Abstract

Purpose: The objective of the present study was to delineate the efficacy of tetrathiomolybdate (TM), a novel antiangiogenic anticancer agent, as a chemopreventative agent.

Experimental Design: Nulliparous Her2/neu transgenic mice were treated with water or TM for 180 days and observed for tumor development during treatment and for 180 days after treatment. Mammary gland composition and architecture were also observed following TM treatment of Her2/neu transgenic and normal FVB mice.

Results: At the 1-year follow-up, 86.7% of control and 40% of TM-treated Her2/neu mice had palpable mammary tumors with a median time to tumor development of 234 days (95% confidence interval, 202-279 days) for control and >460 days for TM-treated mice (P < 0.0005, n = 15). The mammary glands from TM-treated Her2/neu and FVB mice showed a blunted epithelial ductal branching system due to a significant decrease in the number of secondary branches and total number of differentiated mammary epithelial cells. Microvessel density in Her2/neu and FVB mammary glands was lowered by 65.6 ± 6.2% and 50.9 ± 4.5% (P < 0.005), respectively, following TM therapy, consistent with the antiangiogenic effect of TM. Lastly, TM treatment resulted in a 2-fold increase in the absolute number of aldehyde dehydrogenase–positive mammary stem cells in Her2/neu and FVB mammary glands.

Conclusion: Taken together, these results strongly support that TM is a potent chemopreventative agent as a consequence of hypoplastic remodeling of the mammary gland through modulation of the mammary stem cell compartment. (Clin Cancer Res 2009;15(23):7441–6)

Breast cancer affects more than 180,000 women yearly in the United States, and more than 40,000 women each year die of the disease (1). Although the incidence rate seems to have stabilized during the last decade and a decline in the mortality rate has been noted, breast cancer remains a major health problem (2). Recent focus has been on the development of tamoxifen as a chemopreventative strategy against breast cancer. Results from the National Surgical Adjuvant Breast Project and International Breast Cancer Intervention Study trials showed that tamoxifen therapy for 5 years is associated with a significant reduction in breast cancer incidence in high-risk women (3, 4). However, tamoxifen was found to only reduce the incidence of estrogen receptor–positive breast cancers and failed to affect the risk of estrogen receptor–negative breast cancer, a generally more aggressive form of breast cancer. Moreover, the deleterious side effects of tamoxifen, particularly uterine cancer, thromboembolism, and menopausal symptoms, limit its use as a beneficial chemopreventative agent to only a subset of high-risk women with a calculated 5-year Gail risk of 1.7% or higher in whom it is estimated that the benefits outweigh the considerable risks associated with this therapy.

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The development of cancer chemopreventative agents with increased safety profiles is a major priority of cancer research. Tetrathiomolybdate (TM) is a novel anticancer agent that has exhibited antiangiogenic properties in preclinical and clinical studies with minimal adverse events. Here we show that TM retards the development of Her2/neu mammary tumors by hypoplastic remodeling of the mammary gland. In addition to being efficacious, we found that the effects of TM on the normal mammary gland are reversible, and TM-treated mice can still conceive, carry to term, and nurse their pups. This study shows, for the first time, that alterations in the mammary gland ductal morphology and tissue architecture can result in an environment less conducive for carcinogenesis, a principle that can be applied to future design of prevention trials.

Recent work from our laboratory showed that TM significantly reduced microvessel density in the mammary glands (7). In this study, we focused on uncovering the cellular and molecular basis for this novel finding and explored the specific mechanism by which TM protects against Her2/neu-induced breast carcinoma by maintaining these transformed cells as “micro-tumors” in an avascular, dormant-like state (7). In this study, we focused on uncovering the cellular and molecular basis for this novel finding and explored the specific mechanism by which TM protects against Her2/neu-induced breast carcinoma. Mammary glands from TM-treated mice had a decrease in the total number of mammary epithelial cells, a decrease in the complexity of mammary epithelial ductal branching, an increase in the number of aldehyde dehydrogenase-positive (ALDH1) mammary stem cells, and lower microvessel density.

Materials and Methods

Chemoprevention experimental protocol. MMTV-Her2/neu transgenic mice were purchased from The Jackson Laboratory, and a breeding colony was maintained at the University of Michigan Comprehensive Cancer Center. One-hundred-day-old nulliparous female MMTV-Her2/neu mice were randomly assigned to two treatment groups and gavaged with water (control) or 0.75 mg/d TM for 30 d (n = 15 for each group). Mice treated with TM were rendered copper deficient (ceruloplasmin levels <30% of control) after 1 wk of therapy and remained so for as long as TM therapy was administered. After 180 d of treatment, control and TM-treated mice that did not develop mammary tumors were released from therapy and observed for the next 180 d. Mice were monitored weekly for palpable tumors and disease-free survival was calculated as a function of time. The disease-free survival curves for the two groups in this treatment-release protocol were compared using log-rank analysis.

Mammary gland whole mount, histology, and immunohistochemistry. Nulliparous female MMTV-Her2/neu mice (100 d old for both strains) were gavaged with water (control) or 0.75 mg/d TM for 30 d. Mice were euthanized and mammary glands were isolated and processed for whole-mount analysis using carmine red or fixed in 10% buffered formalin. Formalin-fixed glands were embedded in paraffin and processed for H&E staining or immunohistochemistry with anti-CD31 (Dako) or anti-ALDH1 (Abcam). The complexity of mammary ductal branching was quantified using carmine red-stained mammary glands to count the number of secondary branches in three representative fields (×100). Mean number of mammary epithelial cells per field (×100) was calculated and presented as mean ± SEM.

Quantification of microvessel density. Mammary gland microvessel density was assessed with anti-CD31 staining using the vascular hotspot technique (16). Sections were scanned at low power to determine areas of highest vascular density. Within this region, individual microvessels were counted in three separate random fields at high power (×400 magnification). The mean vessel count from the three fields was used. 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. Quantification of microvessel density in the mammary glands was done by a blind observer to eliminate subjectivity of the analysis.

Results and Discussion

Antiangiogenics have shown to be effective and safe anticancer agents in preclinical animal tumor models but have had mixed results in clinical trials with advanced disease. One possible explanation for the lack of efficacy in these clinical trials could be due to the bulky size of tumors in patients with stage III/IV disease. In theory, antiangiogenics would be predicted to be more effective in preventing smaller avascular tumors from activating the angiogenic switch and thus would be an ideal class of compounds for use in a chemopreventative setting. TM is a potent antiangiogenic compound that has completed numerous phase I/II trials for solid tumors. Results from these clinical trials showed that TM is very well tolerated with minimal adverse effects (6, 11–15). The attractive safety profile of TM suggests that it may be amenable to long-term use and thus be developed as a chemopreventative agent.

Recent work from our laboratory showed that TM significantly retards the development of Her2/neu-induced breast carcinoma in transgenic mice (7). To confirm and extend on this exciting finding, we were interested in repeating this experimental protocol with a longer follow-up period. Nulliparous Her2/neu transgenic mice (100 days old) were randomly assigned to two different groups and gavaged with water (control) or TM (0.75 mg/d) for 180 d or until palpable mammary tumors developed. At the end of the 180-day treatment protocol, 66.7% (10 of 15) control Her2/neu transgenic mice but only 13.3% (2 of 15) TM-treated Her2/neu transgenic mice developed palpable tumors (Fig. 1). Control and TM-treated Her2/neu transgenic mice that did not develop mammary tumors were released from therapy and observed for the next 180 d. At the 1-year follow-up (360 days after start of treatment protocol), three additional control mice developed tumors to bring the total of control mice with overt clinical disease to 86.7%. Moreover, 4 of 13 of the TM-treated mice that were released from therapy developed tumors to bring the total of TM-treated mice with palpable disease to only 40%. The median time to
tumor development was 234 days (95% confidence interval, 202-279 days) for control mice. Because 50% of the TM-treated animals did not develop tumors, the median time to tumor development for mice on systemic TM therapy could not be measured, but it is estimated to be >460 days. Using data from the 1-year follow-up, log-rank analysis indicates that the disease-free survival for TM-treated mice was significantly longer than that for controls (Fig. 1; P < 0.0005; n = 15). These observations unequivocally show that chemoprevention with TM was not limited to the time when TM was on board because prolonged protection was observed long after releasing TM-treated mice from therapy. These results add to our exciting initial findings and indicate that further understanding of TM as a chemopreventative agent is warranted.

To determine the effects of TM on adult mammary gland architecture, mammary glands were isolated from control and TM-treated (30 day treatment) Her2/neu and its normal back- ground strain FVB mice for histologic examination. Carmine red–stained whole mounts revealed that the epithelial ductal branching network of mammary glands from TM-treated Her2/neu mice was strikingly less complex as compared with that of glands from untreated Her2/neu mice (Fig. 2A). The difference in the ductal branching network primarily is due to a significant decrease in the number of secondary branches. Moreover, terminal end buds of TM-treated glands were less robust in number and size. It seems that TM is capable of remodeling the adult virgin mammary gland to a state that resembles a prepubertal or early pubertal mammary gland with one notable exception: the lack of actively proliferating terminal end buds. In Fig. 2B, histologic analyses of mammary glands showed that the TM-treated glands had a dramatic decrease (76 ± 6% reduction, P < 0.05) in the total number of mammary epithelial cells, suggesting that TM may be modulating the turnover of mammary epithelial cells, either promoting apoptosis or inhibiting proliferation. The inhibitory effect of TM on mammary epithelial cell number and branching morphogenesis was not limited to Her2/neu-transformed mammary glands because systemic TM therapy had the same effect on normal mammary glands from FVB mice. This suggests that TM is able to remodel the mammary glands regardless of the stage of oncogenic transformation and therefore can be an effective chemopreventative strategy against preneoplastic and neoplastic lesions.

An extensive literature supports that overexpression of Her2/neu in breast cancer patients correlates with poor prognoses and decreased overall survival (17, 18). A study showed that high intratumoral microvessel density is an independent marker of early relapse in node-negative breast cancer and intratumoral microvessel density is highest in breast cancer patients positive for Her2/neu overexpression (19). Indeed, we found that mammary glands from control Her2/neu mice had higher microvessel density than control FVB mice (153 ± 36 versus 58 ± 12 vessels per high-power field), supporting the notion that the Her2/neu oncogene is proangiogenic and perhaps a reason why Her2/neu is a predictor of a more aggressive and metastatic form of breast cancer. Importantly, mean microvessel density in the mammary glands of Her2/neu and FVB mice was significantly lower following systemic TM therapy, 65.6 ± 6.2% and 50.9 ± 4.5% inhibition (P < 0.005) for Her2/neu and FVB mice, respectively (Fig. 3). It is likely that a less vascularized mammary gland environment will make it more challenging for avascular, incipient tumors to activate the angiogenic switch and grow into bulky tumors with metastatic potential. This line of reasoning is supported by our previous finding that TM retarded the development of mammary tumors in Her2/neu transgenics by keeping the transformed “microtumors” in stasis largely devoid of neovascularization (7). However, the results of the present study extend much further and indicate that the chemopreventative action of TM is more complex than simply through attenuating the angiogenic switch of avascular tumors, as TM was found to induce a profound remodeling of the mammary gland resulting in a severe deficit in the total number of ductal epithelial cells. A critical question that remains is whether hypoplastic remodeling of the mammary gland is a consequence of a decrease in mammary gland vascularity. Although our study was not designed to strictly address this question, the observation that the mean microvessel density of TM-treated Her2/neu mammary glands is similar to that of control FVB mammary glands that have normal ductal morphology (52 ± 10 versus 38 ± 12 vessels per high-power field) argues against a direct link between mammary gland vascularization and morphology. Additional work with specific molecularly targeted antiangiogenics, such as vascular endothelial growth factor kinase insert domain-containing receptor inhibitors, will be necessary to adequately address whether the change in mammary gland architecture, namely, a lower number of epithelial cells and secondary branches, is a direct consequence of inhibiting angiogenesis in the mammary gland.

Human normal and tumor mammary epithelial cells with high ALDH activity possess stem cell characteristics (20). Xenotransplantation of ALDH+ mammary epithelial cells isolated from human breast tumors into the cleared mammary glands of recipient nonobese diabetic/severe combined immunodeficient mice was sufficient to induce tumor formation (20). Tumors formed in recipient mice consisted of ALDH+ and ALDH− tumor cells and, thus, recapitulated the heterogeneity of the
parental human breast tumors (20). Moreover, immunohistochemical analysis with ALDH1 is able to identify tumor-initiating cells in fixed paraffin-embedded tumor sections (20). A recent report showed ALDH + as a marker for the identification of mouse tumor mammary stem cells (21). Results from these two groups provide evidence that ALDH can be used as a robust marker to identify human and mouse tumor mammary epithelial stem cells. As shown in Fig. 4, mammary glands from TM-treated Her2/neu transgenic mice had a significant 2-fold increase in the percentage of ALDH + mammary epithelial cells compared with control-treated Her2/neu transgenic mice, 9.5 ± 1.6% versus 20.5 ± 2.4% for ALDH +, respectively (P < 0.004). Similarly, in normal FVB mice, systemic TM treatment resulted in 24.6 ± 1.4% ALDH + mammary epithelial cells compared with 12.2 ± 1.4% ALDH + mammary epithelial cells for the control treatment (P < 0.004).

It is intriguing that systemic TM therapy results in a clear increase in the absolute number of ALDH + mammary stem cells. The current stem cell paradigm indicates that symmetrical cell division, where one stem cell self-renews into two stem cells, is the postulated mechanism to produce a net increase in stem cell number. Thus, our results argue that the mammary gland microenvironment of TM-treated Her2/neu transgenic mice favors the mammary stem cells toward symmetrical rather than asymmetrical cell division. The mammary gland is composed of a network of branching ducts. Ductal branches are composed of terminally differentiated mammary epithelial cells consisting of an inner layer of luminal cells surrounded by an outer layer of myoepithelial cells. Therefore, the mammary gland would be expected to be nascent or less complex in an environment where there is a relative paucity of asymmetrical cell division to yield daughter cells capable of differentiating to luminal or myoepithelial cells. This is entirely consistent with our observation that TM-treated mammary glands are less complex with a lower number of secondary branches. It is unclear how systemic TM therapy leads mammmary stem cells toward symmetrical cell division.

Fig. 2. Systemic TM therapy remodels mammary gland ductal morphogenesis in Her2/neu transgenic and FVB mice. A, mammary gland ductal network. Mammary glands from control and TM-treated Her2/neu and FVB mice were isolated and processed for whole-mount analysis using carmine red staining. The complexity of mammary ductal branching was quantified using carmine red-stained mammary glands to count the number of secondary branches in three representative fields (x100). Columns, mean number of secondary branches per field; bars, SEM. *, P < 0.05. B, mammary gland histology. Mammary glands from control and TM-treated Her2/neu and FVB mice were isolated, fixed, and processed for H&E staining. Mean number of mammary epithelial cells per field (x100) was calculated and presented as mean ± SEM.
division. Does TM directly regulate mammary stem cell fate, or does TM alter the stem cell niche in the mammary gland to indirectly drive symmetrical cell division? It is critical to address these key questions to better understand the mechanism of action of TM as a chemopreventive agent.

It is of interest and further supports the explanation above that the effects of TM on mammary gland development were reversible as a few of the 30-day post-release and a majority of the 60-day post-release mammary glands isolated from TM-treated/released mice had normal breast morphology in terms of the ductal epithelial branching network (data not shown). Moreover, female mice on continuous long-term TM therapy were able to conceive, carry to term, and nurse their pups without apparent difficulty. These results indicate that the effects of TM on the mammary gland are not permanent and that biological signals during pregnancy can override the TM effects by allowing ductal epithelial cells to rapidly proliferate and differentiate to form lactating mammary glands. This observation has important clinical implications in the future development of TM as a chemopreventative agent, as it suggests

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**Fig. 3.** Effects of systemic TM therapy on microvessel density in Her2/neu transgenic and FVB mice. Mammary glands from control and TM-treated Her2/neu and FVB were isolated, fixed, and processed for immunohistochemistry with an anti-CD31 antibody for microvessel density. Columns, mean vessel count per high-power field (×400); bars, SEM. *, P < 0.05.

**Fig. 4.** Systemic TM therapy increases the number of epithelial stem cells in the mammary glands of Her2/neu transgenic and FVB mice. Mammary glands from control and TM-treated Her2/neu and FVB mice were isolated, fixed, and processed for immunohistochemistry with an anti-ALDH1 antibody (arrows, representative stained cells). Columns, mean percent of ALDH1+ mammary epithelial cells per high-power field (×400); bars, SEM. *, P < 0.05.
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that TM, if used in women of reproductive age, may not permanently affect the lactating potential of the adult mammary gland in humans.

In conclusion, TM is preventing the development of breast carcinoma in Her2/neu transgenic mice by hypoplastic remodeling of the mammary gland. This study showed, for the first time, that alterations in the mammary gland ductal morphology and tissue architecture can result in an environment less conducive for carcinogenesis.

Disclosure of Potential Conflicts of Interest

S.D. Merajver was a consultant and has a financial interest in Attenuon, LLC, which has licensed tetrathiomolybdate as an anticancer compound from the University of Michigan.

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References

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