IOSR Journal of Pharmacy
ISSN: 2250-3013, www.iosrphr.org
Ⅲ Volume 2 Issue 5 Ⅲ Sep-Oct. 2012 Ⅲ PP.24-30

Metastatic events of MDA-MB-231 cells induced by angiogenic factors VEGF or MYA1 are inhibited by *Tinospora cordifolia* hexane fraction (tchf)

Sachin Raj M. Nagaraj, Yashaswini Balaraju,
Niharika Shetty and Bharathi P. Salimath*

Department of Studies in Biotechnology,University of Mysore, Manasagangotri Mysore-570006,India

Abstract—Angiogenic growth factors VEGF or MTA1 mediated proliferation, migration and invasion play pivotal role in the process of metastasis. In this paper we have described the preparation of hexane fraction from *Tinospora cordifolia* (Tchf) and have shown that Tchf has an antiangiogenic activity. HUVEC tube formation and Rat cornea angiogenesis assays were used for validation of antiangiogenic activity of Tchf. Our results on VEGF or MTA1 induced proliferation, migration and invasion of MDA-MB-231 cells show that Tchf and SB202190, a MAP kinase inhibitor effectively inhibits these metastatic processes. Both angiogenic factors VEGF or MTA1 could induce expression of VEGF gene in MDA-MB-231 cells, Tchf repressed VEGF gene expression induced either by VEGF or MTA1. These results clearly indicate for the first time that Tchf is a potent inhibitor of metastatic events which are synergistically trigged by VEGF and MTA1.

Keywords—Angiogenesis, Metastatic events, Vascular endothelial factor (VEGF), Metastasis Associated protein (MTA1), *Tinospora cordifolia*, SB202190.

I. INTRODUCTION

Metastasis is a process by which malignant tumor cells from the primary tumors site detaches, escapes into the sprouting capillaries, evades immune clearance, arrest in the capillary bed of a secondary organ, followed by tumor cell proliferation and angiogenesis in the secondary site [1]. The metastatic potential of a tumor may be influenced by angiogenesis [2]. Hence, metastasis presents a major challenge in the cancer therapy. There are several studies citing the correlation between expression of angiogenic factors and metastasis in cancer patients [3]. Vascular endothelial growth factor (VEGF) is one of the most important growth factor in the process of tumor growth and metastasis. VEGF is produced by a variety of tumor cells and, thus, may contribute to tumor expansion associated with neo-vascularization. VEGF exerts its effects after binding with two receptor-tyrosine-kinases, Flt-1 (VEGFR-1) and KDR/Flk-1 (VEGFR-2), present at the surface of endothelial cells. Han and group have shown that VEGF-VEGFR1 signaling pathway is crucial for tumor metastasis and the blockade of VEGF-VEGFR1 pathway may result in inhibition of metastasis [4]. Metastasis Associated Protein 1 (MTA1) [5-6] is representative of a protein family highly conserved through evolution, which also includes the metastasis associated 1-like protein (MTA1- L1), MTA2 and MTA3 [7]. It is known that MTA1 has important and critical roles in the angiogenesis and progression of a wide variety of cancers. Several studies have identified various roles for MTA1 in normal mammary gland development and human breast cancer progression, including cell proliferation and invasiveness [8]. MTA1 also enhances angiogenesis by stabilization of HIF-1α [9].

It is widely studied and understood that angiogenic inhibitors are potential agents in tumor regression and also reduces the vasculature which could aid in tumor metastasis [10]. All well characterized angiogenic inhibitors like, angiostatic steroids, thalidomide, the fumagillin, TNP-470, thrombospondin, angiostatin, endostatin and platelet factor 4 inhibit tumor metastasis [11-18]. The antiangiogenic therapy in combination with chemotherapy is shown to be helpful in anticancer therapy. Some of the anti-angiogenic agents that are already in clinical use such as taxol, tamoxifen and adriamycin have shown better results in combination with conventional chemotherapy in reducing tumor metastasis [19-22]. In triple negative breast cancer where antiangiogenic therapies prove to be beneficial there are few studies at molecular level to project the importance of anti angiogenic molecules in cancer therapy. MDA-MB-231 breast cancer cell line is a triple negative highly metastatic cell line. It is important to understand the mechanism involved in the inhibition of metastatic events in MDA-MB-231 cells. It has been shown that MTA1 is not only associated in regulation of gene expression but also has major role in regulation of tumor angiogenesis. The regulation of metastatic events in triple negative

breast cancer cells by MTA1 is important in order to identify the novel inhibition of metastasis. The concerned actions of angiogenic factors such as VEGF and MTA1 may contribute for angiogenesis in triple negative breast cancer cell line. In this paper we have made an attempt to inhibit growth and metastasis of triple negative cell line MDA-MB-231 cells by using Tchf (*Tinospora cordifolia* hexane fraction) and a known MAP kinase inhibitor SB202190. Tinospora cordifolia, commonly known as guduchi, is widely used in veterinary folk medicine and Ayurvedic system of medicine for its general tonic, antiperiodic, antispasmodic, anti-inflammatory, antiarthritic, anti-allergic and antidiabetic properties [23]. It has been reported that crude extract or hexane fraction of *T. cordifolia* has the proapoptotic potentials [24]. We have also studied that one of the active compound, Octacosanol which was also isolated from *T. cordifolia* has shown to inhibit tumor-induced angiogenesis in vivo by inhibiting VEGF gene expression [25]. In this report, we have shown that VEGF or MTA1 induced proliferation, migration and invasion activities of human breast cancer cell MDA-MB-231 cells are inhibited on by Tchf, an antiangiogenic fraction of *T. cordifolia* or SB202190 an inhibitor of MAP kinase.

II. MATERIALS AND METHODS

MDA-MB-231 cells were purchased from National Centre for Cell Science (NCCS), Pune, INDIA. The cells were cultured in 25 cm^3 tissue culture flask (NUNC, USA) and grown using Dulbeccos's Minimum Essential Medium (DMEM) with 10% Fetal Bovine Serum (FBS), Streptomycin and Penicillin from GIBCO laboratories, Grand Island, NY, USA. Complete medium was prepared according to the manufacturer's protocol. Incubation was carried out in a humidified atmosphere of 5% CO_2 at 37^oC upon reaching to confluency; the cells were passaged after trypsinization. SB202190 specific p38 MAP kinase inhibitor was purchased from Sigma-Aldrich chemicals, St. Louis, USA.

2.1 Plant Material

The mature stems of *T. cordifolia* were collected from University of Mysore campus, Mysore, India. Fresh stems were dried in shade. The voucher specimen was deposited in the herbarium collections maintained in the Department of Botany, University of Mysore, Mysore.

2.2 Preparation of hexane fraction

The hexane fraction from *T. cordifolia* crude extract was essentially prepared as described by Thippeswamy et al. [24]. In brief, Polarity based fractionation and activity guided purification were adopted in order to identify the fraction containing anti angiogenic activity. Shade dried stems (1 kg) were powdered and extracted with different solvents on basis of polarity, using soxhlet apparatus. Solvent such as hexane, benzene, chloroform, ethyl acetate and methanol were used for extraction. In order to follow the fraction containing anti-angiogenic activity the solvents were evaporated using rotary evaporator. The hexane fraction which showed anti-angiogenic activity both *in vitro* and *in vivo* assays, which are described below, was further chosen to verify the anti-metastatic potential.

2.3 Endothelial cell tube formation assay (in vitro)

Formation of tubes by endothelial cells cultured on Matrigel in presence of VEGF, an angiogenic molecule, and further disruption of VEGF induced endothelial cell tube formation by various fractions of T. cordifolia extract was performed. In brief, the assay was performed as described previously [26], Matrigel (50 µl) was coated on a 96-well plate, and was allowed to solidify at 37^oC for 1 h. HUVECs (5x 10^3 cells per well) were seeded on the Matrigel and cultured in EGM media containing VEGF (10 ng/ml) and/or treated with different fractions of T. cordifolia (80 µg/ml). The cells were incubated at 37^oC, for 16 h at 5% CO_2. The next day complete tubes from randomly chosen fields were photographed under an Olympus inverted microscope connected to a digital camera at 40X magnification. All further experiments were continued using the hexane fraction (Tchf) as this fraction showed potent anti-angiogenic activity

2.4 Rat corneal angiogenic assay

A corneal micropocket assay was performed in accordance with the method described previously [27]. In brief, for the pellet preparation, hydron polymer poly- 2-hydroxyethylmethacrylate was dissolved in ethanol to a final concentration of 12%. To the pellet preparation was added PBS (control) or VEGF (1 µg/pellet) with or without Tchf (80 µg/pellet). Aliquots of 10 µl of pellet (Group 1), with cytokine VEGF (Group 2), with VEGF and Taxol, (a clinically proven anti-cancer and anti-angiogenic molecule) (Group 3) and with VEGF and Tchf (Group 4) were placed onto a teflon surface and allowed to air dry for at least 2 h. Male Wister rats weighing 300-350 gms were anesthetized with a combination of xylazine (6 mg/ kg, IM) and ketamine (20 mg/kg, IM). The eyes were topically anesthetized with 0.5% proparacaine and gently proptosed and secured by clamping the upper eyelid with a non-traumatic haemostat. A corneal pocket was made by inserting a 27-gauge needle, with the pocket's base 1 mm from the limbus. A single pellet was advanced into the lamellar pocket to the limbus using corneal forceps. The rats were observed for 24-72 h for the occurrence of non-specific inflammation and localization of the pellets. On day 7, the rats were anesthetized and the corneas were photographed using a CCD camera (40 ×).

2.5 Cell proliferation assay

In order to verify for the *in vitro* effect of Tchf extract on proliferation of MDA-MB-231 cells induced by MTA1 this assay was performed as described earlier [28]. Briefly MDA-MB-231 cells ($1x10^5$ cells per well) were seeded in a six-well plate and cultured in DMEM medium supplemented with 10% FBS, 1 mg/ml penicillin/streptomycin and grown in 5% CO_2 at 37°C for two days. ^{3}[H] thymidine (1 µCi/ml of medium) were added prior to addition of VEGF (10 ng/ml) or MTA1 (10 ng/ml) ± Tchf (60 µg/ml) at different time intervals (0, 6, 12, 24 & 48 h). Similarly in other set of experiment, the MDA-MB-231 cells were treated with VEGF (10 ng/ml) or MTA1 (10 ng/ml) ± Tchf at different concentrations (0, 10, 20 40 & 60 µg/ml). After 48 h, the cells were trypsinized and processed for liquid scintillation counting. In another group of experiment the MDA-MB-231 cells were treated with VEGF or MTA1 (10 ng/ml) or with SB202190 (10 µM) respectively prior to addition of ^{3}[H] thymidine (1 µCi/ml of medium). After 48 h, the cells were trypsinized and processed for liquid scintillation counting.

2.6 Wound healing assay

In order to verify the effect of Tchf or SB202190 on migration of breast cancer MDA-MB-231 cells induced by VEGF or MTA1 the wound healing assay was performed. The assay was performed as described earlier [29]. In brief, MDA-MB-231cells ($2x\ 10^5$ cells per well) were seeded in a six-well plate in complete medium and incubated overnight at 37°C and 5% CO_2. The cells were serum starved overnight and a wound was scratched on the monolayer using a sterile pipette tip. The plates were washed with PBS twice to remove any detached cells. The cells were treated with mitomycin C (10 ng/ml) for 2 h prior to addition of growth factors with SFM and VEGF or MTA1 (10 ng/ml) or treated with SB202190 (10 µM) or Tchf (60 µg/ml) respectively in basal media. The wound was photographed subsequently at 6 h, 12 h and 24 h to visualise the closure of the wound area. The distance moved by cells into the wounded area was enumerated.

2.7 Migration and invasion assay

In order to verify the effect of SB202190 on MTA1 induced migration and invasion of MDA-MB-231 cells the transwell migration assay was performed as described earlier [30]. Briefly MDA-MB-231 cells ($2x\ 10^4$ cells per well) treated with mitomycin C (10 ng/ml) for 2 h was seeded onto the top chamber of transwell which was precoated with 0.1% of gelatin for 30 min at 37°C. The bottom chamber of transwell was filled with basal media and was treated with growth stimulators with or without VEGF (10 ng/ml) or MTA1 or SB202190 (10 µM) respectively, followed by overnight incubation at 37°C, 5% CO_2. The non migrated cells were swabbed using cotton bud and fixed with 4% ice cold paraformaldehyde for 30 min. Then the cells that had migrated into membrane were stained using haematoxylin and counted manually. The cells were photographed under an Olympus inverted microscope (CKX40; Olympus, New York, USA) connected to a digital camera at 40X magnification.

2.8 VEGF gene expression studies using VEGF gene promoter-luciferase reporter

In order to verify the effect of Tchf on VEGF gene expression in MDA-MB-231 cells induced by VEGF or MTA1 assay was performed as described previously [31-32]. MDA-MB-231cells (2×10^5 cells per well) were seeded in six-well plates and cultured at 37°C with 5% CO_2 to 60-70% confluency. On the subsequent day, cells were transfected (calcium phosphate transfection kit, Promega, USA) with 2 µg of VEGF promoter–luciferase reporter constructs containing the 5' flanking region (-2068 bp) of human VEGF gene promoter coupled to promoter-less luciferase gene in vector backbone pcDNA3 and 2 µg of the β-galactosidase expression vector RSV-β-gal as an internal control. The transfected cells were incubated 37°C with 3% CO_2 prior to addition of either VEGF (10 ng/ml) or MTA1 (10 ng/ml) ± Tchf (60 µg/ml). Cells were washed once with PBS and were serum starved for 48 h. Cells were washed once again with PBS and lysed with reporter lysis buffer. Luciferase (Luc) activity of the cell extract was determined using the luciferase assay system as per manufactures instruction. β-galactosidase (β-Gal) activity was determined by measuring hydrolysis of O-nitrophenyl β-D-galactopyranoside using 50 µL of cell extract at 37°C for 2 h. Absorbance was measured at A_{405} and normalized. Luciferase activity was determined using 50 µL of cell extract. The reaction was initiated by injection of 100 µL of luciferase assay substrate. Relative luciferase activity (defined as VEGF reporter activity) was calculated as RLU (relative light units per 50 µL cell extract)/β-Gal activity (A_{405} per 50 µL cell extract per 2 h).

III. STATISTICAL ANALYSIS

Unless stated otherwise, all experiments were performed in triplicates. Wherever appropriate, the data were expressed as the mean ± SD and means were compared using one-way analysis of variance. Statistical significance of differences between control, VEGF and MTA1 treated cells were determined by Duncan's multiple range test (DMRT). For all tests, $P < 0.05$ was considered statistically significant. All of the analyses were performed using the SPSS for Windows, version 13.0 (SPSS Inc.).

IV. RESULTS

During polarity based fractionation, an activity-guided selection of fraction with an antiangiogenic activity was chosen for all further experiments. Two angiogenic assays, (endothelial cell tube formation assay and corneal micropocket assay) were used to identify the fractions containing antiangiogenic potential. The hexane fraction showed antiangiogenic activity.

Tchf inhibits differentiation of HUVECs

HUVECs adhered to the Matrigel surface within 20-24 h and form branching, anastomoses network of capillary like tubules with multicentric junctions over 24-48 h. As shown in Fig.1, VEGF (10ng) induced tube formation and Tchf inhibited the formation of tubular networks induced by VEGF on Matrigel in a dose-dependent manner. A remarkable inhibition of tube formation was observed in the presence of Tchf (1 µg/ml) and complete inhibition was obtained at Tchf (10 µg/ml). At the latter concentration, most of the cells appeared as unorganized cell aggregates.

Angio-inhibitory effect of Tchf

The rat cornea assay is a commonly used *in vivo* validation assay for studying angioinhibitory activity of antiangiogenic molecules. Result as shown in Fig.2, indicates that Tchf inhibits angiogenesis in an in vivo model system. When compared to the extensive angiogenesis seen in VEGF treated rat cornea, angiogenesis at the site of the application of Tchf was significantly reduced, Taxol also showed anti-angiogenic activity and it is a molecule which is a clinically proven anti cancer and antiangiogenic drug.

Tchf and SB202190 inhibits VEGF or MTA1 induced proliferation in MDA-MB-231 cells in vitro

In order to verify the *in vitro* effect of Tchf on either VEGF or MTA1 facilitated proliferation, a proliferation assay was performed using human breast metastatic cancer MDA-MB-231 cells. As is shown in fig. 2A, there was time dependent increase in the proliferation of MDA-MB-231 cells in presence of VEGF or MTA1. However VEGF or MTA1 induced proliferation was highest at 48 h when compared to other time periods. Inclusion of Tchf (60 µg/ml) in proliferation assay along with VEGF or MTA1, significantly reduced proliferation of tumor cells. Like wise data in fig. 2B clearly indicates that Tchf inhibits proliferation in a dose dependent manner. Similarly to verify the *in vitro* effect of SB202190 on either VEGF or MTA1 facilitated proliferation, a proliferation assay was performed. As is shown in fig. 2C, there was increase in the proliferation of MDA-MB-231 cells in presence of VEGF or MTA1. But addition of SB202190 (10 µM) along with VEGF or MTA1, significantly reduced proliferation of MDA-MB-231 cells. However VEGF or MTA1 induced proliferation was highest when compared to other test conditions.

Tchf and SB202190 inhibits VEGF or MTA1 induced cell migration in MDA-MB-231 cells in vitro

Wound healing or scratch assay is considered to be an assay to verify the migration of endothelial cells and it is useful to validate antiangiogenic effects of chemical inhibitors. The data shown in fig. 3A clearly indicates that there is wound closure at 24 h in presence of VEGF or MTA1. However cell migration induced by either VEGF or MTA1 could be effectively inhibited by Tchf (60 µg/ml) and SB202190 (10 µM) a kinase inhibitor. Quantitative analysis of wound healing assay (Fig. 3B) showed that MTA1 treated MDA-MB-231 cells showed more number of cells in closing the wound as either compared to that of SFM or VEGF.

SB202190 inhibits VEGF or MTA1 induced cell migration and invasion in MDA-MB-231 cells *in vitro*

In order to verify the in vitro effect of SB202190 on either VEGF or MTA1 induced cell migration and invasion transwell migration assay was performed in MDA-MB-231 cells.The result indicated that MTA1 or VEGF enhances invasion of MDA-MB-231 cells. Recombinant MTA1 protein (10 ng/ml) showed higher invasion of cells when compared with VEGF treated. In migration assay, responding to MTA1 protein, the number of cells that migrated to bottom side of the membrane was much higher when compared to VEGF (10 ng/ml). In contrast, the SB202190 (10 µM) treated significantly reduced the rate of migration and invasion induced by MTA1 protein (Fig. 4A).

Tchf inhibits VEGF or MTA1 induced VEGF gene expression in MDA-MB-231cells

The effect of Tchf extract on VEGF or MTA1 induced VEGF gene expression in metastatic breast cancer cell line MDA-MB-231 cells was studied by using VEGF promoter-luciferase reporter assay. Results showed that, expression of VEGF was substantially increased on treatment with VEGF or MTA1 (10 ng/ml) protein (Fig. 4B). Subsequently, treatment with Tchf extract (60 µg/ml) along with VEGF or MTA1 (10 ng/ml) reduced the VEGF gene expression in breast cancer MDA-MB-231 cells.

V. DISCUSSION

In this paper we have attempted to fractionate *T. cordifolia* crude extract and identified antiangiogenic molecules by validating the antiangiogenic potential of fractions obtained during polarity based fractionation of *T. cordifolia* crude extract. We have adopted an *in vivo* (Rat cornea assay) and an *in vitro* (HUVECs tube

formation) assay for identification and separation of an antiangiogenic fraction from *T. cordifolia* crude extract. The data shown is the result indicates that *T. cordifolia* hexane fraction has antiangiogenic potential. Angiogenic inhibitors block any of the steps in the angiogenic cascade, including proliferation and attachment of endothelial cells to the extracellular matrix proteins, proliferation, migration and invasion through the matrix, which is required for the capillary sprouting and morphogenesis in a thin tube meshwork and stabilization [33]. Cancer and metastasis are dependent on the formation of new blood vessels [34]. Further we have tried to prove the hypothesis that an antiangiogenic molecule is also antimetastatic. In our present results on closure of wound by VEGF or MTA1 indicates that MTA1 is has as potent as VEGF to induce proliferation and migration of cells this suggest that it is essential to inhibit both VEGF or MTA1 induced movement of the cells during metastastic events. Inhibition of migration of MDA-MB-231 cells induced either by VEGF or by MTA1 is targeting the pathways of migration irrespective of the activating factors. This result indicates that both MTA1 and VEGF may adopt the same signalling pathways that control the migration of cells in cancer. Tchf inhibits the proliferation of MDA-MB-231 cells induced by VEGF or MTA1 *in vitro*. In support of this, Nawa et al., has shown that the concentration of MTA1 is associated with the rate of proliferation of metastatic and non-metastatic cell lines, where cells expressing more MTA1 are also more metastatic [35-36]. Likewise increased production of VEGF is associated with increased metastasis [37].

The stimulation of p38 by VEGF is of great physiological significance since as shown by Rousseau et al., that the p38 MAP kinase pathway is an important component of the signaling network which transduces the migratory signals generated by VEGF suggesting that it may play an important role in regulating angiogenesis [38]. Inhibition of migration and invasion by SB202190 p38MAP kinase inhibitor indicates that p38 MAP kinase is involved in metastasis promoted both by VEGF and MTA1. MAP kinase is involved in metastasis promoted both by VEGF and MTA1. Our result on VEGF gene expression studies indicates that MTA1 per se regulates the expression of VEGF. Our data also reveals that in MDA-MB-231 cells there is an autocrine regulation of VEGF gene expression by VEGF. These results suggest that MTA1 acts synergistically with VEGF to regulate angiogenesis during metastasis. Further, inhibition of either MTA1 or VEGF induced expression of VEGF by Tchf indicates that Tchf via inhibition of p38 MAP kinase regulates VEGF gene expression. We have recently shown that *G. glabra* F6 (G1) has similar activity on MTA1 induced VEGF gene expression [33].

Further characterization of the antimetastatic molecules in Tchf will prove to be useful for large scale production and validation of antimetastatic drug development. In conclusion, inhibition of MTA1 and VEGF induced angiogenesis is inhibited by Tchf reveals that it is essential to suppress synergistic activity of the prometastatic molecules MTA1 and VEGF to achieve best results on the spreading of metastatic tumor than targeting the VEGF pathway alone.

VI. ACKNOWLEDGEMENTS

This work is supported by funding from Department of Biotechnology (DBT) New Delhi, India (No. BT/PR 11026/Med/30/123/2008); DAE, New Delhi; IOE, University of Mysore, Mysore and Council for Scientific and Industrial Research (CSIR)-SRF, New Delhi, India for the fellowship. There is no conflict of interest among authors.

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LEGENDS

Fig.1 VEGF induced angiogenesis (*in vitro*) is inhibited by Tchf. HUVECs ($5X10^3$ cells) was seeded onto the Matrigel. Cells were treated either with VEGF (10ng), or with Tchf (1/10 µg). After incubation for 24 h at 37^0C, capillary networks were photographed at 40x magnification.

Fig.2 VEGF induced angiogenesis in rat cornea (*in vivo*) is inhibited by Tchf and taxol. Photographs of VEGF-induced neovascularization as observed in rat cornea. The pellets containing VEGF with or without either taxol or Tchf were inserted under sterile condition into a vascular rat cornea. After 7 days of insertion, the corneas were photographed at 40 × magnification.

Fig. 3(A, B) Effect of Tchf on VEGF or MTA1 induced proliferation of MDA-MB-231 cells. As described in detail in "materials and methods", MDA-MB-231 cells ($1x10^5$ cells per well) were cultured *in vitro* in a six-well plate and processed for proliferative activity of recombinant VEGF or MTA1 (10 ng/ml) with inhibitor Tchf in a time dependent manner (0, 6, 12, 24, 48 & 72 h) respectively. And Tchf in dose-dependent manner (0, 10, 20 40, 60 µg/ml) respectively, using ^{3}[H] Thymidine (1 µCi/ml of medium). The data shown is the mean ± SD of three independent experiments. a= statistically significant at $P < 0.05$ when MTA1 compared with VEGF and b= statistically significant at $P < 0.05$ when MTA1+Tchf compared with VEGF+ Tchf. **3C** Effect of SB202190 on VEGF or MTA1 induced proliferation of MDA-MB-231 cells. As described in detail in materials and methods, MDA-MB-231 ($1x10^5$ cells per well) were cultured in vitro in a six-well plate and processed for proliferative activity of recombinant VEGF or MTA1 (10 ng/ml) with inhibitor SB202190 (10 µM) respectively, using ^{3}[H] Thymidine (1 µCi/ml of medium). The data shown is the mean ± SD of three independent experiments. a= statistically significant at $P < 0.05$ when MTA1 compared with VEGF and b= statistically significant at $P < 0.05$ when MTA1+SB202190 compared with VEGF+SB202190.

Fig. 4 Effect of Tchf and SB202190 on VEGF or MTA1 on closure of wounded area in MDA-MB-231 cells. (**A**) As described in detail in "materials and methods", MDA-MB-231 cells (2×10^5 cells per well) were seeded in a six-well plate and cultured in DMEM. The cells were serum starved overnight and a scratch was made on the cell monolayer. Cell debris was washed and the cells were cultured in medium containing with or without VEGF or MTA1 (10 ng/ml) or Tchf (60 µg/ml) or SB202190 (10 µM). The wound closure was photographed at different time intervals (0, 12 & 24 hr). **B**. Quantification of the cells involved in wound closure. The cells that moved in the wounded area was counted and expressed as movement of control.

Fig. 5A Effect of SB202190 on VEGF or MTA1 on migration and invasion of MDA-MB-231 cells. As described in detail in "materials and methods" of transwell migration assay, inactivated MDA-MB-231 cells ($2x 10^4$ cells per well) treated with mitomycin C (10 ng/ml) for 2 h was seeded onto the top chamber of transwell for 30 min at 37°C with growth stimulators with or without VEGF or MTA1 (10 ng/ml) in the lower chamber. The non migrated cells were stained using haematoxylin and counted manually. The cells migrated in the transmemberane was counted and expressed as migrated cells. Data are the mean ± SD of three independent experiments. a= statistically significant at $P < 0.05$ when MTA1 compared with VEGF and b= statistically significant at $P < 0.05$ when MTA1+SB202190 compared with VEGF+SB202190. **5B** Effect of Tchf on VEGF or MTA1 **i**nduced VEGF gene expression. As described in detail in "materials and methods", MDA-MB-231 cells (2×10^5 cells per well) was seeded in six-well plates and transiently transfected with 2 µg of VEGF promoter-luciferase reporter construct. Cells were treated either with VEGF (10 ng/ml) or MTA1 (10 ng/ml) or Tchf (60 µg/ml). Forty-eight hours later, cells were assayed for luciferase activity (luc), and β-Galactosidase (β-Gal) activity was used to normalize as internal control. The data shown is the mean ± SD of three independent experiments. a= statistically significant at $P < 0.05$ when MTA1 compared with VEGF and b= statistically significant at $P < 0.05$ when MTA1+ Tchf compared with VEGF+ Tchf.

Figures

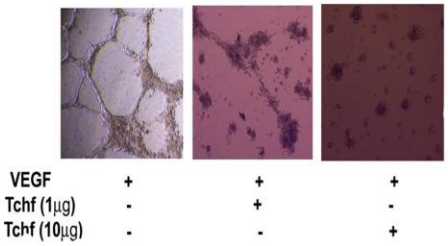
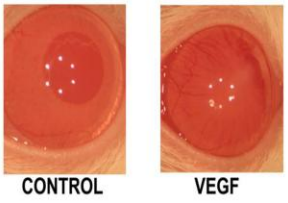


Fig. 1



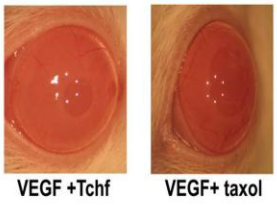


Fig. 2

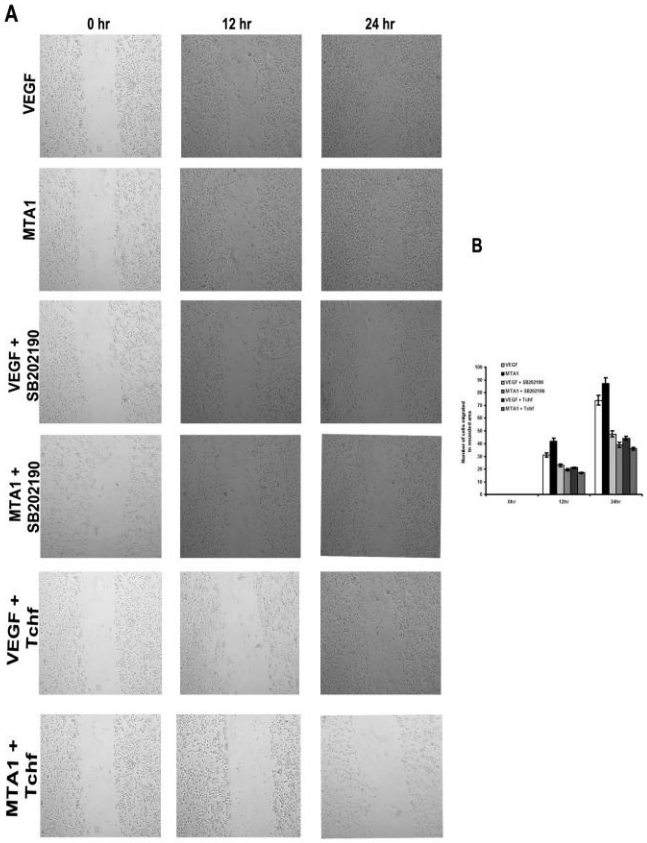


Fig. 4

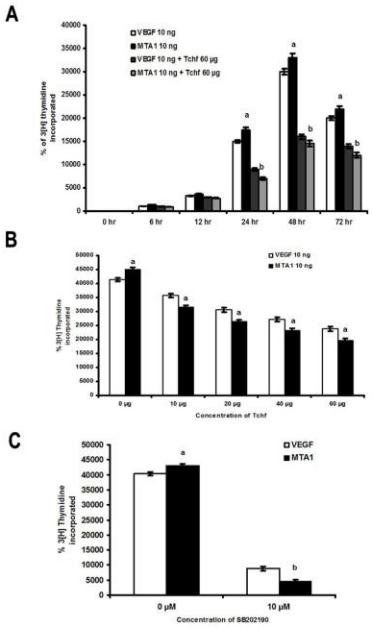


Fig. 3

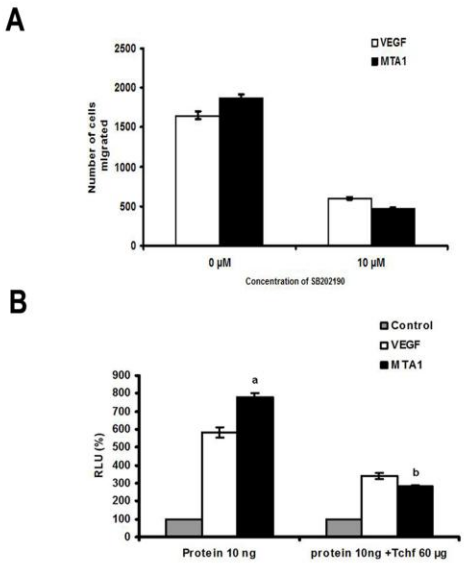


Fig. 5