Thiostrepton selectively targets breast cancer cells through inhibition of forkhead box M1 expression

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Abstract

Elevated expression or activity of the transcription factor forkhead box M1 (FOXM1) is associated with the development and progression of many malignancies, including breast cancer. In this study, we show that the thiazole antibiotic thiostrepton selectively induces cell cycle arrest and cell death in breast cancer cells through down-regulating FOXM1 expression. Crucially, our data show that thiostrepton treatment reduced FOXM1 expression in a time- and dose-dependent manner, independent of *de novo* protein synthesis and predominantly at transcriptional and gene promoter levels. Our results indicate that thiostrepton can induce cell death through caspase-dependent intrinsic and extrinsic apoptotic pathways as well as through caspase-independent death mechanisms, as observed in MCF-7 cells, which are deficient of caspase-3 and caspase-7. Cell cycle analysis showed that thiostrepton induced cell cycle arrest at G_1 and S phases and cell death, concomitant with FOXM1 repression in breast cancer cells. Furthermore, thiostrepton also shows efficacy in repressing breast cancer cell migration, metastasis, and transformation, which are all downstream functional attributes of FOXM1. We also show that overexpression of a constitutively active FOXM1 mutant, ΔN-FOXM1, can abrogate the antiproliferative effects of thiostrepton. Interestingly, thiostrepton has no affect on FOXM1 expression and proliferation of the untransformed MCF-10A breast epithelial cells. Collectively, our data show that FOXM1 is one of the primary cellular targets of thiostrepton in breast cancer cells and that thiostrepton may represent a novel lead compound for targeted therapy of breast cancer with minimal toxicity against noncancer cells. [Mol Cancer Ther 2008;7(7):2022–32]

Introduction

Forkhead box M1 (FOXM1) belongs to the forkhead box transcription factor family, which is characterized by the forkhead box domain (1). FOXM1 is a critical regulator of cell cycle progression, and loss of FOXM1 is associated with G_2-M phase cell cycle arrest and loss of mitotic spindle integrity *in vitro* and is embryonic lethal *in utero* due to a failure to enter mitosis (2, 3). Conversely, an increase in the activity or expression of FOXM1 is associated with the development and progression of numerous cancers of the breast, liver, prostate, brain, and lung, whereas the ectopic expression of the FOXM1 accelerated the development, proliferation, and tumor growth in mouse models of prostate cancer (4). Chromosomal amplification of the *FOXM1* gene locus is frequently observed in multiple tumor types (5) and enhanced activity of FOXM1 may also occur through the deregulation of upstream kinases, which regulate the activity and stability of FOXM1 during the cell cycle progression. FOXM1 expression increases during G_1 to S phase and nuclear translocation occurs before G_2-M phase following cyclin E/cyclin-dependent kinase 2–mediated (6) and Raf/MEK/extracellular signal-regulated kinase (ERK)–mediated (7) phosphorylation. Phosphorylation of the retinoblastoma protein pRb by cyclin D1/cyclin-dependent kinase 4 may also be required to relieve the repression of FOXM1 by pRb through disrupting their direct interaction (8). An increase in FOXM1 activity may promote tumorigenesis through driving proliferation and cell cycle by regulating downstream targets such as cyclin B1 (9), Skp2-Csk1 ubiquitin ligase complex (10), CDC25b phosphatase, and Polo-like kinase-1 (11). Moreover, FOXM1 can also activate other genes involved in metastasis and angiogenesis such as the matrix metalloproteinase (MMP)-2, MMP-9, and vascular endothelial growth factor (12).

Several studies have shown that a reduction in FOXM1 expression results in dramatic decrease in tumor growth. For instance, knockdown of FOXM1 expression in prostate cancer cell lines resulted in proliferation and anchorage-independent cell growth on soft agar (4), whereas knockout mice with *foxm1*$^{-/-}$ genotype also showed a significant reduction in the number of lung adenoma cells (13). Remarkably, loss of the *foxm1* gene in hepatocytes of 6-week-old transgenic mice resulted in resistance to the induction of hepatocellular carcinoma following exposure

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to carcinogens (14). In pancreatic cell lines, small interfering RNA knockdown of FOXM1 expression lead to a decrease level of MMP-9, MMP-2, and vascular endothelial growth factor, thereby decreasing metastasis and angiogenesis respectively (12).

Within the last decade, an emerging class of naturally occurring thiopeptide antibiotics, which are characterized by highly complex sulfur-containing heterocyclic rings, have shown a wide range of physiologic activities including antibacterial, antiparasitic, and anticancer properties. A thiazole ring containing antibiotic, thiostrepton (Fig. 1A), originally isolated from *Streptomyces azureus* ATCC 149215 (15) has been reported to possess antitumor activities (16). Despite the discovery that in prokaryotic cells thiostrepton is an inhibitor of protein translation through binding to the 23sRNA with L11 ribosomal protein (17), little is known about its mechanism of action, its cellular target(s), and its specificity in eukaryotic cells. We have therefore investigated the anticancer potential of thiostrepton using breast carcinoma and untransformed cell lines as models. In this study, we have identified thiostrepton as a potential anticancer agent, which selectively induces cell death in breast cancer cells through down-regulation of FOXM1 expression.

Materials and Methods

Plasmids

The original human *FOXM1* promoter constructs *Apa*I, *Hin*dIII, *Pvu*I, and FOXM1_wt in pFlash reporter vectors were gifts from Prof. Rene H. Medema (University Medical Center Utrecht) and have been described previously (18). These *FOXM1* promoter constructs were then subcloned into the pGL3-basic reporter vector (Promega) using their original cloning sites. The FOXM1 expression vector contains a full-length FOXM1 cDNA cloned into pCDNA3.1 (Invitrogen) that has been described previously in ref. 19. The *FOXO3a* promoter was amplified using primers 5′-CTAGCTAGCTCTCTCCTTCCTTAGAATTT-TACTCG-′ and 5′-GCACACAGATATTGAAACACGAC-GAGAGCTCGCC-3′ from genomic DNA extracted from MCF-7 cells. The amplified DNA was then digested with restriction enzymes *Nhe*I and *Xho*I and subcloned into pGL3-basic reporter vector (Promega).

Cell Lines and Culture Conditions

All the cell lines used in this study, including MCF-10A, MCF-7, SKBR3, BT474, T47D, and ZR-75-1, originated from the American Type Culture Collection. MCF-10A was cultured in DMEM/Ham's F-12 (1:1 mix; Sigma) supplemented with 5% (v/v) horse serum, 10 mg/mL insulin, 5 mg/mL hydrocortisol, 100 ng/mL cholorotoxin, 20 ng/mL epidermal growth factor, 100 units/mL penicillin, and 100 μg/mL streptomycin (all supplements from Sigma UK). All other cell lines were cultured in DMEM (Sigma) supplemented with 10% (v/v) FCS, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C.

Western Blot Analysis and Antibodies

Cells were lysed and SDS-PAGE was done as described previously (19). The antibodies against FOXM1 (H-300), β-tubulin (H-235), CDC25b (C-20), Polo-like kinase-1 (F-8), and cyclin D1 (CRI-24) were purchased from Santa Cruz Biotechnology (Autogen Bioclear). Anti-p14^{ARF} (R562) antibody was acquired from Abcam, and total FOXO3a (06-951) was from Upstate. Antibodies against phospho-Akt (Ser^{473}), total Akt, phospho-FOXO3a (Thr^{32}), phospho-ERK1/2, and total ERK1/2 were from Cell Signaling Technologies. Primary antibodies were detected using horseradish peroxidase–linked anti-mouse, anti-goat, or anti-rabbit conjugates as appropriate (DAKO) and visualized using the enhanced chemiluminescence detection system (Amersham Biosciences).

Real-time Quantitative PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen), and cDNA was prepared using the SuperScript III reverse transcriptase and random primers (Invitrogen). For real-time quantitative PCR (RTQ-PCR), 100 ng cDNA was added to SYBR Green Master Mix (Applied Biosystems) and run in 7900 HT Fast Real-time PCR System (Applied Biosystems). The cycling program was 95°C for 20 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was assayed in triplicates, and the results were normalized to the level of ribosomal protein L19 RNA. The forward and reverse primers used were FOXM1-F, 5′-TGCAGCTAGGGATGTGAATCTTC-3′ and FOXM1-R, 3′-GGAGCCCAGTCCATCAGAACT-5′, FOXO3a-F, 5′-CCCAGCCTAACCAGGGAAGT-3′ and FOXO3a-R, 3′-AGCGCCCTGGGTTTGG-5′, and L19-F, 5′-GCGGAGAGGGTACAGCCAAT-3′ and L-19-R, 3′-GCAGCCGGCGCAAA-5′.

Luciferase Assay

Cells were seeded to a confluency between 30% and 50% on the day of transfection. Cells were then incubated at 37°C, 10% CO_2 for 6 h with a master mix of transfection reagents containing Fugene-6 (Qiagen) and the plasmid DNA in a ratio of 3:1 was dissolved in Opti-MEM. Fresh medium was then added to replace the transfection reagent and cells were allowed to grow overnight. In titration experiments, thiostrepton of varying concentrations from 0 to 20 μmol/L was added to the cells for 24 h. To determine the activity of the reporter genes, the Perkin-Elmer Luclite assay system was used. Following this, the reading of *Renilla* luciferase was obtained as a transfection and expression efficiency control for the experiments. Luminescence was then read using the 9904 Topcount Perkin-Elmer plate reader.

Sulforhodamine B Assay

Cells (~3,000) were seeded in each well of the 96-well plates. After culture, 100 μL trichloroacetic acid was added to each well and incubated for 1 h at 4°C. The plates were then washed with deionized water for three times before incubation at room temperature for 1 h with 0.4% sulforhodamine B in 1% acetic acid. The plates were then washed with deionized water and air-dried. Tris (10 mmol/L) was then added to the wells to solubilize the bound sulforhodamine B dye, and the plates were then read at 492 nm using the Anthos 2001 plate read (Jencons Scientific).

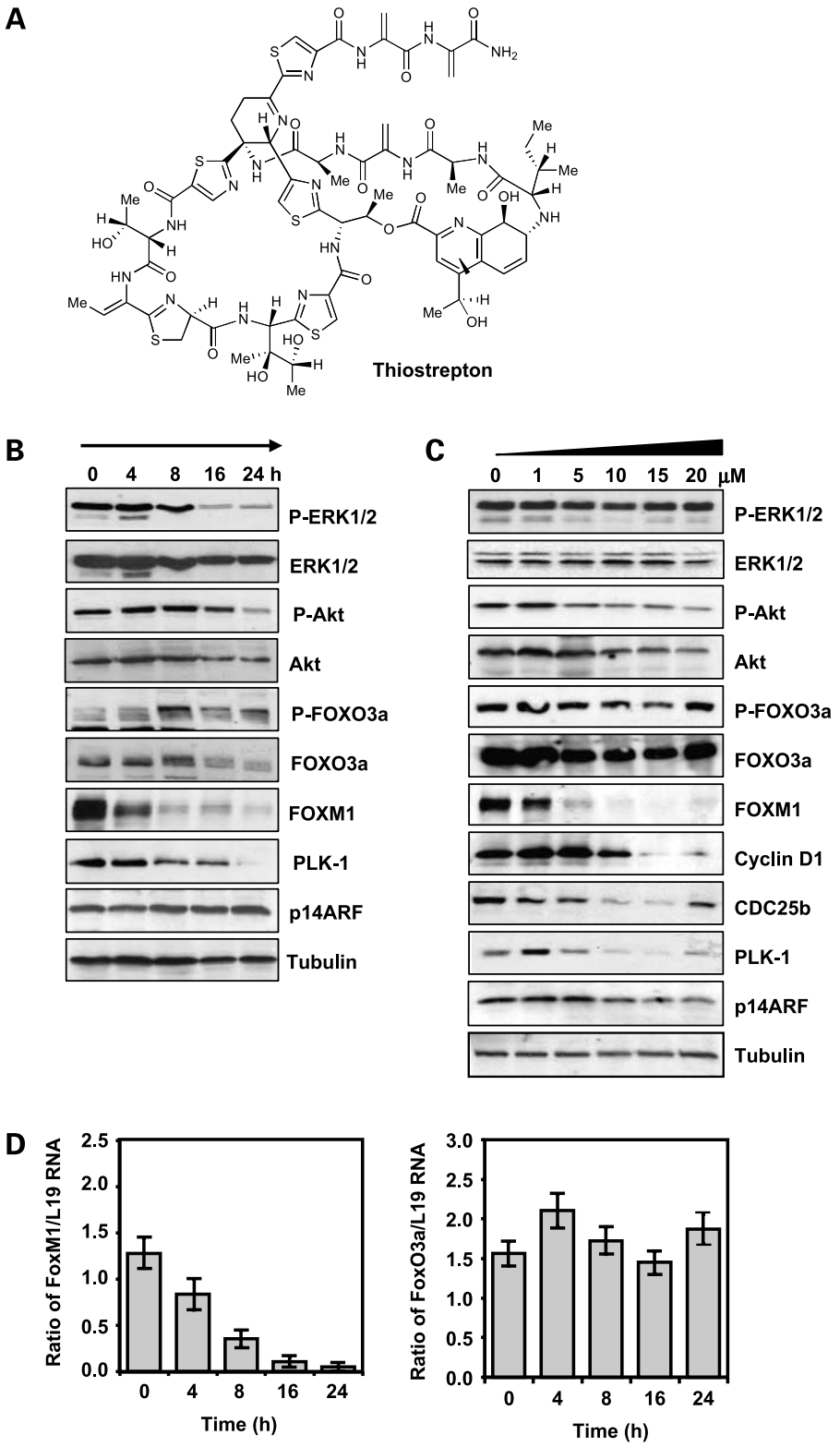

Figure 1. Thiostrepton reduces FOXM1 protein and mRNA expression. **A,** molecular structure of thiostrepton. MCF-7 cells were treated with thiostrepton for (**B**) 10 μmol/L for 0 to 24 h or (**C**) with 0 to 20 μmol/L for 24 h, and protein levels were determined by Western blot analysis. **D,** MCF-7 cells were treated with 10 μmol/L thiostrepton for 0 to 24 h. FOXM1 and FOXO3a mRNA transcript levels were determined by RTQ-PCR. Results of two independent experiments in triplicate. Mean ± SD.

Caspase Activity Assay

Caspase assays were done using the Caspase-Glo 8 and Caspase-Glo 9 assay kits (Promega) according to the manufacturer's protocol. Luminescence was read by the Perkin-Elmer 9904 Topcount plate reader.

Wound-Healing Assay

Cells were seeded to complete confluence in a monolayer in six-well plates. A wound was created by scratching firmly with a 2 μL pipette tip. Automatic time-lapsed images were taken by the ImageXpress Micro (Molecular Devices) where the plates were incubated at 10% CO_2 at 37°C. Subsequently, images were then analyzed by the ImageXpress 2.0 software (Molecular Devices).

Soft Agar Colony Assay

Soft colony assay was done using the Cell Transformation Detection Kit (Millipore). Cells (~6,000) were seeded into each 96-well plate containing 0.8% agarose base layer and 0.4% agarose top layer. Cell culture medium was changed periodically every 2 to 3 days. Following 28 days of incubation, cells were stained with the quantification solution for 1 h. Readings at 492 nm were taken using the Anthos 2001 plate reader.

MMP-9 Assay

Cells were seeded to 50% confluency before treatment with DMSO or thiostrepton. Supernatant was then harvested at specific time points. MMP-9 activity assay was then done using the MMP-9 ELISA kit (Merck Biosciences).

Cell Cycle Analysis

Cell cycle analysis was done by combined propidium iodide and bromodeoxyuridine (BrdUrd) staining or propidium iodide staining alone as described previously in ref. 20. Subconfluent cells with or without drug treatment were labeled for 2 h with 10 μmol/L BrdUrd (Sigma UK). Cells were trypsinized, collected by centrifugation,

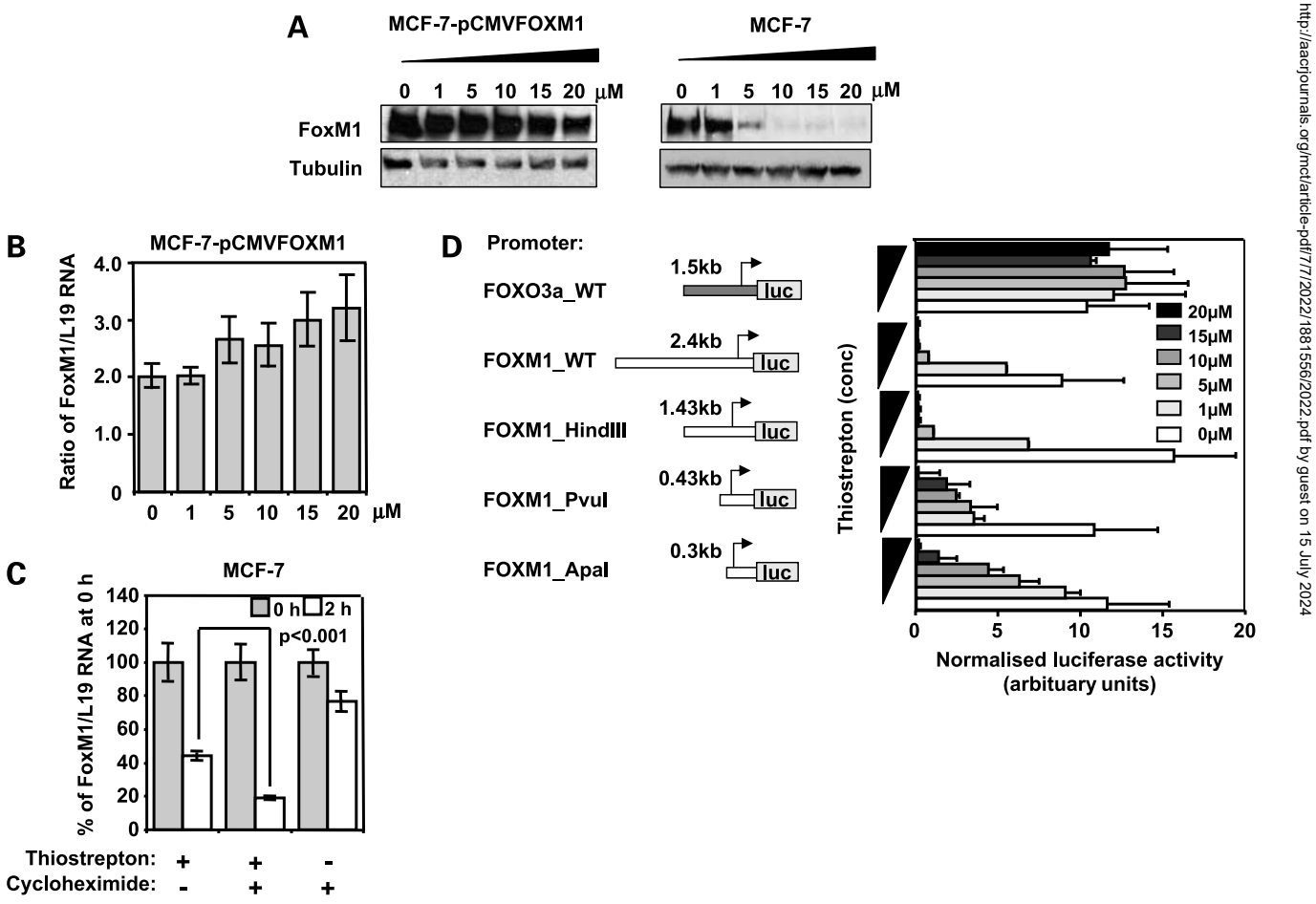

Figure 2. Thiostrepton has no effect on exogenous FOXM1 expression and targets the FOXM1 expression at promoter level. MCF-7 cells were either transfected with FOXM1 under the control of the CMV promoter or empty vector and 24 h later treated with 0 to 20 μmol/L thiostrepton for 24 h. **A,** FOXM1 expression was determined by Western blot analysis. **B,** relative FOXM1 mRNA level was determined by RTQ-PCR analysis. Experiments were done in triplicate. Mean ± SD. **C,** MCF-7 cells were treated with cycloheximide (100 μmol/L) or vehicle and incubated for 30 min before being treated with 10 μmol/L thiostrepton or vehicle for 0 and 2 h and FOXM1 mRNA was determined by RTQ-PCR analysis. Experiments were done in triplicate. Mean ± SD. **D,** MCF-7 cells were transfected with a series of *FOXM1* luciferase reported constructs containing the wild-type, truncated *FOXM1* promoters (containing *Hin*dIII, *Pvu*I, or *Apa*I fragments), or wild-type *FOXO3a* promoter. Cells were incubated for 24 h and treated with 0 to 20 μmol/L thiostrepton for 24 h and promoter activity was determined by luciferase detection and normalized to *Renilla*. Experiments were done in triplicate. Mean ± SD.

and resuspended in PBS before fixing in 90% ethanol. The fixed cells were incubated first with 2 N HCl and then with 0.5% Triton X-100 for 30 min at room temperature and with FITC-conjugated anti-BrdUrd antibodies (Becton Dickinson UK) at 1:3 dilution in 0.1 mol/L sodium borate (pH 8.5) for 30 min, with PBS washes between each treatment. The cells were incubated with 5 μg/mL propidium iodide, 0.1 mg/mL RNase A, 0.1% NP-40, and 0.1% trisodium citrate for 30 min before analysis using a Becton Dickinson FACSCanto analyzer. The cell cycle profile was analyzed using Cell Diva software (Becton Dickinson UK).

Results

Thiostrepton Inhibits FOXM1 Protein and mRNA Expression

Thiostrepton (Fig. 1A) has been reported previously to show cytotoxic effects toward several cancer cell lines in a compound screen of thiazole antibiotics (16). However, the mechanism of action of thiostrepton remains unknown. To define its mechanism of action, we first treated the breast carcinoma cell line MCF-7 with 10 μmol/L thiostrepton over a time course lasting 24 h (Fig. 1B). The activity of Akt and ERK1/2, as revealed by their phosphorylation status, decreased marginally after 16 to 24 h of thiostrepton treatment. Similarly, there were no significant changes in expression and phosphorylation status of FOXO3a, a direct downstream target of both Akt and ERK1/2. In contrast, there was a pronounced decrease in FOXM1 expression as early as 4 h after thiostrepton treatment, suggesting that FOXM1 could be one of the primary cellular targets of thiostrepton. Consistent with this, the expression of Polo-like kinase-1, a downstream target of FOXM1, decreased after the repression of FOXM1 expression by thiostrepton. It is notable that there were no significant changes in the expression of $p14^{ARF}$ and activity of FOXO3a and ERK1/2, all known regulators of FOXM1 expression. To assess further the relationship between thiostrepton and FOXM1 expression, we next examined the dose-dependent effect of thiostrepton on the expression of FOXM1, its upstream regulators and downstream effectors. To this end, MCF-7 cells were treated with various doses of thiostrepton from 0 to 20 μmol/L for 24 h before harvesting for Western blot analysis (Fig. 1C). FOXM1 expression decreased with increasing thiostrepton concentrations, and a significant FOXM1 reduction was observed at a concentration of 5 μmol/L. In concordance with our earlier results, we observed no dramatic changes in the expression and phosphorylation of upstream regulators, including $p14^{ARF}$, Akt, ERK1/2, and FOXO3a, associated with increasing levels of thiostrepton. Similarly, the repression of FOXM1 by increasing doses of thiostrepton was again mirrored by down-regulation of the expression of FOXM1 targets, including cyclin D1, Polo-like kinase-1, and CDC25b (11). We next investigated whether FOXM1 is down-regulated by thiostrepton at transcriptional level and studied the expression of FOXM1 mRNA by RTQ-PCR following thiostrepton treatment over 24 h. The RTQ-PCR analysis showed that the abundance of FOXM1 transcripts decreased with time starting from 4 h after thiostrepton treatment (Fig. 1D) and correlated closely to its protein levels (Fig. 1A), suggesting that thiostrepton represses FOXM1 expression primarily at transcriptional level. By comparison, no significant change in FOXO3a mRNA level was observed throughout the time course (Fig. 1D).

Thiostrepton Inhibits FOXM1 Expression Predominantly at Gene Promoter Level

To investigate further the mechanism by which thiostrepton down-regulates FOXM1 expression, we established a MCF-7 cell line, MCF-7-pCMVFOXM1, expressing a cytomegalovirus (CMV) promoter-driven FOXM1. This MCF-7-pCMVFOXM1 cell line was then treated with various doses of thiostrepton in parallel with the parental MCF-7 cell line (Fig. 2A). Western blot analysis showed that in response to increasing levels of thiostrepton FOXM1 expression was down-regulated in the parental MCF-7 cell line, whereas the expression level of the ectopic FOXM1 remained relatively constant in the MCF-7-pCMVFOXM1 cells, indicating that thiostrepton inhibits FOXM1 expression primarily at gene promoter level and not through mechanisms modulating protein and mRNA stability (Fig. 2A). Consistently, RTQ-PCR assays showed that increasing levels of thiostrepton failed to repress the expression level of the exogenous FOXM1 mRNA in the MCF-7-pCMVFOXM1 cells (Fig. 2B). We next sought to determine if *de novo* protein synthesis is required for the thiostrepton-mediated repression of FOXM1 mRNA expression. To this end, MCF-7 cells were pretreated with or without cycloheximide for 30 min and then stimulated with or without 10 μmol/L thiostrepton for an extra 2 h (Fig. 2C). Consistent with earlier results, RTQ-PCR analysis showed that thiostrepton repressed FOXM1 mRNA expression at 2 h. The RTQ-PCR result also revealed that cycloheximide treatment alone reduces FOXM1 mRNA levels (Fig. 2C). Nevertheless, cycloheximide pretreatment failed to abrogate the additional reduction in FOXM1 transcript level in MCF-7 cells treated with thiostrepton. These data therefore suggest that thiostrepton-mediated FOXM1 repression is, at least partially, independent of *de novo* synthesis of other transcription factors. Notably, we were not able to extend the time course of the experiment, as lengthier cycloheximide treatment abolishes almost all FOXM1 mRNA expression (data not shown). Finally, we transiently transfected various *FOXM1* promoter/reporter constructs into MCF-7 cells and incubated these transfected cells with increasing amounts of thiostrepton (Fig. 2D). The transfection results showed that all *FOXM1* promoter constructs are responsive to thiostrepton-mediated repression and that *FOXM1* gene promoter activity can be effectively repressed by thiostrepton in a dose-dependent manner. In contrast, the *FOXO3a* promoter activity is not affected by increasing levels of thiostrepton.

Thiostrepton Inhibits the Migration and Transformation Ability of Breast Cancer Cells

Besides cell proliferation, FOXM1 is also needed for cell migration and transformation (10, 12), and as such, we reasoned that thiostrepton should repress cell migration and transformation in breast cancer cells through the

down-regulation of FOXM1 expression. To test this hypothesis, we did wound-healing assays on MCF-7 breast cancer cells in the presence of 0, 10, and 20 μmol/L thiostrepton (Fig. 3A). The level of wound healing was measured by the average decrease in distance between the edges of the wounds at least at five different points. The wound-healing assays revealed that MCF-7 cells not treated with thiostrepton healed effectively with wounds decreasing to ~30% of the original distance after 24 h. Treatment with 20 μmol/L thiostrepton completely repressed wound healing in MCF-7 cells, whereas those treated with 10 μmol/L thiostrepton showed some degree of healing in the first 12 h, probably reflecting the time required for the drug to become fully effective. These wound-healing assays showed that thiostrepton can indeed inhibit breast cancer cell migration *in vitro*. To further confirm this observation, we studied the effect of thiostrepton on the activity of the metastatic marker MMP-9 (12), a known direct target of FOXM1 (Fig. 3B). MMP-9 assays done indicated that MMP-9 activity decreases with increasing concentrations of thiostrepton, further supporting the wound-healing results. We next studied the effect of thiostrepton on cell transformation by soft agar colony formation assays using the highly transformed MDA-MB-231 breast carcinoma cell line. MDA-MB-231 cells treated with either 10 or 20 μmol/L thiostrepton resulted in ~30% reduction in the number of colonies formed (Fig. 3C). Student's *t* test comparing the number of colonies between 0 μmol/L and either 10 or 20 μmol/L thiostrepton revealed a value of $P < 0.01$. Together, these findings suggest that

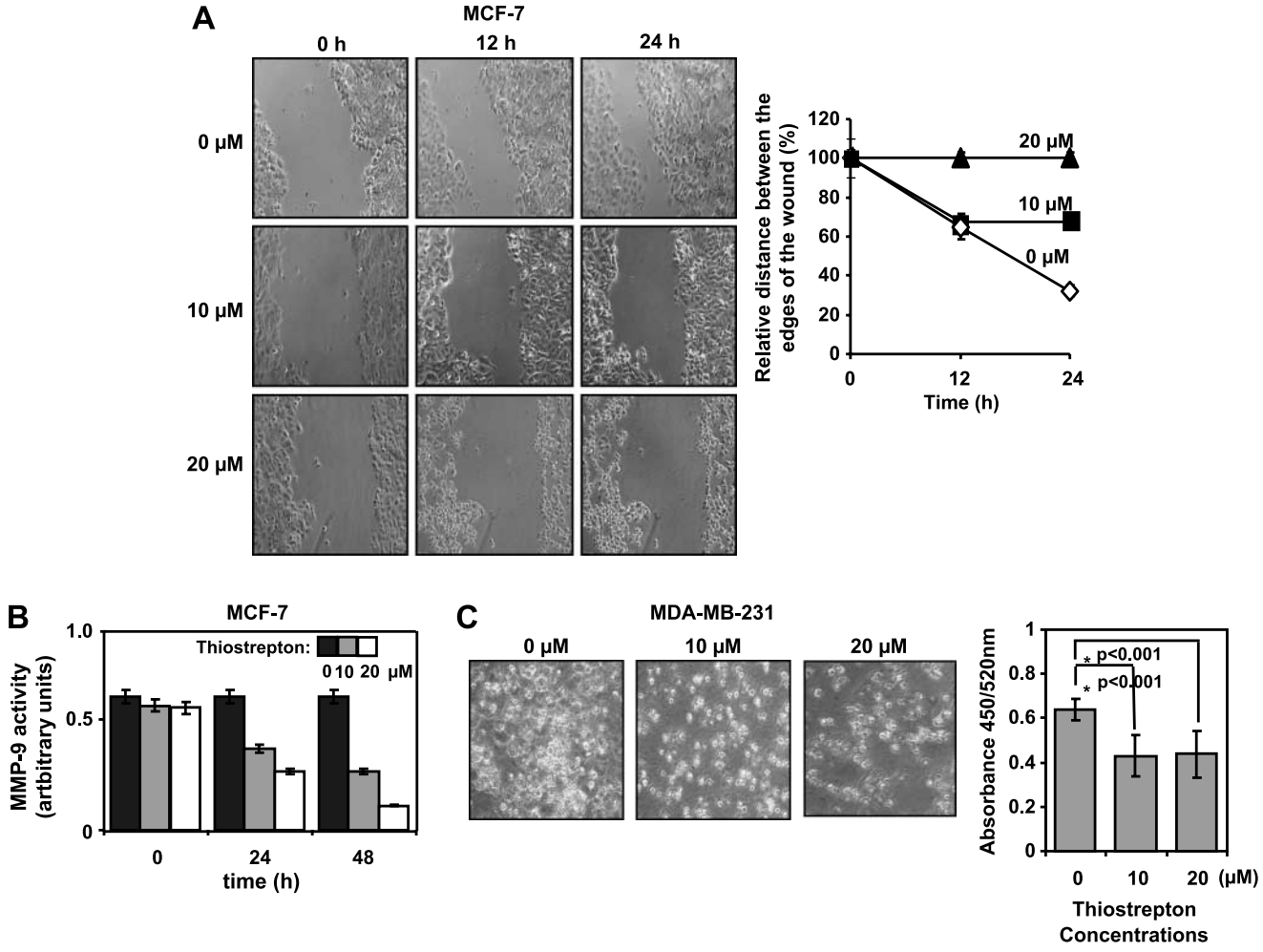

Figure 3. Thiostrepton reduces the migration, invasiveness, and transformation activity of breast cancer cells. **A,** MCF-7 cells were seeded to complete confluence in a monolayer and a wound was created by scratching firmly with a 2 μL pipette tip. Cells were then treated with thiostrepton (0, 10, and 20 μmol/L; 0, 12, and 24 h) and automatic time-lapsed images were taken (*left*). The average relative distances between the edges of the wounds at least at five different points were shown. Results of two independent experiments. Mean ± SD. **B,** MMP-9 activity was determined in MCF-7 cells following treatment with 0, 10, or 20 μmol/L thiostrepton for 0, 24, and 48 h. Results of two independent experiments in triplicate. Mean ± SD. **C,** MDA-MB-231 colony formation was determined following continuous exposure to thiostrepton (0-20 μmol/L) or vehicle using bright-field microscopy (magnification, ×20) and quantitative measurement of colony number at relative absorbance of 450/520 nm. Results of two independent experiments in triplicate. Mean ± SD. Statistical analysis was done using Student's *t* test. *, $P < 0.001$, very significant.

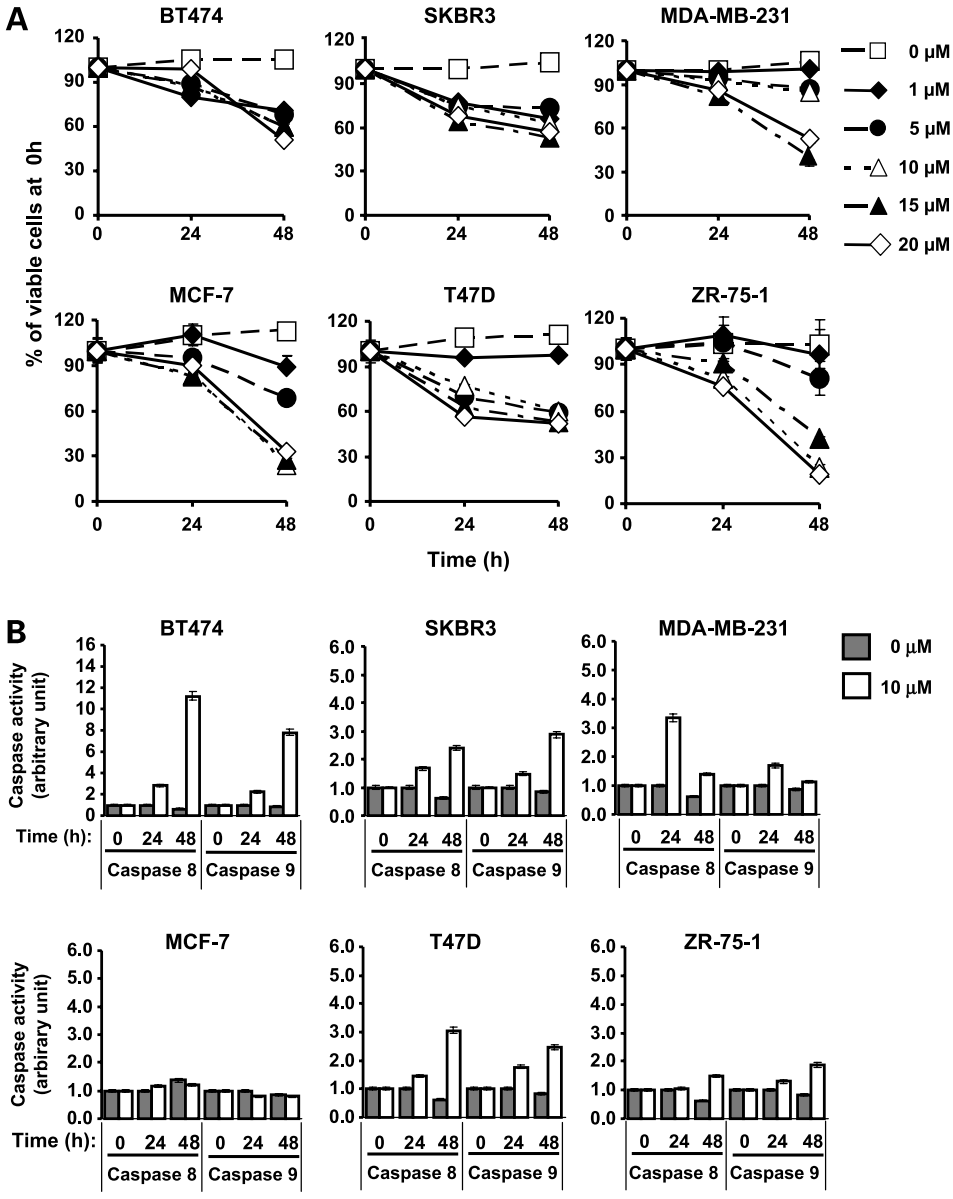

Figure 4. Thiostrepton reduces proliferation and induces cell death in breast cancer cell lines. **A,** BT474, SKBR3, MDA-MB-231, MCF-7, T47D, and ZR-75-1 cells were treated with thiostrepton (0-20 μmol/L; 0-48 h) and proliferation was determined by sulforhodamine B assay. Results of experiments in triplicate. Mean ± SD. **B,** caspase-8 and caspase-9 activity assays were done in BT474, SKBR3, MDA-MB-231, MCF-7, T47D, and ZR-75-1 cells following treatment with 0 or 10 μmol/L thiostrepton for 0, 24, and 48 h. Results of two independent experiments in triplicate. Mean ± SD.

thiostrepton can significantly repress FOXM1 expression and reduce breast cancer cell migration, invasiveness, and transformation ability by down-regulating FOXM1 target gene expression such as the metastatic regulator, MMP-9.

Thiostrepton Inhibits Breast Cancer Cell Proliferation and Induces Caspase-Dependent and Caspase-Independent Cell Death

To further characterize the antiproliferative effects of thiostrepton, sulforhodamine B proliferation assays were done on a panel of breast carcinoma cell lines, including BT474, SKBR3, MCF-7, MDA-MB-231, T47D, and ZR-75-1, following treatment with various doses of thiostrepton over a time course of 48 h (Fig. 4A). All the breast cancer cell lines tested showed a decrease in the rate of proliferation in a dose-dependent manner over 48 h, indicating that thiostrepton is effective in inhibiting breast cancer cell proliferation. Next, caspase-8 and caspase-9 assays were done to determine whether thiostrepton induced apoptosis via the intrinsic or extrinsic cell death pathways (Fig. 4B). All the breast cancer cell lines, apart from MCF-7, showed both caspase-8 and caspase-9 activation in the presence of thiostrepton, indicating thiostrepton causes caspase-dependent apoptosis in these breast cancer cell lines. Cell lines MDA-MB-231, SKBR3, and T47D showed a 3- to 5-fold activation in peak caspase-8 and caspase-9 activity. BT474 showed a remarkable peak of 16- and-8 fold activation in caspase-8 and caspase-9 activation, respectively. This indicates that thiostrepton triggered both extrinsic and intrinsic pathways of apoptosis to cause cell death in breast cancer cells. However, MCF-7

cells showed no activation in caspase activity. This is because MCF-7 cells are known to be deficient in caspase-3 and caspase-7 expression (21); therefore, alternative death pathways must have been invoked to induce cell death (see Fig. 5). Nonetheless, these data together showed that thiostrepton is capable of inhibiting breast cancer cell proliferation and induces apoptosis in a caspase-dependent and caspase-independent manner.

Thiostrepton Specifically Inhibits the Proliferation of MCF-7 but Not MCF-10A Cells

To compare the effect of thiostrepton on cell proliferation and survival of cancerous and untransformed breast epithelial cells, we analyzed the cell cycle phase distribution of MCF-7 and MCF-10A cells following treatment with 10 μmol/L thiostrepton over a time course of 72 h. The results showed that thiostrepton can cause cell cycle arrest and cell death in MCF-7 breast carcinoma cells but has little effects on the cell cycle progression and cell death of MCF-10A. Following thiostrepton treatment, a significant number of MCF-7 cells started to accumulate at G_1 from 8 h, and this was accompanied by a decrease in cells in S and G_2-M phases and an increase in a population of cells with sub-G_1 (2N) DNA content, indicative of cell death (Fig. 5A). By 48 and 72 h following thiostrepton treatment, the majority of cells had sub-G_1 DNA content. In contrast, there was little change in cell cycle distribution of MCF-10A cells following thiostrepton treatment, signifying that MCF-10A cells are resistant to thiostrepton. The flow cytometric analysis also indicated that the untransformed MCF-10A cells progressed through the cell cycle with slower kinetics, with the majority of the cells in G_1 and G_2-M phases. To confirm these results, we next did BrdUrd incorporation analysis on MCF-7 and MCF-10A cells before and after 24 h of 10 μmol/L thiostrepton treatment. The result showed that thiostrepton treatment almost completely abrogated BrdUrd uptake in MCF-7 cells, but it had little effect on BrdUrd incorporation in MCF-10A cells. Consistent with a slower rate of proliferation, the level of BrdUrd

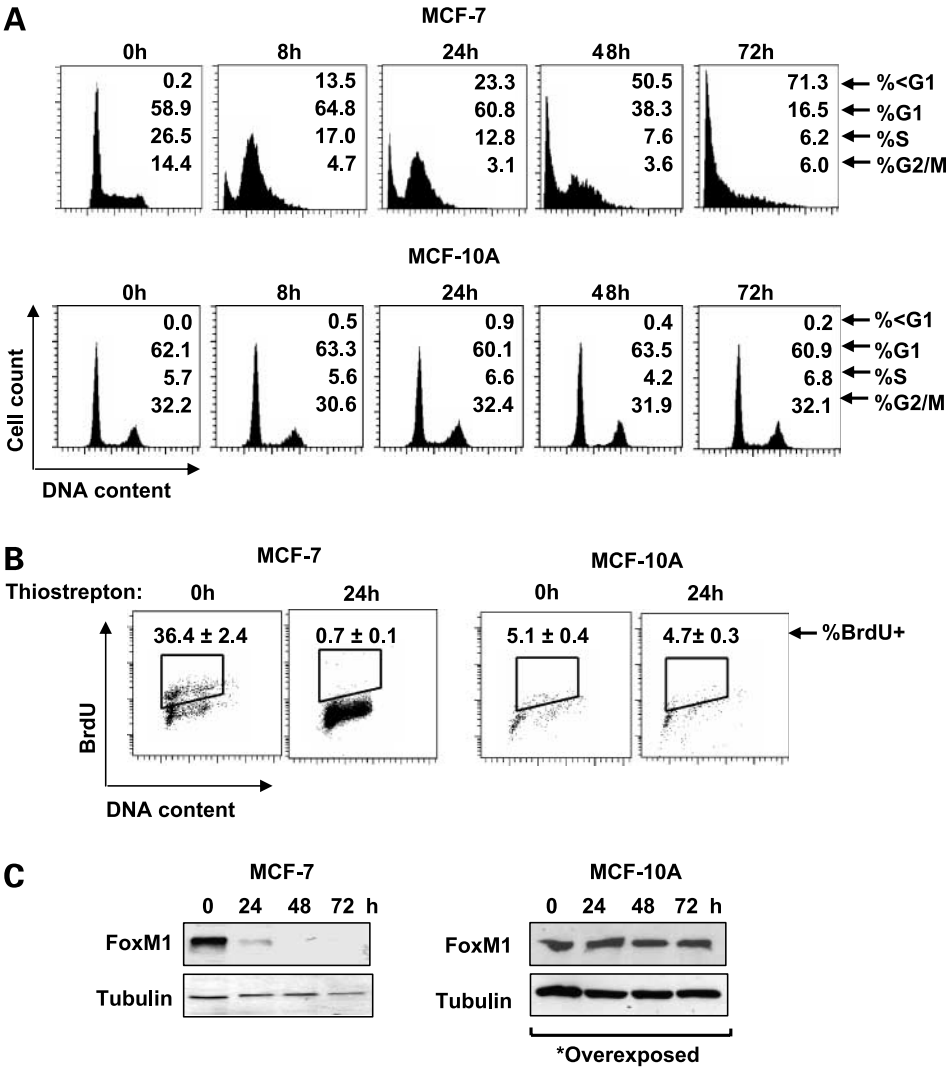

Figure 5. Thiostrepton selectively induces cell cycle arrest in breast cancer cell lines but not in untransformed breast epithelial cells. **A,** MCF-7 and MCF-10A cells were treated with 10 μmol/L thiostrepton for 0 to 72 h and cell cycle analysis was done after propidium iodide staining. **B,** MCF-7 and MCF-10A cells were treated with 10 μmol/L thiostrepton for 0 and 24 h and S-phase cells were determined by BrdUrd and propidium iodide staining and fluorescence-activated cell sorting analysis. Results of experiments in triplicate. Mean ± SD. **C,** MCF-7 and MCF-10A cells were treated with 10 μmol/L thiostrepton for 0 to 72 h and FOXM1 protein level was determined by Western blot analysis. *, MCF-10A blot was overexposed to allow for visualization of FOXM1.

uptake in MCF-10A cells was low compared with MCF-7 cells. Collectively, these data indicate that thiostrepton can effectively block DNA incorporation as well as S-phase and G_2-M-phase entry in MCF-7 but not MCF-10A cells (Fig. 5B). They also suggested that thiostrepton specifically induces cell cycle arrest and cell death in breast carcinoma cells but not in untransformed breast epithelial cells. We then explored if FOXM1 has a role in mediating the cytostatic and cytotoxic effects of thiostrepton in breast cancer cells and studied the expression pattern of FOXM1 in both MCF-7 and MCF-10A following 10 μmol/L thiostrepton treatment by Western blot analysis. Consistent with our earlier observation that MCF-10A has a slow proliferative rate, Western blot analysis also revealed that FOXM1 was expressed at lower levels in MCF-10A cells compared with MCF-7 cells. Nonetheless, we found that FOXM1 expression level remained unchanged following thiostrepton treatment in MCF-10A cells in contrast to MCF-7 cells, which displayed drastically reduced levels of FOXM1 after thiostrepton treatment. These data together suggest that FOXM1 has a role in mediating the antiproliferative function of thiostrepton in breast cancer cells.

Expression of a Constitutively Active FOXM1 Circumvents the Antiproliferative Effects of Thiostrepton in MCF-7 Cells

To show definitively that FOXM1 is a crucial cellular target of thiostrepton, MCF-7 cells were either untransfected or transfected with the empty expression vector or an expression vector encoding for a constitutively active NH_2-terminal truncated form of FOXM1, ΔN-FOXM1, and then stimulated with 10 μmol/L thiostrepton for 24 h. Western blot analyses showed that the endogenous FOXM1 was expressed in the untreated transfected MCF-7 cells, but its expression was repressed by thiostrepton (Fig. 6A). However, the CMV promoter-driven ΔN-FOXM1 was expressed at high levels before and after thiostrepton treatment (Fig. 6A). Proliferative assays done on these cells showed that cell proliferation was significantly repressed by thiostrepton in the mock-transfected and empty vector-transfected MCF-7 cells but not in the MCF-7 cells expressing the constitutively active ΔN-FOXM1 (Fig. 6B). It is notable that we have done the overexpression experiment with the wild-type FOXM1 construct, but it was ineffective in blocking the antiproliferative effects of thiostrepton.

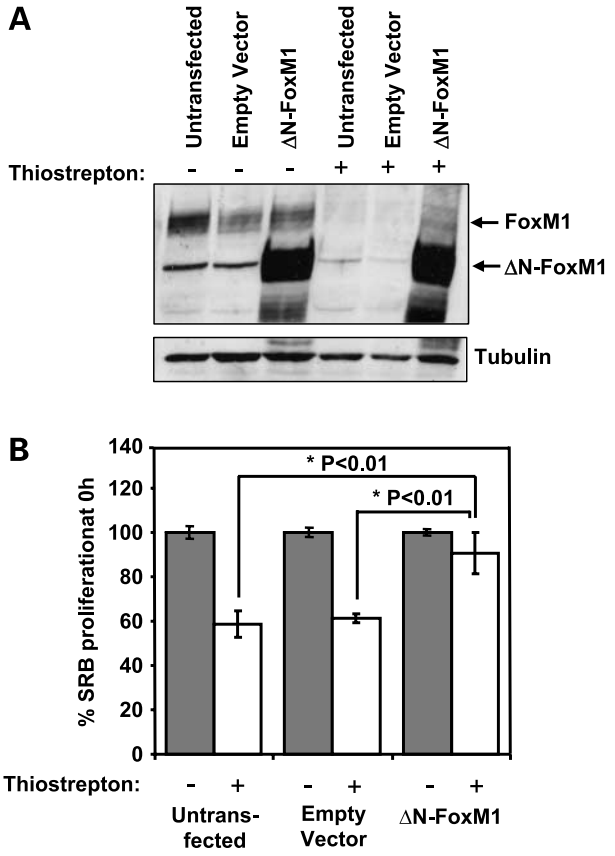

Figure 6. Expression of a constitutively active FOXM1 overcomes the antiproliferative effects of thiostrepton in breast cancer cells. MCF-7 cells were either untransfected or transfected with empty vector or ΔN-FOXM1, a constitutively NH_2-terminal truncated FOXM1 mutant, and 24 h later untreated or treated with 10 μmol/L thiostrepton for 24 h. **A,** FOXM1 and ΔN-FOXM1 expression was determined by Western blot analysis. **B,** cell proliferation was measured by sulforhodamine B assay. Two independent experiments each in triplicate were done. Mean ± SD. Statistical analysis was done using Student's *t* test. *, $P < 0.01$, significant.

Discussion

This study shows for the first time that the novel thiazole antibiotic thiostrepton selectively induces cell death in breast cancer cells through the down-regulation of FOXM1 expression. Furthermore, thiostrepton treatment, accompanied by a loss of FOXM1 expression, results in a reduction in the proliferation, invasiveness, and transformation ability of breast cells. Critically, thiostrepton has no affect on the proliferation of nontransformed breast epithelial cells.

Thiostrepton has been identified previously as an anticancer agent in a study of thiazole antibiotics and derivatives (16). We sought to identify the mechanism by which thiostrepton may induce cell death in breast cancer cells and determined the expression level and activity of a series of cell fate regulators in response to thiostrepton treatment. No change was observed in the phosphorylation status of the prosurvival factors Akt and ERK, whereas phosphorylation of FOXO3a, which we have reported previously as a critical mediator of chemotherapeutic drug-induced cell death (22), remained unchanged by thiostrepton. However, thiostrepton induced a dose-dependent, rapid, and sustained loss of FOXM1 protein expression and mRNA transcript level, suggesting that FOXM1 may be a key target of thiostrepton. FOXM1 has been reported previously to regulate key effectors of G_1-S-phase and G_2-M-phase transition, and loss of FOXM1 following thiostrepton treatment was followed by a concomitant decrease in target gene expression, including

Polo-like kinase-1 and *CDC25b*. However, no change in upstream regulators of FOXM1, such as ERK1/2, $p14^{ARF}$, FOXO3a, or Akt, was observed before the reduction FOXM1 levels, suggesting that thiostrepton may affect the expression of FOXM1 at the transcriptional level. Consistent with this hypothesis, thiostrepton failed to repress an ectopic FOXM1 driven by a CMV promoter. It is also significant that the cotreatment of breast cancer cells with thiostrepton and cycloheximide, an inhibitor of translation, did not prevent the repression of FOXM1 mRNA levels by thiostrepton, indicating that the repression of the FOXM1 promoter activity is not dependent on *de novo* protein synthesis.

Cell cycle analysis showed that thiostrepton induces cell cycle arrest at G_1 and S phase, whereas BrdUrd staining showed a reduction in DNA synthesis following treatment with thiostrepton. This can lead to a block of cells at the G_1-S boundary and cells entering G_2-M from S phase, culminating in an apparent accumulation of cells at G_1 and S phase and a corresponding decrease in G_2-M cell population. Our data also showed that thiostrepton can induce cell death through caspase-dependent intrinsic and extrinsic apoptotic pathways as well as caspase-independent death mechanisms, as observed in MCF-7 cells, which are deficient of caspase-3 and caspase-7 (23) and show no detectable activation of caspase-8 and caspase-9. These findings are consistent with the role of FOXM1 in cell proliferation and survival. Furthermore, in *in vitro* assays, thiostrepton has shown efficacy in repressing breast cancer cell migration, metastasis, and transformation, which are all functional attributes of FOXM1. The finding that overexpression of a constitutively active form of FOXM1, which lacks the NH_2-terminal repressor domain (ΔN-FOXM1), can block the antiproliferative function of thiostrepton establishes FOXM1 as a critical cellular target of thiostrepton. This hypothesis is further supported by the observation that the nontransformed breast epithelial cells, MCF-10A, which show no change in FOXM1 expression in response to thiostrepton, also display no alteration in cell cycle status in response to thiostrepton.

Crucially, our data also indicate that thiostrepton induces cytostatic and cytotoxic effects specifically in breast cancer cell lines, whereas nontransformed MCF-10A breast epithelial cells are refractory to thiostrepton. The resistance of MCF-10A cells to thiostrepton suggests that compound toxicity may be within tolerable limits of noncancer cells. Breast cancer cells often express high levels of FOXM1, and as such, the susceptibility of these cells to repression of FOXM1 expression may be higher than untransformed cells. However, the exact mechanism by which thiostrepton represses FOXM1 expression in breast cancer cells but not in MCF-10A cells remains to be elucidated. Studies of thiostrepton in prokaryotes have shed some light on the potential mechanism of action of thiostrepton. At high concentrations, thiostrepton binds to the 23S subunit of rRNA to inhibit translation and protein synthesis (17, 24). However, this is unlikely to be the primary mode of action of thiostrepton in breast cancer cells because this would lead to a global nonspecific down-regulation in the expression of all proteins and not specifically FOXM1. Although this mechanism does not confer specificity to FOXM1, it raises the possibility that thiostrepton may be used to increase the efficacy of DNA-damaging agents by preventing protein translation and tumor cell recovery. However, the enhanced repression of FOXM1 mRNA by cotreatment with cycloheximide and thiostrepton versus cycloheximide alone would suggest that in mammalian cells thiostrepton has additional mechanisms of action in addition to inhibition of protein translation. FOXM1 has been reported previously to be a valid therapeutic target in cancer (25); the use of a cell-penetrating ARF peptide inhibitor of FOXM1 has been shown to selectively induce apoptosis in human hepatocellular carcinoma cell lines and mouse models (14). In addition, a related antibiotic thiazole compound, siomycin A, has been reported to down-regulate the transcriptional activity and expression of FOXM1 (26). It is interesting to note that Radhakrishnan et al. have shown that overexpression of wild-type FOXM1 is sufficient to block the antiproliferative effects of thiostrepton, whereas we found here that only expression of a constitutively active ΔN-FOXM1, but not the wild-type FOXM1, can rescue cells from the antiproliferative effects of thiostrepton. These differences may reflect the cell lines used or discreet mechanisms of action of the thiazole antibiotics; however, it remains intriguing to speculate that other members of the thiazole antibiotics including sporangiomycin, mircococcin, and cyclodidemnamide B might display similar anticancer properties through the inhibition of FOXM1 expression (27). Moreover, FOXM1 has also been shown to be a downstream target of another forkhead box transcription factor FOXO3a, a physiologic target for several chemotherapeutic drugs, including paclitaxel, anthracyclins, and gefitinib, in breast and other cancers (22, 28, 29). Based on these observations, it is possible that thiostrepton and other thiazole antibiotics may synergize with chemotherapeutic drugs in combinatorial anticancer therapies to improve the efficacy of currently available treatments. In conclusion, our study shows that thiostrepton can specifically inhibit FOXM1 expression at the transcriptional level to selectively target cancer but not untransformed cells and that it can be a candidate for anticancer drug development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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