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Effects of thiostrepton alone or in combination with selumetinib on triple-negative breast cancer metastasis

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Abstract

Objective FoxM1 transcription factor contributes to tumor metastasis and poor prognosis in many cancers including triple-negative breast cancer (TNBC). In this study, we examined the effects of FoxM1 inhibitor Thiostrepton (THIO) alone or in combination with MEK inhibitor Selumetinib (SEL) on metastatic parameters *in vitro* and *in vivo.*

Methods Cell viability was determined by MTT assay. Immunoblotting and immunohistochemistry was used to assess metastasis-related protein expressions in 4T1 cells and its allograft tumor model in Balb/C mice. *In vivo* uPA activity was determined by enzymatic methods.

Results Both inhibitors were effective on the expressions of FoxM1, ERK, p-ERK, Twist, E-cadherin, and Vimentin alone or in combination *in vitro*. THIO significantly decreased 4T1 cell migration and changed the cell morphology from mesenchymal-like to epithelial-like structure. THIO was more effective than in combination with SEL in terms of metastatic protein expressions *in vivo*. THIO alone significantly inhibited mean tumor growth, decreased lung metastasis rate and tumor foci, however, no significant changes in these parameters were observed in the combined group. Immunohistochemically, FoxM1 expression intensity was decreased with THIO and its combination with SEL in the tumors.

Conclusion This study suggests that inhibiting FoxM1 as a single target is more effective than combined treatment with MEK in that TNBC allograft model, and targeting FoxM1 via THIO may provide therapeutic benefits in the subset of TNBC tumors with high metastatic capacity.

Introduction

Breast cancer is the most common cancer type in women all over the world [1]. Although triple-negative breast cancer (TNBC) accounts for about 15–20% of all breast cancers, the heterogeneous phenotypic structure and aggressive clinical course of triple-negative tumors cause shorter survival rates in patients than other subtypes [2, 3]. Also, the poor prognosis of TNBC and its resistance to current treatments cause TNBC mortality rates to be quite high [4, 5]. Therefore, more studies are required to identify new molecular targets to develop effective treatment options for a better prognosis and increase survival rates.

Forkhead box protein M1 (FoxM1) is an oncogenic transcription factor involved in a wide variety of cellular processes, including cell cycle progression, proliferation, differentiation, migration, metabolism, and DNA damage response [6–8]. FoxM1 has been over-expressed in many cancers, including TNBC [9, 10]. It was demonstrated that the increased FoxM1 expression has a negative impact on breast cancer patient survival [11, 12]. FoxM1 has also been reported to activate DNA repair pathways, leading to the development of resistance to genotoxic agents used in breast cancer treatment [13, 14]. Also, its overexpression is associated with epithelial- mesenchymal transition and tumor metastasis [15]. In the litareture, there are some studies that have evaluated the potential of the FoxM1 targeting in breast cancer cells. Ziegler *et al.*(2019) identified a new class of compounds inhibited FoxM1 activity in breast

cancers and Dey et al. (2020) investigated the effectiveness of a novel class of 1,1-diarylethylene FoxM1 inhibitory compounds in suppressing TNBC cell growth and metastasis [16, 17]. Thiostrepton (THIO), a natural Thiazole antibiotic, is another agent that has been shown to specifically bind FoxM1 and prevent its activity [18, 19]. Kwok et al. showed anti-proliferating, apoptotic and anti-metastatic effects of THIO in FoxM1-overexpressing cells [20]. It was demonstrated that FoxM1 could be a specific marker for TNBC and inhibiting FoxM1 using THIO could suppress breast cancer tumorigenesis [21]. Although THIO is widely used as a FoxM1 inhibitor in some cancer cell lines, there are few studies showed its *in vivo* efficacy on metastasis. Therefore, the potential of FoxM1 targeting with THIO and in combination with chemotherapeutic drugs for reducing tumor progression and metastatic outgrowth in TNBC should be investigated.

The mitogen-activated protein (MAP) kinase cascade has a crucial role in all stages of cancer, from the onset of the tumor to its progression [22, 23]. Increased MAPK activity has been shown to be associated with breast cancer metastasis [24]. Overexpression of ERK1/2, which is a downstream target of the MAPK pathway, has been reported to have prognostic significance on the survival of breast cancer patients [25–27]. Furthermore, it has been reported that the activity of Raf/MEK/MAPK signaling pathway is necessary for nuclear translocation of FoxM1[28, 29]. Selumetinib (SEL) is a highly potent MEK inhibitor and has phase I and phase II clinical studies on some cancers such as melanoma and non-small-cell lung cancer [30, 31]. It has recently been approved for the treatment of children with neurofibromatosis type 1 (NF1) [32]. Also, SEL reduced the cell growth and migration in TNBC cell lines [33]. Therefore, SEL might be potential drug in reducing tumor progression and metastatic outgrowth in TNBC.

The main purpose of this study was to investigate the potential of FoxM1 inhibition as a therapeutic target for metastasis in 4T1 cells and its orthotopic mouse model. We used THIO as FoxM1 specific inhibitor alone and in combination with MEK inhibitor SEL. Our data show that THIO alone effectively suppressed the expression of FoxM1 and its direct or indirect metastatic protein targets.

Material And Methods

Cell culture and treatment procedure

In this study, we used 4T1 mouse TNBC cell line (ATCC, LGC Promochem, Rockville, MD, USA) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO2. THIO and SEL (AZD6244) were purchased from Enzo Life Sciences and Selleckchem, respectively. Both agents were dissolved in DMSO to prepare stock solution (10 mM) afterward diluted concentrations were used in all analysis.

Cell Viability Assay

In order to determine the cytotoxic effect of THIO, SEL and combined treatment of THIO and SEL on 4T1 cells, MTT cell viability analysis was conducted. For this purpose, the equal number of 4T1 cells ($2x10^3$ cells/well) were cultured in 96 well plates. After 24 h, the cells were treated with the various concentrations of THIO (1, 2, 4, 8 and 16 μM) and SEL (1, 2, 4, 8 ,16 and 32 μM) alone for 24 and 48 h. Additionally, 4T1 cells were treated with different combined concentrations of THIO and SEL (1 μM THIO + 8 μM SEL, 1 μM THIO + 16 μM SEL, 1 μM THIO + 32 μM SEL, 2 μM THIO + 8 μM SEL, 2 μM THIO + 16 μM SEL and 2 μM THIO + 32 μM SEL). After the treatment of THIO, SEL and combined concentrations of THIO and SEL, the cells were incubated with 20 μl MTT solution at 37°C for 3–4 h. Then, cell medium was removed and 100 μl DMSO was added. Afterwards the absorbance of each well was measured at 570 nm using Spectramax M3 microplate reader (Molecular Devices, Silicon Valley, California, USA). All experiments were performed in quadruplicate for each dose and repeated a minimum of three times independently. Half-maximal inhibitory concentration (IC50) was calculated by linear regression analysis.

Wound healing assay To evaluate cell migration *in vitro*, cells were seeded in 24-well plates ($5x10^4$ cells per well). After cells reached 90–95% confluency, a wound was generated in the vertical direction with a 1 ml pipette tip, and the cells were gently washed at least 2 times with PBS. The cells were then incubated with a medium containing 2% FBS and treatment concentrations for 24h. After incubation time, the medium was removed, wells were washed with PBS gently again. Cells were then fixed with 4% PFA and kept in a solution containing 1% crystal violet for 15 minutes. Images were taken in 10X lens magnification under the Olympus IX70 microscope. By analyzing at least four different image areas with the Image J program, average and standard error values are indicated in %.

In vivo **experiments with 4T1 tumor-bearing mouse model** 6–8 week old BALB/c female mice (Kobay Inc., Ankara, Turkey) were housed under animal care protocols. Tumor inoculation was carried out according to the modified method of Pulaski et al. [34]. Briefly, cultured 4T1 cells were harvested and resuspended in serum-free media. $5x10^4$ cell suspensions prepared in PBS were inoculated subcutaneously to the left inguinal mammary fat pad of BALB/c mice. After the development of palpable tumors, mice were divided into following four groups: 1) vehicle control (DMSO, intraperitoneally (i.p) n = 7), 2) Thiostrepton (25mg/kg body weight (BW), i.p. n = 9) 3) oral Selumetinib (25 mg/kg BW n = 7), 4) combined group (25 mg/kg BW Thiostrepton i.p. and 25 mg/kg BW oral Selumetinib n = 9). Treatments were given 3 days a week for 2 weeks (weekend off). Tumor size was evaluated as width (W) length (L) with the help of a caliper, and tumor sizes were calculated with the formula (W^2xL)/2. At the end of the experiment, primary breast tissue, lung and blood samples were collected for further analysis.

This experiment was performed in accordance with Turkish Law for the protection of animals and was approved by Gazi University Animal Experiments Local Ethics Committee (G.Ü. ET 15.068).

Western blot analysis Cell pellets and tissues were lysed with RIPA buffer (Thermo Fisher, USA) supplemented with a protease and phosphatase inhibitor cocktail (Biovision, USA). Total protein samples, quantified with the BCA kit (Thermo Fisher, USA), were prepared for loading. For this purpose, 2X sample loading buffer (Sigma-Aldrich, USA) was added on the same amount with proteins. Thus, protein samples

were diluted 1:1 to obtain working concentrations. Then, the protein samples were kept in the heater block set at 95°C for 5 minutes, and after the samples reached room temperature, the samples were loaded into the wells. Protein samples in the gel were run for 90 min at 120 V and 250 mA with 1X running buffer. The gel was transferred to the PVDF membrane (Santa Cruz, USA) by the wet transfer method. Membranes were blocked in 5% nonfat milk in tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1 h, washed and then incubated with the E-cadherin (CST; 3195S), ERK1/2 (CST; 9102S), p-ERK1/2 (CST; 4370S), FoxM1 (Abcam; ab180710), MMP-2 (Santa Cruz, sc13594), MMP-9 (Santa Cruz, sc13520), Twist (Abcam; ab49254), Vimentin (CST; 5741S), Beta actin (Abcam; ab25894) primary antibodies overnight at + 4ºC. Membranes were then washed 3 times and then incubated with Goat IgG Anti-Rabbit (HRP) (Abcam; ab97051), Goat IgG Anti-Mouse (HRP) (Bio-Rad; 1706516) secondary antibodies for 1 hour at room temperature. To observe blotting bands, the membrane was visualized with a chemiluminescent substrate (Biovision, USA) using the Gel Logic 2200 Pro Carestream program. Bands were photographed and analyzed with the Image J Software (NIH, Maryland). The relative levels of indicated protein bands were detected with B-actin as the standard.

uPA (Urokinase Plasminogen Activator) analysis in mouse plasma Enzyme-linked immunosorbent assay (ELISA) was used to identify the level of uPA in the mice plasma. Intracardiac blood was collected from mice at the end of the *in vivo* experiment period. The blood was taken into purple capped tubes containing EDTA. As soon as blood was taken, 15 minutes centrifugation was performed at 1000g. Then, the plasma was placed in sterile 1.5 mL tubes and stored at -20°C. The following steps were carried out according to the uPA Elisa kit (Abcam; ab198512) protocol.

Histopathological and Immunohistochemical Examinations All mice were sacrificed after the treatment period ended. Primary tumor tissue and lungs were resected and sent to the pathology laboratory in 10% formalin solution. After tissue processing, all samples were embedded in paraffin. Then, 4 µm sections were cut from each paraffinized tissue block, and slides were prepared for staining. Samples were stained for routine hematoxylin and eosin (H&E) for histopathological examination. Histopathologically, metastatic nodules within lungs were counted under the light microscope (Olympus CX41, USA). To determine the expression of FoxM1 (rabbit polyclonal primary antibody 1: 200 dilution) immunohistochemically, Ventana Benchmark XT immunostainer (Ventana Medical Systems, Tucson, USA) was used.

Statistical Analysis Statistical analysis was performed using Graphpad Prism 8.0.2. Treatment groups were compared with those of the control groups by using Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of THIO and SEL alone and in combined treatments on 4T1 cell viability and morphology. MTT test was used to analyze the effects of THIO and SEL on cell viability (Fig. 1A-1C). THIO significantly decreased the cell viability in both 24 and 48 h treatments at 4 µM, 8 µM and 16 µM concentrations

(Fig. 1A). According to the dose response curve, the IC50 value of THIO was found to be 4.98 μM for 24 h and 4.08 μM for 48 h treatments. To determine the cytotoxic effect of SEL, 4T1 cells were treated with varying concentrations (1–32 μM) for 24 and 48 hours. As shown in Fig. 1B, SEL decreased cell viability significantly in 8 μM at 48 hours and in 16–32 μM concentrations at both treatment times. We determined that the IC50 value of SEL was 8.71 μM for 24 h and 6.88 μM for 48 h. Effects of THIO and SEL combined treatments on cell viability were shown in Fig. 1C. After 24 h treatment, the lowest cell viability (46.08%) was observed in the combined treatments of 1 μM THIO and 32 μM SEL. It was determined that 2 μM THIO and 32 μM SEL treatments decreased cell viability below 50% in both 24 h and 48 h significantly. Combined treatments led to a further decrease in cell viability compared to alone treatments of inhibitors. Therefore, two inhibitors may have synergistic effects on 4T1 cells. 2 and 4 μM of THIO and 8 and 16 μM of SEL concentrations were chosen for all of the subsequent *in vitro* experiments. Phase-contrast microscopy images were obtained to investigate the effects of THIO and SEL on cell morphology (Fig. 1D). Especially after THIO treatment, it was observed that cell morphology changed from mesenchymal-like structure to epithelial-like structure. This effect was also observed in combined treatments.

THIO significantly reduces 4T1 cell migration. Wound-healing assays revealed that treatment with THIO significantly inhibited the migration of 4T1 cells (Fig. 2A, 2D). 8 μM SEL did not show any significant change on cell migration, while the 16 μM concentration decreased the cell migration significantly compared to the 24 h control (Fig. 1B). In combined groups, it was observed that the decrease in cell migration was statistically significant compared to 24 h control (Fig. 2C).

THIO has negative effects on the expression of metastasis-related proteins through FoxM1 inhibition in vitro. As seen in Figs. 3A and 3B, FoxM1 inhibition was observed after 2 and 4 μM THIO at 6 h, however, 4 μM concentration of THIO was more efficient on FoxM1 inhibition at 24 h. SEL alone did not decrease FoxM1 expression. 2 μM THIO and 16 μM SEL combined treatments were decreased FoxM1 expression at 6 and 24 h. SEL provided sufficient inhibition on p-ERK at both concentrations. It was observed that this effect persisted in combined treatments as well. However, 4 μM THIO decreased p-ERK expression at 24 h. Twist was reduced by THIO alone and in combination with SEL in all concentrations during both treatment times. SEL alone increased the expression of E-cadherin and this effect was also observed in combined treatments at both treatment times. Vimentin expression was decreased at 4 μM THIO after 6 and 24 h treatment.

THIO significantly changes the protein expressions involved in metastatic processes in vivo. FoxM1 protein expression was significantly reduced after 25 mg/kg THIO, while SEL treatment did not change FoxM1 expression in primary tumor tissues (Fig. 4A-4D). 25 mg/kg oral SEL administration to mice, sufficiently suppressed ERK phosphorylation and this effect was observed to continue in the combined group. Twist protein expression was significantly decreased in treatment groups compared to the control group. MMP-2 expression was decreased in all treatment groups. However, MMP-9 protein expression was significantly reduced in THIO and combined group. E-cadherin expression increased significantly at all treatment groups. Besides vimentin expression changed in only THIO treatment groups. Urokinase-

type plasminogen activator (uPA) level in mice plasma was shown in Fig. 4E. THIO treatment significantly decreased uPA activity compared to control. However, decreases in SEL and combined treatment groups were not significant ($p > 0.05$).

THIO inhibits tumor growth and shows less metastatic nodules in the lungs. After 14 days of treatment protocol, tumor growth was only decreased in THIO treated mice significantly compared with the vehicle group (Fig. 5A). 25 mg/kg oral treatment of SEL had no significant effect on tumor growth. In the combined group, mice did not show any ability of tumor growth regression compared to vehicle control. During the 4-week experimental period, 2 of 7 mice died in the control group. While all mice were alive until the end of the experiment in the SEL treatment group, 1 of 9 mice have died in the THIO group. In the combined group, 2 of 9 mice died during the experimental period (Fig. 5B). The percentage of metastases in the control group was about 57.14% (4 out of 7 mice) in comparison with 33.33% (2 out of 6 mice) in THIO treated mice and 40% (2 out of 5 mice) in combined group (Fig. 5C). It was observed that THIO and combined group mice had less metastatic foci on lungs in the H&E staining images. Furthermore, multiple subpleural malign epithelial tumor metastasis were observed in control and SEL group lungs. According to immunohistochemical staining results, THIO and the combined group showed decreased expression of FoxM1 expression (Fig. 5D). This finding also confirms our immunoblot data and shows that administration of 25 mg/kg THIO to mice adequately inhibits FoxM1 expression. Also, FoxM1 was stained more in lymphoid cells, whereas cytoplasmic and membranous staining was observed as localization.

Discussion

In the current study, we have shown for the first time how FoxM1/ERK axis affects metastasis in 4T1 cells and its allograft BALB/c mouse model. For this purpose, we used THIO as a FoxM1 specific inhibitor and MEK inhibitor SEL. The possible mechanisms of action of inhibitors, alone or in combination, on metastatic processes were evaluated.

Thiostrepton is an antibiotic derived from *Streptomyces azureus* that shows significant inhibition of cell proliferation against cancer cells using a combination of direct and indirect inhibition of the transcriptional activity of FoxM1 [19, 20, 35]. In previous studies, 4–10μM concentrations of THIO were reported to adequately inhibit FoxM1 protein expression in different cancer cell lines [20, 36]. Consistent with the literature, our results showed that 4μM concentration of THIO reduced FoxM1 protein expression *in vitro*. We have given THIO to mice at a lower dose than reported in previous studies. Siraj et al. used 40 mg/kg, whereas Tan et al. used 50 mg/kg THIO dose in a MDA-MB-231 xenograft animal model [21, 37]. Our study demonstrated that 25mg/kg THIO was found to be effective and sufficient to suppress FoxM1 protein expression on the 4T1 allograft breast cancer mouse model.

It was demonstrated that FoxM1 is one of the upstream effectors on the ERK signaling pathway in hepatocellular carcinoma and regulating the ERK signal in breast cancer cells [38, 39]. Furthermore, FoxM1 is activated by cyclin-CDK and ERK-mediated phosphorylation. Phosphorylation of FoxM1 by ERK

and p38 from the serine at 331 and 704 positions or other possible regions stimulates the nuclear translocation of FoxM1 [40, 41]. In our *in vitro* and *in vivo* treatments MEK inhibitor Selumetinib suppressed ERK phosphorylation (p-ERK) sufficiently. It was also observed that 4 μM Thiostrepton inhibited p-ERK at 24 h *in vitro*, however 25 mg/kg THIO did not change p-ERK protein expression *in vivo*.

We have obtained considerable findings on the proteins involved in the epithelial-mesenchymal transition (EMT) which is one of the crucial processes of metastasis. E-cadherin, an epithelial marker in EMT, was increased especially in SEL treatment groups *in vitro* and *in vivo.* Previously, p-ERK was reported to be a negative regulator of E-cadherin which is supported by a positive correlation between loss of E-cadherin and ERK activation [42, 43]. Wierstra demonstrated that the DNA-binding domain of FoxM1c binds to the murine and human E-cadherin promoters *in vitro* [44]. Contrary to this finding, loss of E-cadherin is observed in many cancer cells with increased FoxM1 expression. Park et al. explained this discrepancy with the predominance of Snail-mediated inhibition of E-cadherin expression in tumor cells [39]. However, our *in vitro* and *in vivo* findings suggest that E-cadherin expression was mainly affected by ERK inhibition.

We showed that Vimentin, as a mesenchymal marker that supports invasive features in the EMT process, decreased significantly in THIO treatment groups. According to our *in vitro* study, Vimentin expression decreased in 4 μM THIO and combined treatments in 6 and 24 h, while SEL treatment did not change Vimentin expression. Therefore, we can conclude that the decrease observed in the combined treatments may be due to the effect of FoxM1 inhibitor THIO. There are studies showing that reduced Vimentin expression in FoxM1 knock-out (KO) mouse models or after silencing FoxM1 expression in various human cancers [15, 45]. In a study investigating the role of FoxM1 in the EMT process in hepatocellular carcinoma, it was reported that Vimentin expression increased with the FoxM1 overexpression, that Vimentin was a target of FoxM1 directly or indirectly [46]. Therefore, in our study, the decrease in Vimentin expression in THIO treated groups could be associated with FoxM1 inhibition. The exact molecular mechanism of THIO on Vimentin should be investigated in further studies.

Twist protein expression, which is another marker of epithelial-mesenchymal transition and metastasis, was found to be suppressed both *in vitro* and *in vivo* in all treatment groups. Qian et al. showed that the human FoxM1 gene promoter region contains a binding site for the Twist protein, and can be uniquely linked to the FoxM1 promoter of Twist by ChIP analysis. Thus Twist has been reported to be a direct transcriptional target of FoxM1 [47]. Besides, Twist's largest phosphorylation region was stated to be phosphorylated by ERK from serine 68, thereby maintaining its stability but its expression decreased during the inhibition of MAPK activities [48]. In this context, together with FoxM1 and MEK inhibition, could have a strong effect in suppressing Twist expression which is the master regulator of tumor metastasis.

The expression and activity of the matrix metalloproteinases (MMP) increase in almost all types of breast cancer and is associated with advanced stage of the tumor and poor prognosis [49, 50]. The expression of MMP-2 and MMP-9 proteins isolated from our *in vitro* experiments could not be determined by the

Immunoblot method. Although MMPs are connected with survival and expansion of cancer cells, they are produced in very small quantities [51]. Cancer cells in a paracrine manner, stimulate tumor microenvironment to supply sufficient MMPs from them [52]. This phenomenon may explain why we could observe the expression of MMP-2 and MMP-9 in protein samples isolated from tissues, but could not observe in protein samples isolated from *in vitro* experiments. Our *in vivo* findings demonstrated that MMP-2 expression was decreased significantly in all groups as compared to the control, whereas MMP-9 expression was decreased only in THIO and combined treatment groups. Wang et al. (2007) reported that a decrease in FoxM1 expression reduced MMP-2, MMP-9 and VEGF expression in pancreatic cancer cells, thereby reducing the metastasis ability of these cells [53]. It is demonstrated that FoxM1 increases its activity by directly connecting to MMP-2, and indirectly regulates MMP-9 via downstream target of JNK1[54].

Malignant tumors have the capacity to break down the extracellular matrix (ECM) with controlled proteolysis. One of the proteolytic systems that play a role in these processes is the urokinase type plasminogen activator (uPA) system. Studies have demonstrated that the expression of FoxM1 is positively correlated with urokinase-type PA(uPA), MMP-2 and MMP-9 expressions [55, 56]. Previously, Ma et al reported that overexpression of FoxM1 and the uPA system were associated with gastric cancer progression and poor prognosis [57]. We evaluated uPA activity in mice plasma using an Elisa kit and found a significant decrease in uPA level only in the THIO group. According to the findings of this study, the decreases in the level of uPA, MMP-2 and MMP-9 protein expression via THIO confirm that FoxM1 regulates ECM proteolysis through this system.

In this study, a wound healing assay was carried out to evaluate cell migration of 4T1 cells. According to our results, while both concentrations of THIO significantly inhibited cell migration, only 16μM concentration of SEL caused a decrease in cell migration. We also found a significant reduction in cell migration in combined treatments. These results are consistent with our *in vitro* findings, such as the increase in E-cadherin protein expression, decrease in Vimentin protein expression, and decrease in Twist expression, which are the markers that encourage cell motility.

4T1 mouse breast cancer cells are highly invasive, so they can spontaneously form metastases [34]. When 4T1 cells are implanted into BALB/c mice, they spread to distant organs such as the lung, liver, brain [58]. In our allograft breast cancer tumor model, the percentage of metastasis in the control group was 57.14% in the control group, whereas this ratio decreased in THIO (33.33%) and the combined group (40%). THIO may have reduced distant organ spread by altering protein expression associated with metastasis. Administration of mice 25 mg/kg THIO to the mice every other day decreased tumor volume significantly, however no significant decrease in tumor size was observed in the combined group. We observed that the combined group of mice has the shortest survival rate. Despite its promising effects at the molecular level on metastasis, we suggest that THIO should be followed up with more groups of mice and prolonged days to analyze survival and tumor growth.

As a conclusion, our study revealed that FoxM1 inhibitor THIO alone was more promising on proteins involved in metastasis steps as compared to SEL-alone treatment. However, both inhibitors affected EMT with different molecular processes and that their combined usage had a stronger effect on EMT. THIO is an FDA-approved antibiotic used for the treatment of dermatological diseases in veterinary applications. However, it has no application for human use. The difficulty of its synthesis, degradation potential and its hydrophobic form make it difficult to use in the clinic. Therefore, in order to overcome these problems, we think that THIO should be formulated in nanoparticle or microparticle drug delivery systems and should be investigated *in vitro* and *in vivo* considering the toxicological and genotoxic effects of these formulations.

Declarations

Authors' Contributions FDK, IDT, AE participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; FDK and GE conducted the experiments, FDK wrote the manuscript, and IDT, AE edited the manuscript.

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Conflict interest The authors declare that they have no conflicting interests.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants

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References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F (2021) Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 0:1–41
2. Gelmon K, Dent R, Mackey JR, Laing K, McLeod D, Verma S (2012) Targeting triple-negative breast cancer: optimising therapeutic outcomes. Ann Oncol 23:2223–2234
3. Lehmann BD, Jovanović B, Chen X, Estrada M V., Johnson KN, Shyr Y, Moses HL, Sanders ME, Pietenpol JA (2016) Refinement of Triple-Negative Breast Cancer Molecular Subtypes: Implications for Neoadjuvant Chemotherapy Selection. PLoS One 11:e0157368
4. O'Reilly EA, Gubbins L, Sharma S, et al (2015) The fate of chemoresistance in triple negative breast cancer (TNBC). BBA Clin 3:257–275

5. Jerusalem G, Collignon J, Schroeder H, Lousberg L (2016) Triple-negative breast cancer: treatment challenges and solutions. Breast Cancer Targets Ther 93
6. Yu C, Chen L, Yie L, Wei L, Wen T, Liu Y, Chen H (2015) Targeting FoxM1 inhibits proliferation, invasion and migration of nasopharyngeal carcinoma through the epithelial-to-mesenchymal transition pathway. Oncol Rep. https://doi.org/10.3892/or.2015.3834
7. Liao G Bin, Li XZ, Zeng S, Liu C, Yang SM, Yang L, Hu CJ, Bai JY (2018) Regulation of the master regulator FOXM1 in cancer. Cell Commun Signal 16:1–15
8. Raychaudhuri P, Park HJ (2011) FoxM1: A Master Regulator of Tumor Metastasis: Figure 1. Cancer Res 71:4329–4333
9. Hamurcu Z, Delibaşı N, Nalbantoglu U, et al (2019) FOXM1 plays a role in autophagy by transcriptionally regulating Beclin-1 and LC3 genes in human triple-negative breast cancer cells. J Mol Med 97:491–508
10. Xu N, Jia D, Chen W, et al (2013) FoxM1 Is Associated with Poor Prognosis of Non-Small Cell Lung Cancer Patients through Promoting Tumor Metastasis. PLoS One 8:e59412
11. Dai J, Yang L, Wang J, Xiao Y, Ruan Q (2015) Prognostic Value of FOXM1 in Patients with Malignant Solid Tumor: A Meta-Analysis and System Review. Dis Markers 2015:1–10
12. Lu X-F, Zeng D, Liang W-Q, Chen C-F, Sun S-M, Lin H-Y (2018) FoxM1 is a promising candidate target in the treatment of breast cancer. Oncotarget 9:842–852
13. Khongkow P, Gomes AR, Gong C, et al (2016) Paclitaxel targets FOXM1 to regulate KIF20A in mitotic catastrophe and breast cancer paclitaxel resistance. Oncogene. https://doi.org/10.1038/onc.2015.152
14. Zona S, Bella L, Burton MJ, Nestal de Moraes G, Lam EWF (2014) FOXM1: An emerging master regulator of DNA damage response and genotoxic agent resistance. Biochim Biophys Acta - Gene Regul Mech 1839:1316–1322
15. Kong FF, Qu ZQ, Yuan HH, et al (2014) Overexpression of FOXM1 is associated with EMT and is a predictor of poor prognosis in non-small cell lung cancer. Oncol Rep 31:2660–2668
16. Ziegler Y, Laws MJ, Sanabria Guillen V, et al (2019) Suppression of FOXM1 activities and breast cancer growth in vitro and in vivo by a new class of compounds. npj Breast Cancer 5:1–11
17. Dey P, Wang A, Ziegler Y, Kim SH, El-Ashry D, Katzenellenbogen JA, Katzenellenbogen BS (2020) Suppression of Tumor Growth, Metastasis, and Signaling Pathways by Reducing FOXM1 Activity in Triple Negative Breast Cancer. Cancers (Basel) 12:2677
18. Tabatabaei-Dakhili SA, Aguayo-Ortiz R, Domínguez L, Velázquez-Martínez CA (2018) Untying the knot of transcription factor druggability: Molecular modeling study of FOXM1 inhibitors. J Mol Graph Model 80:197–210
19. Chen Y, Ruben EA, Rajadas J, Teng NNH (2015) In silico investigation of FOXM1 binding and novel inhibitors in epithelial ovarian cancer. Bioorganic Med Chem 23:4576–4582

20. Kwok JMM, Myatt SS, Marson CM, Coombes RC, Constantinidou D, Lam EWF (2008) Thiostrepton selectively targets breast cancer cells through inhibition of forkhead box M1 expression. Mol Cancer Ther 7:2022–2032
21. Tan Y, Wang Q, Xie Y, Qiao X, Zhang S, Wang Y, Yang Y, Zhang B (2019) Identification of FOXM1 as a specific marker for triple-negative breast cancer. Int J Oncol 54:87–97
22. Deschênes-Simard X, Kottakis F, Meloche S, Ferbeyre G (2014) ERKs in Cancer: Friends or Foes? Cancer Res 74:412–419
23. Braicu, Buse, Busuioc, et al (2019) A Comprehensive Review on MAPK: A Promising Therapeutic Target in Cancer. Cancers (Basel) 11:1618
24. Adeyinka A, Nui Y, Cherlet T, Snell L, Watson PH, Murphy LC (2002) Activated mitogen-activated protein kinase expression during human breast tumorigenesis and breast cancer progression. Clin Cancer Res 8:1747–1753
25. Gee JMW, Robertson JFR, Ellis IO, Nicholson RI (2001) Phosphorylation of Erk1 / 2 Mitogen-Activated Protein Kinase Is Associated With Poor Response To Anti-Hormonal Therapy and. Int J cancer 95:247–254
26. Mueller H, Flury N, Eppenberger-Castori S, Kueng W, David F, Eppenberger U (2000) Potential prognostic value of mitogen-activated protein kinase activity for disease-free survival of primary breast cancer patients. Int J Cancer 89:384–388
27. Esteva FJ, Sahin AA, Smith TL, Yang Y, Pusztai L, Nahta R, Buchholz TA, Buzdar AU, Hortobagyi GN, Bacus SS (2004) Prognostic significance of phosphorylated P38 mitogen-activated protein kinase and HER-2 expression in lymph node-positive breast carcinoma. Cancer 100:499–506
28. Ma RYM (2005) Raf/MEK/MAPK signaling stimulates the nuclear translocation and transactivating activity of FOXM1c. J Cell Sci 118:795–806
29. Chan DW, Hui WWY, Cai PCH, Liu MX, Yung MMH, Mak CSL, Leung THY, Chan KKL, Ngan HYS (2012) Targeting GRB7/ERK/FOXM1 Signaling Pathway Impairs Aggressiveness of Ovarian Cancer Cells. PLoS One 7:e52578
30. Metro G, Chiari R, Baldi A, De Angelis V, Minotti V, Crinò L (2013) Selumetinib: a promising pharmacologic approach for KRAS-mutant advanced non-small-cell lung cancer. Future Oncol 9:167–177
31. Haslett K, Koh P, Hudson A, Ryder WD, Falk S, Mullan D, Taylor B, Califano R, Blackhall F, Faivre-Finn C (2021) Phase I trial of the MEK inhibitor selumetinib in combination with thoracic radiotherapy in non-small cell lung cancer. Clin Transl Radiat Oncol 28:24–31
32. Campagne O, Yeo KK, Fangusaro J, Stewart CF (2021) Clinical Pharmacokinetics and Pharmacodynamics of Selumetinib. Clin Pharmacokinet 60:283–303
33. Zhou Y, Lin S, Tseng KF, Han K, Wang Y, Gan Z hua, Min D liu, Hu H yan (2016) Selumetinib suppresses cell proliferation, migration and trigger apoptosis, G1 arrest in triple-negative breast cancer cells. BMC Cancer. https://doi.org/10.1186/S12885-016-2773-4

34. Pulaski BA, Ostrand-Rosenberg S (2000) Mouse 4T1 Breast Tumor Model. Curr Protoc Immunol 39:1–16
35. Hegde NS, Sanders DA, Rodriguez R, Balasubramanian S (2011) The transcription factor FOXM1 is a cellular target of the natural product thiostrepton. Nat Chem 3:725–731
36. Jiang L, Wang P, Chen L, Chen H (2014) Down-regulation of FoxM1 by thiostrepton or small interfering RNA inhibits proliferation, transformation ability and angiogenesis, and induces apoptosis of nasopharyngeal carcinoma cells. Int J Clin Exp Pathol 7:5450–5460
37. Siraj AK, Pratheeshkumar P, Parvathareddy SK, Qadri Z, Thangavel S, Ahmed S, Al-Dayel F, Tulbah A, Ajarim D, Al-Kuraya KS (2018) FoxM1 is an independent poor prognostic marker and therapeutic target for advanced Middle Eastern breast cancer. Oncotarget 9:17466–17482
38. Calvisi DF, Pinna F, Ladu S, et al (2009) Forkhead box M1B is a determinant of rat susceptibility to hepatocarcinogenesis and sustains ERK activity in human HCC. Gut 58:679–687
39. Park HJ, Gusarova G, Wang Z, et al (2011) Deregulation of FoxM1b leads to tumour metastasis. EMBO Mol Med 3:21–34
40. Laoukili J, Alvarez M, Meijer LAT, Stahl M, Mohammed S, Kleij L, Heck AJR, Medema RH (2008) Activation of FoxM1 during G2 Requires Cyclin A/Cdk-Dependent Relief of Autorepression by the FoxM1 N-Terminal Domain. Mol Cell Biol 28:3076–3087
41. Lüscher-Firzlaff JM, Lilischkis R, Lüscher B (2006) Regulation of the transcription factor FOXM1c by Cyclin E/CDK2. FEBS Lett 580:1716–1722
42. Conacci-Sorrell M, Simcha I, Ben-Yedidia T, Blechman J, Savagner P, Ben-Ze'ev A (2003) Autoregulation of E-cadherin expression by cadherin–cadherin interactions. J Cell Biol 163:847–857
43. Bae G-Y, Choi S-J, Lee J-S, Jo J, Lee J, Kim J, Cha H-J (2013) Loss of E-cadherin activates EGFR-MEK/ERK signaling, which promotes invasion via the ZEB1/MMP2 axis in non-small cell lung cancer. Oncotarget 4:2512–22
44. Wierstra I (2013) FOXM1 (Forkhead box M1) in Tumorigenesis. In: Cell Cycle. pp 191–419
45. Bao B, Wang Z, Ali S, Kong D, Banerjee S, Ahmad A, Li Y, Azmi AS, Miele L, Sarkar FH (2011) Over-expression of FoxM1 leads to epithelial-mesenchymal transition and cancer stem cell phenotype in pancreatic cancer cells. J Cell Biochem 112:2296–2306
46. Meng F-D (2015) FoxM1 overexpression promotes epithelial-mesenchymal transition and metastasis of hepatocellular carcinoma. World J Gastroenterol 21:196
47. Qian J, Luo Y, Gu X, Zhan W, Wang X (2013) Twist1 Promotes Gastric Cancer Cell Proliferation through Up-Regulation of FoxM1. PLoS One 8:e77625
48. Hong J, Zhou J, Fu J, He T, Qin J, Wang L, Liao L, Xu J (2011) Phosphorylation of Serine 68 of Twist1 by MAPKs Stabilizes Twist1 Protein and Promotes Breast Cancer Cell Invasiveness. Cancer Res 71:3980–3990
49. Pellikainen JM, Ropponen KM, Kataja V V., Kellokoski JK, Eskelinen MJ, Kosma V-M (2004) Expression of Matrix Metalloproteinase (MMP)-2 and MMP-9 in Breast Cancer with a Special

Reference to Activator Protein-2, HER2, and Prognosis. Clin Cancer Res 10:7621–7628

50. Li H, Cao D, Liu Y, et al (2004) Prognostic value of matrix metalloproteinases (MMP-2 and MMP-9) in patients with lymph node-negative breast carcinoma. Breast Cancer Res Treat 88:75–85
51. Jabłońska-Trypuć A, Matejczyk M, Rosochacki S (2016) Matrix metalloproteinases (MMPs), the main extracellular matrix (ECM) enzymes in collagen degradation, as a target for anticancer drugs. J Enzyme Inhib Med Chem 31:177–183
52. NOEL A, JOST M, MAQUOI E (2008) Matrix metalloproteinases at cancer tumor–host interface. Semin Cell Dev Biol 19:52–60
53. Wang Z, Banerjee S, Kong D, Li Y, Sarkar FH (2007) Down-regulation of Forkhead Box M1 Transcription Factor Leads to the Inhibition of Invasion and Angiogenesis of Pancreatic Cancer Cells. Cancer Res 67:8293–8300
54. Wang I-C, Chen Y-J, Hughes DE, Ackerson T, Major ML, Kalinichenko V V., Costa RH, Raychaudhuri P, Tyner AL, Lau LF (2008) FoxM1 Regulates Transcription of JNK1 to Promote the G1/S Transition and Tumor Cell Invasiveness. J Biol Chem 283:20770–20778
55. Yue M, Li S, Yan G, Li C, Kang Z (2018) Paeoniflorin inhibits cell growth and induces cell cycle arrest through inhibition of FoxM1 in colorectal cancer cells. Cell Cycle 17:240–249
56. Ai C, Zhang J, Lian S, Ma J, Győrffy B, Qian Z, Han Y, Feng Q (2020) FOXM1 functions collaboratively with PLAU to promote gastric cancer progression. J Cancer 11:788–794
57. Ma J, Qi G, Xu J, Ni H, Xu W, Ru G, Zhao Z, Xu W, He X (2017) Overexpression of forkhead box M1 and urokinase-type plasminogen activator in gastric cancer is associated with cancer progression and poor prognosis. Oncol Lett 14:7288–7296
58. Fantozzi A, Christofori G (2006) Mouse models of breast cancer metastasis. Breast Cancer Res 8:212

Figures

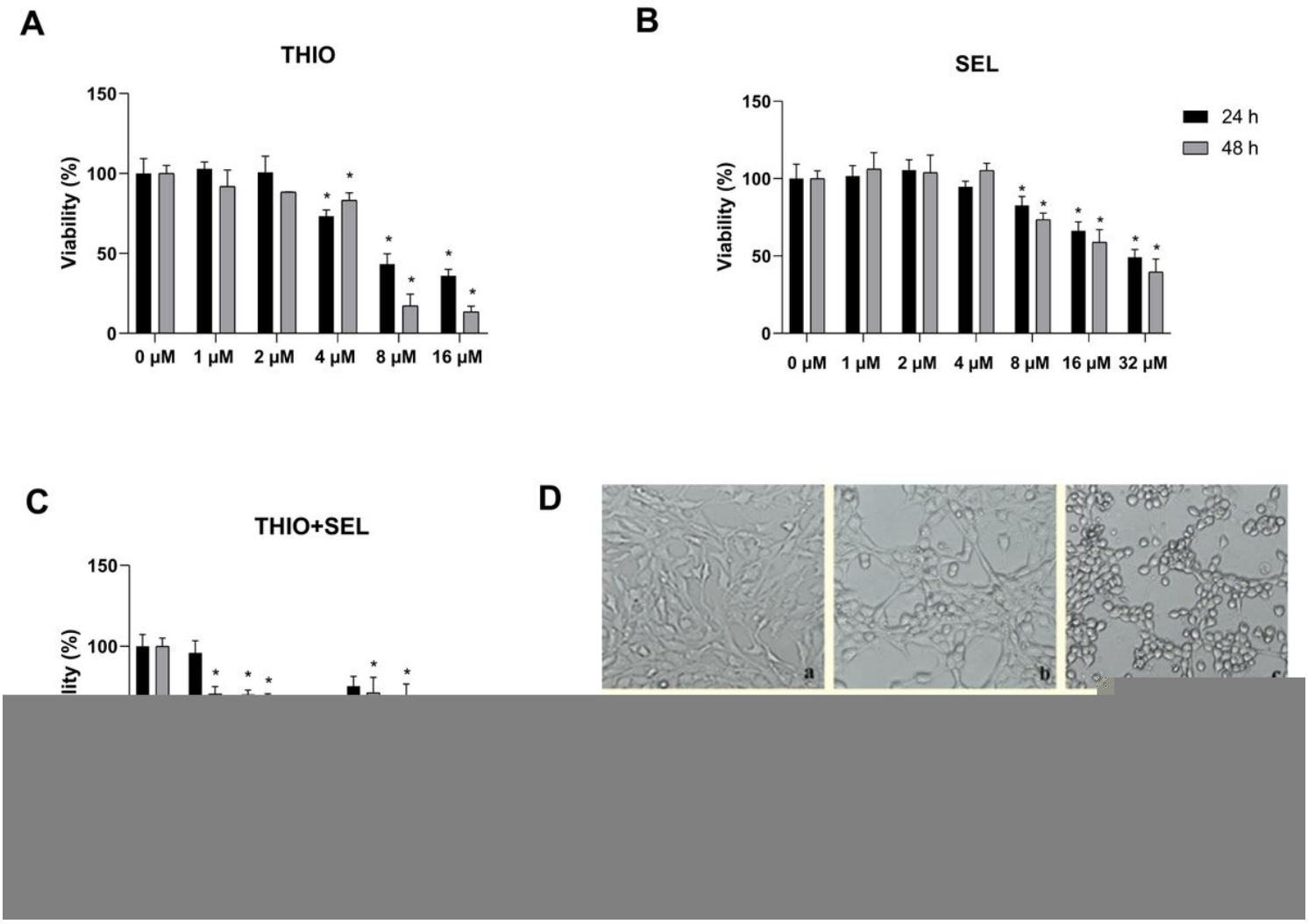


Figure 1

Effects of THIO (A), SEL (B) and selected concentrations of their combination (C) on 4T1 cell viability. Each value represents the mean ± SD of four experiments (n = 4). (D) Cell morphology images after THIO and SEL treatments for 24 hour by phase contrast microscopy (20x). (a; Control, b; 2 µM THIO, c; 4 µM THIO, d; 8 µM SEL, e;16 µM SEL, f; 2 µM THIO +8 µM SEL)

Figure 2

Wound healing assay of 4T1 cells after treatment with THIO (A), SEL (B) and their combinations (C). Cells were grown in 6 well plates, a wound was created when the cells grew into 90-95% confluence. After incubation for 24 h the cells were fixed and imaged. Images were taken at 0 and 24 h by an inverted microscope (Olympus, IX70, Japan). (D) All data represents mean±SD as percentage. The difference

between 0-hours control group and 24-hours control group is shown with α. The difference between the 24-hour control group and the 24-hour treatment group is indicated by * (p≤0.05).

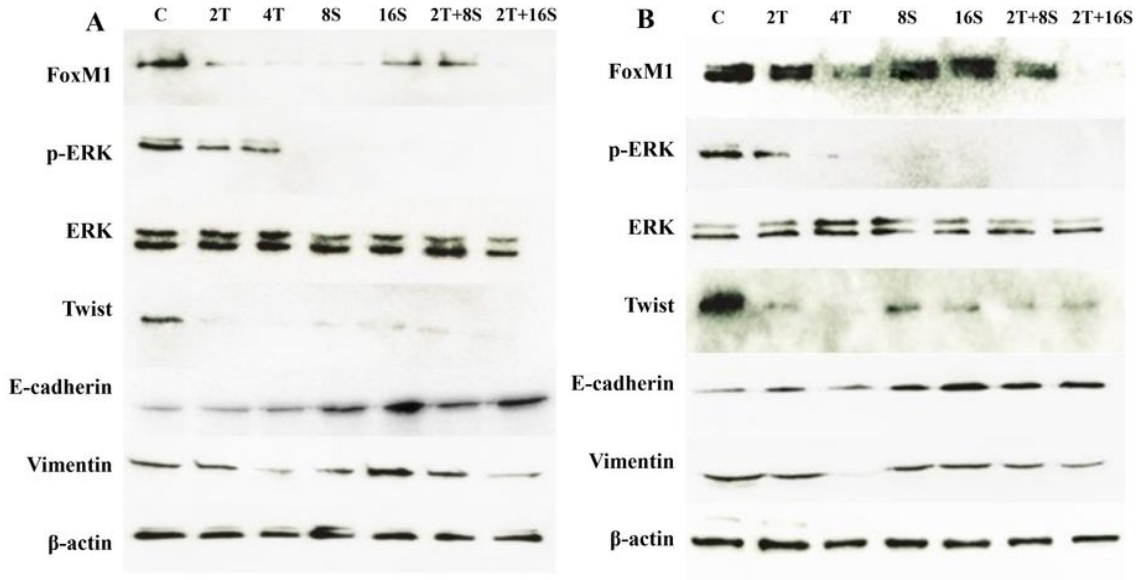


Figure 3

Protein expressions in 4T1 cells treated with THIO, SEL alone and their combination for 6 hours (A) and 24 hours (B) (p-ERK; phospho-ERK, 2T; 2 μM THIO, 4T; 4 μM THIO, 8S; 8 μM SEL, 16S; 16 μM SEL, 2T+8S; 2 μM THIO+8 μM SEL, 2T+16S; 2 μM THIO+16 μM SEL)

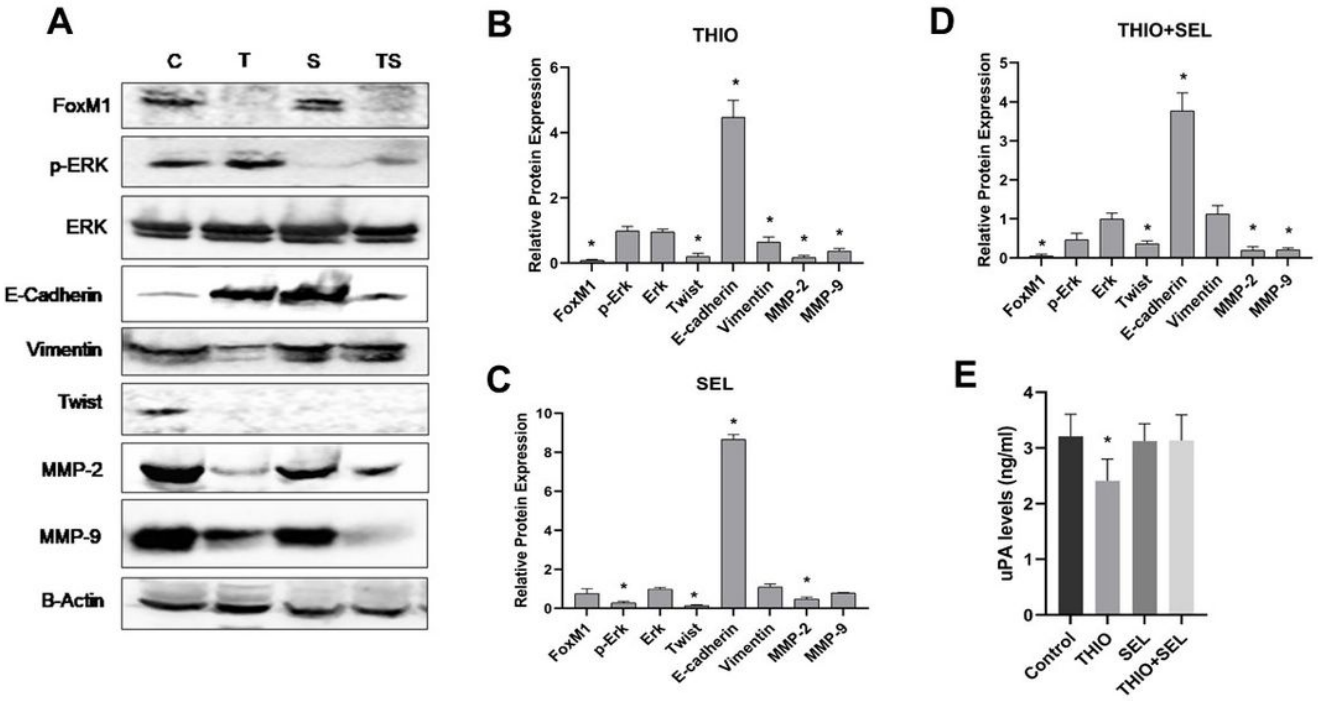


Figure 4

(A) Western blot images of the metastasis related proteins in primary tumor tissue. Relative protein expressions in tumor tissues from mice treated with THIO (B), SEL (C) and THIO+SEL (D) groups. Graphs represent fold change of indicated proteins at least 3 different mice for each group (*p <0.05). (E) uPA levels in mice plasma. The results shown are representative of the three independent experiments (*p< 0.05).

Figure 5

(A) The effect of *in vivo* treatments on tumor growth. Following inoculation of the 4T1 cells, mice were divided into 4 group: Control (Sham group; DMSO ip injection, n=6); Thiostrepton (25mg/kg, ip injection, n=6); Selumetinib (25mg/kg oral, n=6); Combined group (25mg/kg Thiostrepton ip+25mg/kg Selumetinib oral, n=6). Tumor volumes were measured by using a caliper every 3 days. Graph represents the mean ±SEM volume of mice in each group day by day. (B) Kaplan-Meier survival analysis of mice (C) Metastasis percentage in lungs and the number of metastatic colonies (D). Immunohistochemical staining of FoxM1 in primary tumor tissue in 4T1 allograft mice model (400×).