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附件一

行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告 □期中進度報告

Terbinafine 的抗血管增生與抗癌作用之研究

計畫類別：■ 個別型計畫　□ 整合型計畫
計畫編號：NSC　94－2320　－B　－038　－　005　－
執行期間：　2005　年 08　月　01　日至 2006　年 07　月　31　日

計畫主持人：李文森
共同主持人：何元順
計畫參與人員：

成果報告類型(依經費核定清單規定繳交)：□精簡報告　■完整報告

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
□涉及專利或其他智慧財產權，□一年■二年後可公開查詢

執行單位：臺北醫學大學醫學研究所

中　華　民　國　95　年　9　月　18　日

中文摘要

關鍵詞：抗血管增生、抗腫瘤生長、TB、內皮細胞

我們的先前研究發現臨床上治療皮膚病的用藥 Terbinafine (TB)可藉由抑制內皮細胞增生進而抑制血管增生。本研究我們繼續證明 TB 可以抑制內皮細胞的粘附及遷移，且其抑制作用隨著 TB 濃度的增加而漸增。利用西方點墨法我們證實 TB 可以抑制細胞內 Ras 蛋白質的含量以及結合到細胞膜上面 Rho 的含量。同時當細胞給予 farnesol (FOH) 及 geranylgeraniol (GGOH)等藥物，可以避免 TB 所引起的內皮細胞遷移的抑制作用。然而這些因爲 FOH 及 GGOH 所引起的作用卻可被 Ras inhibitor peptide 及 ROCK inhibitor (Y27632) 所終止。這樣的結果讓我們認爲細胞內 geranylgeranyl pyrophosphate 的耗損會抑制蛋白質的 geranylgeranylation 及 farnesylation (這兩個作用是 Rho GTPases 及 Ras 的活化所必須的)，進而抑制內皮細胞的遷移。我們同時也觀察到 TB 藥物會降低內皮細胞的磷酸化 focal adhesion kinase (FAK)及 paxillin 兩種蛋白質的含量，以及 matrix metalloproteinase (MMP)2 及 MMP 9 的 mRNA 含量。綜合這些研究結果，我們認爲 TB 對內皮細胞遷移作用的抑制現象可能是藉由抑制了 Rho 所調控的訊息傳遞所造成。

英文摘要

Keywords: Anti-angiogenesis, anti-tumorigenesis, TB, endothelial cells

Our previous studies have demonstrated that terbinafine (TB), a newly synthesized oral antimycotic drug, exerts anti-tumorigenesis and anti-angiogenesis activities. TB treatment caused cell cycle arrest at the G0/G1 phase through up-regulation of the p53 protein, which in turn caused an increase in p21 expression, and finally inhibited the cyclin-dependent kinase 4 (CDK4) activity in various cancer cells including colon and liver cancer cell lines. Moreover, administration of TB reduced the growth of tumors derived from human colon cancer cells in an *in vivo* setting. (Lee et al. 2003). In the human vascular endothelial cells, treatment with TB also caused growth inhibition through up-regulation of p53 and p21 protein, which in turn inhibited CDK2 kinase activity, and finally arrested cell cycle at the G0/G1 phase. Using tube formation and CAM assays, we further demonstrated that TB exerts anti-angiogenic activity (Ho et al. 2003). Taken together, we results strongly suggest the potential applications of TB in the treatment of human cancer. Although we are very happy with these exciting findings, several important issues still need to be further addressed before it can be applied for the clinical uses. Accordingly, the proposed grant application is aimed to apply the cellular and molecular biology techniques to further study the anti-cancer activity of TB in detail. In the present study, we found that TB dose-dependently inhibited adhesion and migration of HUVEC. The levels of phosphorylated ERK and FAK were downregulated in the TB-treated HUVEC. Pretreatment of HUVEC with TB prevented TB-induced inhibition of [3H]thymidine incorporation. Taken together, our data suggest that RAS, ERK and FAK might be involved in the TB-induced inhibition of angiogenesis. Using RT-PCR technique, we also demonstrated that the p21 mRNA levels were up-regulated in HUVEC after 6 hr treatment with TB, suggesting that TB-induced increase of p21 protein is at the transcriptional level.

Introduction

TB is a newly synthesized oral antimycotic drug in the allylamines class: a fungicidal agent that inhibits ergosterol synthesis at the stage of squalene epoxidation. It shows a good safety profile and relatively few drug interactions. Recently, we have demonstrated that TB at a range of concentrations (0-120 μM) dose-dependently decreased cell number in various cultured human malignant cells. Flow cytometry analysis revealed that TB interrupts the cell cycle at the G0/G1 transition (Lee et al. 2004). The TB-induced cell cycle arrest in colon cancer cell line (COLO 205) occurred when the cyclin-dependent kinase (CDK) system was inhibited just as the levels of p53, p21 and p27 protein were augmented. In the TB-treated COLO 205, the binding between p53 protein and p53 consensus binding site in p21/Cip1 promoter DNA probe was increased. Pre-treatment of COLO 205 with p53-specific antisense oligodeoxynucleotide decreased the TB-induced elevations of p53 and p21/Cip1 protein, which in turn led to arrest the cell cycle at the G0/G1 phase. Moreover, in the p53 null cells, HL60, TB treatment did not induce cell cycle arrest. Taken together, these results suggest an involvement of the p53-associated signaling pathway in the TB-induced antiproliferation in COLO 205. We further examined whether administration of TB could affect the growth of tumors derived from human colon cancer cells in an *in vivo* setting. COLO 205 cells implanted subcutaneously in nude mice formed solid tumor; subsequent intraperitoneal injections of TB (50 mg/kg) led to obvious decline in tumor size of these tumors of up to 50-60%. In these tumors, increases in the p21, p27, and p53 protein and the occurrence of apoptosis were observed.

We also demonstrated that TB (0-120 μM) inhibited DNA synthesis and decreased cell number in cultured human vascular endothelial cells in a dose-dependent manner (Ho et al. 2004). TB was not cytotoxic at the concentrations used in the studies of cell growth and [3H]thymidine incorporation and this indicates that they may have an inhibitory effect on cell proliferation in the subcultured human vascular endothelial cells. Moreover, studies of [3H]thymidine incorporation revealed that treatment of the human vascular endothelial cells with TB decreased DNA synthesis and arrested the cells at the G0/G1 phase of the cell cycle. Western blot analysis demonstrated that the protein levels of cyclin A, but not cyclins B, D1, D3, and E, CDK2 and CDK4, decreased after TB treatment. The TB-induced cell cycle arrest in HUVEC occurred when the cyclin-dependent kinase 2 (CDK2) activity was inhibited just as the protein level of p21 was increased and cyclin A was decreased. Pretreatment of HUVEC with a p21 specific antisense oligonucleotide reversed the TB-induced inhibition of [3H]thymidine incorporation. Taken together, these results suggest an involvement of the p21-associated signaling pathway in the TB-induced antiproliferation in HUVEC. Capillary-like tube formation and chick embryo chorioallantoic membrane (CAM) assays further demonstrated the anti-angiogenic effect of TB. These findings demonstrate for the first time that TB can inhibit the angiogenesis.

Results

Effect of TB on migration of HUVEC

Previously, we demonstrated that TB exerts an anti-angiogenic activity through inhibiting the growth of ECs [8]. To examine whether inhibition of EC migration is also involved in the TB-induced inhibition of angiogenesis, a Transwells migration assay was performed in HUVEC. As illustrated in Figure 1a, TB at a range of concentrations (30-120 μM) dose-dependently inhibited the migration of HUVEC, and the migration activity was completely stopped when the TB concentration reached 120 μM.

Inhibition of EC attachment to matrix caused by TB

Since the attachment and adhesion of ECs to ECM are critical steps for the process of angiogenesis and would affect the ECs migration, we further examined whether TB could affect the cell adhesion on different matrices. Pretreatment of HUVEC for 24 hr with TB at a concentration of 120 μM, but not 60 μM or below, caused a significant decrease of cell number attached on the plate coated with collagen (Fig. 1b, left panel) or fibronectin (Fig. 1b, right panel).

Effect of TB on the levels of phosphorylated FAK and paxillin

Since the focal adhesion complex is one of the important components promoting cell motility, the adhesive interactions between cells and ECM can influence the attachment and transmigration across the surrounding cells [17]. We evaluated the effects of TB on the formation of focal adhesion complexes by examining the levels of tyrosine phosphorylations of focal adhesion kinase (FAK) and paxillin, two elementary proteins for forming stable adhesion complexes and transducing the survival/motility signals, in the TB-treated HUVEC by using Western blot analysis. After 24 hr pretreatment of the cells with TB (0-120 μM) followed by 1 hr challenged with 10% FCS, the ratios of both p-FAK/FAK (Fig. 2a) and p-paxillin/paxillin (Fig. 2b) were decreased in the TB-treated HUVEC as compared with vehicle-treated HUVEC (control). These TB-induced inhibitions were in a dose-dependent manner.

Effect of TB on the levels of Rho and Ras

To further delineate the molecular mechanisms underlying TB-induced migration inhibition in HUVEC, we examined the protein levels of Rho and Ras in the TB-treated HUVEC. As shown in Figure 3a, TB at a range of concentrations (0-120 μM) dose-dependently decreased the total protein levels of RhoB, but not Rho A and RhoC, in HUVEC. Since translocation of Rho GTPases from the cytosol to the cytoplasmic membrane is required for their activations and functions, we further examined the effect of TB treatment on membrane translocation of Rho GTPases. As illustrated in Figure 3b, TB (0-120 μM) dose-dependently increased the protein levels of RhoA in the cytosolic fraction of HUVEC. In contrast, RhoB is mainly located in the membrane fraction and TB did not affect the subcellular distribution of RhoB. The membrane translocation of RhoC was increased by TB treatment. The absence of G3PDH

immunoreactivity in the membrane fraction indicated the success of subcellular fractionation. TB also dose-dependently inhibited the protein levels of Ras (Fig. 3c). These results indicate that the interference of prenylation might be involved in the inhibition of HUVEC migration caused by TB.

Involvement of suppressions of farnesylation and geranylgeranylation in TB-induced migration inhibition in HUVEC

Since farnesylation and geranylgeranylation are important for subcellular distribution and functions of Ras and Rho, we further examined the involvement of farnesylation of the Ras proteins and geranylgeranylation of the Rho proteins in regulating the HUVEC migration. To examine whether prenylation was involved in the inhibition of migration induced by TB, we pre-treated HUVEC with isoprenoid pyrophosphate precursors (FOH & GGOH) followed by TB treatment. As illustrated in Figure 4, pretreatment of HUVEC with FOH (30 μM) partially prevented the TB-induced migration inhibition in HUVEC. This prevention effect caused by FOH treatment was abolished by Ras inhibitor peptide, (VPPPVPPRRR). Ras inhibitor alone caused a slight decrease in HUVEC migration. On the other hand, pretreatment of HUVEC with GGOH (30 μM) completely prevented the TB-induced migration inhibition in HUVEC (Fig. 5). This prevention effect caused by GGOH was abolished by 5 μM Y-27632, a ROCK (a kinase associated with RhoA for transducing RhoA signaling) inhibitor. Moreover, treatment of HUVEC with Y-27632 without TB caused migration inhibition of HUVEC. Taken together, these data suggest that suppressions of farnesylation and geranylgeranylation might be involved in the TB-induced migration inhibition in HUVEC.

Effects of TB on MMP expression

The activity of matrix metalloproteniases (MMPs) is also associated with the process of angiogenesis. To evaluate the effects of TB on the expression of MMP-2 and MMP-9, total RNA was isolated from HUVEC and RT-PCR analysis was conducted. The RT-PCR products of G3PDH were used as an internal control. TB at a range of concentrations (30-120 μM) dose-dependently inhibited the mRNA levels of MMP-2 (Fig. 6a) and MMP-9 (Fig. 6b), suggesting that inhibition of MMP-2 and MMP-9 might be involved in the TB-induced anti-angiogenic activity.

Discussion

Previously, we showed that TB at a range of concentrations (30-120 μM) dose-dependently inhibited angiogenesis by suppressing the proliferation of HUVEC and capillary-like tube formation *in vitro* and sprouting angiogenesis *in vivo* [8]. Here, we further demonstrated that TB (30-120 μM) dose-dependently inhibited HUVEC adhesion and migration. The findings of the present study suggest that the Rho-mediated pathway might be involved in the signal transduction leading to the migration inhibition caused by TB in cultured HUVEC. To our knowledge, this is the first demonstration that TB inhibits HUVEC migration through a Rho-dependent pathway.

Vascular endothelial cell adhesion (on the substratum by integrins) is one of the principal requirements for cell migration and proliferation [18] and by which multiple integrins with distinct combinations of α/β subunits have been recognized [19]. Integrin α1β1 and α2β1 mediate cell adhesion on collagen, which is a critical step in initiating tube formation of ECs [20], whereas recruitment of integrin α5β1, the fibronectin receptor, is required for cell migration in the process of angiogenesis. In the present study, we showed that TB at a concentration of 120 μM caused a 50% inhibition in the adhesion of ECs on collagen- or fibronectin-coated plates (Fig. 1b). In examination of the effect of TB on cell migration, we found that TB at a concentration of 120 μM completely inhibited HUVEC migration (Fig. 1a). These findings suggest that inhibition of cell adhesion could contribute only partially to endothelial migration inhibition induced by TB treatment. Moreover, our previous study demonstrated that TB at a concentration of 120 μM did not cause any cell death in HUVEC [8], suggesting that the TB-induced endothelial migration inhibition was not due to cytotoxicity.

In cholesterol synthesis pathway, squalene epoxidase converts squalene to 2,3-oxidosqulene. As an inhibitor of squalene epoxidase, TB might alter the protein prenylation by changing the expression of farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), which are the upstream molecules of squalene and regulate protein post-translational modification. The importance of the protein prenylation is underscored by the nature of the estimated 300 prenylated proteins in the human proteome; many of them participate in a multitude of signal transduction pathways related to cell growth, differentiation and migration [21]. Here, we showed that FOH (a precursor of FPP) partially and GGOH (a precursor of GGPP) completely reversed the TB-induced migration inhibition in HUVEC (Figs. 4 & 5), suggesting that synthesis inhibition or depletion of FPP and GGPP, which is essential for protein farnesylation and geranylgeranylation respectively, occurred in TB-induced migration inhibition in HUVEC. It has been shown that inhibition of squalene epoxidase could induce the accumulation of squalene [22]. Surprisingly, we found that TB, an inhibitor of squalence epoxidase, caused a decrease of the FPP and GGPP, which in turn inhibited endothelial cell migration. One of the possible explanations is that TB might directly inhibit the synthesis of FPP and GGPP or promote their degradation. However, the exact mechanisms underlying

TB-induced inhibition of polyisoprenyl pyrophophates deserve further investigation.

It has been recognized that the post-translational modification of proteins by the addition of isoprenoids is a key physiological process for facilitating cellular protein-protein interactions and membrane-associated protein trafficking [21]. The results from the present study suggest that TB could suppress the cell migration through altering the prenylation. Prenylation serves as important lipid attachments for the posttranslational modification of a variety of proteins, including small GTP-binding proteins belonging to the family of Ras, Rho, Rap, and Rab GTPases. In general, Rho family proteins, which regulate cell motility, require modification with geranylgeranyl pyrophophate [23]. Blockade of farnesyl biosynthesis leads to an inhibition in the Ras-mediated regulation of proliferation and migration in primary cultured human cells [24]. Tyrosine phosphorylation of focal adhesion kinase (FAK) triggers downstream signaling events including phosphorylation of paxillin, which is required for the regulation of Rho-family GTPases (Rho, Rac and Cdc42) and Pak (a downstream effector of Rac and Cdc42) [25]. To be functionally active, Rho proteins must be localized to the cell membrane by posttranslational modification through addition of isoprenyl groups from isoprenoid pyrophosphate substrates [26]. Rho GTPases play an important role in growth factors-stimulated cell migration and cytoskeletal organization, membrane trafficking, cell cycle control, and transcriptional activation [27-29,30,31]. To test whether TB suppressed HUVEC migration through regulating polyisoprenyl pyrophophates synthesis, we examined the effect of TB on the expression of isoprenylated protein. TB at a range of concentrations (30-120 μM) dose-dependently decreased the protein levels of Rho B, but not RhoA and RhoC protein (Fig. 3a). On the other hand, TB dose-dependently decreased the levels of membrane-bound RhoA and accumulated the RhoA in the cytosolic compartment (Fig. 3b). In contrast, TB increased the levels of membrane-bound of RhoC and did not affect the levels of membrane bound of RhoB. These data suggested that TB might interfere with cell migration via suppressing the prenylation of RhoA. Although various Rho GTPase family members (such as RhoA, RhoB and RhoC) are highly homologous, our present data suggest that inhibition of RhoA/ROCK signaling is critical for suppressing migration activity in the TB-treated HUVEC. Unlike RhoA protein, which is located in plasma membrane for regulating actin stress fiber formation and integrin signaling, RhoB is located in the endosome and nuclear membranes with a unique function in intracellular trafficking of growth factor receptors such as the epidermal growth factor (EGF) receptor [32]. RhoB plays an inhibitory role during cell cycle regulation and is upregulated in response to stress stimuli [33]. It has been indicated that RhoA and RhoC have overlapping functions including promotion of cell motility, cytoskeletal alterations, and metastasis [34]. Treatment of HUVEC with TB induced an increase instead of a decrease of RhoC translocation from cytosol fraction to particulate fraction, suggesting that RhoC might not be involved in the TB-induced inhibition of HUVEC migration. In the present study, we also showed that TB (30-120 μM) dose-dependently inhibited the levels of Ras protein (Fig. 3c). Pre-treatment of HUVEC with FOH partially prevented the TB-induced migration inhibition in HUVEC. This prevention effect mediated by FOH was abolished by pre-treated HUVEC with Ras inhibitor (Fig. 4a),

suggesting that Ras-mediated pathway was involved in the TB-induced migration inhibition in HUVEC.

Proteolytic breakdown of the basement membrane, which results from secretion and activation of MMPs in response to exogenous signals such as cytokines, growth factors and cell–matrix interactions, is also essential for endothelial cell migration [5]. During angiogenesis, the extracelluar proteolytic activity in the endothelial pericellular environment was significantly upregulated. The phenomenon of a temporal sequence of matrix dissolution and assembly is replaced by a more mature collagenous matrix with time, in which fibrin provided a temporary matrix scaffold for migrating ECs. It was demonstrated that the matrix metalloproteniases (MMPs) might mediate the basement membrane degradation. The zinc-dependent enzymes MMPs can be divided into secreted MMPs and membrane-type MMPs (MT-MMPs) by their structural difference. Secreted MMPs include collagenases (eg, MMP-1), gelatinases (MMP-2; MMP-9), stromelysins (eg, MMP-3), and other MMPs (eg, MMP-7). The catalytic activity of secreted MMPs is tightly regulated. The secreted inactive proenzymes (zymogens) are later activated in the extracellular compartment. MT1-MMP is endowed with two characteristics important for cell migration; association with the plasma membrane, which focuses matrix digestion close to the cell surface, and the capacity to catalytically activate the precursor of MMP-2, which allows for amplification of the degradative process [35]. It has been shown that Rho family can regulate the expression and activation of MMP-2 and -9. RhoA triggers signalling pathways, that upregulate expression of MMP-9 at specific membrane localizations and may confer a highly invasive phenotype to ECs [36]. Rac1 mediates MMP-2 activation during the encounter between invading tumor cells and collagen-rich stroma, and thereby facilitated collagenolysis and cell invasion [37,38]. Treatment of HUVEC with TB caused a decrease in the mRNA levels of MMP-2 and -9 (Fig. 6). We presumed that the reduced expressions of MMP-2 and -9 might also contribute to the inhibition of cell migration in TB-treated HUVEC, and Rho might be one of the key targets of TB for down-regulating the expression or inactivation of MMP-2 and -9.

In the present study, we showed that TB suppressed the phosphorylation of FAK and paxillin (Fig. 3). The phosphorylation state and activity of FAK are tightly related to cell adhesion to the extracellular matrix through integrin receptors [39]. Paxillin is a downstream target of FAK. It has been suggested that tyrosine phosphorylation of FAK and paxillin is able to influence cellular events either that are dependent on cell adhesion (such as cell proliferation and survival), or that require modulation of cell adhesion (such as cell migration) [40]. The tyrosine phosphorylation of FAK and paxillin is necessarily and sufficiently mediated by Rho [25,41]. Our results suggest that TB might inhibit endothelial cell migration through inhibiting the activation of FAK and paxillin via Rho-mediated pathway.

In conclusion, this study provides the evidence that TB suppressed the prenylation of Rho, which in turn caused inactivaton of FAK and paxillin, and eventually resulted in the inhibition of

endothelial cell adhesion and migration. Taken together, our previous and current findings strongly suggest the potential applications of TB as an anti-angiogenic agent because it is capable of retarding the cell cycle of ECs, reducing the adhesion and migration of ECs, interrupting the tube formation, and inhibiting sprouting angiogenesis. This study also provides new insights into the regulation of endothelial cell behaviors by medical products, which regulate the cholesterol synthesis process.

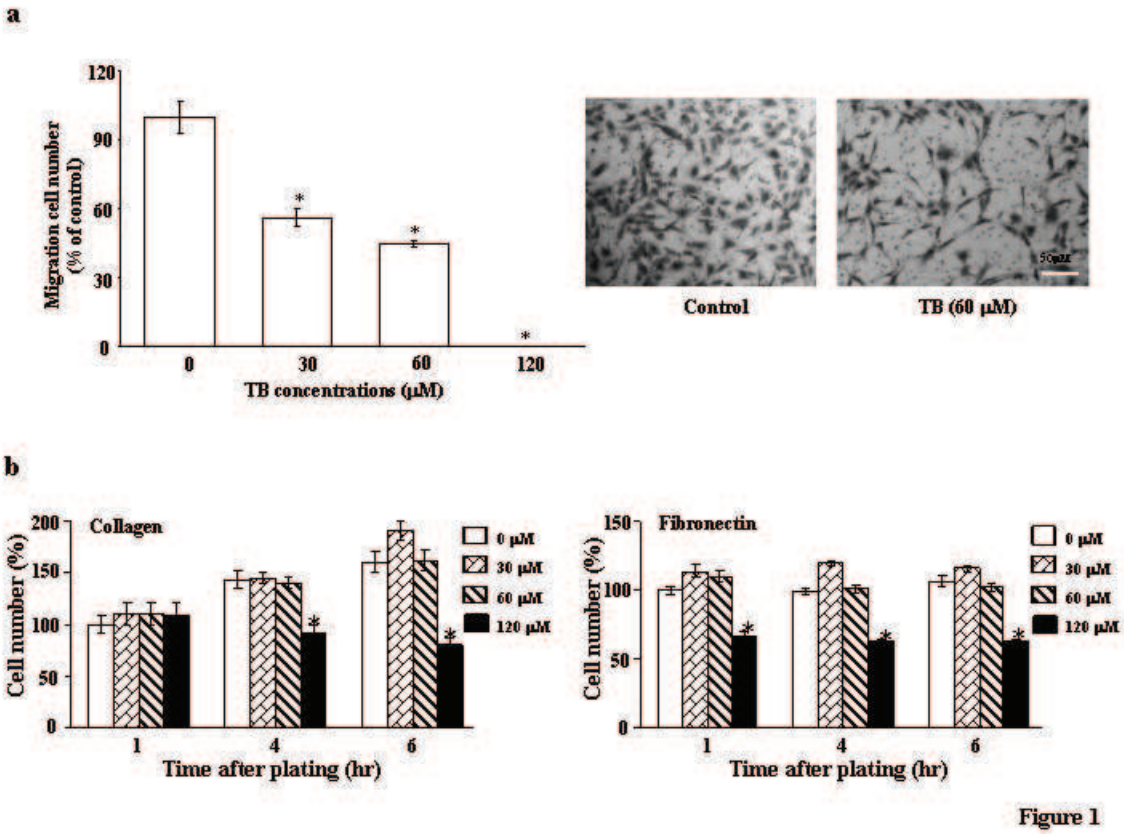


Figure 1

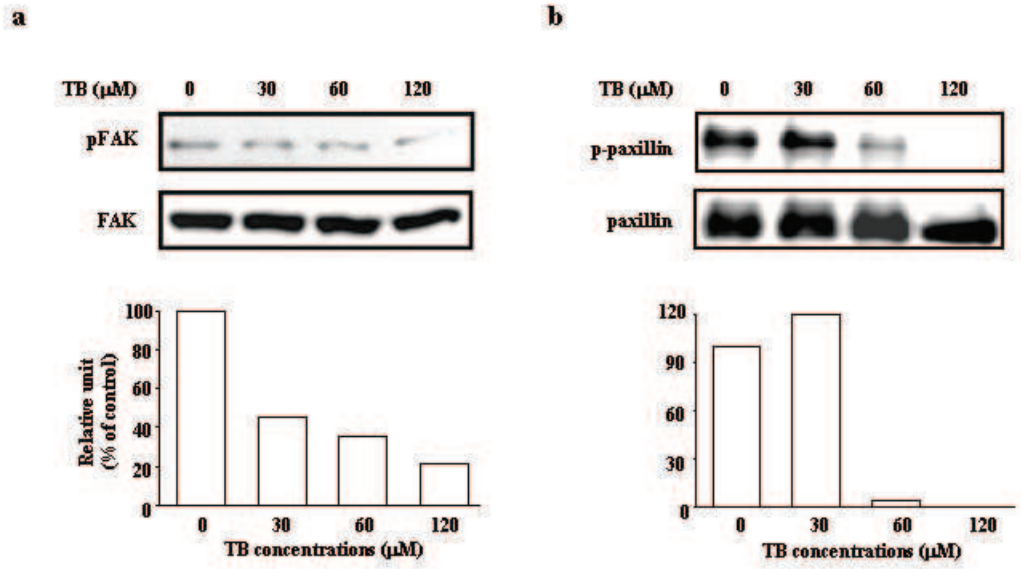


Figure 2

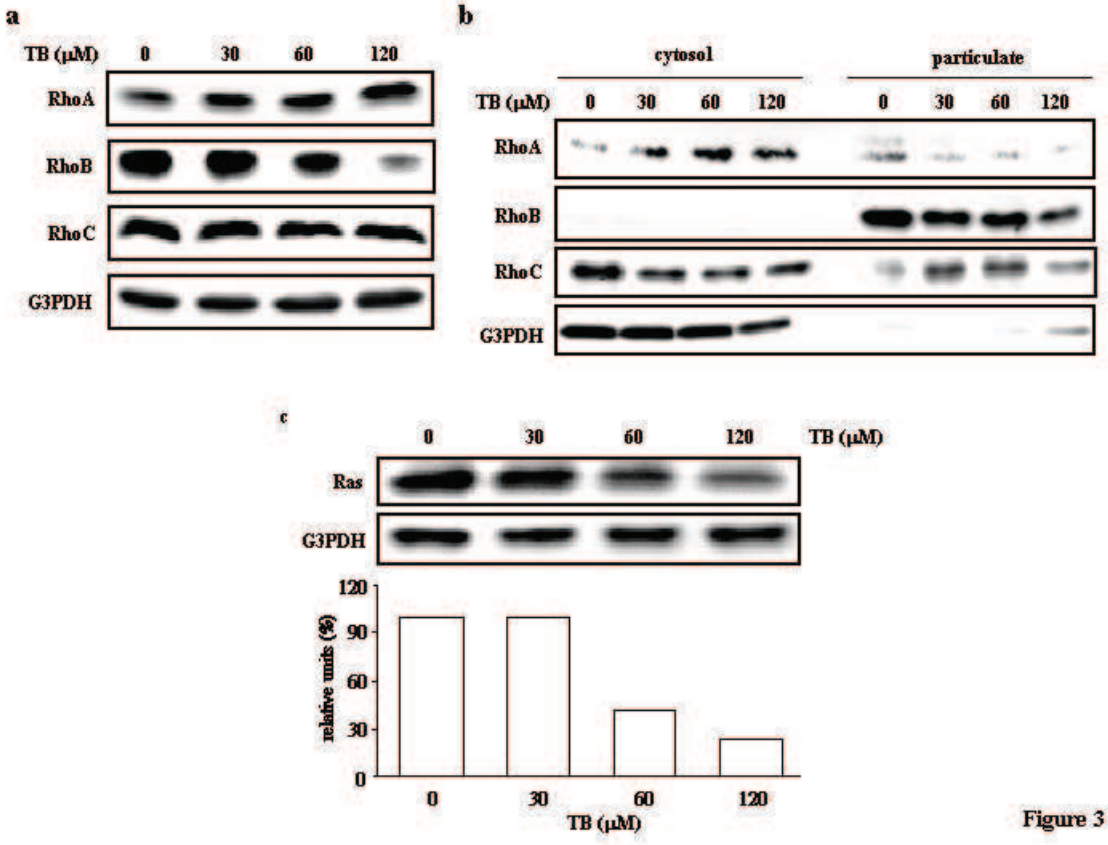


Figure 3

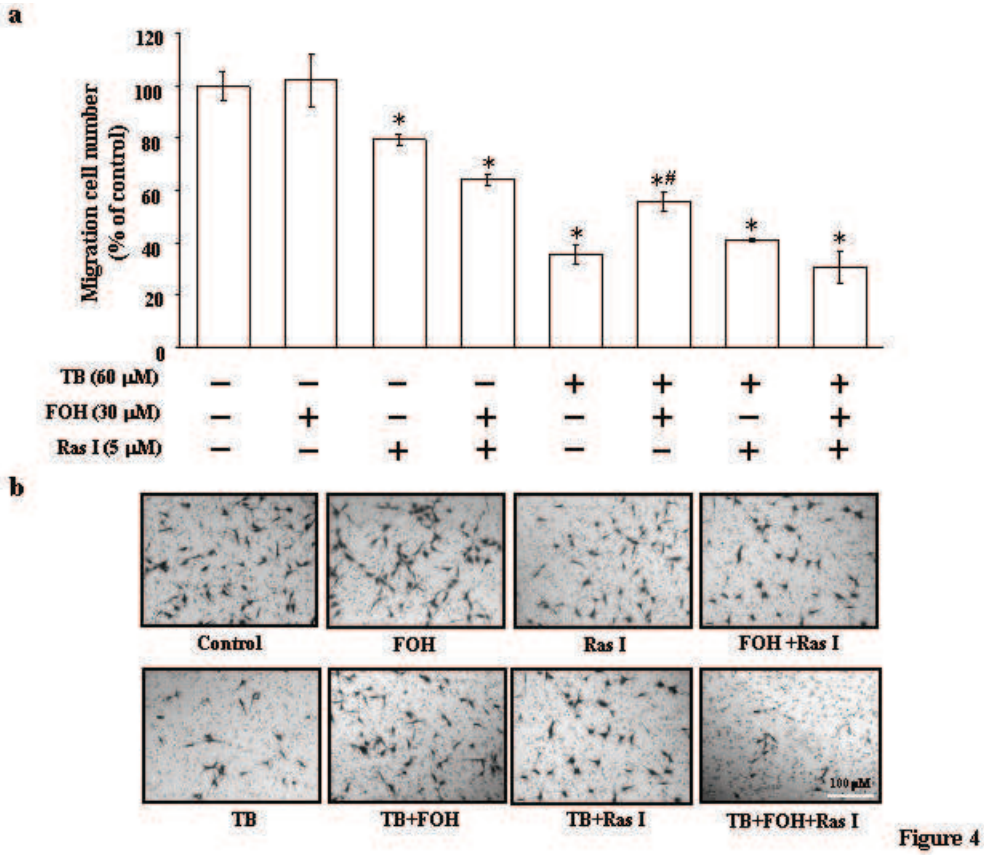


Figure 4

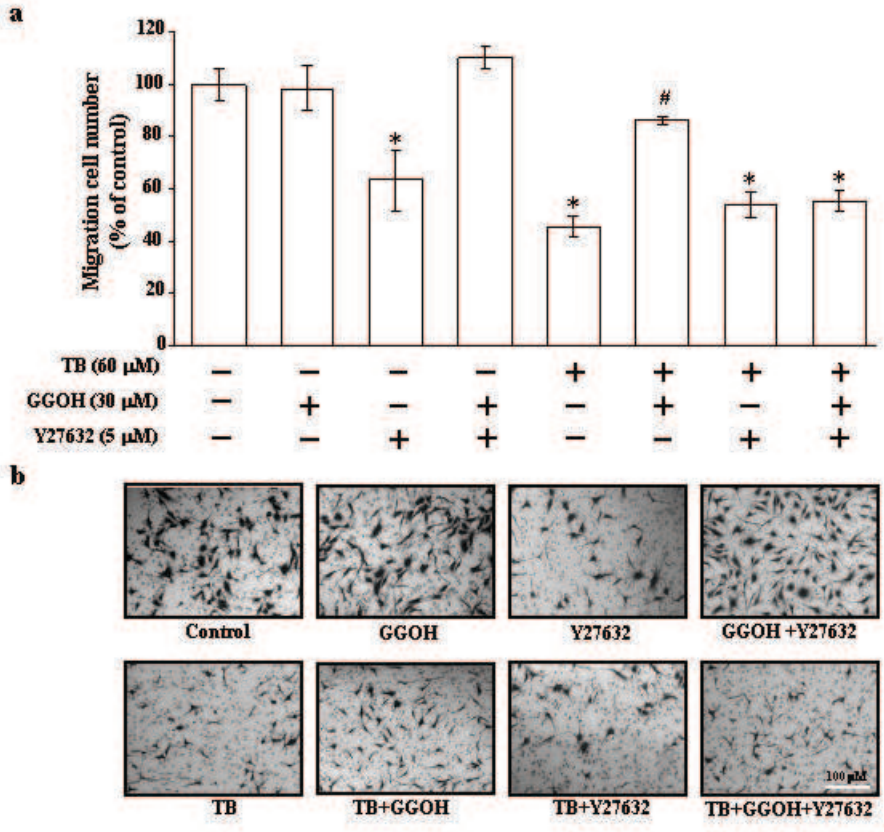
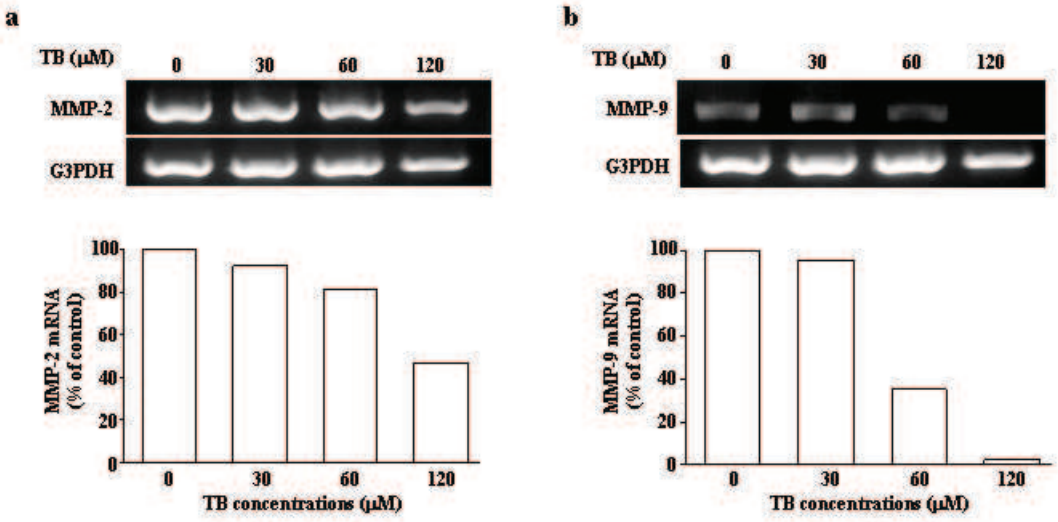


Figure 5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TB (μM)
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MMP-2
G3PDH
MMP-2 mRNA (% of control)
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TB concentrations (μM)
b
TB (μM)
0
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120
MMP-9
G3PDH
MMP-9 mRNA (% of control)
100
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60
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20
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TB concentrations (μM)

Figure 6

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