Tranilast inhibits cell proliferation and migration and promotes apoptosis in murine breast cancer

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The malignant transformation of breast epithelium involves a number of cellular pathways, including those dependent on signaling from TGF β. Tranilast [N-(3, 4-dimethoxycinnamonyl)-anthranilic acid] is a drug that is used in Japan to control allergic disorders in patients, and its mechanism of action involves TGF β. In view of the multiple roles of TGF β in tumor progression, we hypothesized in this study that tranilast impacts cell proliferation, apoptosis, and migration. Using the mouse breast cancer cell line 4T1, our studies showed that tranilast increases AKT1 phosphorylation and decreases ERK1/2 phosphorylation. Alterations in the cell cycle mediators' cyclin D1, p27, cyclin A, pRB, cyclin B, and Cdc2 were observed after exposure to tranilast, favoring cell arrest beyond the G1/S phase. Tranilast reduced tumor cell proliferation even when it was amplified by exogenous TGF β. TGF β-neutralizing antibody did not cause a significant decrease in cell proliferation. Tranilast treatment upregulates p53, induces PARP cleavage *in vitro*, consistent with a promotion of tumor cell apoptosis. TGF β-neutralizing antibody downregulates endoglin and matrix metalloproteinases (MMP)-9 levels *in vitro* indicating that the tranilast effect is mediated through TGF β modulation. Tranilast treatment results in the inhibition of cell migration and invasion. Western blot analysis of tumor lysates from tranilast-treated mice shows decreased levels of TGF β1, endoglin, and significantly higher levels of p53 and cleaved PARP. Cleaved caspase 3 expression is significantly elevated in tranilast-treated mouse breast tumors. To conclude, tranilast induces cellular and molecular changes in murine breast cancer that can be exploited in preclinical therapeutic trials. *Anti-Cancer Drugs* 21:351–361 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

The refinement of multimodal therapies of breast cancer, including the introduction of therapies targeting the Her2/neu oncogene, has resulted in significant improvements in prognosis. However, distant metastasis continues to be a challenge in addressing therapeutic failures. Tumor growth at the primary site and metastatic dissemination depend on a large number of cellular and extracellular factors [1]. Among them, TGF β plays a complex role: while having a tumor suppressor function in the early phase of carcinogenesis it switches to a tumor-promoting function at later stages of the cancer progression [2]. This latter role of TGF β has been attributed to a number of transduced functions such as increase in cell migration, and increased survival of cancer cells, in addition to causing conducive extracellular microenvironments that promote tumor growth [3–5]. As there are several pathways that TGF β signaling transduces [6], the choice of a therapeutic drug needs to be related to the balance between the tumor-promoting and tumor suppression function of TGF β.

On account of its known inhibitory effect on TGF β [7,8], tranilast [N-(3,4-dimethoxycinnamonyl)-anthranilic acid] is a drug that has potential for the biology of the breast cancer cells. Tranilast has been used as an antiasthmatic drug for a long time in Japan with few adverse reactions [9,10]. Tranilast has also been reported to be a potential therapeutic agent for reducing invasiveness of human malignant glioma cells [11], antitumor effect in experimental pancreatic cancer [12] and for its antiproliferative effect on uterine leiomyoma cells [13]. Although tranilast has been implicated as an antagonist of TGF β action in the above-mentioned tumor studies, the mechanisms of antitumor action downstream to TGF β have not been clearly identified. Using a murine model of orthotopic breast cancer, we have recently reported that tranilast markedly inhibits the growth of the primary tumor and its metastatic dissemination [14].

In this study, we hypothesized that tranilast impacts cell proliferation, apoptosis and migration through cellular and molecular mechanisms that explain the therapeutic effects observed earlier in the mouse model. We observed that tranilast downregulates the endogenous levels of TGF β1 both *in vitro* and *in vivo*. This effect was accompanied by changes consistent with an inhibitory effect on the cell cycle and a promotion of apoptosis.

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Furthermore, tranilast was found to inhibit breast cancer cell motility, invasion and the expression of biomarkers related to invasion.

Materials and methods

Antibodies

Rabbit anti-cyclin A, cyclin B1, Cdc2, pRb, cyclin E, Cdk2, p53, p21, p27, matrix metalloproteinases (MMP)-9, endoglin and mouse anti-phospho-AKT1 (Santa Cruz Biotechnology, Santa Cruz, California, USA), mouse anti-human β-actin (Sigma-Aldrich, St. Louis, Missouri, USA), rabbit anti-TGF β1, phospho-p44/42 MAPK, PARP, cleaved caspase 3 (Cell Signaling Technology, Beverly, Massachusetts, USA), and neutralizing anti-TGF β1, β2, β3 antibody (R & D systems, Minneapolis, Minnesota, USA) were used in the western blot and immunohistochemistry studies. Rabbit and mouse IgG controls for both the studies were from Santa Cruz Biotechnology. Secondary antibodies were goat anti-rabbit HRP and goat anti-mouse HRP (Promega, Madison, Wisconsin, USA) and anti-rabbit-labeled polymer HRP (Dako, Carpenteria, California, USA).

Cell line and in-vitro culture

4T1 is an estrogen-independent breast carcinoma cell line, originally developed from a spontaneously arising breast tumor in Balb/cfC3H mice [15] and was obtained from ATCC (Manassas, Virginia, USA). The cells were grown in DMEM supplemented with 10% FBS and antibiotics in a complete medium (CM). Tranilast (800 μmol/l; a kind gift from Dr Richard Gilbert, St Michael's Hospital, Toronto, Ontario, Canada) in 0.8% dimethyl sulfoxide (DMSO) was added to the cells in culture for the indicated time periods. The DMSO vehicle (0.8%) alone served as control. For all in-vitro experiments, cells in culture were G0 arrested for 24 h using a serum-free medium before being released by the addition of CM. For cells in culture, human recombinant TGF β1 growth factor (R & D systems) was added at a final concentration of 2 ng/ml. For cells in culture, TGF β1,2,3 neutralizing antibody was added at a final concentration of 20 μg/ml.

Effect of TGF β and anti-TGF β1,2,3 neutralizing antibody on the growth of 4T1 cells in culture

Cell proliferation assays were performed as described earlier [14]. Briefly, 4T1 cells (5×10^3/well) were allowed to attach to the wells of a flat-bottom 96-well plate. Recombinant human TGF β1, anti-TGF β1,2,3 neutralizing antibody, tranilast (dissolved in DMSO), or DMSO were added to the cells at concentrations mentioned above and cultured for 48 h. Cell growth was measured by the MTT assay read at 540 nmλ.

Cell lysate preparation and western blot analysis

Cell lysates were prepared and western blotting performed as described [16]. Briefly, the cells were lysed in lysis buffer (50 mmol/l Tris pH 7.6, 150 mmol/l NaCl, 0.1% NP-40) containing a cocktail of protease inhibitors (phenylmethylsulphonyl fluoride, leupeptin, pepstatin, and aprotinin) and 5X concentrated Laemmli sodium dodecyl sulfate (SDS) sample buffer containing β-mercaptoethanol was added to the cell lysates and incubated for 5 min in a boiling water bath, vortexed, and an appropriate amount was loaded onto a 12% SDS–polyacrylamide gel electrophoresis for western blot analysis.

Animal experiments

The 4T1 cells were transplanted orthotopically in breast fat pads (5×10^5 cells/pad/mouse in 50 μl) of 6-week-old syngeneic mice (Balb/c from Charles River Laboratories, Wilmington, Massachusetts, USA) under anesthesia as described elsewhere [14,17]. The mice were given tranilast by gavages daily at the dose of 300 mg/kg body weight, or vehicle from day 0 (day of cancer cell transplantation) to the end of the treatment as described [14]. Tranilast was dissolved in 1% $NaHCO_3$ for this purpose. Neutralized 1% $NaHCO_3$ served as the vehicle. On day 28 of treatment, the mice were killed and the tumors were excised and processed for different experiments.

Tissue lysate preparation and immunoblotting

Breast tumors were excised from the mice and processed for immunoblotting as described [18]. Briefly, the freshly excised tumors were washed twice in sterile PBS, pH 7.4, and snap-frozen in liquid nitrogen. We weighed the specimen (approximately 80 mg), crushed it into fine pieces in a mortar and pestle, and lysed in 300 μl of Tris–EDTA buffer containing protease inhibitors and homogenized on ice. The lysates were vortexed and kept on ice for 30 min. The tubes were vortexed and spun at 14 000 rpm for 10 min at 4°C. The supernatant was then used for immunoblotting with appropriate primary antibodies as described above.

Immunohistochemistry

Paraffin sections of mouse breast tumors were dewaxed and rehydrated and processed for immunohistochemistry as described [19]. Briefly, antigen unmasking was done in a 0.01 mol/l sodium citrate buffer (pH 6.0). Sections were blocked in 5% normal goat serum followed by incubation with rabbit polyclonal anticleaved caspase 3 for 2 h at room temperature. Negative control slides were incubated with isotype-matched rabbit IgG. Incubation with anti-rabbit HRP polymer secondary antibody was for 30 min. A signal was developed using 3,3′-diaminobenzidine tetra hydrochloride (Vector Laboratories, Burlingame, California, USA) and counterstained with hematoxylin. Images were acquired using an Olympus BX50 microscope and an UplanSapo × 40 lens.

Wound assay

In-vitro wound assay for the quantification of cell motility was performed using 4T1 cells in culture that was treated

with 800 µmol/l tranilast or DMSO vehicle control. The wound migration assay was performed as described earlier [20]. A 1-mm-wide wound was made across the center of each well with a micropipette tip. For each well, five areas along the length of the wound were chosen randomly for photography with phase contrast microscopy. After photography, the cells were incubated at 37°C and allowed to migrate. Photographs of the exact wound areas chosen on day 0 were again taken at 24, 48, 72, and 96 h. Photographs taken at the various time points were used for analyses of cell motility and morphometric quantitation of the width of the wound, using image J software (NIH, Bethesda, Maryland, USA).

Matrigel invasion assay

Invasion assay was carried out using matrigel-coated Falcon fluoro Blok 24-well plates (BD Biosciences, Mississauga, Ontario, Canada) according to the manufacturer's protocol. Briefly, 4T1 cell suspensions (1×10^5 cells/ml) in serum-free DMEM, containing DMSO vehicle control or tranilast, were added to the top of the each insert plates, respectively. The lower chambers with 20% serum-containing medium served as a chemoattractant along with DMSO or tranilast. After incubation for 48 h, the insert plate was transferred to a second 24-well plate containing 0.5 ml/well of 4 µg/ml Calcein AM (BD Biosciences) in Hanks buffered saline. The plates were incubated for 1 h at 37°C. Cell invasion into the coated filter was quantified using a plate flourimeter (Thermo Lab Systems, Franklin, Massachusetts, USA). Invasion is expressed as a percentage of the vehicle control.

Results

Tranilast upregulates AKT1-P and alters MAPK phosphorylation levels in 4T1 cells

Western blot analysis of cell lysates from the 4T1 cells treated with tranilast showed an increase in the phosphorylation of AKT1 at the end of 24 h (Fig. 1a). The same set of lysates showed a remarkable decrease in the levels of ERK1/2 phosphorylation after tranilast treatment (Fig. 1b) suggesting that AKT1 phosphorylation negatively regulates ERK1/2 phosphorylation.

Tranilast treatment modulated the cell cycle regulator proteins

As AKT1 and ERK1/2 affect cell proliferation and in view of the changes noted above, we then asked whether tranilast alters the expression of cell cycle regulators in the 4T1 cells. Compared with the vehicle-treated controls, we observed changes in the family of proteins as early as 24 h and persisting to 48 h in culture after the addition of tranilast. Among the cell cycle regulator changes observed were downregulation in the levels of cyclin D1 and Cdk4 (Fig. 1c), upregulation of cyclin A (Fig. 1d), and cyclin B1/cdc2 levels (Fig. 1e). Levels of p27 were downregulated by tranilast with no effect on p21 levels (Fig. 1f). Dephosphorylation of Rb occurred at the end of 48 h in tranilast-treated 4T1 cells (Fig. 1g). Overall, these results indicate that tranilast caused a G1/S arrest of the 4T1 cells, with the remaining cells in cycle undergoing a G2/M arrest.

Tranilast inhibits 4T1 cell proliferation even in the presence of TGF β stimulation

As tranilast treatment resulted in a majority of the 4T1 cells undergoing G1/S arrest, we tested the effect of TGF β on cell proliferation. Using the MTT assay for cell proliferation, 4T1 cells in culture showed a significant increase in cell proliferation after the addition of exogenous TGF β compared with the vehicle control, and tranilast reduced cell proliferation even in the presence of TGF β1 stimulation (1H). As cell proliferation increased significantly on TGF β addition, we did the corollary experiment to see whether anti-TGF β1,2,3 neutralizing antibody would reduce cell proliferation. Addition of TGF β1,2,3 neutralizing antibody to the cells in culture did not result in a significant decrease in cell proliferation. However, a significant decrease in cell proliferation was noticed when tranilast was added to the TGF β antibody-treated cells (Fig. 1i).

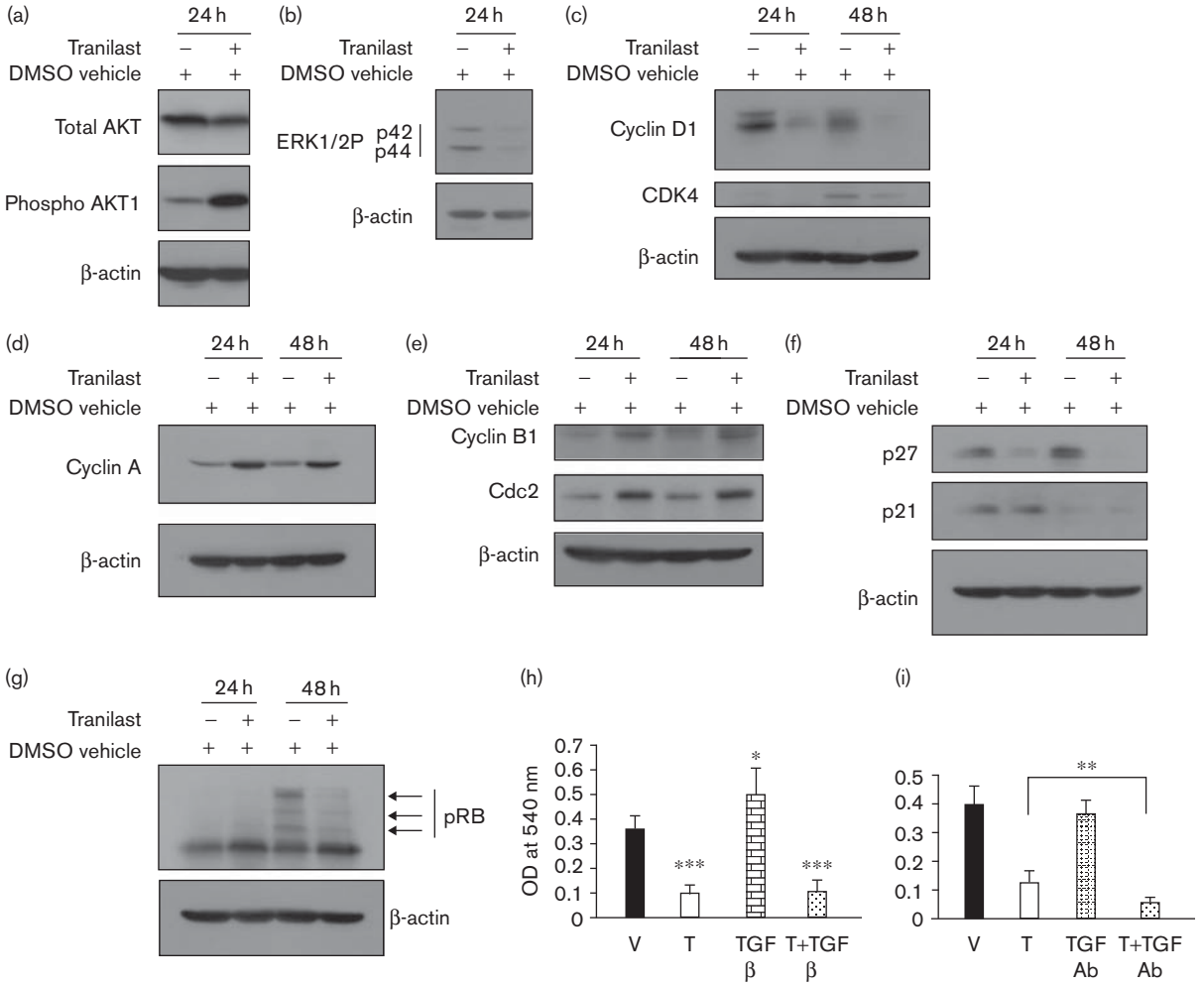
Tranilast upregulates p53 and PARP cleavage in 4T1 cells

Cell lysates from the 4T1 cells treated with tranilast showed an increase in the levels of p53 (Fig. 2a). A densitometric study of the western blot data normalized to β-actin from Fig. 2a showed a 28.5% increase in the levels of p53, 48 h after the addition of tranilast (Fig. 2b). Western blot analysis of the tranilast-treated cell lysates showed increased levels of cleaved PARP at the end of 48 h (Fig. 2c). These results indicate that tranilast treatment of the breast cancer cells induces them to undergo apoptosis.

Tranilast downregulates metastatic marker endoglin and MMP-9 levels in 4T1 cells

We then investigated whether tranilast alters the expression of cellular proteins associated with tumor cell invasion. Western blot analysis of cell lysates from the 4T1 cells treated with tranilast showed a decrease in the levels of endoglin at 24 h, which became undetectable at 48 h (Fig. 2d). MMP-9 levels were downregulated in tranilast-treated cell lysates at 48 h (Fig. 2e). To investigate whether tranilast mediates its effect on endoglin and MMP-9 through TGF β, 4T1 cells were treated with a TGF β growth factor (2 ng/ml) in addition to tranilast. Western blot analysis of the cell lysates showed an upregulation of endoglin and MMP-9 levels after the addition of TGF β growth factor, with the levels being downregulated by tranilast treatment, respectively (Fig. 2f and g). The corollary experiments on 4T1 cells using a neutralizing antibody to TGF β1,2,3 and western blot analysis showed a 29.7% decrease in the levels of endoglin (Fig. 2h) and 44.2% decrease in MMP-9 levels (Fig. 2i), respectively, suggesting that tranilast mediates

Fig. 1



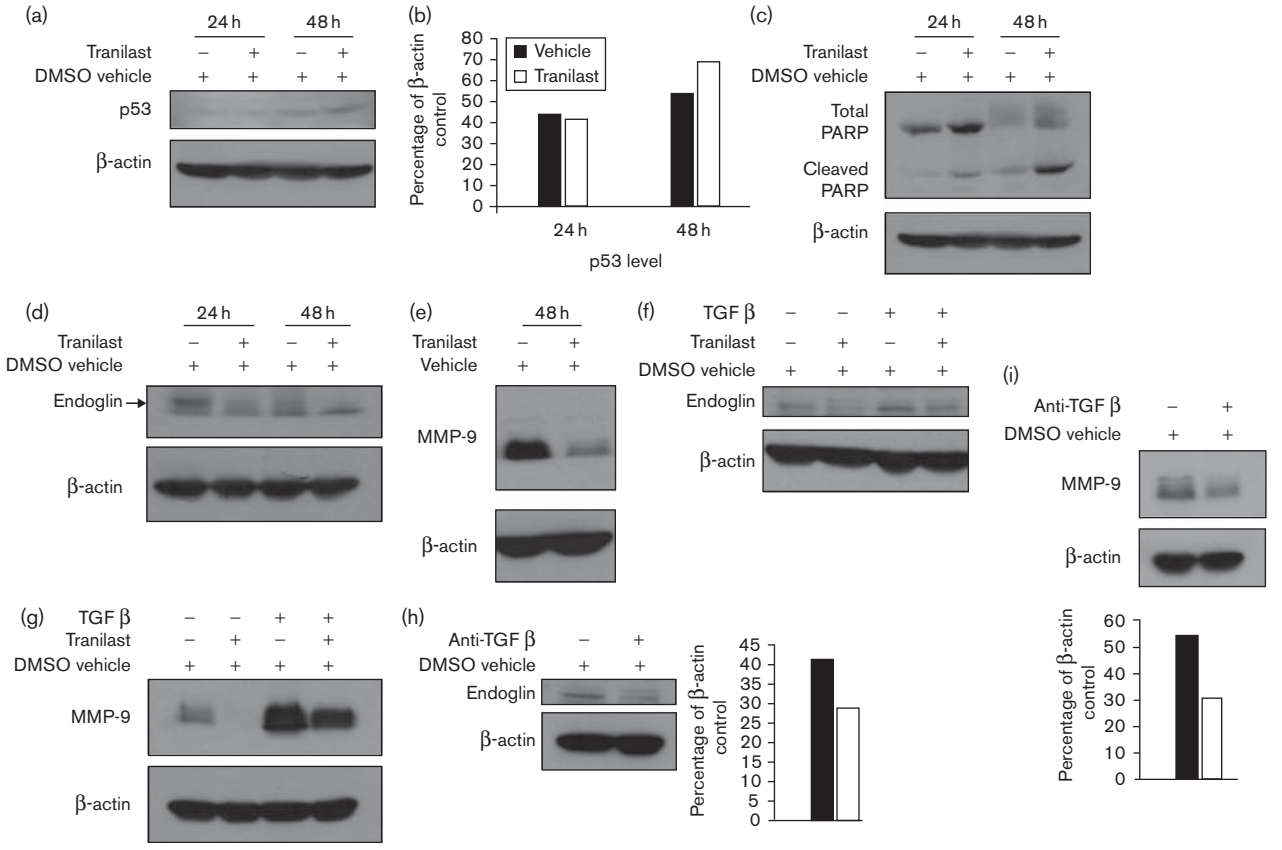
Tranilast treatment modulates AKT1, ERK1/2 phosphorylation, and cell cycle regulators: western blot analyses of 4T1 cells lysates, prepared as detailed under Materials and methods, were performed to investigate the levels of phosphorylation of AKT1 and ERK1/2 after tranilast treatment. Tranilast increases AKT1 phosphorylation by 24 h in culture (a). ERK1/2 phosphorylation is decreased after 24 h of tranilast treatment (b). 4T1 cell lysates were also tested by western blot for regulators of cell cycle after tranilast treatment. There is a decrease in the levels of cyclin D1 and Cdk4 as early as 24 h (c, lane 2 vs. 1), persisting at 48 h (c, lane 4 vs. 3). Cyclin A, cyclin B1, and Cdc2 are increased after 24 and 48 h of tranilast treatment (d and e). At the two time periods, p27 levels are downregulated after tranilast treatment (f, lanes 2 and 4), whereas the levels of p21 are unaffected (f). Tranilast treatment of 4T1 cells causes dephosphorylation of retinoblastoma (RB) protein by 48 h compared with the dimethyl sulfoxide (DMSO) control (g, lane 4 vs. 3). MTT proliferation assay of 4T1 cells in culture shows that cell proliferation is increased by TGF β1 (*$P<0.05$, h). Tranilast reduces cell proliferation even in the presence of TGF β1 stimulation (h). No significant reduction in cell proliferation occurs when anti-TGF β1,2,3 neutralizing antibody was added to the cells compared with the vehicle control (i). Addition of tranilast to TGF β antibody-treated cells reduces cell proliferation when compared with cells treated with tranilast and not TGF β antibody (i). V, vehicle; T, tranilast; TGF Ab, Anti-TGF β1,2,3 neutralizing antibody. **$P<0.001$; ***$P<0.0001$.

its effect through the regulation of TGF β. The above data thus suggest that tranilast has the potency to alter the ability of breast cancer cells to invade and metastasize.

Tranilast decreases cell migration of 4T1 cells

As one of the basic cellular behaviors of cancer cells is the ability to activate a motility program that leads to tumor invasion and metastasis; we investigated whether tranilast has an effect on tumor cell migration. This was evaluated after wound induction in monolayers of 4T1 cells in culture. In tranilast-treated 4T1, cells migrated at a slower pace to close the wound when compared with vehicle-treated control even at the end of 96 h (Fig. 3a, day 4). The linear wound in the control cell monolayers closed rapidly, starting as early as 3 h after wound induction, and was completed at 24 h. In contrast, there was a marked inhibition of the wound closure when 4T1 cells were exposed to tranilast, as shown by the failure of cells to close the wound 96 h after it had been created (Fig. 3a and b: days 1–4). This result shows the ability of tranilast treatment to inhibit cell migration in breast cancer *in vitro*.

Fig. 2



Tranilast treatment increases apoptosis and reduces endoglin and matrix metalloproteinases (MMP)-9 levels in 4T1 cells: western blot analysis of 4T1 cell lysates for p53 expression shows upregulated levels in the tranilast treated cells at 48 h (a). Densitometric analysis of the western blots shows upregulation of p53 (28.5%) at 48 h (b). Western blot analysis of 4T1 cell lysates shows upregulation of the apoptosis marker, cleaved PARP in tranilast treated cells as early as 24 h in culture when compared with dimethyl sulfoxide (DMSO) vehicle, persisting at 48 h (c). Western blot analysis of 4T1 cell lysates shows downregulation of endoglin after 48 h exposure to tranilast (d) and MMP-9 (e). In 4T1 cells not exposed to tranilast, TGF β induces an upregulation of endoglin and MMP-9 (lanes 3 vs. 1 in f and g). Addition of TGF β neutralizing antibody to 4T1 cells is accompanied by a 29.7% decrease in endoglin and 44.2% decrease in MMP-9 levels when compared with the vehicle control (h and i).

Tranilast treatment inhibits invasion of 4T1 cells

Cell migration in response to chemical stimuli is central to wound repair and tumor metastasis. Tranilast effectively inhibited cell migration as shown by the wound assay. Hence, using a matrigel assay system we asked whether tranilast could inhibit the invasive ability of 4T1 cells *in vitro*. From the invasion assay we observed that tranilast effectively inhibited the invasion of 4T1 cells by nearly 65% when compared with the vehicle-treated control at the end of 48 h (Fig. 3c). Globally, these results thus show the ability of tranilast to inhibit cell motility and invasion of breast cancer cells *in vitro*.

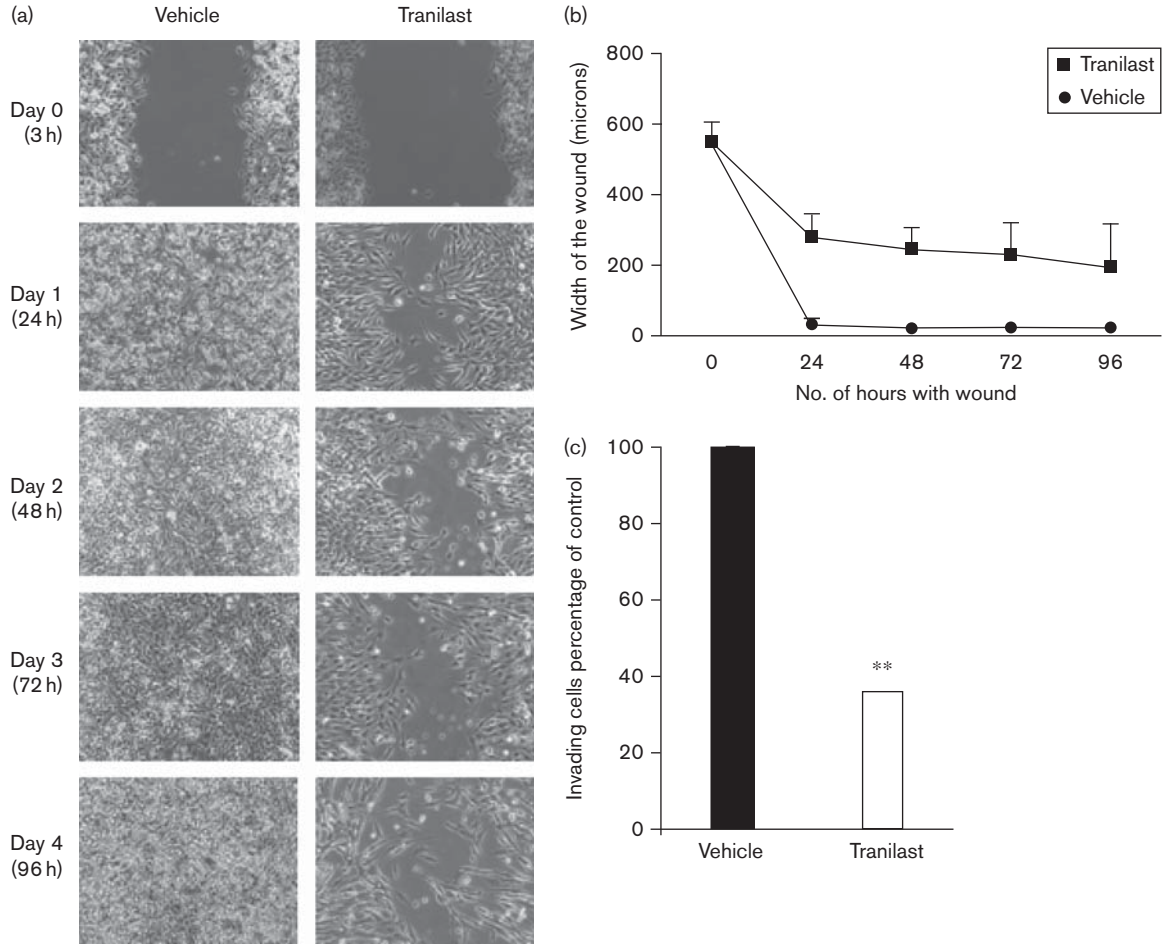
Tranilast treatment alters TGF β1 levels in breast tumors

On the basis of our earlier demonstration of the effect of tranilast on the expression of TGF β1 in cultured 4T1 cells [14], we investigated whether this effect was also observed in the orthotopic breast tumors, following oral administration of tranilast to the mice. Western blot analysis of tumor lysates from tranilast-treated mice showed a 36% decrease in the unprocessed levels of TGF β1 when compared with vehicle-treated control tumors, which were confirmed by densitometric analysis (Fig. 4a and b). This result suggests that tranilast treatment alters TGF β1 levels, a key signaling molecule involved in cell proliferation and motility.

Tranilast treatment upregulates p53 and cleaved PARP and altered endoglin levels in breast tumors

Western blot analysis of tumor lysates from mouse breast tumors after tranilast treatment showed a significant 49.5% increase in p53 levels compared with the vehicle-treated control group (Fig. 4c and d). Tranilast treatments also significantly upregulated the levels of cleaved PARP by 117.7% in the tumors tested when compared with the vehicle control (Fig. 4e and f). These results suggest that tranilast treatment can effectively induce tumor suppression and cell death *in vivo*. In addition, tranilast

Fig. 3



Tranilast slows the migration and invasion of mouse breast cancer cells: a wound was created using 4T1 cells in culture as described under Materials and methods. Pictures of the wound closure were taken at various time intervals for 4 days as indicated and subjected to image analysis. The cells in the dimethyl sulfoxide (DMSO) vehicle starts migrating to close the wound as early as 3 h after wound tracing, and was completed at 24 h (a). In contrast, tumor cell migration is markedly inhibited by tranilast exposure, and the wound is still open after 4 days. Quantitation of cell migration by image analysis is illustrated in (b). A matrigel invasion assay using 4T1 cells in culture was carried out as described under Materials and methods. Tranilast treatment resulted in a significant reduction of cell invasion (65%, $^{**}P<0.001$) using Student's *t*-test when compared with the vehicle-treated control (c).

treatment downregulated the levels of endoglin by 24% in breast tumors tested when compared with the vehicle control (Fig. 4g and h), suggesting that tranilast plays an important role in the regulation of breast cancer metastasis.

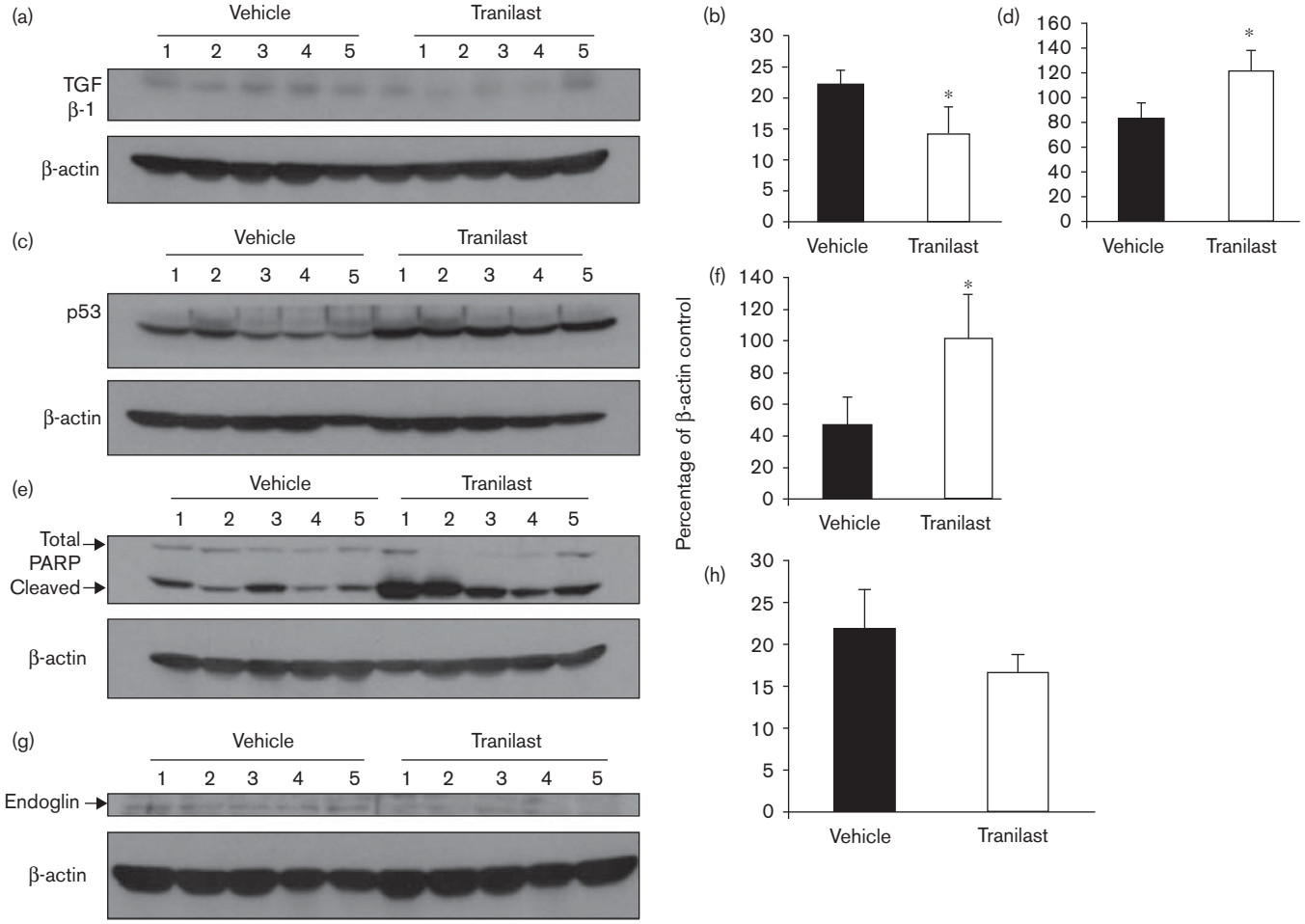
Tranilast treatment causes cleaved caspase 3 levels to be overexpressed in breast tumors signifying apoptosis

The earlier result is an indication that the oral administration of tranilast promotes apoptosis in the tumor cells. To show that this effect involves cancer cells in the orthotopic tumor, we performed immunohistochemistry staining of cleaved caspase 3 in paraffin tissue sections of the excised tumors (Fig. 5a). We observed that cleaved caspase 3 was present in cells that had the morphological features of cancer cells within the tumor and that the number of positive cells was increased compared with the vehicle control (Fig. 5b). This result thus shows that tranilast treatment limits proliferation and causes breast tumor cell death.

Discussion

In the area of breast cancer treatment, the last two decades have been characterized by the emphasis on biology-based therapies, most notably treatments based on the inhibition of Her2 [21,22]. Currently, there are several drugs of choice and treatment protocols available for breast cancer in terms of chemotherapy, biological therapy, hormonal therapy, and radiation therapy [21–25]. Several other drug/treatment protocols are also being evaluated including those targeting TGF β signaling. This is based on the knowledge of the functional targets of TGF β: cell cycle effectors, stromal–epithelial

Fig. 4



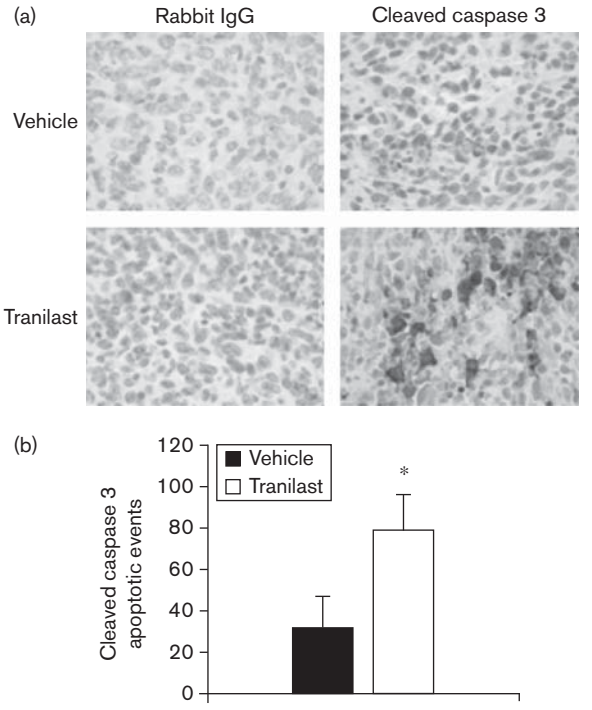
Tranilast treatment alters the levels of TGF β1 and p53, induces apoptosis, and reduces endoglin levels in mouse breast tumors: western blot analysis of mouse breast tumor lysates showed TGF β1 downregulation in the tranilast-treated group when compared with the vehicle control (a). Densitometric analysis of the western blot shows that tranilast treatment significantly reduced TGF β1expression using Student's *t*-test (36%, $*P<0.05$) in the tumor lysates compared with the vehicle control (b). Western blot analysis shows that p53 levels are upregulated in the mouse tumor lysates after tranilast treatment when compared with the vehicle control (c). Densitometric analysis of the western blot shows that tranilast treatment increased (49.5%, $*P<0.05$) p53 expression significantly using Student's *t*-test in the tumor lysates compared with the vehicle control (d). Western blot analysis of mouse breast tumor lysates shows that the levels of the apoptosis marker cleaved PARP are upregulated in the tranilast-treated tumor lysates compared with the vehicle control (e). Densitometric analysis of the western blot shows that tranilast treatment increased (117.7%, $*P<0.05$) cleaved PARP expression significantly using Student's *t*-test in the tumor lysates compared with the vehicle control (f). Western blot analysis of mouse breast tumor lysates shows decreased expression of endoglin in the tranilast treated tumor lysates compared with the vehicle control (g). Densitometric analysis of the western blot shows tranilast treatment reduced (24%) endoglin expression in the tumor lysates compared with the vehicle control (h).

interactions, epithelial-to-mesenchymal transitions (EMT), regulation of breast cancer stem cells (reviewed in [21–26]. Targeting TGF β can be predicted to impact Smad and non-Smad pathways, such as phosphotidylinositol-3 kinase (PI3K), extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase, p38 MAPK and Rho GTPases reviewed in Refs [3,5].

In our earlier study using tranilast, we have shown the efficacy of this drug in significantly reducing tumor growth and lung metastasis in syngeneic mice receiving subcutaneous injections of 4T1 breast cancer cells [14]. This study explores the mechanisms involved in the use of tranilast as a drug to prevent and treat breast cancer and metastasis. Tranilast has been used in the treatment of asthma patients in Japan with no significant adverse drug reaction or side effects even when orally administered for longer periods.

In this study using 4T1 cells, we show that tranilast upregulates AKT1 phosphorylation and suppresses ERK1/2 phosphorylation. Furthermore, in tranilast-treated 4T1 cells, we observed that cell migration after wound induction was slower than in the control cells. We have shown earlier that tranilast treatment prevents EMT in 4T1 cells [14]. Activated AKT1 has been reported to

Fig. 5



Tranilast treatment enhances the expression of cleaved caspase 3 in mouse breast tumors: immunohistochemistry shows that cleaved caspase 3 expression in the breast tumors is present in more tumor cells of the tranilast-treated mice, as compared with vehicle-treated control mice (a, × 400). Rabbit IgG was used as a negative control in the immunohistochemical analyses. Cell counting analysis of tumor tissue sections immunostained for cleaved caspase 3 was done in five individual fields per tumor, and 5 mice per group (vehicle vs. tranilast treated) of the tumor cells. The number of cleaved caspase 3 immunostained cells is significantly higher (*$P<0.05$) in breast tumors of the mice that received tranilast treatment using Student's *t*-test when compared with the vehicle control (b).

block the in-vitro migration and invasion of breast cancer cells [27], suppress ERK activity, and regulate the transcription genes involved in motility and invasion [28] and are reviewed in Ref. [29]. Irie *et al.* [30] have also shown that overexpression of AKT1 suppresses EGF-stimulated migration and ERK activation in MCF 10A breast cancer cells. In addition, the same study showed that AKT1 downregulation induced migration and EMT in MCF cells. Thus, our earlier studies on the effect of tranilast on EMT [14] and our present observation that tranilast inhibits cell migration led us to attribute the slower migration of 4T1 cells to the upregulation of AKT1 and downregulation of ERK activity by tranilast.

Important rate-limiting steps to unhindered tumor cell proliferation, despite the presence of growth-inhibiting stimuli of cells in cycle, are the control mechanisms that prevent G1/S transition. After the release from G0, we looked at the effect of tranilast on key cell cycle regulators. Our data showed that tranilast downregulates the expression of G1/S cyclin. Cyclin D1 and Cdk4 in 4T1 cells were suppressed by tranilast as early as within 24 h of drug exposure and the effect was sustained up to 48 h. Cyclin E and Cdk2 levels were not altered by tranilast (data not shown). pRB had decreased phosphorylation by 48 h. Taken together, these results suggest that tranilast has a growth-inhibiting role in breast cancer cells at the G1/S phase of the cell cycle. Studies have shown that in breast cancer, cyclins D1 and E play important roles in the acquisition of uncontrolled cell proliferation during breast oncogenesis [31]. Thus, in the event of drug-induced growth inhibition, the cells undergo downregulation of transcription of cyclin D1, resulting in unphosphorylated state of RB, sequestration of E2Fs, repression of E2F target genes and ultimately cell cycle arrest or exit from the cell cycle [32,33].

We further observed G2/M cyclins B1, A, and Cdc2 levels being elevated from 24 h in tranilast-treated 4T1 cells, suggesting that the remaining cells in the cycle are further inhibited at the G2/M phase. Studies have reported such a cell inhibition at the G2/M phase (reviewed in Ref. [34]). Further, we saw no effect of tranilast on p21 levels, whereas p27 levels decreased in response to tranilast. Studies have attributed a decrease in p27 levels to subcellular localization and proteosomal degradation brought by AKT phosphorylation [35–37]. In this study, we did observe an increase in AKT phosphorylation in response to tranilast treatment. Earlier studies do caution that p21 and p27 can behave as context-dependent inhibitors or promoters of cell cycle progression in breast epithelium (reviewed in Refs [31,38]). The present data observed are thus the first on the role of tranilast on the major effectors of cell cycle using a breast cancer line. Recently, tranilast was found to have similar G1 arrest and apoptosis in prostate cancer cells using LNCap and PC-3 cells [39]. We found that the addition of TGF β1 to 4T1 cells increased its proliferation. These data extend the findings of an earlier study in which TGF β addition caused an increase in cell proliferation of 4T1 cells [40]. However, we found that the inhibiting action of tranilast was not affected by the proliferative effect of TGF β in these cells. Alternately, on using an anti-TGF β1,2,3 neutralizing antibody, 4T1 cells showed no significant reduction in cell proliferation compared with the vehicle control. We have earlier reported that tranilast treatment reduces the levels of TGF β1 in 4T1 cells [14]. Globally, these data indicate that, although tranilast reduces the cellular content of TGF β, its inhibitory effect on cell proliferation involves other pathways not dependent on signaling from TGF β. Alternate signaling pathways do exist in the regulation of cell proliferation by AKT and MAPK and are not necessarily mediated by TGF β [41,42].

Results from wound induction assays further support our cell cycle data on growth inhibition by tranilast. The drug induced an inhibition of cell migration as early as 3 h extending even up to 96 h of culture. Western blot analysis of the cell and breast tumor lysates from syngenic

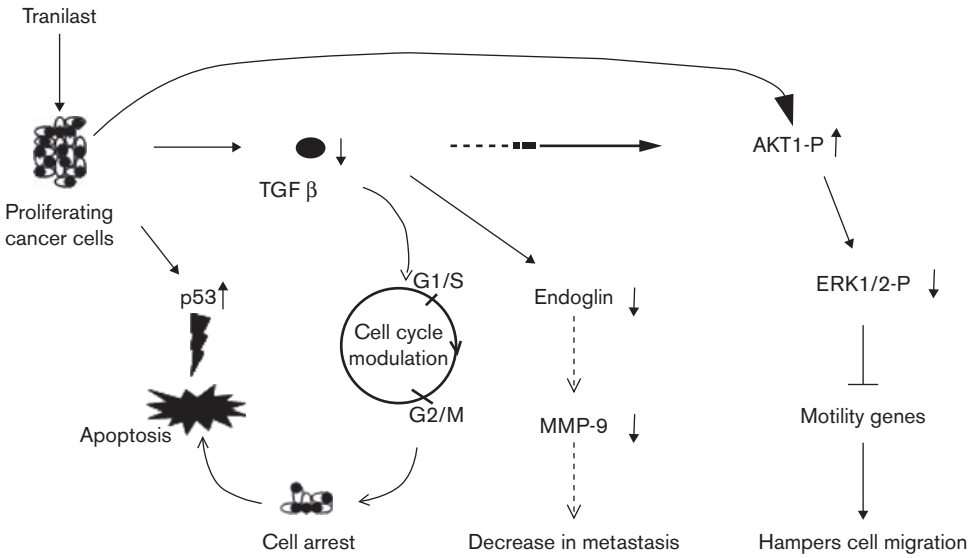
mice bearing 4T1 tumors after tranilast treatment showed significantly increased levels of p53 favoring the extension of the G1/S arrest of the cells and apoptosis, in keeping with the known functions of p53 [43].

Further mechanisms relevant to tumor cell apoptosis were explored in this study. PARP is a DNA-binding protein and also a substrate and marker for the apoptosis effector, caspase 3. Activated caspase 3 and cleaved PARP are signs of apoptosis [44]. In this study, tranilast-treated cells showed upregulation of cleaved PARP. In the tranilast-treated tumors, we observed a significant upregulation and expression for cleaved PARP and activated caspase 3, respectively, suggesting that tumors undergo apoptosis.

Breast cancers commonly metastasize to distant organs such as bone, lung, liver and brain accounting for the most of the patient's mortality. Hence, discovery of new markers or genes responsible for such metastasis is clinically important. Endoglin (CD105), a cell surface adhesion protein and a co-receptor for TGF-β, has been reported to be a useful marker in assessing breast carcinoma patients for increased risk of metastasis and poor outcome [45]. Endoglin expression is increased during metastatic invasion to the brain, and it contributes to the formation of invadopodia [46]. Invadopodia are finger-like projections from the tumor cell that can break down and invade the extracellular matrix because of, perhaps, higher expression of MMPs. Among the different MMPs, higher expression of MMP-9 has been correlated directly with invasiveness and metastasis in breast cancer [47–49]. In addition, recently, TGFβ has been implicated in invadopodia formation in breast cancer [50].

In our study, we observed a downregulation of TGF β1 after tranilast treatment but did not have any effect on the expression of TGF β receptors, namely TGF βRI, TGF βRII, and TGF βRIII that were studied (data not shown). Overall, the implication of TGF β1 downregulation in the mechanism of action of tranilast and the mechanics associated with other downstream-signaling targets of TGF β1 can be diverse: (i) TGF-β-dependent ERK downregulation and activation of MMP; (ii) TGFβ-driven EMT and invadopodia formation. Hence, tranilast by its ability to block TGFβ, ERK1/2 phosphorylation, and EMT, has the potential to prevent invadopodia formation. From our western blot data on 4T1 mouse breast cancer cells in culture, we observed that tranilast treatment reduces the expression of endoglin as early as 24 h and extends upto 48 h of exposure to the drug. Levels of MMP-9 expression in the tranilast-treated 4T1 cells were also found to be downregulated at the end of 48 h. Further, an increase in the levels of endoglin and MMP-9 were shown to be mediated by TGF β and by using a neutralizing antibody to TGF β; the levels of expression of the endoglin and MMP-9 were greatly reduced suggesting that tranilast mediates this effects through the regulation of TGF β expression. We have earlier shown that tranilast downregulates the endogenous expression of TGF β1 in 4T1

Fig. 6



Proposed mechanisms of action of tranilast in breast cancer. The figure depicts the possible mechanism of the inhibitory effect of tranilast on 4T1 murine breast cancer cells and corresponding tumor growth, as derived from this study. Tranilast may regulate the levels of AKT1/MAPK phosphorylation and cell motility independent of TGF β1 (↷) and/or may downregulate the endogenous expression level of TGF β1 contributing to several cascading effects (▪▪▪▶) depending on the signaling pathways: (a) Cell cycle modulation with induction of arrest at the G1/S phase. (b) Upregulation of AKT1 phosphorylation leading to inhibition of cell motility through the MAPK pathway. (c) Upregulation of p53 levels leading to cell death. (d) Downregulation of endoglin and matrix metalloproteinases (MMP)-9 expression resulting in decreased metastatic potential for the cancer cells.

cells [14]. Further, western blot analysis of tumor lysates obtained from syngenic mice bearing 4T1 tumors showed downregulation of endoglin after tranilast treatment compared with the vehicle control. Our earlier study showed a significant reduction in metastases in mice after tranilast treatment [14]. Along with this data with endoglin and MMP-9, these results indicate that tranilast inhibits breast tumor cell invasion and metastasis by interfering with TGF β signaling. The matrigel invasion assay showed the ability of tranilast to inhibit the invasion of 4T1 cells in culture. Thus, the result from the invasion assay supports our conclusion drawn from a wound assay with regard to cell migration as well as regulation of MMP-9 and endoglin levels in terms of invasion and metastasis. In addition, endoglin and MMP-9 can also serve as useful markers to assess the progression of the disease after tranilast treatment.

To conclude, the data of this murine cell culture and orthotopic tumor study indicate that tranilast is a candidate anticancer drug in breast cancer. Its effects on tumor cell signaling result in restricting cancer cell migration, regulating the cell cycle effectors, and causing cell arrest and apoptosis. We summarize the various pathways that tranilast might target in Fig. 6.

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