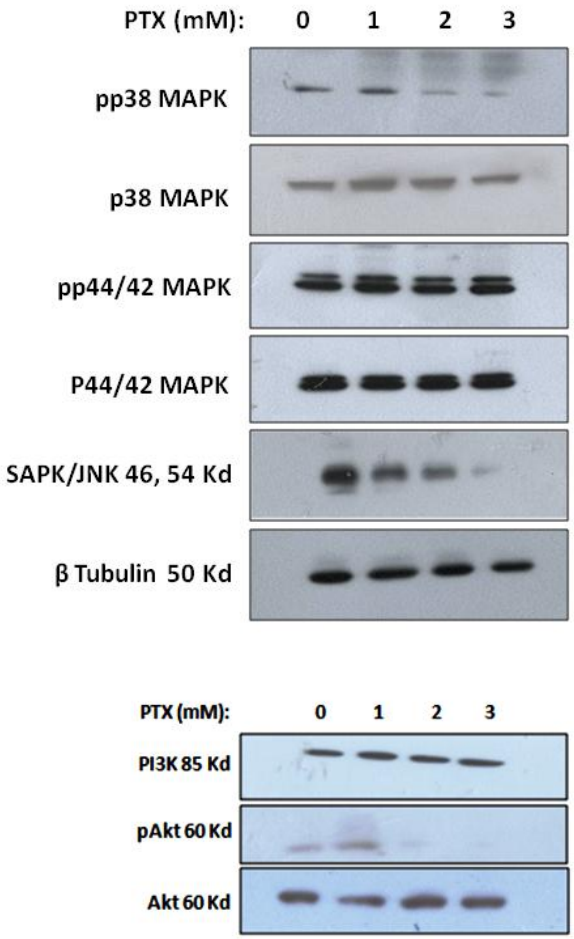

Figure 3.22: PTX inhibits pp38, SAPK/JNK and pAkt expression in A375 melanoma cells. *A375 cells were treated with sub-toxic doses of PTX (0, 1, 2 and 3 mM) for 24 h. Whole cell extracts were prepared and subjected to western blotting to determine level of* ***(a)*** *pp38, p38, pp44/42, p44/42 and SAPK/JNK.* ***(b)*** *PI3K, pAkt and Akt .*

3.10 DISCUSSION

3.10 Discussion

STAT3 was initially discovered as a DNA-binding factor that selectively binds to IL6-responsive element in the promoter of acute phase genes. It is activated by diverse cytokines and growth factors and acts as a transducer of signals from cell surface receptors to the nucleus. The biological functions of STAT3 are very broad and mostly derived from STAT3 knockout mice and/or by tissue-specific deletions. These studies demonstrate that STAT3 signaling is critical for various normal physiological processes such as embryonic development, organogenesis, cell differentiation, growth and innate and adaptive immune function (174).

Under normal physiological conditions, STAT3 activation is transient and tightly controlled. However, constitutive activation of STAT3 has been detected at high frequency in diverse human cancer cell lines and tissues (127). Studies to date provide strong evidence that aberrant STAT3 signaling participates in tumor growth and metastasis by enhancing cellular proliferation, invasion, migration and angiogenesis (132). Studies have also revealed that altered STAT3 activation could itself be responsible for cellular transformation and oncogenesis (225). High STAT3 expression is correlated with the most invasive phenotype of cancer cells. Furthermore, STAT3 expression is upregulated in highly metastatic tumors as compared to low metastatic or non-metastatic tumors (267). Since there is substantial evidence of STAT3 involvement in tumor progression and metastasis, large efforts are underway to target this protein. Suppression of constitutive STAT3 signaling results in the inhibition of tumor growth *in vitro* as well as *in vivo* with little or no effects on normal cells (255). Although, inactivation of STAT3 leads to embryonic lethality in mice, many normal adult tissues are unaffected by loss of STAT3 (255). In addition, functions of STAT3 are dispensable in many adult tissues (255). Thus, it is regarded as druggable target for cancer therapy.

PTX is a safe, non-toxic as well as well tolerated drug and has been widely used in the treatment of peripheral vascular disease (73). In our previous objective, we have shown that PTX inhibited metastasis of A375 human melanoma cells *in vitro* by inhibiting cellular proliferation, adhesion, invasion and migration. In this objective we attempted to uncover the underlying molecular mechanism(s) for antimetastatic or antiangiogenic activity of PTX.

Our results showed that PTX inhibited constitutive as well as IL-6-inducible STAT3 activation (STAT3 phosphorylation) in A375 human melanoma cells in a dose dependent manner. PTX also inhibited STAT3 phosphorylation in A549 lung carcinoma and U87 glioblastoma. Interestingly, PTX did not alter STAT3 activation in normal cells such as HEK-293. It is thus clear that inhibitory effect of PTX is specific for cancer cells. Earlier studies from our laboratory already have demonstrated that PTX inhibits the enzyme phosphodiesterase resulting in the increase of cAMP level in melanoma cells (108). Hence, we hypothesized that the same might be occurring in our model system i.e., inhibition in phosphorylation of STAT3 by PTX might be due to increase in cAMP level in the cells.

Since PTX is engaged in dephosphorylation of STAT3, we evaluated if the change in expression of upstream kinases could be attributed to the process of dephosphorylation. Various kinases such as JAKs, non-receptor tyrosine kinase Src and receptor with intrinsic tyrosine kinase activity such as VEGFR or EGFR have been reported to regulate STAT3 phosphorylation. PTX inhibited pJAK1 and pJAK2 and VEGFR2 expression in a dose dependent manner. In contrast, PTX had no effect on non-receptor tyrosine kinase Src.

STAT3 phosphorylation has also been shown to be regulated by protein tyrosine phosphatases. We used sodium orthovanadate, a known broad range tyrosine phosphatase inhibitor to test if it had any effect on pSTAT3 expression. It was observed that sodium orthovanadate reversed the effect of PTX by increasing pSTAT3 expression, suggesting a contributive role for protein tyrosine phosphatases in PTX-induced inhibition of STAT3 activation. We therefore, decided to explore exactly which phosphatases are affected by PTX. Here we showed that PTX increased the expression of pSHP2 phosphatase. In agreement with this observation, decrease in SHP2 expression has been shown to enhance JAK/STAT signaling in hepatocellular carcinoma (249). These results confirm that PTX inhibited STAT3 phosphorylation by downregulating upstream kinases (such as pJAK1, pJAK2 and VEGFR2) as well as by upregulating pSHP2 phosphatase.

Like other transcription factors, nuclear translocation of activated STAT3 is essential for transcriptional activity. Since, phosphorylation of STAT3 at Tyr 705 is essential for its nuclear translocation. We used an anti-phospho STAT3 (Tyr 705) antibody for immunoflorescence study. Consistent with the results of western blot, we found that PTX treatment inhibited pSTAT3 expression in cells in a dose dependent manner. Although Tyr phosphorylation of STAT3 is required for nuclear translocation, some reports suggested that nuclear import of STAT3 takes place constitutively and independently of Tyr phosphorylation (268). Yang and co-workers has reported that unphosphorylated STAT3 is required for the constitutive expression of certain oncoproteins (e.g. MET and MRAS) that do not respond directly to pSTAT3 (269). Further mechanistic studies revealed that unphosphorylated STAT3 can stimulate expression of pro-inflammatory and pro-oncogenic genes either independent or

dependent on binding to NFκB (270). Therefore irrespective of its state of tyrosine phosphorylation, STAT3 must gain entrance to the nucleus to impact transcription. In this study we observed that PTX treatment inhibited STAT3 nuclear translocation in a dose dependent manner.

In nucleus, binding of STAT3 to the promoter of target genes is essential for initiation of transcription. By using ELISA-based DNA binding assay we showed that PTX inhibited binding of STAT3 to DNA-promoter sequences. It thus confirmed that PTX has potential to inhibit STAT3 activation.

Angiogenesis, the formation of new blood vessels from pre-existing vasculature is essential step for tumor growth and metastasis. The most potent angiogenic molecule is VEGF (271). VEGF secreted from tumor cells binds to transmembrane receptor tyrosine kinases of endothelial cells and participates in neovascularisation. STAT3 is regarded as a direct transcriptional activator of VEGF gene (263). Niu and co-workers reported that constitutive activation of STAT3 up-regulates VEGF expression and tumor angiogenesis in melanoma cells (272). Other reports also confirmed the role of STAT3 in regulation of VEGF and angiogenesis in various cancers. In contrast, targeting STAT3 blocks VEGF expression and angiogenesis (273). Consistently, we found that PTX treatment inhibited VEGF expression at transcriptional as well as translational level. Further, by using ELISA we showed that PTX treatment inhibited VEGF secretions in culture supernatant in a dose dependent manner. Furthermore, by using different inhibitors such as STAT3 inhibitor (WP1066) and JAK2 inhibitor (AG490), we confirmed that PTX inhibited VEGF expression by targeting STAT3 signaling.

VEGFR2 or kinase insert domain-containing receptor (KDR) is predominantly located on the surfaces of endothelial cells and contributes to the almost all biological responses to VEGF. VEGF/VEGFR2 autocrine or paracrine signaling exists between endothelial cells and tumor cells and play a role in tumor angiogenesis (274). In this study, we observed that PTX inhibited expression of both VEGF and VEGFR2, thus suggesting that at least in some cellular context PTX disrupts VEGF/VEGFR2 signaling. However, additional studies with PTX are still required to clarify the mechanism. Besides VEGF, STAT3 has also been reported to induce expression of HIF1α, another key mediator of angiogenesis (127). Our data revealed that PTX treatment inhibited HIF1α expression in a dose dependent manner. Taken together, these findings, for the first time, describe the mechanism(s) for anti-angiogenic effect of PTX.

STAT3 activation plays a critical role in proliferation and survival of tumor cells. Both cyclin D1 and cMyc are required for regulation of G1 phase of cell cycle (275). In mammalian cells, cyclin D1 complex with CDK4/CDK6 and control G1/ S phase transition. Evidences indicate that constitutive STAT3 signaling is associated with upregulation of cyclin D1 and cMyc expression, contributing to accelerated cell cycle progression (127). Our data revealed that PTX treatment inhibited cyclin D1, CDK6 and cMyc expression in a dose dependent manner. In our earlier objective we have shown that PTX inhibited cellular proliferation and G1/S phase transition. Thus, here our results clarify the mechanism that PTX inhibits cellular proliferation and G1/S phase transition by inhibiting cyclin D1/CDK6 and cMyc expression.

In our earlier objective, we have also shown that PTX can induce apoptosis in A375 melanoma cells. Here we observed that PTX treatment inhibited expression of anti-

apoptotic protein BclXL and activated caspase 3 and caspase 9 indicating one of the probable mechanisms by which PTX induces apoptosis.

IL-6 is an immunosuppressive cytokine which has been shown to be overexpressed in response to infection, inflammation and injury. Many tumor cells have been found to produce excess amounts of IL-6 or alternatively, express an IL-6 receptor which helps them in maintaining tumor microenvironmental conditions (239). The binding of IL-6 to its receptor leads to the activation of the Janus kinase family which in turn activate STAT3 signaling. Dendritic cells are known to be involved in anti-tumor activity. However, the dendritic cells surrounding the tumor microenvironment are partially differentiated and lack MHC class II molecules (276). The tumor secreted factors such as IL-6, IL-10 and VEGF are responsible for this partial differentiation of dendritic cells, and hence reducing their antigen presenting ability (256). Thus, tumor cells with activated STAT3 secrete IL-6 and other immunosuppressive factors that act on immune cells leading to immune suppression around tumor microenvironment. Clinically, IL-6 is shown to be elevated in serum levels of patients with certain carcinoma such as breast, lung and lymphoma and also in advanced prostate cancer (266, 277). This high level of IL-6 expression is correlated with poor clinical prognosis. Given the reported involvement of IL-6 and its downstream targets in the regulation of tumorigenesis, various reports highlighted molecular rationale for the development of anti–IL-6 therapeutics. IL-6 or IL-6 receptor antagonists (i.e., siltuximab, a human-mouse chimeric antibody to human IL-6, and tocilizumab, humanized anti–IL-6 receptor antibody) are currently in either phase I or phase II clinical trials in a small subset of cancers (278). Our data revealed that PTX treatment inhibited IL-6 secretions in a dose dependent manner.

STAT1 is another member of STAT family protein that exerts a complex array of functions on both tumor cells and the immune system and is usually considered as a tumor suppressor (257). In contrast, STAT3 is considered as an oncogene and its constitutive activation is reported in nearly 70% of solid and hematological tumors (233). In many types of tumors STAT1 induces antiproliferative and proapoptotic genes that directly hamper tumor growth while STA3 promotes tumor growth by enhancing cellular proliferation and inhibition of apoptosis. Thus, the balance between activated STAT1 and STAT3 may play a role in directing cells towards proliferation or apoptotic death. Care should be taken to plan therapeutic intervention using compounds or inhibitors that could unbalance finely tuned equilibria between STAT1 and STAT3. Ideally, a pharmacological compound should have the property to inhibit STAT3 signaling without altering STAT1 activation. Several strategies have been developed to inactivate STAT3. However, a few compounds have been reported that maintain finely tuned equilibria between STAT1 and STAT3 so far. Bill and co-workers showed that FLLL32 curcumin analog inhibit STAT3 activation without altering STAT1 signaling in melanoma cells (279). In line with this, in the present study we reported that PTX only inhibited STAT3 signaling without altering STAT1 activation. This fact further strengthens the potential of PTX as a STAT3 inhibitor.

Cancer is a complex disease in which multiple signaling pathways are altered. These signaling pathways work either independently, in parallel, and/or through interconnections to promote cancer growth and development. Thus, maximally effective therapy may need such regimens that target multiple signaling pathways and have minimal effects on normal cells. MAP kinase and PI3K/Akt pathway has been shown to cross-react with STAT3 signaling and are frequently activated in a wide range of

human cancers. p38 MAP kinase activates STAT3 signaling by tyrosine phosphorylation (280), while SAPK/JNK activate STAT3 with concomitant activation of Akt signaling (281). Our data revealed that PTX treatment inhibited phospho p38, phospho Akt and SAPK/JNK expression in a dose dependent manner.

Taken together, our data for the first time revealed the molecular mechanism(s) by which PTX exerts anticancer or antimetastatic effect.

CHAPTER 4

Effect of PTX *in vivo*

4.1 MATERIALS AND METHODS

Animal study

Animal studies were conducted with the approval of Institutional Animal Ethics Committee (IAEC), ACTREC.

Sub-cutaneous xenograft model: A375 cells were grown to 70 to 80% confluency in complete medium and harvested using trypsin-EDTA. Six-eight week old female NOD-SCID mice were injected sub-cutaneously with 100 µl of cell suspension containing $1.5x10^6$ cells in plane medium. The mice were randomised (5 mice/group) and divided into three groups viz, PBS alone, 40 mg/kg PTX and 60 mg/kg PTX. Mice were injected PTX by intra-peritoneal (i.p) route from day 9^{th} when tumors were visible (around 20 to 25 mm^3). Treatment continued from day 9 to day 17 i.e. total 9 days. Animals were sacrificed on 30^{th} day. Tumor size was determined alternate day by vernier caliper and volume was calculated using the formula $0.5ab^2$, where a is the long diameter and b is the short diameter.

Intra-dermal angiogenesis model: A375 cells were grown to 70 to 80% confluency in complete medium and harvested using trypsin-EDTA. One million cells were injected intra-dermaly into 6- to 8-week-old female NOD-SCID mice. The mice were randomised (5 mice/group) and divided into three groups viz, PBS alone, 40 mg/kg PTX and 60 mg/kg PTX. Mice were injected PTX by i.p route from day 5^{th} when tumors were visible. Treatment continued from day 5 to day 13 i.e. total 9 days. Animals were sacrificed on day 18^{th} and the skin containing incipient solid tumors were collected, spreaded and stapled on a whatmann sheet. The number of angiogenic blood vessels converging around the tumor was counted manually.

4.2 RESULTS AND DISCUSSION

4.2.1 PTX inhibits sub-cutaneously implanted A375 tumor growth *in vivo*

In order to evaluate the effect of PTX on tumor growth in vivo, A375 cells were injected sub-cutaneously into 6 to 8 week old female NOD-SCID mice. Tumor latency period observed was 9 days. Thereafter, mice were treated for 9 days (i.e. from day 9 to day 17) intra-peritoneally with a dosage of 40mg/kg and 60 mg/kg PTX. It was observed that mean volume of tumor treated with PTX was significantly decreased as compare to tumors treated only with PBS **(Figure 4.1a and 4.1b)**. The PTX was well tolerated and did not show any visible toxicity. Further, PTX treated tumor tissue had lower pSTAT3 expression as compared to untreated control as shown by western blot **(Figure 4.1c)**.

4.2.2 PTX inhibits angiogenesis *in vivo*

As PTX inhibited tumor growth and decreased the expression of various angiogenic molecules such as VEGF and HIF1α. We investigated antiangiogenic efficacy of PTX *in vivo*. A375 cells were injected intra-dermaly into 6 to 8 week old female NOD-SCID mice. Tumor latency period observed was 4 days. Treatment was carried out for 9 days intra-peritoneally with a dosage of 40mg/kg and 60 mg/kg PTX. It was observed that mean volume of tumor treated with PTX was significantly decreased as compare to tumors treated only with PBS **(Figure 4.2a)**. Animals weight was not affected by PTX treatment **(Figure 4.2b)**. Furthermore, the number and the thickness of the blood vessels converging towards the tumors were reduced in PTX treated mice as compare to control **(Figure 4.2c and 4.2d)**.

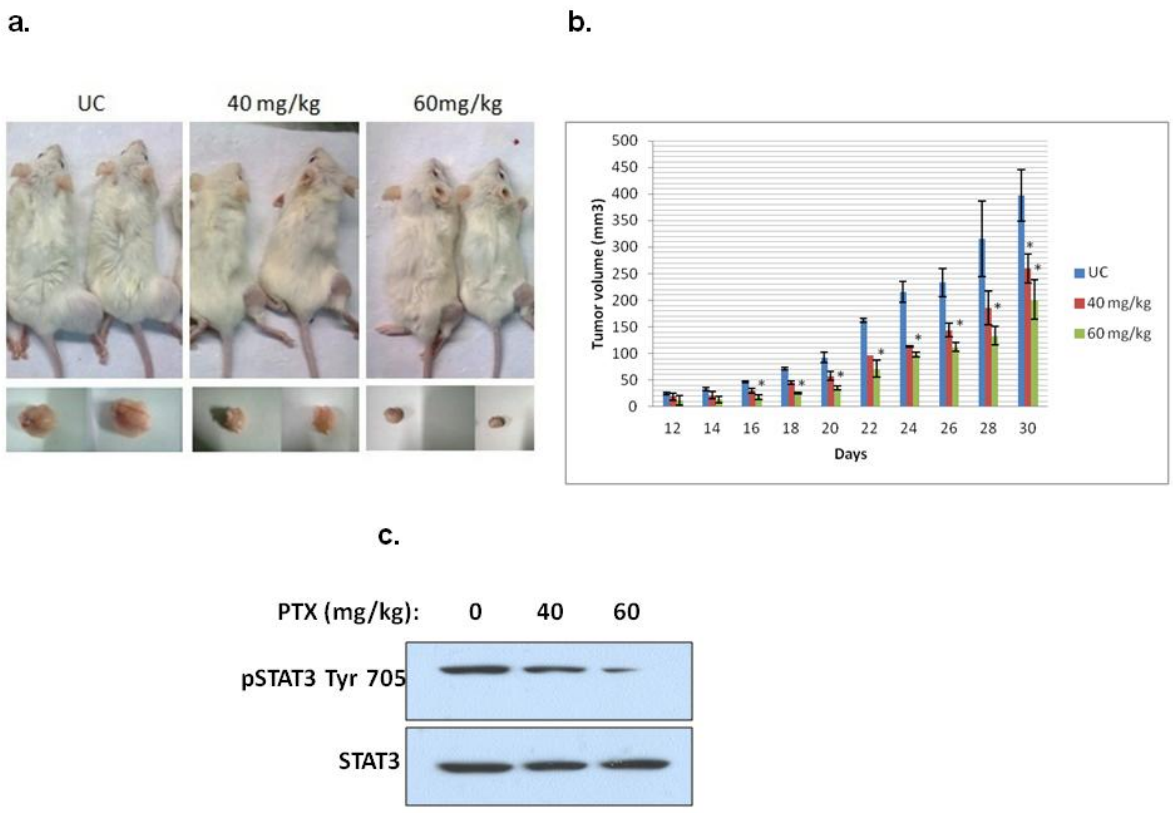

Figure 4.1: PTX inhibits A375 tumor xenograft in vivo. *A375 cells (1.5 x10^6) were injected sub-cutaneously into 6 to 8-week-old female NOD-SCID mice. Mice were injected with PTX at 40 mg/kg and 60 mg/kg by i.p route for 9 days.* ***(a)*** *Photograph of mice and dissected tumors from untreated control mice and mice treated with PTX 40mg/kg and 60mg/kg respectively.* ***(b)*** *Tumor growth inhibition induced by the administration of 40mg/kg PTX or 60mg/kg PTX compared with untreated control.* ***(c)*** *Western blot from tumor tissue treated with 40mg/kg PTX and 60mg/kg PTX. Values are expressed as mean ± SE of two independent experiments. *p < 0.05 compared with the untreated control.*

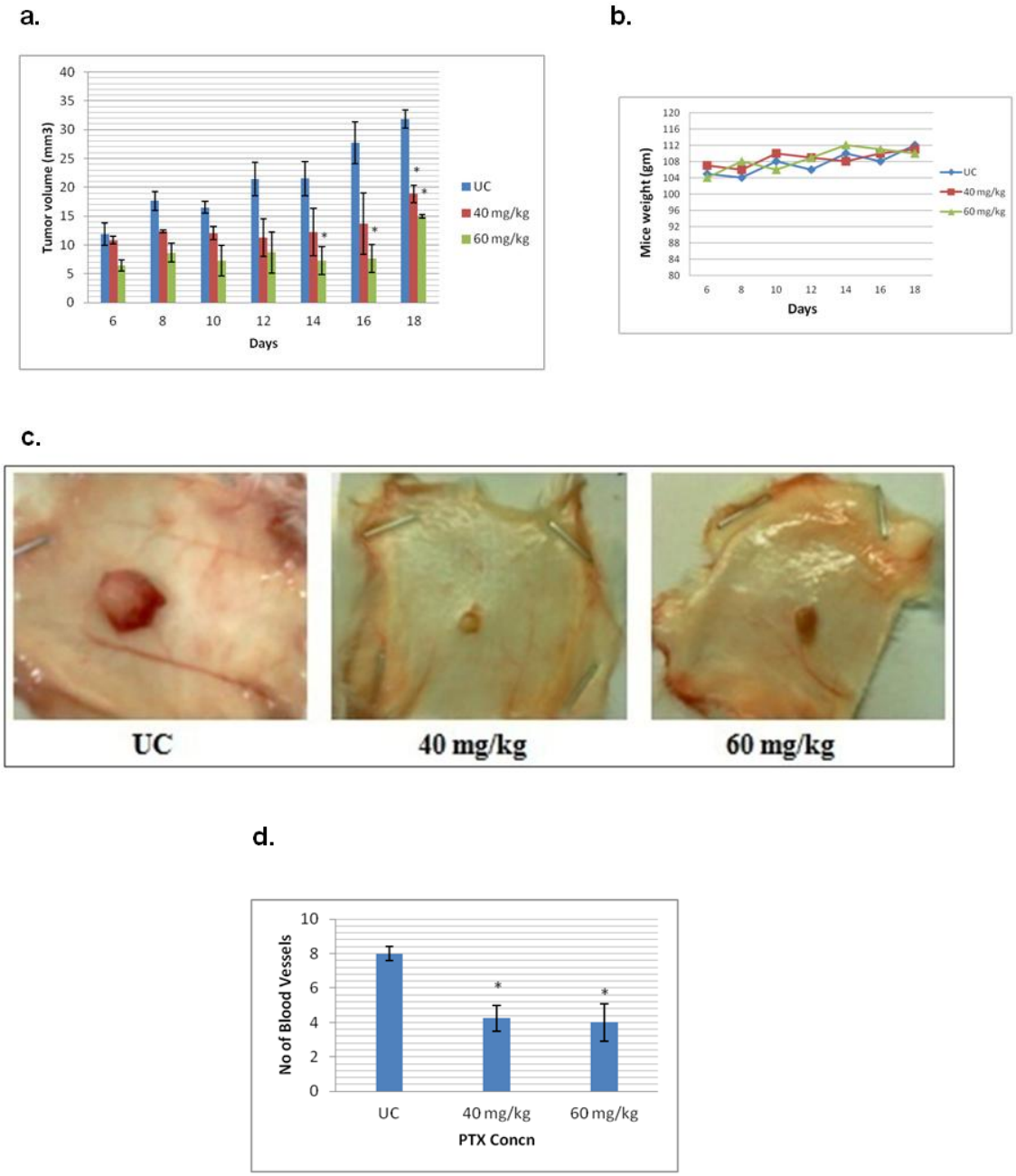

Figure 4.2: PTX inhibits angiogenesis in vivo. *A375 cells (1.0 x10^6) were injected intra-dermaly into 6- to 8-week-old female NOD-SCID mice. Mice were injected with PTX at 40 mg/kg or 60 mg/kg by i.p route for 9 days. (a) Tumor growth inhibition induced by the administration of 40mg/kg PTX or 60mg/kg PTX compared with untreated control. (b) Mice weight. (c) Mice skin sections containing the intra-dermal implanted tumor with converging blood vessels towards tumor. (d) Quantitative representation of blood vessels touching to the tumor. Values are expressed as mean ± SE of two independent experiments. *p < 0.05 compared with the untreated control.*

SUMMARY AND CONCLUSION

Summary and conclusion

The most deadly aspect of cancer is its ability to spread, or metastasize therefore search for an antimetastatic compound is a crucial aspect of anticancer drug research. However, drug development requires an average of 13 to 17 years of research and an investment of US$1.8 billion to bring a single drug from the bench to a patient's bedside (103). In addition, more than 90% of drug candidates for human testing are likely to fail, due most commonly to lack of desired activity or unacceptable toxicity. When compared to other types of drugs (e.g. for infectious diseases or heart disease), cancer drugs fail more often. This is due at least in part to the complexity of cancer itself.

One way to reduce this time investment and failure is to look for already-approved drugs that could lessen or stop metastases. In recent years "Drug repurposing" or "Drug repositioning" i.e., the practice of looking for new clinical uses of existing drugs, has gained considerable attention. Recently, metformin, a FDA approved drug for the treatment of diabetes, has been shown to have anticancer activity. The therapeutic potential of metformin in prostate, breast, endometrial, and pancreatic cancers is currently being evaluated in several clinical trials, some of which have advanced to Phase III (e.g., NCT01101438, NCT01864096) (282, 283). Thus, it is likely that the same drug can be therapeutic for more than one disease, because of the common molecular origins of diverse diseases.

Based on similar approach, our laboratory is investigating the antimetastatic and antiangiogenic activity of an old drug known as Pentoxifylline (PTX). PTX is a FDA approved drug for the treatment of peripheral vascular disease. In the present study, we

have investigated the antimetastatic and antiangiogenic activity of PTX with underlying molecular mechanisms.

The first objective was to study the effect of PTX on different steps of metastatic cascade. Various *in vitro* assays which mimic each step in the metastatic cascade were carried out. The results showed that PTX at sub-toxic doses inhibited cellular growth and proliferation of cancer cells by inhibiting cell cycle progression from G1/S phase and also by induction of apoptosis. Sub-toxic doses of PTX showed minimal toxicity to immortalized non-tumorigenic cell lines HaCaT and NIH3T3 fibroblast. Cells treated with PTX showed an inhibition in adhesion to ECM substrate matrigel and type IV collagen. Activity of MMP2 and MMP9 was decreased on PTX treatment. Cell treated with PTX showed abnormalities in cytoskeletal structures and impaired cellular migration. These results suggest that PTX has potential to inhibit metastasis by affecting each of the individual steps of the metastatic process.

Numerous signaling pathways whose aberrant activation triggers metastasis formation have been redefined in recent years. One such pathway is the Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) pathway. Many studies suggest that STAT3 is constitutively activated in many tumor tissues and thus place it at the central node in the development and progression of cancer growth and metastasis. In fact, STAT3 signaling plays an important role in each and every steps of metastasis such as cellular proliferation, migration, invasion and angiogenesis. Thus, targeting the JAK/STAT3 pathway can be an excellent therapeutic approach to overcome tumor growth and metastasis.

Despite minimal cytotoxicity against normal cells, our data revealed that PTX suppressed STAT3 activation, STAT3 nuclear translocation and binding of STAT3 to

the DNA in a dose-dependent manner. Also, PTX inhibited phosphorylation of the upstream kinases JAK1 and JAK2 and increased the expression of pSHP2 phosphatase. Expression of various STAT3 regulated gene products, such as cylin D1, CDK6, cMyc, BclXl, Bcl2, caspase 3, caspase 9, VEGF, VEGFR2 and HIFα were downregulated following PTX treatment. Tumor microenvironment favours tumor growth and metastasis. PTX altered tumor microenvironment by limiting the secretion of IL-6 and TNFα and also disrupted VEGF-VEGFR2 autocrine/paracrine signaling. Further investigation of the underlying mechanism revealed that PTX inhibited pp38 MAPK, SAPK/JNK and pAkt expression.

STAT1 is the founding member of STAT family transcription factors. In contrast to the growth stimulatory and anti-apoptotic functions of STAT3, STAT1 is generally known to have a tumor suppressor function. In the present study, it was observed that PTX only inhibited STAT3 signaling without altering STAT1 activation. This fact further strengthens the potential of PTX as a STAT3 inhibitor. Finally, PTX treatment significantly inhibited solid tumor growth induced by sub-cutaneous route *in vivo* and also inhibited tumor induced angiogenesis in intra-dermal xenograft model without having any visible toxicity.

In conclusion, our data suggest that PTX has potential to inhibit tumor growth, metastasis and angiogenesis *in vitro* as well as *in vivo* by targeting STAT3 signaling pathway. These findings, for the first time, explain the molecular mechanism(s) by which PTX exerts antimetastatic and antiangiogenic activity.

FUTURE DIRECTIONS

Future directions

Taking into consideration, the current understanding and the knowledge of the inhibitory role of PTX in cancer growth and metastasis, its imminent usefulness in the clinics is predictable. There are an adequate number of reports from our laboratory and other groups which confirmed the antimetastatic and antiangiogenic activity of PTX in different cancers. The preclinical experience with PTX warrants clinical trials with PTX as a single agent or in direct comparison with other agents. However, a double-blind randomized trial suggested, PTX as protective agent in both early and late radiation-induced lung toxicity in patients with breast and lung cancer (93). In this regard, PTX might be an attractive prophylactic agent. We strongly believe that PTX could both precede and be given concurrently with chemotherapy or radiation therapy, in an attempt to increase their effectiveness, decrease the required doses and limit the toxicity of standard therapies. Clinical trials with PTX will need to carefully define the maximally tolerated and biologically active dose. Furthermore, the pharmacokinetics in the cancer population will need to be defined.

The therapeutic concept of administering cytotoxic agents continuously at lower doses is known as ‘metronomic therapy’, is increasingly being recognized as an experimental option for treating cancer. PTX is a safe, non-toxic, anti-inflammatory and immuno-modulatory drug that has been in used for decades. Recent data have confirmed the functional relationship between inflammation and cancer and anti-inflammatory therapy is efficacious towards early neoplastic progression and malignant conversion. Therefore, a good initiative would be to design metronomic therapy with PTX as an innovative therapeutic strategy for the treatment of cancers.

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