# Copper Binding by Tetrathiomolybdate Attenuates Angiogenesis and Tumor Cell Proliferation through the Inhibition of Superoxide Dismutase 1

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**Abstract**

**Purpose:** A second-generation tetrathiomolybdate analogue (ATN-224; choline tetrathiomolybdate), which selectively binds copper with high affinity, is currently completing two phase I clinical trials in patients with advanced solid and advanced hematologic malignancies. However, there is very little information about the mechanism of action of ATN-224 at the molecular level.

**Experimental Design:** The effects of ATN-224 on endothelial and tumor cell growth were evaluated in cell culture experiments *in vitro*. The antiangiogenic activity of ATN-224 was investigated using the Matrigel plug model of angiogenesis.

**Results:** ATN-224 inhibits superoxide dismutase 1 (SOD1) in tumor and endothelial cells. The inhibition of SOD1 leads to inhibition of endothelial cell proliferation *in vitro* and attenuation of angiogenesis *in vivo*. The inhibition of SOD1 activity in endothelial cells is dose and time dependent and leads to an increase in the steady-state levels of superoxide anions, resulting in the inhibition of extracellular signal-regulated kinase phosphorylation without apparent induction of apoptosis. In contrast, the inhibition of SOD1 in tumor cells leads to the induction of apoptosis. The effects of ATN-224 on endothelial and tumor cells could be substantially reversed using Mn(III)tetrakis(4-benzoic acid)porphyrin chloride, a catalytic small-molecule SOD mimetic.

**Conclusions:** These data provide a distinct molecular target for the activity of ATN-224 and provide validation for SOD1 as a target for the inhibition of angiogenesis and tumor growth.



Angiogenesis is the process whereby new blood vessels are induced to sprout from preexisting vessels and plays a critical role in the transition of tumors from a dormant state to a malignant state (1). Copper has long been recognized as being important to the ability of an animal to mount an angiogenic response (2), and endothelial cells are induced to become more mobile when incubated with copper (3). Ceruloplasmin, the predominant copper-carrying protein in plasma, is angiogenic only when bound to copper (4), and copper bound to either heparin or a tripeptide composed of glycine-histidine-lysine can also be angiogenic (4).

Despite these observations, the precise molecular mechanisms of the role of copper in angiogenesis remain unclear. Ammonium tetrathiomolybdate is a copper-binding compound that has been shown to have efficacy as an antiangiogenic and antitumor agent against several types of cancer in mouse models (5, 6). Tetrathiomolybdate has also recently been evaluated as an anticancer therapy in several clinical trials (7, 8), where it has been suggested to deplete systemic copper, leading to effects on copper-dependent cellular processes (9). However, no specific cellular target for the antiangiogenic effects of copper depletion has been identified. Tetrathiomolybdate has been shown to inhibit the activities of a variety of cuproenzymes, including Cu/Zn superoxide dismutase 1 (SOD1; ref. 10) and cytochrome *c* oxidase (11), but the biological effects of these inhibitions have not been investigated.

In the present study, we have used a second-generation orally available choline salt of tetrathiomolybdate (ATN-224), which is being developed for the treatment of cancer, to probe the role of copper in angiogenesis and tumor cell proliferation. Using ATN-224, we show that SOD1, which has been implicated previously in both angiogenesis (12) and cancer (13), is central to the proangiogenic effects of copper. SOD1 has not been shown previously to be a target for a clinically relevant anticancer compound and has never been validated as a therapeutic target for the treatment of cancer. In this study, we present data validating SOD1 as a therapeutic target for the inhibition of angiogenesis and the induction of tumor cell apoptosis and describe downstream effects of SOD1 inhibition in both endothelial and tumor cells.

## Materials and Methods

***Reagents.*** ATN-224 (choline tetrathiomolybdate) was manufactured under current good manufacturing process, a proprietary manufacturing process with >99% purity. ATN-224 stocks (50 mg/mL) were

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prepared in water, aliquoted, and frozen until use. ATN-224 was diluted to the desired concentration using PBS or medium just before use. Fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF) were purchased from Research Diagnostics, Inc. (Flanders, NJ). Heparin and polyethylene glycol (PEG)-SOD were acquired from Sigma (St. Louis, MO). Mn(III)tetrakis(4-benzoic acid)-porphyrin chloride (Mn-TBAP) was obtained from A.G. Scientific (San Diego, CA). Mn-TBAP was prepared by adding 0.1 N NaOH to produce a 100 mmol/L solution, which was then diluted to a 10 mmol/L concentration in 20 mg/mL bovine serum albumin (pH 7.5). This solution was sterile filtered and frozen until use.

***Cells and proliferation assays.*** Human umbilical vein endothelial cells (HUVEC; Cascade Biologics, Portland, OR) were maintained in M200/LSGS medium (Cascade Biologics) and cells were used between passages 2 and 4 for all experiments. For proliferation assay, cells were plated at 3,000 per well on 0.1% gelatin in M200/2% FBS for 4 hours and then stimulated with 2 ng/mL FGF-2 in the presence or absence of drug up to 48 hours. HUVEC proliferation was determined using either the Alamar Blue (Biosource International, Camarillo, CA) or the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (14). Multiple myeloma MM1S cells were a kind gift from Dr. Stanton Gerson (Case Western Reserve University, Cleveland, OH) and grown in RPMI 1640 with 10% fetal bovine serum and 2 mmol/L L-glutamine. HL-60 promyelocytic leukemia cells and MOLT-4 acute myeloblastic leukemia cells were purchased from American Type Culture Collection (Manassas, VA) and cultured according to the instructions of the American Type Culture Collection. Cells were plated at 400,000/mL in T-75 flasks and incubated for 48 to 96 hours for proliferation assays. MMS1 cell proliferation was determined using calcein AM according to the manufacturer's instructions (Molecular Probes, Eugene, OR).

***Subcellular fraction preparation.*** Cells were harvested by centrifugation. Cytoplasmic and nuclear fractions were prepared as described (15). Mitochondria were prepared from fresh mice livers using the Mito-ISO kit (Sigma). Mitochondrial fractions were used on the day of preparation.

***Matrigel plug.*** Cold Matrigel (500 μL) was mixed with 800 ng/mL FGF-2 or 300 ng/mL VEGF and heparin (50 μg/mL). Negative control plugs did not contain the proangiogenic factors. The Matrigel mixture was injected s.c. into 4- to 8-week-old female BALB/c nude mice. In some experiments, either ATN-224 (94 μmol/L) with or without Mn-TBAP (100 μmol/L) or water was added directly to the Matrigel plug in the treated and negative control groups, respectively. Alternatively, mice were treated by oral gavage either with distilled water or ATN-224 everyday from Monday to Friday. Animals were sacrificed and the plugs were recovered 5 days after plug injection. The plugs were then minced and homogenized with a tissue homogenizer, and hemoglobin levels in the plugs were determined using Drabkin's solution according to the manufacturer's instructions (Sigma). All animal studies were done according to the Institutional Animal Care and Use Committee–approved protocols.

***SOD assays.*** SOD1 activity in lysed cells was assayed by measuring the inhibition of reduction of the water-soluble tetrazolium salt, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium, monosodium salt, which produces a water-soluble formazan dye on reduction with a superoxide anion (Dojindo Molecular Technologies, Gaithersburg, MD). Superoxide anion is generated by xanthine oxidase. Bovine SOD (Sigma) was used to generate a standard curve.

***Cytochrome c oxidase assay.*** Cytochrome *c* oxidase was assayed using the CYTOCOX1 kit (Sigma) according to the manufacturer's instructions.

***Copper accumulation and depletion studies.*** ATN-224 concentration in cells and in medium was determined by measuring molybdenum using inductively coupled plasma-mass spectrometry (ICP-MS). Cell extracts or whole tissues were sent to Elemental Research, Inc. (Vancouver, British Columbia, Canada) for analysis. Cells and tissues were digested using 6 N $HNO_3$ to completely release all metals before analysis. The concentration of ATN-224 in an endothelial cell was calculated from the normalized (ng molybdenum/mg total protein) molybdenum content in a known amount of HUVEC (where $2 \times 10^6$ HUVEC contain ~100 μg protein) using an average volume of 1,827 fL/cell (16). To measure copper depletion from pure SOD1, ATN-224 was incubated with SOD1 protein for 30 minutes and the protein was repurified by gel filtration chromatography using a PD10 (Bio-Rad, Hercules, CA) rapid separation column. The copper content of the protein was also determined using ICP-MS (Elemental Research).

***Dihydroethidine staining.*** HUVEC (plated in a six-well plate) were stimulated with 2 ng/mL FGF-2 in the presence or absence of 1 μmol/L ATN-224 for up to 72 hours. Cells were then rinsed in PBS and incubated in 5 μmol/L dihydroethidine (Molecular Probes) for 45 minutes. Cells were visualized using a Nikon microscope (Melville, NY) with a Spot RT camera. HUVEC treated as indicated above were trypsinized and incubated for 15 minutes with 1.5 μmol/L dihydroethidine and immediately analyzed by flow cytometry. Dihydroethidine is specifically oxidized by superoxide to form a fluorescent product (17).

***Extracellular signal-regulated kinase studies.*** Cells were plated in a six-well format (100-300,000 per well) and incubated with ATN-224 at indicated concentrations and for indicated times. Cells were then stimulated with 10 ng/mL FGF-2 for various times and lysed. Lysates were subjected to Western blot analysis using an antibody specific for phosphorylated p44/42 mitogen-activated protein kinase ($Thr^{202}$/$Tyr^{204}$; Cell Signaling Technologies, Beverly, MA) with appropriate signal correction using an antibody specific for p44/42 mitogen-activated protein kinase.

***Apoptosis studies.*** Cells were harvested by centrifugation in the presence or absence of ATN-224. Cytoplasmic and nuclear fractions were prepared as described above. Fractions were analyzed for cleaved poly(ADP-ribose) polymerase or activated caspase-3 (Apoptosis Sampler kit, Cell Signaling Technologies) by Western blot analysis.

***Statistical analysis.*** GraphPad software was used for all statistical analysis. Data are presented as mean ± SD. Data were analyzed using unpaired, two-tailed *t* tests when comparing two variables. ANOVA with Tukey's post-test was used to compare data in experiments where more than two variables were compared simultaneously.

## Results

***ATN-224 inhibits endothelial cell proliferation and angiogenesis.*** ATN-224 has a specific and high affinity for copper ions ($10^8$ mol/$L^{-1}$) and showed no binding to calcium, iron, magnesium, zinc, or manganese ions at concentrations up to 1 mmol/L as determined by isothermal titration calorimetry (data not shown). ATN-224 inhibited the proliferation of both HUVEC (Fig. 1A; $IC_{50}$ = 1.4 ± 0.3 μmol/L; $n$ = 5) and human microvessel endothelial cells (data not shown) but had little effect on the cell number of confluent, quiescent HUVEC (data not shown), suggesting a selectivity for proliferating cells.

ATN-224 also significantly ($P < 0.05$) inhibited angiogenesis in the Matrigel plug model in mice either when added directly to the plug (data not shown) or when given by oral gavage (Fig. 1B). Inhibition of angiogenesis when ATN-224 was given by oral gavage occurred before there was measurable depletion of copper in either plasma (data not shown) or copper from the Matrigel plug (Fig. 1C). This result showed that ATN-224 inhibited angiogenesis independently of copper depletion.

***Cu/Zn SOD (SOD1) is a target for ATN-224.*** A previous study suggested that ammonium tetrathiomolybdate can be sequestered by cells and causes an inhibition in the copper-mediated release of FGF-1 and interleukin-1α during the vascular response to injury (18). We observed that endothelial cells take up (Fig. 1D) and retain ATN-224 (Fig. 1E). The intracellular concentration of ATN-224 per HUVEC was

calculated (as described in Materials and Methods) to be ~411 μmol/L at the highest concentration of ATN-224 tested (20 μmol/L), suggesting that ATN-224 can accumulate in the cell. Therefore, we compiled a list of intracellular cuproenzymes that could be potential targets for a copper-binding drug. This list included SOD1 and cytochrome *c* oxidase. SOD1 is a major cuproenzyme in cells responsible for catalyzing the conversion of superoxide to hydrogen peroxide (19). Cytochrome *c*

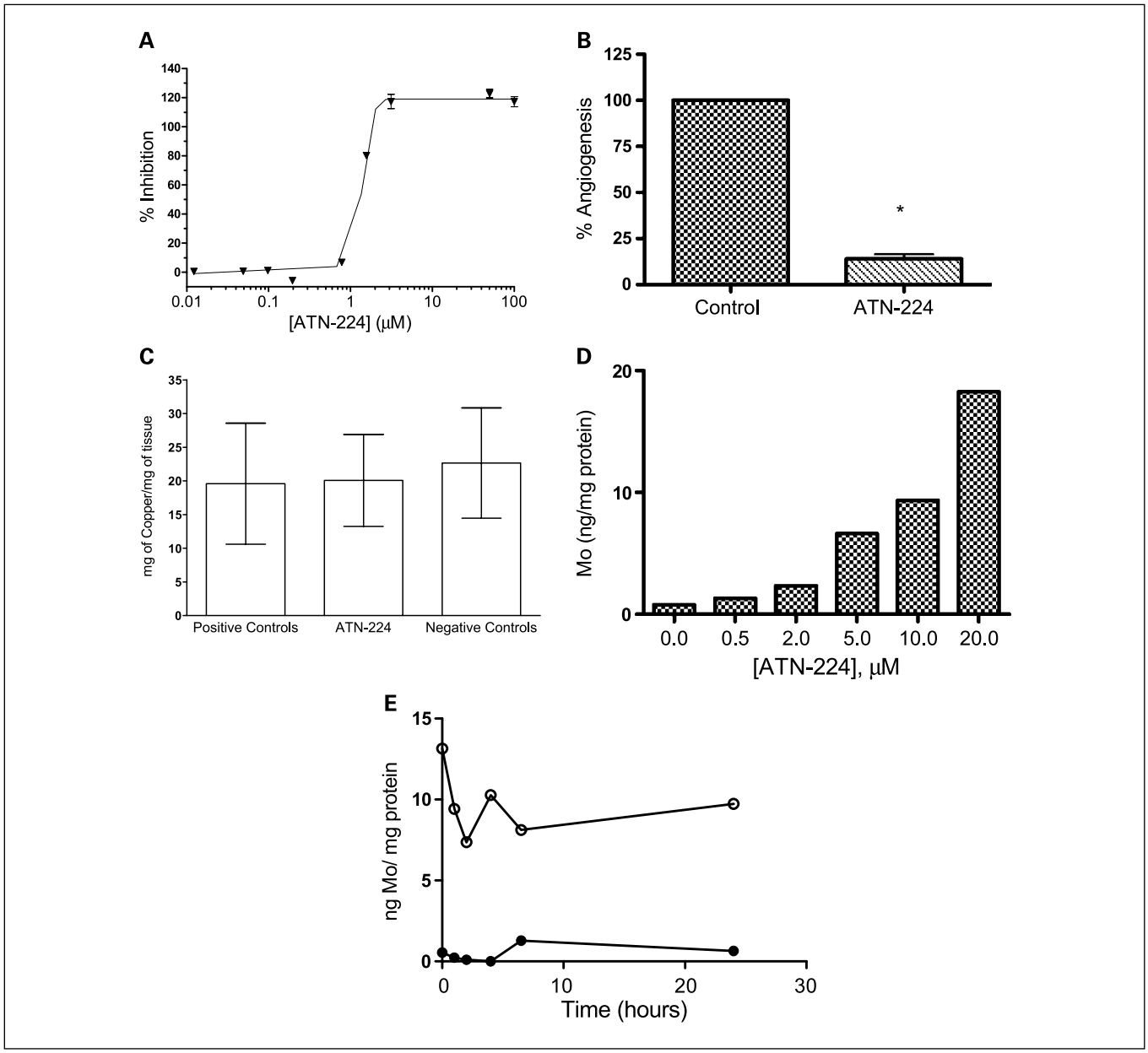


**Fig. 1.** ATN-224 inhibits HUVEC proliferation *in vitro* and angiogenesis *in vivo* and accumulates within HUVEC. *A,* ATN-224 inhibits HUVEC proliferation. HUVEC proliferation was measured as described in Materials and Methods. ATN-224 inhibited HUVEC proliferation with an $IC_{50}$ of 1.4 ± 0.3 μmol/L. Points, mean; bars, SD ($n = 5$). *B,* ATN-224 inhibits growth factor – driven angiogenesis in the Matrigel plug. ATN-224 was tested in the Matrigel plug model ($n = 3$ per group) as described in Materials and Methods. FGF-2 and VEGF were used to stimulate angiogenesis. The amount of angiogenesis in the FGF-2/VEGF-containing plugs was corrected by subtracting the amount of angiogenesis measured in the negative control plugs (Matrigel only, no FGF-2/VEGF) and this value was set as 100% angiogenesis (*Control*). Matrigel plugs from ATN-224–treated animals were corrected in a similar manner and then scored as a percentage of inhibition relative to the FGF-2/VEGF-containing plugs (*ATN-224*). Matrigel plugs had significantly ($P = 0.05$) less angiogenesis than the positive control Matrigel plug as determined by *t* test analysis. *C,* ATN-224 treatment does not alter the copper content in the Matrigel plug. Matrigel plugs ($n = 3$ per group) from the experiment described in (*B*) were also analyzed for copper content using ICP-MS as described in Materials and Methods. There were no significant differences in copper levels among the Matrigel plugs containing only FGF-2/VEGF (positive control), plugs from ATN-224–treated animals (ATN-224), and plugs from animals treated with vehicle alone (negative controls) as determined by one-way ANOVA analysis. *D,* ATN-224 is taken up by HUVEC in a dose-dependent manner. Cells were incubated with ATN-224 for 16 hours and then harvested, washed extensively, and lysed. Cell lysates were analyzed by ICP-MS for molybdenum content (molybdenum concentrations are a surrogate for ATN-224 uptake). Data are normalized to the total protein content in the HUVEC extract. The coefficient of variation of the ICP-MS technique is ~15%. Representative of a typical experiment ($n = 3$). *E,* ATN-224 is retained by HUVEC for at least 24 hours after removal of the drug from culture medium. Cells were incubated with ATN-224 (20 μmol/L) for 16 hours, at which point drug was removed from the medium and the cells were washed extensively. Cells were incubated for an additional 24 hours and then harvested, lysed, and assayed for molybdenum content using ICP-MS at the indicated time points (○). Control HUVEC (●) that were not incubated with ATN-224 did not contain appreciable amounts of molybdenum. The molybdenum concentrations were normalized to the total protein contained in the HUVEC extract. Representative of a typical experiment ($n = 3$).

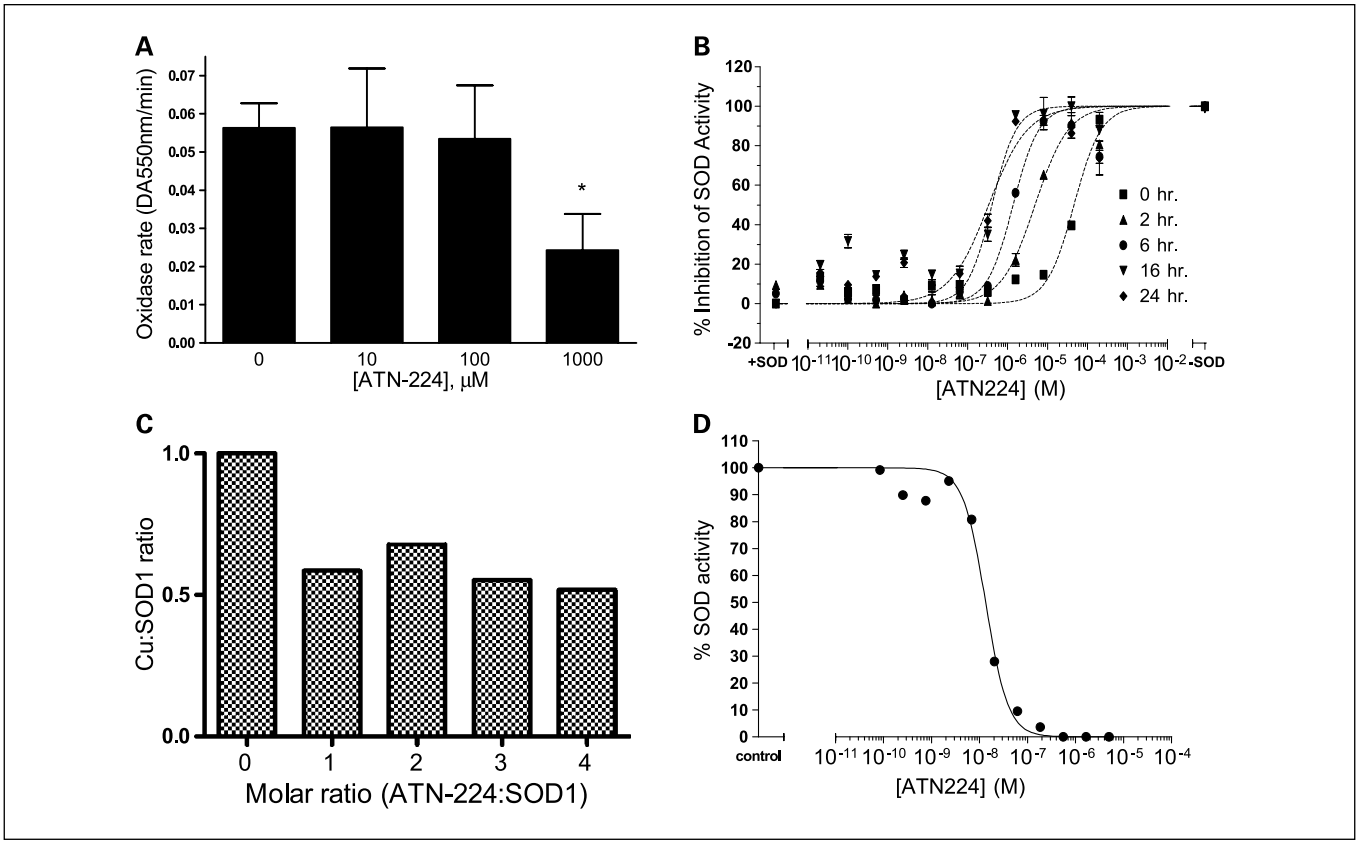


**Fig. 2.** ATN-224 is an inhibitor of SOD1. *A,* ATN-224 is a poor inhibitor of cytochrome *c* oxidase. The effect of ATN-224 on cytochrome *c* oxidase activity was measured using isolated mitochondria ($n = 3$ per experiment) as described in Materials and Methods. The only significant inhibition of activity by ATN-224 was observed at 1,000 μmol/L ($P = 0.03$). One-way ANOVA with a Tukey's post-test was used to evaluate the data. *B,* ATN-224 inhibits SOD1 activity *in vitro* in a time- and dose-dependent manner. SOD1 activity was assayed as described in Materials and Methods. Prolonged exposure of SOD1 to ATN-224 is required to observe maximal inhibition SOD1 activity, with the most potent inhibition only observed after 16 hours of incubation. The apparent $IC_{50}$ also shifts as a function of time with the maximal $IC_{50}$ at $0.33 \pm 0.03$ μmol/L also observed after incubation of bovine SOD1 with ATN-224 for ≥16 hours. *C,* ATN-224 removes copper from SOD1. Various concentrations of ATN-224 were incubated with SOD1 for 30 minutes and separated using size-exclusion chromatography as described in Materials and Methods. The concentration of SOD1 was then determined using a Bradford assay and the protein was analyzed for copper and molybdenum content using ICP-MS. No molybdenum was found to be associated with SOD1 and partial copper depletion under these conditions was observed. Additional depletion of copper from SOD1 was not observed when greater than a 1:1 molar ratio of ATN-224/SOD1 was tested. Representative of a typical experiment ($n = 3$). *D,* ATN-224 inhibits intracellular (HUVEC) SOD1 activity. HUVEC were treated with different concentrations of ATN-224 for 16 hours and intracellular SOD1 activity was measured as described in Materials and Methods. ATN-224 inhibited intracellular SOD1 with an $IC_{50}$ of $0.0175 \pm 0.0037$ μmol/L.

oxidase is a mitochondrial cuproenzyme with a vital role in cellular respiration. Tetrathiomolybdate has been shown to inhibit the activity of purified cytochrome *c* oxidase (10) with an $IC_{50}$ of 2 μmol/L and to reduce the activity of cytochrome *c* oxidase by ~25% to 30% in tissue homogenates obtained from sheep dosed i.v. with tetrathiomolybdate (2.6 mg/kg) for 80 days (11). However, in our hands, ATN-224 did not affect mitochondrial cytochrome *c* oxidase activity in an hepatic mitochondrial preparation at concentrations up to 100 μmol/L (Fig. 2A) even after 16 hours of incubation with the drug at 4°C, although some inhibition (50%) of cytochrome *c* oxidase activity was observed at 1 mmol/L ATN-224. The inhibition of purified and RBC SOD1 activity by ammonium tetrathiomolybdate has also been described previously (10, 11), and ATN-224 was also able to inhibit the activity of purified bovine SOD1 (Fig. 2B) with an $IC_{50}$ of $0.33 \pm 0.03$ μmol/L after 24 hours of incubation. The SOD1 inhibition by ATN-224 is time dependent, reaching maximal inhibition at ~16 hours (Fig. 2B). The inhibition of human SOD1 by ATN-224 is almost identical to that observed for bovine SOD1; thus, bovine SOD1 was used for all experiments reported here. Control experiments in the absence of ATN-224 show that SOD1 and ATN-224 are completely stable for the entire time course of this experiment (data not shown). ATN-224 seems to inhibit SOD1 by depleting the enzyme of copper (Fig. 2C). The incomplete removal of copper for SOD1 in this experiment is consistent with the time-dependent inhibition observed in Fig. 2B. In addition, ATN-224 was able to inhibit SOD1 activity in endothelial cells, an effect that was dose dependent with an $IC_{50}$ of $17.5 \pm 3.7$ nmol/L (Fig. 2D). Endothelial cell SOD1 inhibition is also time dependent, similar to the results observed with purified enzyme, reaching maximal inhibition at ~16 hours (data not shown). The $IC_{50}$ for endothelial cell SOD1 is considerably lower than that for purified SOD1 and may reflect time-dependent accumulation of drug inside the cell, consistent with the data presented in Figs. 1D and 2B. The $IC_{50}$ for the inhibition of proliferation is ~70-fold higher, suggesting that SOD1 activity must be inhibited to a large extent before an effect on proliferation is observed. Western blot analysis of endothelial cell lysates showed that levels of SOD1 protein remain unaffected by ATN-224 over the course of these experiment (data not shown), indicating that the

reduction of activity is due to inhibition of the enzyme rather than down-regulation of the levels of SOD1 protein.

The cell-permeable SOD mimetic Mn-TBAP abrogated the ATN-224-mediated inhibition of proliferation in both HUVEC (Fig. 3A) and human microvessel endothelial cells (data not shown), providing further support for the hypothesis that SOD1 inhibition mediates the antiproliferative effects by ATN-224. The buffer used to reconstitute Mn-TBAP did not have any

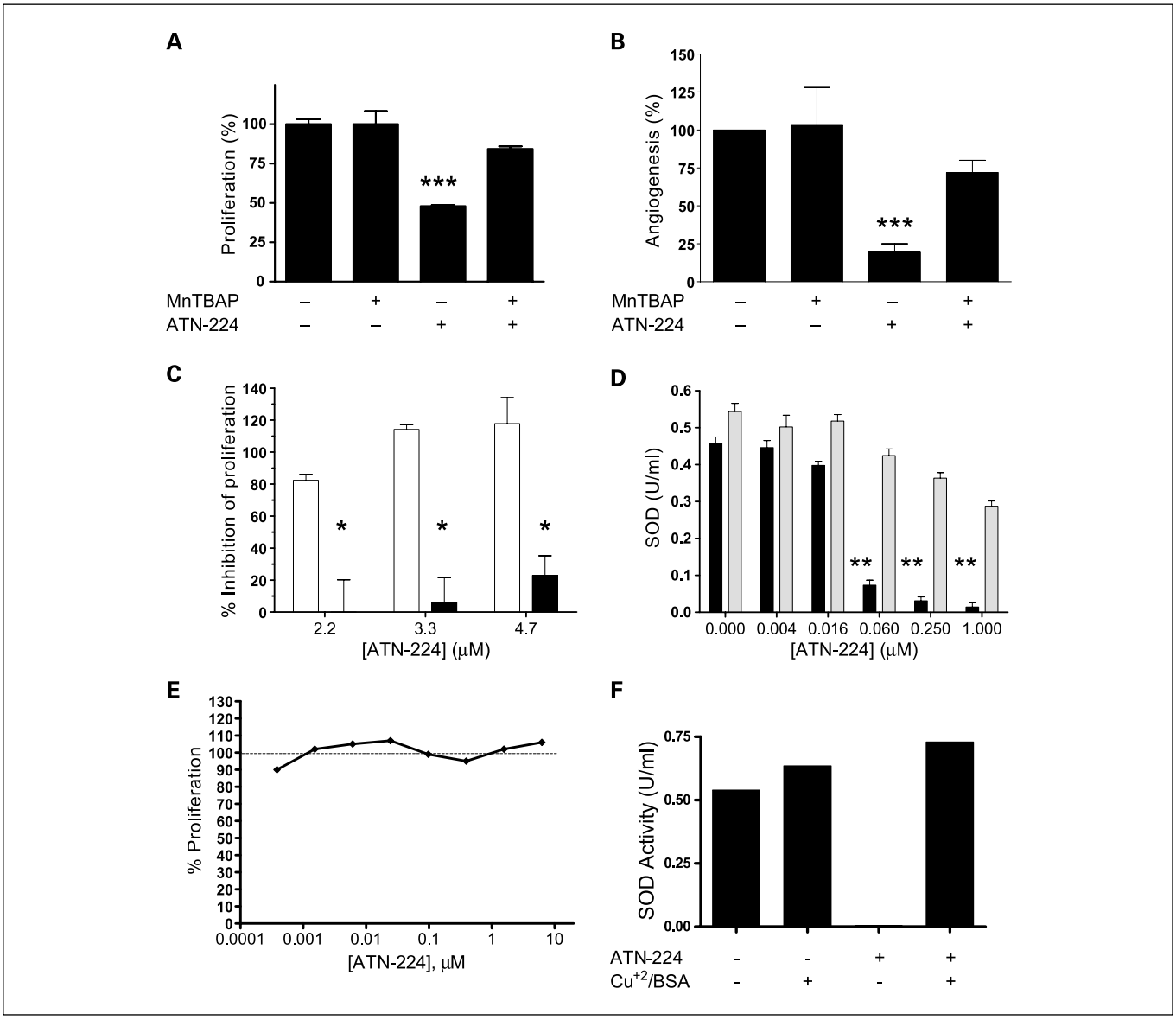


**Fig. 3.** A small-molecule SOD mimetic (Mn-TBAP), PEG-SOD1, and copper reverse the effects of ATN-224. *A,* Mn-TBAP abrogates the ATN-224-mediated inhibition of HUVEC proliferation. HUVEC proliferation was stimulated with FGF-2 (10 ng/mL) in the presence of ATN-224 (1 μmol/L) and Mn-TBAP (100 μmol/L) and proliferation was measured after 16 hours. ATN-224 significantly inhibits proliferation of HUVEC ($P < 0.0001$) and a significant portion of this inhibition could be recovered in the presence of Mn-TBAP. $P < 0.001$, ATN-224-treated cells compared with cells treated with both ATN-224 and Mn-TBAP. Mn-TBAP-treated HUVEC were not significantly affected compared with control HUVEC. All comparisons were determined by one-way ANOVA analysis with Tukey's post-test. *B,* Mn-TBAP abrogates the ATN-224-mediated inhibition of angiogenesis in the Matrigel plug. FGF-2 (800 ng/mL), VEGF (300 ng/mL), and Mn-TBAP (500 μmol/L final concentration) alone and in combination with ATN-224 (100 μmol/L) were added to the Matrigel before s.c. injection. Data have been corrected for background angiogenesis measured in plugs that contained Matrigel alone (no FGF-2/VEGF). Mn-TBAP alone did not significantly affect angiogenesis compared with control Matrigel plugs. ATN-224-treated plugs had significantly less angiogenesis compared with control plugs ($P < 0.0001$). Treatment with Mn-TBAP significantly reversed the effects of ATN-224 ($P < 0.001$) to levels that were not significantly different than the control plugs. *C,* addition of PEG-SOD to HUVEC overcomes the antiproliferative effects of ATN-224. HUVEC were treated with 0.5 mg/mL PEG-SOD (*black columns*) or control buffer (*white columns*) overnight. HUVEC were then washed extensively, trypsinized, and plated on 96-well plates. A proliferation assay in the presence of the indicated amounts of ATN-224 was carried out as described in Materials and Methods ($P = 0.013$). Columns, mean of three measurements; bars, SD. *D,* addition of PEG-SOD to HUVEC overcomes the inhibition of intracellular SOD1 by ATN-224. HUVEC were treated with 0.5 mg/mL PEG-SOD or control buffer overnight. HUVEC were then washed extensively, trypsinized, and plated in six-well plates. SOD activity in the presence of the indicated amounts of ATN-224 was carried out in 10 μg whole-cell extract as described in Materials and Methods. Columns, mean of three measurements; bars, SD. $P = 0.004$. *E,* copper and albumin abrogate the inhibition of HUVEC proliferation by ATN-224. HUVEC were incubated with various concentrations of ATN-224, $CuSO_4$, and bovine serum albumin (100 μmol/L) for 48 hours and proliferation stimulated with FGF-2 (10 ng/mL). Proliferation was assessed as described in Materials and Methods and percentage of proliferation was determined by assigning 100% proliferation to HUVEC treated with FGF-2 alone. Representative of three separate experiments. *F,* copper and albumin abrogate the ATN-224-mediated inhibition of HUVEC SOD1. HUVEC were incubated with ATN-224 (7 μmol/L) alone, $CuSO_4$, and bovine serum albumin (*BSA*; 100 μmol/L) alone or in combination with ATN-224 for 48 hours. Cells were harvested and lysed. SOD1 activity was assayed as described in Materials and Methods.

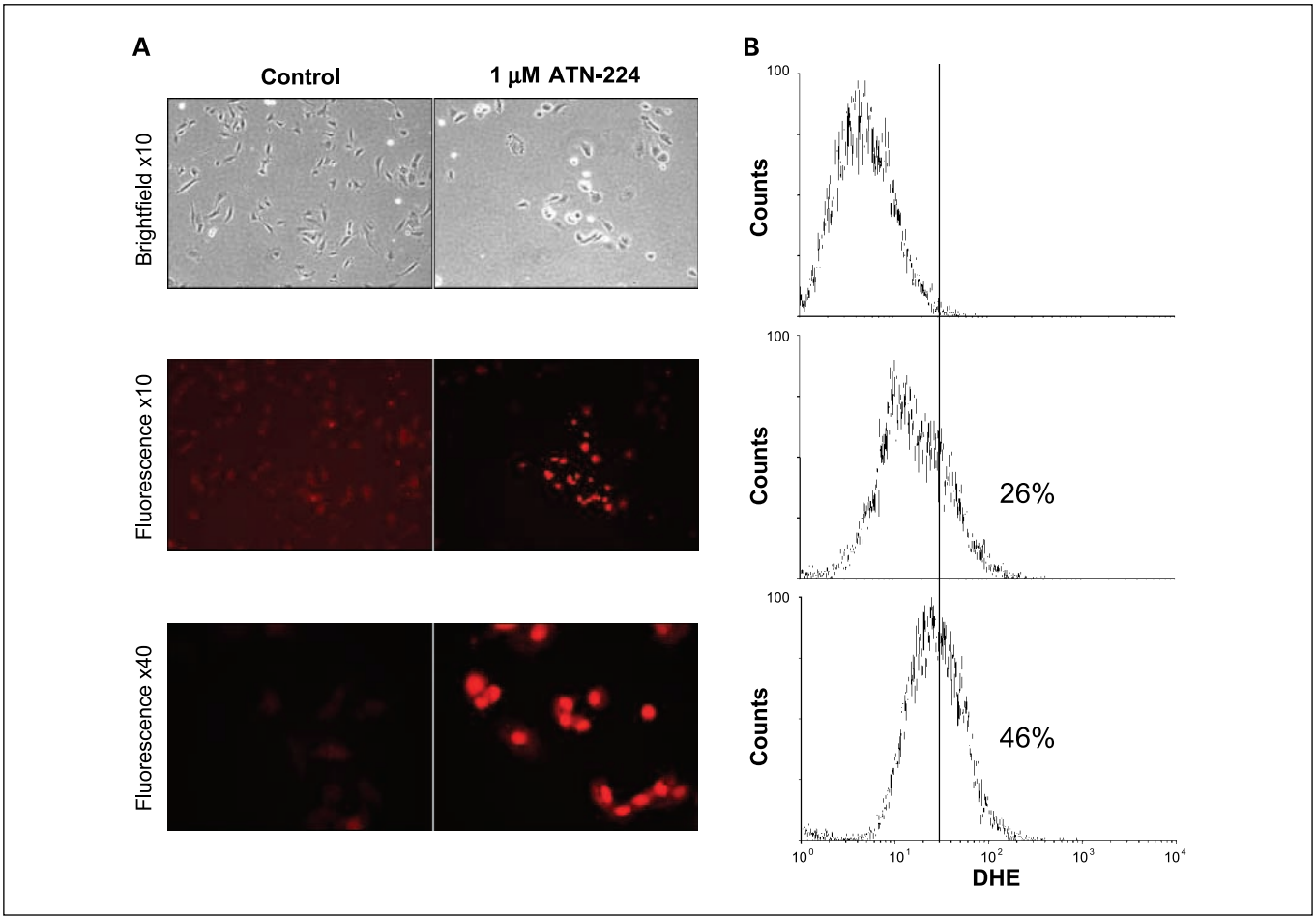


**Fig. 4.** ATN-224 treatment results in an increase in steady-state levels of superoxide anions in HUVEC. *A,* proliferating cells were incubated in the absence or presence of ATN-224 (1 μmol/L) for 72 hours. Cells were then incubated with dihydroethidine as described in Materials and Methods. Images were obtained under bright-field conditions or using a red filter under fluorescent conditions at the magnifications indicated. *B,* HUVEC were treated, trypsinized, and incubated with dihydroethidine (*DHE*) for 15 minutes, and analyzed by flow cytometry. Top, control cells; middle, cells incubated with dihydroethidine alone; bottom, HUVEC and treated with 1 μmol/L ATN-224 and dihydroethidine as indicated above.

measurable effect in this assay (data not shown). Mn-TBAP also reversed the ATN-224-mediated inhibition of angiogenesis *in vivo* in the Matrigel plug model (Fig. 3B). To further validate SOD1 as the target for ATN-224 antiproliferative activity, HUVEC were preloaded with PEG-SOD, which is internalized by cells (20), and the effect of ATN-224 on proliferation was measured. ATN-224 was less efficient in inhibiting proliferation (Fig. 3C) and in inhibiting SOD1 activity (Fig. 3D) in HUVEC preloaded with PEG-SOD than in control cells. SOD activity was increased in preloaded cells by ~20% leading to a 4-fold increase in the $IC_{50}$ for proliferation inhibition. We also evaluated whether preloading ATN-224 with copper ions would abrogate its antiproliferative and SOD1 inhibitory activities. It is not possible to test ATN-224-copper complexes directly because this complex tends to precipitate in aqueous solution in the absence of protein. In the presence of protein and specifically albumin, it is possible to bind copper to ATN-224, which then forms a soluble tripartite complex with albumin (21). Thus, the HUVEC proliferation assay was carried out in the presence of copper and bovine serum albumin (Fig. 3E). Under these conditions, ATN-224 is not antiproliferative and does not inhibit HUVEC SOD1 (Fig. 3F), indicating that the copper-binding activity of ATN-224 is required for these activities.

SOD1 inhibition by ATN-224 would be predicted to lead to an increase in the steady-state levels of superoxide anion in the cell. This was confirmed by using fluorescence microscopy and flow cytometry to evaluate HUVEC that had been treated for 72 hours with 1 μmol/L ATN-224, sufficient to completely inhibit SOD1. An increase in superoxide anion in the ATN-224-treated cells was observed using dihydroethidine, a dye that is selectively oxidized by superoxide anion to generate red fluorescence (Fig. 4).

***SOD1 inhibition by ATN-224 in HUVEC leads to inhibition of extracellular signal-regulated kinase 1/2 phosphorylation.*** To further evaluate how SOD1 inhibition could lead to an inhibition of HUVEC proliferation, we investigated the effects of ATN-224 on various signaling pathways known to be important for endothelial cell growth. The extracellular signal-regulated kinases 1 and 2 (ERK1/2) are mitogen-activated protein kinases that have been implicated in the signal transduction cascade leading to cell proliferation. To evaluate the effects of ATN-224 on ERK1/2, endothelial cells were preincubated with the compound for various times and at

various doses and stimulated with FGF-2 and the cell lysates were analyzed for ERK1/2 phosphorylation. Western blot analysis for phosphorylated ERK1/2 revealed that ATN-224 inhibited FGF-2-induced ERK1/2 phosphorylation in a dose-dependent (Fig. 5A) and time-dependent (Fig. 5B) manner with an $IC_{50}$ between 1.25 and 2.5 μmol/L, consistent with the $IC_{50}$ for the inhibition of proliferation. A decrease in ERK1/2 phosphorylation was observed only after 24 hours of preincubation with 10 μmol/L ATN-224, suggesting that near complete inhibition of SOD1 activity may be required for the inhibition of ERK1/2 phosphorylation. As observed for the antiproliferative activity of ATN-224, the effects of ATN-224 on FGF-2-induced ERK1/2 phosphorylation could be abrogated using Mn-TBAP. Similar effects were also observed on the VEGF-stimulated phosphorylation of ERK, which was also reversible using Mn-TBAP (Fig. 5C).

***ATN-224 inhibits SOD1 and induces apoptosis in multiple myeloma MMS1 cells.*** Poly(ADP-ribose) polymerase cleavage, caspase activation, and nuclear fragmentation have not been observed in ATN-224-treated HUVEC, indicating that ATN-224 only inhibits proliferation in these cells but does not induce cell death. ATN-224 inhibits the proliferation of the multiple myeloma cell line MM1S with an $IC_{50}$ of ~6.5 μmol/L (Fig. 6A) and MM1S SOD1 activity with an $IC_{50}$ of ~0.04 μmol/L (Fig. 6B). However, in contrast to HUVEC, ATN-224 induces apoptosis in MM1S cells as detected by an increase in cleaved poly(ADP-ribose) polymerase and activated caspase-3 (Fig. 6C). Similar results were observed with several other tumor cell lines, including HL-60 and MOLT-4, and these effects could be reversed using Mn-TBAP (data not shown).

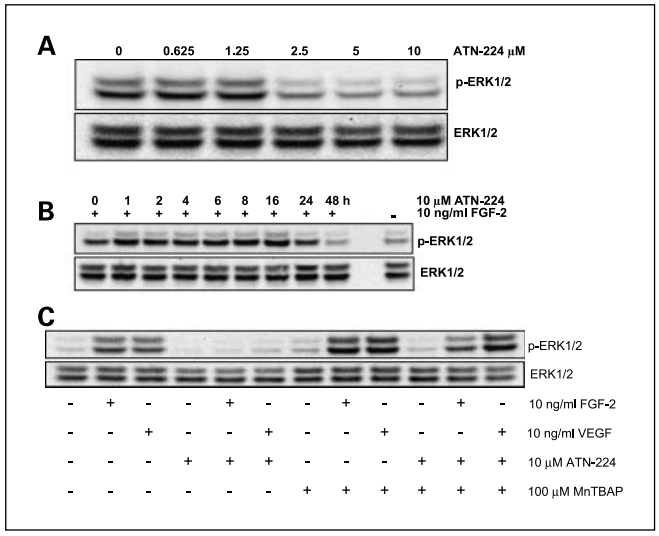


**Fig. 5.** ATN-224 inhibits ERK1/2 phosphorylation in HUVEC. *A,* ATN-224 inhibits FGF-2-mediated ERK phosphorylation in HUVEC in a dose-dependent manner. HUVEC were incubated with different concentrations of ATN-224 for 48 hours followed by stimulation of the cells with FGF-2 (10 ng/mL) as described in Materials and Methods. Cells were then harvested and the cell extracts were analyzed by Western blot for the expression of total and phosphorylated ERK1 and ERK2. A significant inhibition of ERK1/2 phosphorylation was observed at concentrations of ATN-224 greater than 2.5 μmol/L. *B,* ATN-224 inhibits FGF-2-mediated ERK phosphorylation in HUVEC in a time-dependent manner. HUVEC were preincubated with ATN-224 (10 μmol/L) for various times followed by stimulation with FGF-2 (10 ng/mL) as described in Materials and Methods. After harvesting, cell extracts were analyzed by Western blot for expression of total and phosphorylated ERK1 and ERK2. The complete inhibition of phosphorylation was observed by 48 hours of incubation with ATN-224. *C,* Mn-TBAP abrogates the ATN-224-mediated inhibition of ERK phosphorylation. HUVEC were incubated with ATN-224 (10 μmol/L) for 48 hours and then stimulated with either FGF-2 (10 ng/mL) or VEGF (10 ng/mL). Cells were harvested and the extracts were analyzed for total and phosphorylated ERK1 and ERK2 as described in Materials and Methods. ATN-224 inhibited both VEGF- and FGF-2-stimulated ERK1/2 phosphorylation to unstimulated levels, an effect that could be reversed using the SOD mimetic, Mn-TBAP.

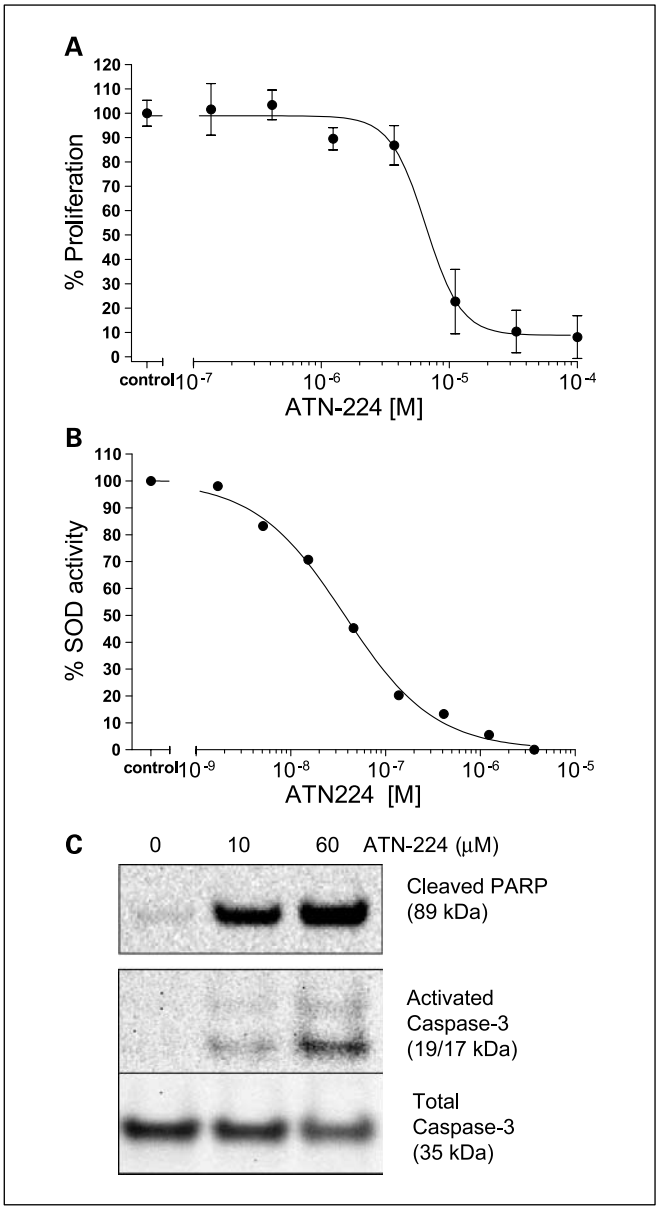


**Fig. 6.** ATN-224 inhibits proliferation and induces apoptosis in tumor cells. *A,* a dose titration of ATN-224 was carried out using multiple myeloma cells MM1S as described in Materials and Methods. ATN-224 inhibited MM1S proliferation with an $IC_{50}$ of ~5 μmol/L. *B,* ATN-224 also inhibited SOD1 activity in MM1S cells ($IC_{50}$ ~ 0.04 μmol/L) in a SOD assay carried out with 10 μg whole-cell extract as described in Materials and Methods. *C,* MM1S cells were treated with the indicated concentrations of ATN-224 for 48 hours and probed with antibodies against cleaved poly(ADP-ribose) polymerase and total and cleaved activated caspase-3.

## Discussion

ATN-224, the choline salt form of tetrathiomolybdate, is a highly specific copper-binding compound. ATN-224 inhibits the proliferation of endothelial and tumor cells and inhibits angiogenesis *in vivo*. The inhibition of angiogenesis is observed

before there are measurable decreases in systemic copper content and SOD1 has been identified as an intracellular target for ATN-224. These results suggest that the induction of systemic copper deficiency may not explain the previously described mechanism of inhibition of angiogenesis by tetrathiomolybdate.

ATN-224 accumulates and is retained by endothelial cells and acts at the intracellular level as an inhibitor of SOD1. ATN-224 seems to inhibit SOD1 by removing copper from the enzyme, although, under the conditions of our studies, only partial copper removal was observed. SOD1 has a very high affinity ($K_d \sim 10^{-14}$ mol/L) for copper, which implies a slow off-rate; thus, prolonged incubation of SOD1 with excess ATN-224 is likely required to completely deplete SOD1 of copper. In support of this hypothesis, the complete inhibition of SOD1 activity (an indirect measure of copper removal from the enzyme) *in vitro* requires at least 16 hours of incubation with ATN-224. This observation has significant pharmacologic implications as well, because it suggests that a cell must be able to take up and retain ATN-224 for long periods of time to achieve the inhibition of SOD1.

Although ATN-224 has the potential to inhibit numerous intracellular cuproenzymes, such as cytochrome *c* oxidase (10, 11), the results presented herein suggest that most of the antiangiogenic effects of ATN-224 are manifested by the ability of this compound to inhibit SOD1 activity in endothelial cells. This does not rule out the possibility that other intracellular targets for ATN-224 exist, but several pieces of data strongly point to SOD1 as the major antiangiogenic target for ATN-224 in endothelial cells. First, a synthetic small-molecule SOD mimetic (Mn-TBAP) almost completely abolishes the antiproliferative effects of ATN-224 on endothelial cells. In addition, delivering exogenous PEG-SOD to HUVEC significantly decreases the ability of ATN-224 to inhibit proliferation and SOD1 activity in these cells.

We describe the time- and dose-dependent inhibition of ERK1/2 phosphorylation in endothelial cells stimulated with angiogenic growth factors. The inhibition takes place after incubation with ATN-224 for longer than 16 hours, with complete inhibition of ERK1/2 phosphorylation observed after 48 hours. The inhibition of ERK phosphorylation by ATN-224 can be nearly completely abrogated by the small-molecule SOD mimetic, Mn-TBAP, consistent with the effects of Mn-TBAP on SOD1 inhibition and HUVEC proliferation, providing further support that SOD1 is central to these effects in HUVEC. In addition to inhibiting HUVEC proliferation and ERK phosphorylation stimulated by FGF-2, ATN-224 also inhibited the VEGF-induced phosphorylation of ERK, suggesting that the inhibition of SOD1 activity by ATN-224 can affect a variety of proangiogenic signaling pathways.

The only three other copper binders that have been shown in the literature to inhibit intracellular SOD1, and in some instances angiogenesis and/or tumor growth, are thiocarbamates: tetraethylthiuram disulfide (disulfiram; ref. 12), tetramethylthiuram disulfide (thiram; ref. 22), and diethyldithiocarbamate (23). The inhibition of SOD1 by these compounds is much weaker than by ATN-224. Dithiocarbamates also bind to a variety of metals in addition to copper and have pleiotropic cellular activities, including the inhibition of aldehyde dehydrogenase, caspases, DNA topoisomerase, and generation of oxygen radicals (24, 25). Thus, the effects on angiogenesis and tumor growth observed with these agents can occur via multiple pathways and cannot be attributed to SOD1 inhibition.

One major issue is how SOD1 inhibition by ATN-224 leads to the growth inhibition of HUVEC and to apoptosis in multiple myeloma cells. At least three different and nonexclusive mechanisms could account for the ATN-224-mediated antiproliferative activity. For example, the increase in steady-state levels of superoxide, which we formally show in HUVEC using dihydroethidine, may lead to oxidative alterations to proteins, lipids, and/or DNA directly by superoxide, by peroxynitrite formed in the reaction of superoxide with nitric oxide, or by other reactive oxygen or nitrogen species that may originate from superoxide reactivity. Alternatively, superoxide may deplete NO, which is involved in mitogen-activated protein kinase signaling in endothelial cells, or may inactivate aconitase-like enzymes, both of which could contribute to an antiproliferative effect. Finally, the inhibition of SOD1 also decreases the production of hydrogen peroxide. Recent data have shown that hydrogen peroxide behaves like a second messenger during mitogen signaling mediated by growth factors, such as VEGF and FGF-2, and hydrogen peroxide has been shown to stimulate ERK activation as well as mediate endothelial cell proliferation and migration *in vitro* (26–29). Studies are ongoing to resolve these various possibilities at the molecular level.

The incubation of endothelial cells with 10 μmol/L ATN-224 for up to 96 hours did not induce nuclear fragmentation or activation of either caspase-9 or caspase-3, although ATN-224 is taken up readily by these cells. Thus, the effect of SOD1 inhibition in endothelial cells seems to be truly antiproliferative. In contrast, ATN-224 affected the apparent growth of multiple myeloma cells (MM1S) by inducing apoptosis. Detectable cleavage of caspase-3 and poly(ADP-ribose) polymerase in these cells was observed after 48 hours of incubation with 10 μmol/L ATN-224. This effect seems to also be mediated by SOD1 because Mn-TBAP abrogated the induction of apoptosis in MM1S cells. This result is consistent with previously published data showing that down-regulation of SOD1 expression using RNA interference can induce apoptosis in tumor cells (30). We are currently investigating the specificity and selectivity of SOD1 inhibition in a panel of tumor cells, with a view to understanding the basis of the selectivity for inducing apoptosis in tumor cells.

The fact that ATN-224 may selectively induce apoptosis in tumor cells but not endothelial cells has important implications in the clinical setting, as ATN-224 might be expected to simultaneously inhibit proliferating endothelial cells and induce apoptosis in the tumor cell compartment, thus affecting distinct compartments of a tumor in different but potentially synergistic ways. Thus, the specific inhibition of SOD1 by ATN-224 reveals a novel agent with potentially pleiotropic antitumor effects. Taken together, the data presented here provide validation of SOD1 as a therapeutic target for the treatment of cancer and provide insight into how copper mediates angiogenesis and tumor growth at the molecular level.

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