Combination Tetrathiomolydate and Radiation Therapy in a Mouse Model of Head and Neck Squamous Cell Carcinoma

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Objective: To assess the effect of combining tetrathiomolydate therapy and radiation treatment (RT) on tumor growth in the mouse head and neck squamous cell carcinoma (HNSCC) model.

Design: One million HNSCC cells were injected subcutaneously into the flanks of C3H/HeJ mice and the tumors grown to an average of 301 mm³ (day 0). Mice were randomized into 4 groups: (a) no therapy, (b) tetrathiomolydate alone, (c) RT alone, or (d) tetrathiomolydate + RT. Data from 3 experiments with these 4 groups were analyzed. A gaussian mixed model was fit to the initialized logarithm of the tumor size counts between days 7 and 16 (linear component), and growth rates were compared. Assays using 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) were conducted on HNSCC cells in culture with varying doses of tetrathiomolydate.

Interventions: Treated mice were given tetrathiomolydate in their water and observed for clinical evidence of toxic effects associated with copper depletion as measured by ceruloplasmin assay. When tumor sizes reached an average of 535 mm³, mice receiving RT were given a single fraction of 750 rad (7.5 Gy), a dose determined in previous experiments to slow but not cure tumor growth, permitting an examination of interaction of radiation with tetrathiomolydate.

Results: Data from 3 separate experiments were analyzed. There were a total of 37 mice in the untreated group, 32 mice in the tetrathiomolydate alone group, 38 mice in the RT alone group, and 46 mice in the tetrathiomolydate + RT group. Ceruloplasmin assays showed that we had obtained adequate copper reduction throughout the experiments to inhibit angiogenesis with minimal toxic effects. The tetrathiomolydate + RT combined therapy group of mice showed a statistically significant decrease in tumor growth compared with both the tetrathiomolydate alone (P = .001) and RT alone groups (P < .001).

Conclusion: The combination of the anti-angiogenic copper chelating agent tetrathiomolydate with RT improved local control of HNSCC in an isogenic mouse model compared with either therapy alone.

Arch Otolaryngol Head Neck Surg. 2006;132:333-338

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The effectiveness of radiation therapy (RT) to combat primary tumors is improved in animal models by the addition of anti-angiogenic agents.1-5 Tetrathiomolydate is a copper level-reducing drug developed for Wilson disease6 that has anti-angiogenic properties resulting from lowering copper availability.7-9 Tetrathiomolydate inhibits angiogenesis in multiple pathways without significant toxic or adverse effects. Previous research has shown that tetrathiomolydate can be combined with RT to improve the efficacy of the RT in a Lewis lung high metastatic carcinoma mouse tumor model.10 Numerous studies have demonstrated that angiogenesis (formation of new blood vessels from preexisting microvasculature) is a critical step in the growth of primary and metastatic tumors.11-13 This has important implications for the treatment of head and neck squamous cell carcinomas (HNSCC), where angiogenesis has been demonstrated to play an important role,14-23 particularly in tumor response and local control after RT.24-28 If angiogenesis is inhibited, tumors remain microscopic. The balance of numerous stimulators and inhibitors of angiogenesis determines whether angiogenesis is turned on or off.29 Inhibition of angiogenesis will require manipulation of multiple steps in this process. Tetrathiomolydate inhibits angiogenesis by affecting several molecules involved in angiogenesis, including vascular endothelial growth factor, basic fibroblast growth factor, nuclear factor-κB, and other well-known proangiogenic molecules.30-33

To test the validity of the conclusions drawn in previous research on a lung can-
cer mouse model, we conducted experiments using tetrathiomolybdate and RT in a mouse model of human HNSCC. We postulated that it was reasonable to apply similar methods to these 2 different types of cancers because anti-angiogenic therapy targets the reasonably homogeneous microvasculature and not the tumor directly. It is also critical to understand the effects of the combination of tetrathiomolybdate and RT on human endothelial cells, as RT is a key component of primary and postoperative treatment of head and neck carcinomas, and there will likely be a patient group where the addition of tetrathiomolybdate will be tested.

**Methods**

### In Vivo Therapy with RT or Tetrathiomolybdate

The SCC cells used are head and neck cancer cells isogenic to C3H/HeJ mice. The SCC cell line was maintained in serial passage in vitro. Male, 6- to 9-week-old C3H/HeJ mice (Jackson Laboratories, Bar Harbor, Me) were used for experiments. The mice were acclimated and caged in groups of 5 or fewer and then were shaved 1 day prior to receiving radiation. All mice were fed a diet of animal chow ad libitum. They were anesthetized using isoflurane delivered through a ventilation chamber prior to all procedures and were observed until fully recovered.

The SCC cells were grown in cell culture and maintained with Dulbecco Modified Eagle medium with 4.5-g/L glucose and 1-glutamine, 10% fetal bovine serum, and 1% penicillin/streptomycin. Cells were harvested from the culture using a tryptic-EDTA solution in Hanks balanced salt solution. After harvesting, these cells were counted on a hemocytometer and spun down in a refrigerated centrifuge at 4°C at 200g. The tumor cells were suspended in phosphate-buffered saline at a concentration of 107 cells/mL, and the suspension was placed on ice. The upper dorsal surface of the leg of each mouse was injected with 105 cells in 0.1 mL of phosphate-buffered saline after cleaning the injection site with ethanol.

Using the same technique as previously determined, initial experiments with 10 mice were carried out to determine the average volume of water consumed per mouse per day. The volume of water imbibed in each 24-hour period was measured for each cage, with each cage holding 5 mice, and the volume of water imbibed in each 24-hour period was measured. The concentration of tetrathiomolybdate will be tested.

### Copper Levels in Mouse Serum

Blood was collected from mice treated with tetrathiomolybdate by anesthetizing the mice with isoflurane and performing cardiac puncture. Blood samples were centrifuged at 3000g at 4°C, and serum samples were collected and frozen at −20°C until the assays were run.

The ceruloplasmin levels were measured per a standard assay. Aliquots of 25 µL of serum from each mouse were placed into 2 tubes, and 375 µL of 0.1M sodium acetate buffer (stored at 4°C; pH 5.0) was immediately added. Tubes were placed in a 30°C water bath. After 5 minutes, 100 µL of o-dianisidine dihydrochloride (7.88 mM; Sigma-Aldrich, St Louis, Mo) reagent (preincubated at 30°C) was placed into each tube using a pipette. After 30 minutes (A30), 1 of the 2 tubes for each group was removed from the water bath, and 1 mL of 9M sulfuric acid was added to quench the reaction. After 45 minutes (A45), the second tube was quenched, and the absorbance of both tubes at 540 nm (A30 and A45, respectively) was analyzed using a 1-cm path length cuvette in a spectrophotometer. The ceruloplasmin concentration in international units was calculated by the formula ceruloplasmin oxidase activity=(A45−A30)×0.625 IU/mL.

### In Vitro Tumor Cell Proliferation Assay

In vitro experiments were carried out on the SCC cell line using a 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT; Sigma-Aldrich) proliferation assay. The MTT reagent stains live cells, and the 959-nm absorbance correlates with cell number. Cell proliferation MTT assays were carried out for 4 days (days 0, 1, 2, and 3) with daily measurements of absorbance. A flat-bottomed, 96-well Falcon microtest tissue culture plate was used for each time point, with 6 replicate wells used for each tetrathiomolybdate concentration. One thousand cells were added to each well. On the following day (day 0), tetrathiomolybdate was added to bring the concentrations to 0, 0.001, 0.005, 0.01, 0.1, and 1.0, and 10 nM well. On days 0 to 3, 50-µL aliquots of MTT were added to each well using a pipette, and the plate was incubated for 2 hours at 37°C. Aliquots of 100 µL of dimethyl sulfoxide were added to each well using a pipette, and the plates were agitated for 5 to 10 minutes. The absorbance at 595 nm was read by a Dynatek MR 3000 plate reader (Dynatek, Chantilly, VA). An absorbance titration curve at 395 nm was generated using data from the MTT assay carried out with a 10-fold increase in cells from 0 to 1 million cells.

### Statistical Analysis

Data from the 3 experiments each containing 4 treatment groups were analyzed separately. A gaussian mixed model (SAS PROC MIXED; SAS Institute, Cary, NC) was fit to the initialized logarithm of the tumor volumes. The logarithm of the tumor volumes were initialized by subtracting the day 0 log tumor volume for each mouse. Although the experiments were run out to day 21, the growth curves for the log tumor volumes were approximately linear only between days 7 and 16. After initial analysis of mean plots, it appeared that the growth curves leveled off for the tetrathiomolybdate group after day 16, and thus a linear model was inappropriate after day 16. The model was fit to the initialized log tumor volumes between days 7 and 16, and the growth rates were compared. Separate intercepts and slopes for each of the 4 treatment groups were used as predictors in the model. At the experiment level, a random intercept for each experiment was included to account for variation between experiments, and at the mouse level, a random slope and intercept were included for each mouse.

### Results

Three experiments were carried out in succession using 80 mice per experiment. One million SCC cells were injected into the flanks of C3H/HeJ mice and tumor volumes allowed to reach an average of 301 mm3 (mean ± SD values of 304 ± 82, 314 ± 71, and 286 ± 52 mm3 in experiments 1, 2 and 3, respectively). On this day (day 0), the mice were randomly separated into 4 treatment...
groups: (a) untreated, (b) tetrathiomolybdate only, (c) RT only, and (d) tetrathiomolybdate + RT. The number of mice for each treatment group for experiments 1, 2, and 3 were, respectively, (a) 10, 11, and 16; (b) 10, 10, and 12; (c) 11, 16, and 11; and (d) 11, 17, and 18.

Radiation was delivered to tumors in groups (c) and (d) on the same day that the tumor volumes averaged 535 mm³ (mean ± SD values, 546 ± 172, 557 ± 201, and 502 ± 110 mm³ in experiments 1, 2, and 3, respectively). A dose of 7500 rad (7.5 Gy) was selected for RT because this dose was shown in previous experiments to inhibit tumor growth but not produce cures, permitting analysis of the interaction of RT and tetrathiomolybdate.

Tetrathiomolybdate therapy was continued for the duration of the experiment in groups (b) and (d). As mice exhibited clinical signs of illness due to copper depletion, the doses of tetrathiomolybdate were adjusted as needed throughout the experiment. In experiment 1, groups (b) and (d) began treatment on day 0 with an initial dose of 0.9 mg per mouse per day of tetrathiomolybdate and on day 2 were switched to 0.45 mg per mouse per day for the duration of the experiment. In experiments 2 and 3, mice in groups (b) and (d) received an initial dose of 1.1 mg per mouse per day of tetrathiomolybdate on day 0 and were switched to 0.6 mg per mouse per day on day 2 for the duration of the experiment. The effectiveness of the tetrathiomolybdate doses was assessed by measuring the copper level (reflected in the ceruloplasmin levels). Between days 16 and 21, the remaining mice were killed by carbon dioxide overdose.

Our experimental data show that tetrathiomolybdate and RT in combination can slow tumor growth more effectively than either treatment alone compared with untreated SCC tumors (Figure 1). The results were the same whether we analyzed all mice or excluded mice with severe ulcerations. The data for the full analysis (all mice included) are summarized in Table 1. The log tumor volumes were approximately linear from day 7 through day 16, which is when the statistical model was applied. All 3 treatments—tetrathiomolybdate, RT,
and tetrathiomolybdate + RT—caused significant reduction in tumor growth compared with untreated tumors ($P<.001$, $P=.002$, and $P<.001$, respectively) (Table 2). The tetrathiomolybdate + RT groups of mice showed statistically significant decrease in tumor growth compared with both the tetrathiomolybdate alone ($P=.001$) and RT alone groups ($P<.001$) (Table 2).

The tetrathiomolybdate alone group appeared to have a more marked slope decrease after day 16, making that curve nonlinear after day 16 (Figure 1). Growth curves for the other groups remained approximately linear after day 16. There were so few data points after day 16 that no statistical significance could be obtained for this late decrease in this part of the tetrathiomolybdate alone curve. For this reason, the statistical analysis included only days 7 through 16 (Table 1 and Table 2) because this was the region where a linear effects model would apply most accurately.

We conducted further experiments to examine the effect of tetrathiomolybdate on the growth of SCC cells in vitro, including MTT assays to examine the growth of tumor in vitro (without a microvasculature). The SCC tumor cells were grown in untreated microtiter wells or in wells containing media treated with various concentrations of tetrathiomolybdate. The MTT assay absorbance data show that the levels of SCC cells on days 0, 1, 2, and 3 showed no statistical difference between all tetrathiomolybdate (TM) concentrations tested (0nM-10nM). Error bars represent standard deviations.

![Figure 2](image_url)

**Figure 2.** Analysis of tumor cell number by the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. Absorbance levels corresponding to number of squamous cell carcinoma cells on days 0, 1, 2, and 3 showed no statistical difference between all tetrathiomolybdate (TM) concentrations tested (0nM-10nM). Error bars represent standard deviations.

![Figure 3](image_url)

**Figure 3.** Ceruloplasmin assay curves. Serum ceruloplasmin levels (as represented by the average absorbance at 540 nm) were measured at various time points in at least 2 tetrathiomolybdate-treated tumor-bearing mice in each group. As a comparison, the top curve (untreated) represents the baseline measurement of ceruloplasmin from 5 tumor-bearing untreated mice.

### Table 1. Statistical Analysis of Curves of Tumor Growth From Day 7 Through Day 16 for All Experiments

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Slope</th>
<th>No. of Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0.0648</td>
<td>37</td>
</tr>
<tr>
<td>TM</td>
<td>0.0441</td>
<td>32</td>
</tr>
<tr>
<td>RT</td>
<td>0.0501</td>
<td>38</td>
</tr>
<tr>
<td>TM + RT</td>
<td>0.0277</td>
<td>46</td>
</tr>
</tbody>
</table>

Abbreviations: RT, radiation therapy; TM, tetrathiomolybdate.

### Table 2. Pairwise Comparison of Tumor Growth From Day 7 Through Day 16

<table>
<thead>
<tr>
<th>Pairwise Comparison</th>
<th>2-Sided P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated vs TM</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Untreated vs RT</td>
<td>.002</td>
</tr>
<tr>
<td>Untreated vs TM + RT</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>TM vs TM + RT</td>
<td>.001</td>
</tr>
<tr>
<td>RT vs TM + RT</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

Abbreviations: RT, radiation therapy; TM, tetrathiomolybdate.

We show herein that the combination of RT with the multiple-target, anti-angiogenic, copper depletion therapy, tetrathiomolybdate, decreases the growth of tumors in an HNSCC mouse model. This combination therapy is superior to monotherapies of tetrathiomolybdate or RT. Tetrathiomolybdate is the most potent copper level reducing agent known and has been used for over a decade in the treatment of the copper storage disorder called Wilson disease, for which there is now an extensive clinical experience with tetrathiomolybdate as a copper-lowering drug. Tetrathiomolybdate forms a stable tripartite complex with copper and protein, creating in patients a negative copper balance immediately. The safety and antitumor effects of tetrathiomolybdate therapy alone in patients with solid tumors have been evaluated in a phase 1 clinical trial, in which tetrathiomolybdate was shown to impair angiogenesis but leave other copper-
dependent cellular processes largely intact, so that no significant clinical toxic effects occur.

The data collected have demonstrated that tetrathiomolybdate treatment can slow the growth of SCC tumors in vivo. We conducted further experiments to support the case that this slowing of tumor growth is owing to the anti-angiogenic action of the drug (via depletion of copper) and not due to direct effects of the drug on the tumor. The MTT (tumor cell proliferation) assays showed that the numbers of SCC cells on all days tested were not significantly different. In other words, SCC cells exposed to all tetrathiomolybdate concentrations tested (0nM-10 nM) were not significantly different from each other over time, and none was significantly different from the untreated SCC cells. Previous estimates (calculations) from patients treated with tetrathiomolybdate showed that the 0.01 nM to 0.1 nM tetrathiomolybdate concentrations are similar to those seen in cancer cells in vivo (unpublished data, 2004). In the MTT assays reported herein, tetrathiomolybdate had no effect even at 1000 times those levels in vitro. This finding supports the concept that it is the copper depletion action of tetrathiomolybdate that slows tumor growth in vivo, accounting for its known anti-angiogenic effects on endothelial cells and not direct action on tumor cells.

Several studies support the use of anti-angiogenic agents with RT.1-5 A problem with several of the agents under study today (for example, anti-vascular endothelial growth factor antibody therapy) is that they attack only 1 anti-angiogenic target at a time. Over time, tumor cells have been known to evolve the ability to secrete several pro-angiogenic molecules, therefore possibly rendering a therapy directed against only 1 target useless. One envisioned way around this is to use a “combination” therapy with multiple anti-angiogenic agents at the same time, as has been done somewhat successfully with some chemotherapeutic agents. Therapy with tetrathiomolybdate presents exactly this scenario but within a single agent. Depletion of copper by tetrathiomolybdate interferes with multiple angiogenic factors at once, making it an ideal multiple-target anti-angiogenic agent.30 This multiple-target anti-angiogenic mechanism for tetrathiomolybdate has also been recently demonstrated in angiogenic in vitro systems relevant to HNSCC.39 The fact that copper depletion therapy facilitated by tetrathiomolybdate has been apparently safely combined with RT in 2 different tumor mouse models (lung cancer19 and the present HNSCC model) supports the notion that tetrathiomolybdate targets the tumor microvasculature, not the tumor itself, and therefore should be applicable to multiple tumor types. The fact that tetrathiomolybdate can be successfully combined with RT to increase the suppression of tumor growth in a HNSCC mouse model has significant implications for the treatment of head and neck cancers, where radiation is a mainstay of therapy for many stages of this cancer, including definitive and postoperative treatment.

Submitted for Publication: May 12, 2005; final revision received July 20, 2005; accepted August 1, 2005.

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Financial Disclosure: Drs Brewer and Merajver have an equity interest in Attenuon LLC, San Diego, Calif, and are paid consultants for this company. Attenuon has licensed the anti-angiogenic uses of tetrathiomolybdate from the University of Michigan, Ann Arbor.

Funding/Sponsor: Research for this article was supported in whole or in part by a Specialized Programs of Research Excellence (SPORE) Grant in Head and Neck Cancer—National Institutes of Health/National Cancer Institute/National Institute of Dental and Craniofacial Research (NIH/NCI/NIDCR), grant 1P50 CA/DE97248-01 (Drs Teknos and Merajver), and NIH R21 grant 7R21CA096314-02 (Drs Khan, Nair, and Kariapper). In addition, Dr Merajver was supported in part by grant CA 77612 from the Burroughs Wellcome Fund and the Breast Cancer Research Foundation; Mss May, Kwitny, and Warnat were supported in part by University of Michigan Summer Research Opportunities grants.

Acknowledgment: We thank Robert Dick, BS, for the supply of tetrathiomolybdate for the studies.

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