Antiangiogenic Tetrathiomolybdate Enhances the Efficacy of Doxorubicin against Breast Carcinoma[1]

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Abstract

Constitutive activation of nuclear factor κB is implicated to be a critical survival mechanism used by carcinoma cells to escape apoptosis. Tetrathiomolybdate (TM), a novel copper chelator, exhibits potent antiangiogenic properties, in part, through suppression of the nuclear factor κB signaling cascade. In this study, we determined whether TM enhances doxorubicin-induced apoptosis in SUM149 inflammatory breast carcinoma cells. Apoptosis was not observed in these cells after TM treatment. Moreover, SUM149 cells were relatively resistant to doxorubicin-induced apoptosis ranging from 9.9 ± 2.9% to 21.5 ± 2.0% apoptotic cells for 0.1 to 2.5 μM doxorubicin treatment. A greater-than-additive increase (33.8 ± 4.6%, 57.5 ± 5.2%, or 83.7 ± 1.0% apoptosis with TM and 0.1, 0.5, or 2.5 μM doxorubicin, respectively) in apoptosis was observed in cells treated with the combination therapy of TM and doxorubicin. In SUM149 xenografts, TM and doxorubicin significantly retarded tumor growth in comparison with either agent administered alone ($P < 0.03$). Tumor cell apoptosis in the combination therapy-treated mice was 113.3 ± 20% greater than that in TM-treated mice and 52.4 ± 14.3% greater than that in doxorubicin-treated mice. These results suggest that TM may enhance the rate of pathological complete response when used in combination with an anthracycline in neoadjuvant therapy of breast carcinoma.

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Introduction

The NF-κB[3]/Rel family of transcription factors is comprised of RelA, RelB, c-Rel, p50 (nfκb1), and p52 (nfκb2; Ref. 1). Numerous reports have characterized various types of solid tumors, including breast, ovarian, colon, pancreatic, thyroid, bladder, and prostate tumors, with deregulated NF-κB activity as a result of constitutive activation of the NF-κB signaling pathway or inactivating mutations of IκB protein members (2–6). Overexpression of p50 and p52 has been observed in colon, prostate, and breast carcinomas (5, 6). Several studies using human breast carcinoma cells have shown that overexpression of p50 results in constitutive NF-κB activity (5, 6). Subsequently, constitutive NF-κB activation was shown to correlate with the conversion of breast carcinoma cells to hormone-independent growth, a hallmark of a more aggressive and metastatic phenotype (5). Taken together, these reports indicate that activation of NF-κB appears to be an event that frequently occurs during malignant transformation and progression of HME cells and other cell types.

There is increasing evidence that deregulated NF-κB activation is linked to the development of resistance to cytotoxic therapy. Tumors with elevated levels of NF-κB are resistant to apoptosis induced by chemotherapy and radiotherapy (7). Several groups reported that inhibition of NF-κB activation increased the sensitivity of resistant carcinoma cells to apoptosis-inducing stimuli (6, 8, 9). TM, a copper chelator, has proven to be a potent antiangiogenic compound in animals and humans (10–13). Recently, TM was found to be an inhibitor of NF-κB activity in SUM149 inflammatory breast carcinoma cells (11). This observation is exciting from a clinical perspective because it suggests that TM may be able to enhance the efficacy of chemotherapeutics against cancers with NF-κB overexpression. In this study, the combination therapy of TM and doxorubicin was more effective at inducing apoptosis and suppressing tumor growth in NF-κB-overexpressing SUM149 cells than either agent administered alone. Our data indicate that TM can enhance the efficacy of doxorubicin and thus suggest a testable clinical hypothesis of using TM in combination with an anthracycline in neoadjuvant therapy of primary or refractory tumors.

Materials and Methods

Cell Line. The SUM149 inflammatory breast carcinoma cell line was developed from a primary inflammatory breast carcinoma nodule and was grown in Ham's F-12 medium sup-

[3] The abbreviations used are: NF-κB, nuclear factor κB; TM, tetrathiomolybdate; HME, human mammary epithelial.

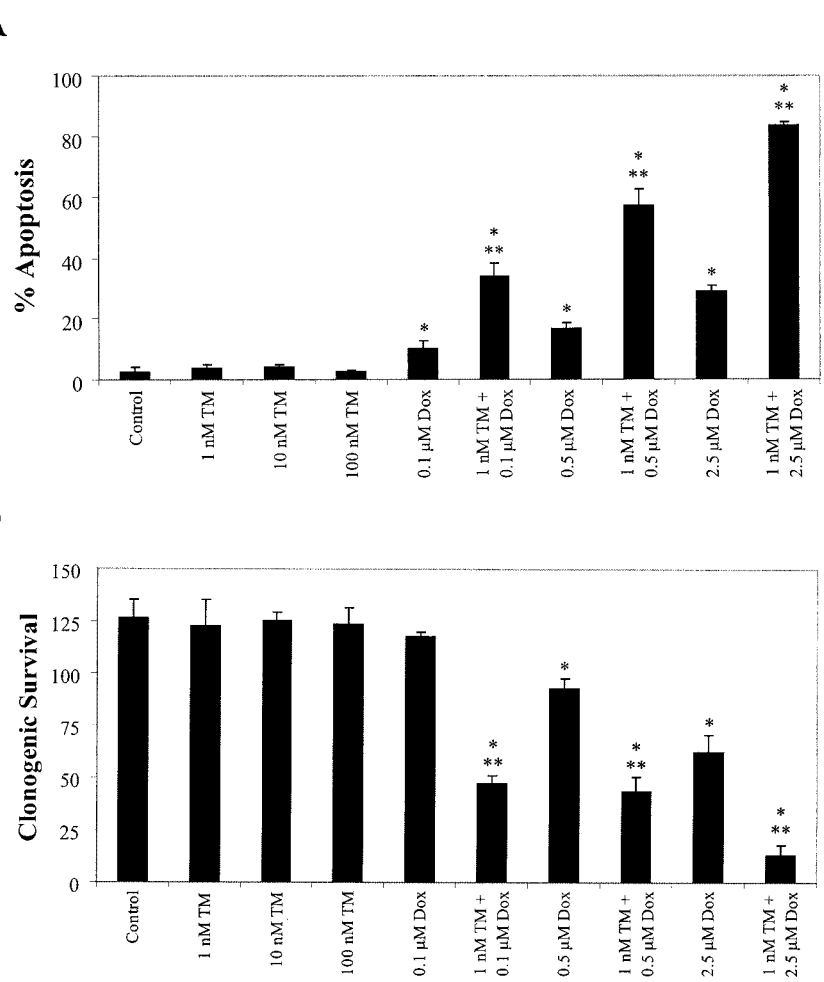

Fig. 1. Combination therapy of TM and doxorubicin enhances apoptosis and decreases clonogenic survival. *A,* flow cytometric analysis of apoptosis. SUM149 cells were treated with control, TM (1, 10, or 100 nM), doxorubicin (0.1, 0.5, or 2.5 μM), or TM (1 nM) and doxorubicin (0.1, 0.5, or 2.5 μM) for 72 h. Apoptosis was determined by the FITC-Annexin V/propidium iodide assay. Data are presented as mean ± SE. *, $P < 0.01$ *versus* control; **, $P < 0.005$ *versus* appropriate single-agent doxorubicin treatment. *B,* clonogenic survival. SUM149 cells were treated with control, TM (1, 10, or 100 nM), doxorubicin (0.1, 0.5, or 2.5 μM), or TM (1 nM) and doxorubicin (0.1, 0.5, or 2.5 μM) for 72 h. After 14 days, cells were stained with trypan blue and counted. Data are presented as mean number of clones ± SE. *, $P < 0.02$ *versus* control; **, $P < 0.003$ *versus* appropriate single-agent doxorubicin treatment.

plemented with 5% fetal bovine serum, insulin, and hydrocortisone (14).

Detection of Apoptotic Cells. SUM149 cells (2.5×10^5) were plated on 100-mm dishes and allowed to grow for 24 h. Subsequently, cells were treated with vehicle (control), TM, doxorubicin, or TM and doxorubicin for 72 h. Cells were harvested, washed with cold PBS, and costained with Annexin V and propidium iodide according to the manufacturer's protocol (ApoAlert Annexin V-FITC Apoptosis Kit; Clontech). Apoptotic cells were analyzed on a FACScan flow cytometer.

Clonogenic Survival Assay. SUM149 cells (250 cells) were plated on 6-well plates and allowed to grow for 24 h. Subsequently, cells were treated with vehicle (control), TM, doxorubicin, or TM and doxorubicin for 72 h. Cells were washed with PBS, and fresh medium was replaced as needed for 14 days. Clones were fixed with methanol and acetic acid, stained with trypan blue dye, and counted.

Orthotopic Xenograft Model of Breast Cancer. SUM149 (1×10^6) cells were orthotopically injected in the upper left mammary pad of 10-week-old female athymic nude mice (Harlan). Cells were trypsinized, washed, and resuspended in HBSS at a density of 1×10^6 cells/100 μl. Mice were anesthetized using 10 mg/ml ketamine, 1 mg/ml xylazine, and 0.01 mg/ml glycopyrrolate, and an incision below the thoracic left mammary fat pad was made. Using a 27-gauge needle, the cell suspension was injected into the exposed mammary fat pad, and the wound was closed with a single wound clip. Mice were monitored daily, and tumor size was measured using a microcaliper. Tumor volume was calculated using the following formula: (length × width^2)/2.

Immunohistochemistry and Quantitation of Microvessel Density and Apoptosis. Mammary gland tumors harvested at autopsy were fixed in formalin and processed for immunostaining. Intratumoral microvessel density was assessed with CD31 staining (PharMingen) using the vascular hot spot technique (15). Sections were scanned at low power to determine areas of highest vascular density for quantitative assessment. Within this region, individual microvessels were counted in 10 separate random fields at high power (×400 magnification), and the mean vessel count from the 10 fields was calculated. A single countable microvessel was defined as any endothelial cell or group of cells that was clearly separate from other vessels, stroma, or tumor cells without the necessity of a vessel lumen. Intratumoral apoptosis was assessed using the ApopTag kit (Intergen). Sec-

tions were scanned at low power to determine areas of highest apoptotic cell density for quantitative assessment. Within this region, the number of apoptotic cells per 500 total cells was counted in 5 separate random fields at high power (×400), and the total percentage of apoptotic cells was calculated. Quantification of intratumoral microvessel density and apoptosis was performed by a blind observer to eliminate subjectivity of the analysis.

Results

Combination Therapy of TM and Doxorubicin Enhances Apoptosis *in Vitro*. Apoptosis was determined by flow cytometry using FITC-Annexin V. As shown in Fig. 1*A*, apoptosis was not observed in these cells after TM (1–100 nM) treatment. A dose-dependent induction of apoptosis was observed with doxorubicin ranging from 9.9 ± 2.9% for 0.1 μM doxorubicin to 21.5 ± 2.0% for 2.5 μM doxorubicin. Doxorubicin (0.1 μM for 72 h) induced 75 ± 3.4% apoptosis in MDA-MB435 cells and 60 ± 2.1% apoptosis in MDA-MB231 cells. This observation indicates that SUM149 cells are relatively resistant to doxorubicin-induced apoptosis when compared with other commonly used breast carcinoma cell lines. In SUM149 cells, the combination therapy of TM and doxorubicin was more effective at inducing apoptosis than either compound administered alone; 33.8 ± 4.6%, 57.5 ± 5.2%, or 83.7 ± 1.0% apoptosis was observed in cells treated with TM and 0.1, 0.5, or 2.5 μM doxorubicin, respectively. Using ordinary least-squares regression framework to model these data, we found that this combination therapy resulted in a significant, greater-than-additive increase in apoptosis ($P < 0.005$; $n = 3$). Consistent with our apoptosis data, the clonogenic survival of cells treated with TM was similar to control (Fig. 1*B*). Doxorubicin induced a dose-dependent decrease in clonogenic survival, ranging from 118 ± 5 clones for 0.1 μM doxorubicin to 63 ± 8 clones for 2.5 μM doxorubicin. Importantly, the combination therapy was significantly more cytotoxic and resulted in fewer clones than single-agent doxorubicin therapy ($P < 0.003$; $n = 3$).

Combination Therapy of TM and Doxorubicin Suppresses Tumor Growth and Enhances Apoptosis *in Vivo*. Female athymic nude mice were transplanted with SUM149 cells (1×10^6), and palpable tumors were allowed to develop without treatment. At 28 days postimplantation, mice with established tumors of approximately 0.5 cm^3 were randomly assigned to four protocols and treated with control (gavaged with water daily and i.v. injection with PBS weekly), TM (1.25 mg/day loading dose for 3 days followed by 0.7 mg/day maintenance dose for the remainder of protocol) by oral gavage, or doxorubicin (5 mg/kg/week) by i.v. injection or cotreated with TM (1.25 mg/day loading dose for 3 days followed by 0.7 mg/day maintenance dose for the remainder of protocol) and doxorubicin (5 mg/kg/week). Although the doubling time of control SUM149 tumors appears to be around 19 days, the experiment was terminated at 28 days posttreatment due to general health concerns because the size of the mammary tumors was bulky enough to impede mice from the control group from eating and drinking. Mice treated with TM as a single agent or in combination with doxorubicin were rendered copper deficient (ceruloplasmin levels < 25 ± 5% of control) after 1 week of therapy and remained so for the rest of the protocol. As shown in Fig. 2, therapy with TM or doxorubicin alone significantly inhibited tumor growth in comparison with control treatment ($P < 0.05$; $n = 6$). More importantly, mice treated with the combination of TM and doxorubicin had complete tumor stabilization without an apparent increase in toxicity as assessed by general health, weight, and behavior ($P < 0.008$ compared with control; $P < 0.03$ compared with TM or doxorubicin; $n = 6$).

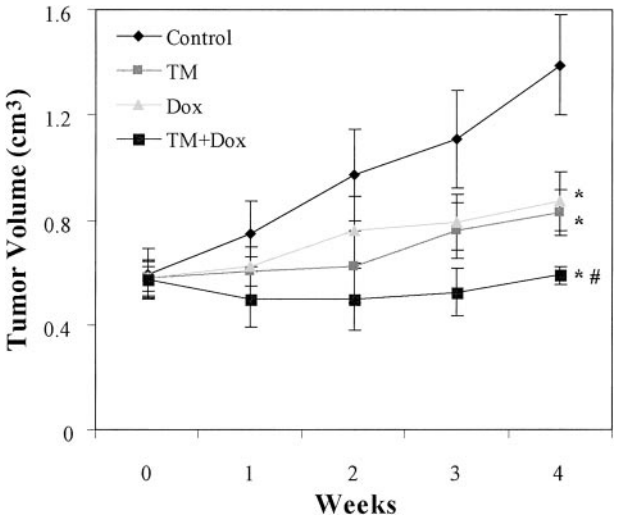

Fig. 2. Combination therapy of TM and doxorubicin inhibits tumor growth in SUM149 xenografts. SUM149 cells (1×10^6) were orthotopically injected in the upper left mammary fat pad of 10-week-old athymic female mice. At 28 days after implantation, mice with established tumors of approximately 0.5 cm^3 were randomly assigned to four protocols: control (gavaged with water daily and i.v. injection with PBS weekly); TM (1.25 mg/day loading dose for 3 days followed by 0.7 mg/day maintenance dose for the remainder of protocol) by oral gavage; doxorubicin (5 mg/kg/week) by i.v. injection; or cotreatment with TM (1.25 mg/day loading dose for 3 days followed by 0.7 mg/day maintenance dose for the remainder of protocol) and doxorubicin (5 mg/kg/week). Tumor volume was calculated as (length × $width^2$)/2 and presented as mean ± SE. *, $P < 0.05$ *versus* control group; #, $P < 0.03$ *versus* TM- or doxorubicin-treated group; $n = 6$/treatment group.

Immunohistochemical analyses of the resected tumors revealed that intratumoral apoptosis was significantly higher than control in all treatment protocols ($P < 0.01$; 50% increase in the TM-treated group, 110% increase in the doxorubicin-treated group, and 220% increase in the combination-treated group). Moreover, tumor cell apoptosis was significantly higher in the combination therapy group than in either single-agent group of TM or doxorubicin ($P < 0.01$). As expected, tumors from the control group were highly vascularized, with a mean vessel count of 33 ± 4 (mean ± SE) per high-power field. In contrast, the smaller tumors resected from TM-treated (19 ± 2; $P < 0.01$), doxorubicin-treated (23 ± 3; $P < 0.05$), or TM and doxorubicin-treated (15 ± 2; $P < 0.01$) mice were significantly less vascularized per high-power field. The difference in microvessel density among these three treatment groups did not reach statistical significance (Fig. 3). Taken together, our data indicate that the combination therapy of TM and doxorubicin is more effective

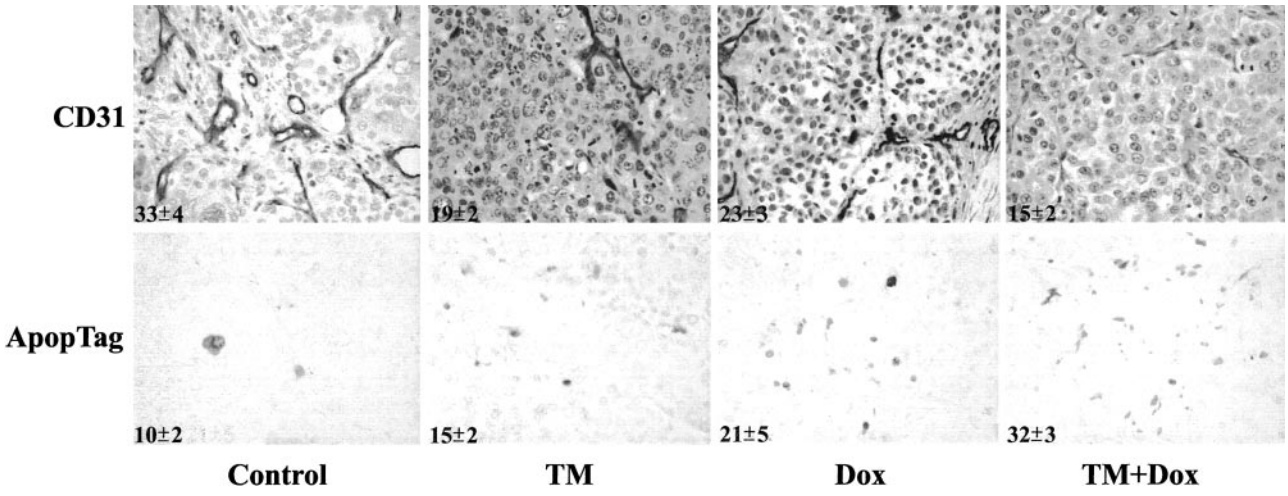

Fig. 3. Immunohistochemical analyses of SUM149 xenografts. Mammary tumors from control, TM-treated, doxorubicin-treated, and TM and doxorubicin combination-treated mice were resected and processed for immunohistochemical analysis. Intratumoral blood vessels were visualized using a CD31 antibody, and intratumoral cell apoptosis was visualized using the ApopTag kit. Mean vessel count/high-power field (×400) and mean number of apoptotic cells/500 total cells in a high-power field (×400) were calculated and presented as mean ± SE.

at suppressing tumor growth due to an increase in intratumoral apoptosis.

Discussion

The SUM149 inflammatory breast carcinoma cell line was derived from a primary tumor nodule in an inflammatory breast cancer patient that was refractory to conventional chemotherapy. Increased levels of NF-κB subunits, including p50, p52, and RelA, were found in SUM149 cells in comparison with immortalized HME cells (data not shown). Moreover, intrinsic NF-κB activity in SUM149 cells was about 2.5-fold higher than that in HME cells (11). Because constitutive activation of NF-κB is linked to resistance to several therapeutic modalities, we surmised that SUM149 cells may be refractory to conventional chemotherapy, consistent with the anthracycline resistance exhibited by the parental tumor in the patient. SUM149 cells were found to be resistant to doxorubicin (0.1 μM for 72 h)-induced apoptosis with about 90% cell survival. Under the same conditions, <40% of MDA-MB231 and <25% of MDA-MB435 breast carcinoma cells were viable. These results indicate that SUM149 cells are inherently extremely resistant to doxorubicin and thus an excellent system in which to examine the efficacy of combination therapies aimed at decreasing resistance.

Several groups demonstrated that inhibition of NF-κB activity sensitizes carcinoma cells with elevated levels of NF-κB to ionizing radiation and chemotherapeutic drugs (16, 17). Adenoviral delivery of dominant-negative IκBα sensitized chemoresistant tumors to the chemotherapeutic drug CPT-11 (18). With resistant lymphoid and leukemic cell lines, suppression of NF-κB resulted in an increased sensitivity to induction of apoptosis by doxorubicin (8). These studies provide direct evidence that inhibition of NF-κB is effective *in vitro* in enhancing therapeutic efficacy against carcinoma cells with elevated NF-κB activity. Our laboratory reported that copper deficiency induced by TM has antiangiogenic properties, in part, through blockade of the NF-κB signaling cascade (11). In SUM149 cells, TM treatment resulted in a significant decrease in NF-κB activation and expression (11). This observation led us to examine whether TM can enhance the efficacy of conventional chemotherapy against SUM149 cells. Our data demonstrate that the combination therapy of TM and doxorubicin was significantly more effective than either agent alone at inducing apoptosis *in vitro*. Using an alternate treatment regimen, cells pretreated with TM (1 nM for 72 h) were about 3-fold more sensitive to doxorubicin-induced apoptosis (data not shown). These results indicate that TM, under cotreatment or pretreatment conditions, enhances SUM149 cells to doxorubicin-induced apoptosis, consistent with TM's known suppression of NF-κB activity.

In our xenograft animal study, single-agent therapy with TM resulted in significantly smaller tumors with a decrease in mean intratumoral vessel density and an increase in intratumoral apoptosis. Surprisingly, *in vivo*, tumor growth was inhibited, and intratumoral apoptosis was increased with doxorubicin treatment. We anticipated that the SUM149 xenografts would be refractory to doxorubicin, based on our *in vitro* data demonstrating the resistance of these cells to doxorubicin-induced apoptosis. Interestingly, doxorubicin lowered mean vessel density to the same extent as TM (about 40% inhibition), indicating that the dose and scheduling of doxorubicin used in this study have a significant antiangiogenic effect. From this result, we suggest that the intratumoral apoptosis observed with doxorubicin is not a direct effect of cytotoxicity but possibly an indirect result of inhibiting tumor angiogenesis, through either inhibition of endothelial cell proliferation or suppression of angiogenic mediators produced by tumor cells. Importantly, the growth of well-established bulky tumors was completely stabilized with the combination therapy of TM and doxorubicin, likely due to the fact that intratumoral apoptosis from the combination treatment group was significantly higher than that

seen with either TM or doxorubicin treatment alone. In a separate study, nuclear proteins extracted from tumors in TM-treated mice showed a significant decrease in NF-κB binding activity.[4] In light of these findings, we postulate that in the combination therapy regimen, TM is sensitizing the established SUM149 tumors by suppressing NF-κB activation, thereby reverting these tumor cells to a phenotype responsive to doxorubicin-induced apoptosis.

Accumulating evidence indicates a therapeutic benefit of combining antiangiogenic compounds with conventional chemotherapy. Antiangiogenic compounds were reported to potentiate antineoplastic therapies against primary and metastatic disease, presumably through increased delivery of antineoplastic agents into the tumor mass (19, 20). It is speculated that an overabundance of endothelial cells in established tumors contributes to the formation of abnormal dilated leaky vessels within the tumor mass (21, 22). Apoptosis of tumor endothelial cells, through targeted direct or indirect antiangiogenic therapies, results in a decrease of immature leaky tumor vessels (23, 24). It is further suggested that pruning of these abnormal vessels leads to a more "normalized" tumor vasculature, thereby allowing for more efficient delivery of therapeutic compounds (25). In keeping with this line of reasoning, we hypothesize that TM, a global inhibitor of proangiogenic mediators including vascular endothelial growth factor, may be normalizing the tumor vasculature, resulting in increased delivery of doxorubicin. It could be argued that the enhanced efficacy observed with the combination therapy is just due to a higher concentration of doxorubicin in the tumor mass. However, if this were the case, then the intratumoral apoptosis observed with single-agent doxorubicin would be similar to the combination therapy. Single-agent doxorubicin resulted in a decrease in intratumoral microvessel density, and therefore, by the same argument, may be remodeling the tumor vasculature to allow for increased delivery of doxorubicin. Beyond the scope of our work, additional experiments such as measurements of intratumoral doxorubicin levels at several time points would be necessary to address this possibility. Nevertheless, the fact that treatment with doxorubicin and TM was significantly more effective at inducing apoptosis than single-agent doxorubicin suggests that TM must have an additional direct effect on the tumor cells, namely, sensitization of these cells to doxorubicin-induced apoptosis.

In conclusion, TM was shown to enhance the efficacy of doxorubicin in mammary carcinoma cells. This study provides strong support for the evaluation of TM in combination with anthracyclines, especially doxorubicin, in future clinical trials of locally advanced breast carcinoma. One suitable clinical end point suggested by this work would be the rate of pathological complete response after multiple cycles of combination therapy with TM and anthracyclines.

[4] Q. Pan, L. W. Bao, and S. D. Merajver, unpublished data.

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