Phase I Study of Copper-Binding Agent ATN-224 in Patients with Advanced Solid Tumors

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Abstract

Purpose: Copper chelation reduces the secretion of many angiogenic factors and reduces tumor growth and microvascular density in animal models. ATN-224 is a second-generation analogue of ammonium tetrathiomolybdate. The aim of our phase I study was to reduce serum copper levels, as measured by ceruloplasmin, to 5 to 15 mg/dL (normal 16-60) in 14 to 21 days, to determine the pharmacokinetic profile of ATN-224 and to evaluate dose-limiting toxicities.

Patients and Methods: Cohorts of patients were treated with escalating oral doses of ATN-224 until copper depletion followed by a titrated maintenance dose.

Results: Eighteen patients received 78 cycles of ATN-224. Mean baseline ceruloplasmin was 39.6 mg/dL. The maximum administered dose was 330 mg/d where grade 3 fatigue was dose-limiting. At the maximum tolerated dose of 300 mg/d, the median time to achieve target ceruloplasmin was 21 days, and toxicities included grade 3 anemia, grade 3 neutropenia, fatigue, and sulfur eructation. ATN-224 treatment caused a significant reduction (>90%) in RBC superoxide dismutase 1 activity and circulating endothelial cells. Pharmacokinetic data indicate greater absorption of ATN-224 and more rapid ceruloplasmin reduction when administered with a proton pump inhibitor. Stable disease of >6 months was observed in 2 patients.

Conclusions: Oral ATN-224 is a well-tolerated therapy and at a loading dose of 300 mg/d leads to a reduction of serum ceruloplasmin levels in 80% patients within 21 days. A loading dose of 300 mg/d for 2 weeks followed by a titrated maintenance dose will be the recommended starting dose for phase II study.

Angiogenesis, the growth of new blood vessels, is recognized as a crucial process in tumor growth (1) and is stimulated by a wide array of angiogenic factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor. VEGF is a key angiogenic factor and targeted therapies have shown benefit in several malignancies, particularly colorectal cancer (2), although not in all tumor types. This may be in part due the redundancy of factors involved in angiogenesis.

Copper is required for normal growth and development in humans as evidenced by Menkes disease, a deficiency of copper arising from a mutation in a copper-transporting ATPase, which leads to stunted growth, neurologic abnormalities, and kinky hair (3). Copper has been found to be essential for the process of angiogenesis (4, 5). Conversely, copper deficiency was found to significantly decrease the size and vascularity of tumors in a brain tumor xenograft model (6).

One of the mechanisms by which copper-lowering therapy is thought to reduce angiogenesis is by inhibiting the secretion and activity of several different growth factors and cytokines (7–10). Copper is also an essential cofactor in many human enzymes including copper/zinc superoxide dismutase (SOD-1), cytochrome oxidase, dopamine β-hydroxylase, and lysyl oxidase, and these may represent targets of copper chelation.

One of the attractions of copper chelation therapy is that well-tolerated metal-specific oral preparations are now available. Tetrathiomolybdate is an oral copper chelator that has been shown to have antitumor and antiangiogenic effects in animal models (7, 11, 12). In a phase I study of tetrathiomolybdate in patients with advanced malignancy, there was an average period of stable disease of 9.5 months (13, 14). However, one of the limitations of this study was the slow onset of copper depletion (50-60 days), during which time many patients experienced progression of their disease. Tetrathiomolybdate also has poor stability, requiring multiple daily dosing and leading to sulfur eructation due to drug breakdown in the stomach.

ATN-224 (choline tetrathiomolybdate) is the bis-choline salt of tetrathiomolybdate (Attenuon). Compared with

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tetrathiomolybdate, ATN-224 has superior stability and can be given once or twice daily. There is evidence from preclinical studies that ATN-224 acts by inhibition of SOD-1 activity (15). The aim of this phase I study was to find the dosing regimen required to lower serum copper levels in 21 days while monitoring for toxicity and tumor response and to investigate the pharmacokinetics and pharmacodynamics of ATN-224 in humans.

Patients and Methods

Eligibility

Eligible patients were ages >18 years, with histologically or cytologically proven solid tumors refractory to conventional therapy or for which no such therapy exists. Other eligibility criteria included a life expectancy of >12 weeks, WHO performance status of 0 to 2, recovery from the toxic effects of prior chemotherapy, and adequate bone marrow, liver, and renal function including a hemoglobin of $\geq$9 g/dL.

Patients were ineligible if they had received radiotherapy, chemotherapy, immunotherapy, endocrine therapy, or major surgery in the previous 4 weeks or if they had brain metastases. The study was approved by Cancer Research UK protocol review committee and our local ethics committee and informed written consent was obtained from patients before enrollment. The first patient was enrolled in April 2004 and the last patient entered the study in January 2006.

Drug formulation and administration

ATN-224 was supplied by Attenuon as 30 mg white size 0 V-cap capsules in child-resistant Termoform blister packs, sealed in an argon atmosphere, and stored at 2-8°C. Patients were instructed to take the drug ~30 min after a meal either in two daily doses during the loading phase or in one or two daily doses during the maintenance phase. Patients kept treatment diaries to assess compliance, and unused capsules were returned and counted.

Treatment and dose escalation

The starting dose of ATN-224 (150 mg/d) was calculated as 50% of the recommended dose of tetrathiomolybdate (180 mg/d) identified in previous studies (150 mg/d ATN-224 contains equimolar amounts of molybdate to 94 mg tetrathiomolybdate; refs. 13, 16). This dose is ~1/10th of the oral maximum tolerated dose (MTD) in rats and 1/7th of the MTD of dogs given tetrathiomolybdate orally once daily for 28 days. The loading dose was administered for 2 weeks, after which ATN-224 was reduced to 90 mg/d. The protocol was subsequently amended (patient 6 onwards) to continue loading with ATN-224 until the ceruloplasmin fell to 15 mg/dL and then titrate the maintenance dose to keep ceruloplasmin in the target range. One patient was entered per dose level. The dose levels were 1 (150 mg/d), 2 (210 mg/d), 3 (240 mg/d), 4 (270 mg/d), 5 (300 mg/d), and 6 (330 mg/d). ATN-224 was taken 30 min after the morning and evening meals in two equally divided doses if possible or the larger dose was taken in the evening. A cycle was 28 days of treatment. In the event of a dose-limiting toxicity (DLT), the dose level was expanded to include 6 patients. The final dose level was that which achieved the desired reduction in the plasma ceruloplasmin level within 21 days in all patients or the MTD.

Ceruloplasmin is a copper-binding plasma protein secreted by the liver, which, in the absence of copper, is rapidly degraded (17). Ceruloplasmin levels are a reflection of tissue copper levels and were used as a surrogate marker for copper status because the ceruloplasmin assay is rapid and clinically available. A target ceruloplasmin range was chosen as 5 to 15 mg/dL because previous trials of tetrathiomolybdate have shown that ceruloplasmin levels above 5 mg/dL could be maintained for extended periods without significant toxicity and preclinical data showed this degree of reduction was associated with activity *in vivo* (13, 16).

DLT and MTD

The DLT and MTD were defined using the National Cancer Institute Common Toxicity Criteria version 2.0. DLT was defined as almost certainly/probably drug related: anemia grade 4; neutropenia grade 4 for >5 days duration; febrile neutropenia; infection with grade 3/4 neutropenia; thrombocytopenia grade 4 for $\geq$5 days, associated with active bleeding or requiring platelet transfusion; grade 3/4 non-hematologic toxicity (excluding grade 3 nausea and grade 3/4 vomiting or diarrhea in patients who have not received antiemetics or antidiarrheals) or death. The MTD was defined as the dose below that at which >30% (2 of up to 6 patients) of the patient population suffered DLT due to the drug. The dose that would be recommended for phase II trials would be either the dose that fulfilled the primary pharmacodynamic endpoint or the MTD, whichever was the lower.

Patient evaluation

Before enrollment and within 1 week before the pharmacokinetic dose, each patient had a complete history and physical examination done and electrocardiogram, chest X-ray, urinalysis, and WHO performance status assessed. Laboratory investigations were done 1 week before treatment and thereafter two weekly, except the full blood count and ceruloplasmin, which were done weekly for the first two cycles.

Toxicity was assessed by the National Cancer Institute Common Toxicity Criteria version 2.0. Measurable lesions were documented by computed tomography or magnetic resonance imaging scan within 3 weeks before the pharmacokinetic dose and thereafter every two cycles (8 weeks). Lesions were measured by Response Evaluation Criteria in Solid Tumors.

Table 1. Patient demographic data

	n
Total patients	
Enrolled	18
Treated for >21 d	16
Age (y), Mean	56 (37-78)
Ceruloplasmin	
Mean screening (mg/dL)	39.6
Cycles of ATN-224 received	
Total	78
Mean cycles per patient	4.3
Gender	
Female	12
Male	6
WHO performance status	
0	3
1	9
2	6
Tumor type	
Breast	4
Colon	2
Renal	2
Melanoma	2
Other (prostate, spinal ependymoma, nasopharyngeal, ovarian, endometrial, mesothelioma, pancreatic, carcinoma of unknown primary)	8
Previous therapies	
Chemotherapy, no. patients	16
Mean no. previous chemotherapies per patient	2
Radiotherapy	12
Hormone/biological therapy	7

Pharmacokinetic studies

A week before commencing the ATN-224 loading regime, each patient received a single pharmacokinetic dose of ATN-224 after a standardized breakfast. This was 60 mg for the first patient and increased by 30 mg for each subsequent dose level.

For the initial patients, 2.7 mL whole blood was taken at baseline and at 1, 2, 3, 4, 5, 6, 8, 24, 30, 48, 72, and 168 h following the pharmacokinetic dose. Steady-state pharmacokinetic samples were taken on days 15 and 21 of the first cycle. Plasma was analyzed using a validated inductively coupled plasma-mass spectrometry assay for total and free molybdenum. Total and free plasma copper were measured on the same samples using inductively coupled plasma-mass spectrometry. The data were analyzed in the Kinetica 2000 software package using noncompartmental analysis.

Biomarker studies

Blood samples were taken for biomarker estimation at pretreatment, on days 1 and 15 of the first two cycles, and at the first day of cycles 3 to 6.

Copper/zinc SOD-1 activity assay. SOD-1 activity was assayed on plasma and blood cells using the Dojindo SOD assay (Dojindo Molecular Technologies).

Circulating endothelial cells. A WBC preparation was obtained by lysing the red cells in the blood sample and washing the remaining cells by centrifugation. Purified WBC were then analyzed by flow cytometry using fluorescently labeled antibodies. Circulating endothelial cells (CEC) were defined as $CD45^-$/weak, $CD34^+$, $CD144^+$, and VEGF receptor 2^+. Endothelial progenitor cells (EPC) were defined as $CD45^-$/weak, $CD133^+$, $CD144^+$, and VEGF receptor 2^+. On some occasions, activated CECs were identified using anti-CD62E. The number of CEC and EPC was determined by reference to the mononuclear cell number gated by flow cytometry and the mononuclear cell concentration was obtained by the hematology analyzer.

Cytokines. Plasma cytokine measurements were done by the analytic testing service of R&D Systems using the Quantakine HS Immunoassay.

Results

Eighteen patients received a total of 78 cycles of ATN-224 and their demographic data are shown in Table 1.

Drug toxicities. Overall therapy was well tolerated. A summary of the toxicities experienced with ATN-224 is contained in Table 2. The most frequently occurring drug-related adverse events were fatigue, hematologic adverse events, and sulfur burps.

Drug-related hematologic adverse events grade ≥ 3 were anemia grade 3 (11% of patients), neutropenia grade 3 (22% of patients) and grade 4 (6% of patients), and thrombocytopenia grade 3 (6% of patients). The mean hemoglobin of patients at dose levels 4 to 6 is shown in Fig. 1. At the MTD, dose level 5, the mean baseline hemoglobin was 12.1 g/dL. The reduction in hemoglobin became significant at day 14 (mean, 11 g/dL; $P = 0.046$, paired t test) and fell to a nadir of 9.6 g/dL ($P = 0.01$; range, 7.3-12.1 g/dL) at day 28. The hemoglobin slowly recovered after this but remained significantly reduced at day 42. Two of the 5 patients (16 and 17) at dose level 5 received blood transfusions for grade 3 anemia on day 1 of cycle 2. Both these patients had anemia at baseline (grades 1 and 2, respectively) and both received >2 weeks of loading with ATN-224. One of these patients experienced both grade 3 anemia and neutropenia (patient 17).

Table 2. Display of drug-related adverse events with ATN-224

Adverse event	Maximum severity (Common Toxicity Criteria grade), *n* (%)					
	2		3		4	
Hematologic						
Lymphopenia	10	(55.6)	0	(0)	0	(0)
Neutropenia	3	(16.7)	4 (4,5,6)	(22.2)	1 (6)	(5.6)
Thrombocytopenia	0	(0)	1 (6)	(5.6)	0	(0)
Anemia	8	(44.4)	2 (5)	(11.1)	0	(0)
Blood/bone marrow, other	10	(55.6)	0	(0)	0	(0)
Gastrointestinal						
Nausea	2	(11.1)	0	(0)	0	(0)
Emesis	2	(11.1)	1 (4)	(5.6)	0	(0)
Diarrhea	1	(5.6)	0	(0)	0	(0)
Sulfur eructation	2	(11.1)	0	(0)	0	(0)
Hepatic						
Alkaline phosphatase	0	(0)	1 (5)	(5.6)	0	(0)
Aspartate aminotransferase	0	(0)	1 (5)	(5.6)	0	(0)
γ-Glutamyl transferase	3	(16.7)	0	(0)	0	(0)
Bilirubin	1	(5.6)	0	(0)	0	(0)
Other nonhematologic						
Fatigue	4	(22.2)	4 (4,6)	(22.2)	0	(0)
Infection, urinary	1	(5.6)	0	(0)	0	(0)
Hypophosphatemia	4	(22.2)	0	(0)	0	(0)
Hypokalemia	0	(0)	1 (3)	(5.6)	0	(0)
Neurology, other	0	(0)	0	(0)	1* (6)	(5.6)
Pain	1	(5.6)	0	(0)	0	(0)

NOTE: The maximum severity of each adverse event per patient graded according to National Cancer Institute Common Toxicity Criteria version 2.0. Grades 2 to 4 drug-related (possibly, probably, or certainly related) toxicities are shown. Number of patients and percentage of patients experiencing each toxicity are shown. Number in parentheses shows the dose level at which grades 3 and 4 toxicities occurred.
*Cerebrovascular accident (ischemic) in a patient with progressive disease with metastatic pancreatic carcinoma 4 d after withdrawal of ATN-224 (patient 14, dose level 6).

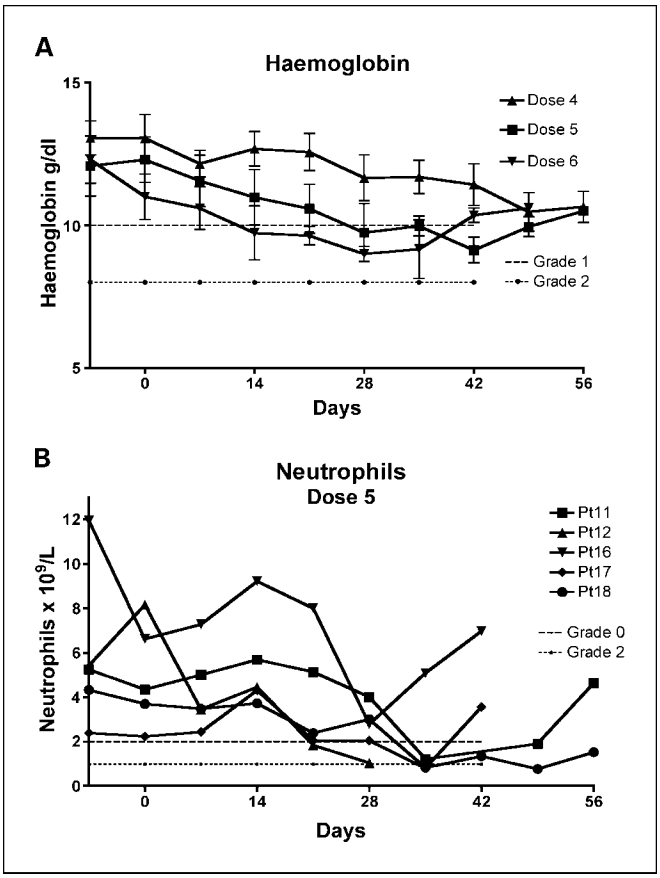

Fig. 1. Effect of ATN-224 on hematologic parameters. *A,* mean hemoglobin (*Hb*) in patients at dose levels 4 (6 patients), 5 (5 patients), and 6 (3 patients). *Bars,* SE. *Top dotted line,* lower limit of grade 1 anemia (hemoglobin 10 g/dL); *bottom dotted line,* lower limit of grade 2 anemia (hemoglobin 8 g/dL). *B,* individual neutrophil counts of patients on ATN-224 300 mg/d loading dose followed by titrated ATN-224 (dose 5, MTD). *Top dotted line,* lower limit of normal neutrophils (2×10^9/L); *bottom dotted line,* lower limit of grade 2 neutropenia (1×10^9/L). The dose shown is the loading dose level.

Grade 3 to 4 neutropenia was seen in 5 patients. There was no associated fever or infection. One patient at dose level 4 experienced grade 3 neutropenia during the fourth cycle when the ceruloplasmin was at the lower limit of the target range (5.5 mg/dL). This responded to cessation of ATN-224 for 1 week. Four patients (2 patients at the MTD at two at dose level 6) experienced neutropenias at the beginning of the second cycle when ceruloplasmin levels >10 mg/dL, and ATN-224 has been reduced to a maintenance dose. The two patients (17 and 18) who developed grade 3 neutropenia at the MTD had received loading for 3 weeks. The neutropenia responded to a reduction in ATN-224 dose and resolved by the end of the second cycle. The individual neutrophil counts of patients at the MTD are shown in Fig. 1.

Grade 3 drug-related fatigue was reported in 4 (22%) patients and these patients also had some degree of anemia. Patient 5 treated at 270 mg/d ATN-224 developed grade 3 fatigue and grade 3 vomiting on day 2 of cycle 1 and ATN-224 was stopped. The fatigue resolved in 6 days and the vomiting in 2 days. Patients 13 to 15 who received 330 mg/d ATN-224 developed grade 3 fatigue rapidly around day 14. These 3 patients all had grade 2 anemia. The fatigue responded to a reduction in drug dose, despite ceruloplasmin levels being maintained in the target range throughout.

The most severe adverse event was a cerebrovascular accident in 1 patient. This patient had metastatic pancreatic cancer with liver metastases and had been withdrawn from the study 4 days before the cerebrovascular accident due to progressive disease. Computed tomography scan showed no brain metastases; magnetic resonance imaging scan suggested some cerebral ischemia. Although the patient had ceased to take ATN-224 before the cerebrovascular accident and was at risk of thrombotic events due to the underlying malignancy, a contribution of ATN-224 cannot be excluded.

Grade 1 to 2 sulfur burps occurred in 11 patients (61% of patients) treated at all dose levels. The concomitant use of lansoprazole was introduced to help reduce this toxicity.

Reduction in ceruloplasmin with ATN-224. The mean baseline ceruloplasmin was 39.6 (range, 22-63 mg/dL). The patients received ATN-224 loading doses at 1 patient per dose level, commencing at 150 mg/d. The second patient was withdrawn at day 15 due to clinically progressive disease (spinal cord compression) and was replaced by a new patient at dose level 2.

Review of the data from the first 3 patients revealed that 2 weeks of loading with ATN-224 at dose levels 1 to 2 was insufficient to lower ceruloplasmin levels and ceruloplasmin thereafter rebounded on the maintenance dose (Fig. 2). The protocol was therefore amended to allow loading to continue until the upper target ceruloplasmin of 15 mg/dL was reached followed by a titrated maintenance dose.

A single patient was enrolled at dose level 3 and achieved the desired ceruloplasmin in 21 days.

Six patients were enrolled at dose level 4 (270 mg/d) due to one DLT (grade 3 fatigue). At this dose level, there was a median time to reduction of ceruloplasmin of 28 days.

Two patients were enrolled at dose level 5. The first achieved target ceruloplasmin reduction in 21 days and the second in 14 days. At dose level 6 (330 mg/d), 2 patients achieved target ceruloplasmin levels in 14 days and 1 patient in 21 days. However, these patients also experienced grade 3 fatigue on day 14; therefore, recruitment of the final 3 patients was made to the lower dose of 300 mg/d. Of the 5 patients at the MTD of 300 mg/d, the median time to reach the target ceruloplasmin was 21 days (Fig. 2) and there were no DLTs.

Effect of proton pump inhibitors. Review of the data from the first 5 patients revealed greater ATN-224 absorption, more rapid reduction in ceruloplasmin, and decreased nausea and sulfur burps in those patients taking concomitant proton pump inhibitors (PPI; patients 2 and 4). A possible explanation for this effect was reduced gastric acidity leading to decreased degradation of ATN-224 in the stomach. The protocol was therefore amended to include medication of all patients with 30 mg lansoprazole before breakfast, starting the day before the pharmacokinetic dose. This amendment was commenced for patient 8 onwards, and patients 6 and 7 commenced lansoprazole on day 21 of treatment. Concomitant medication with a PPI lead to more rapid reductions in ceruloplasmin and higher plasma molybdenum concentrations (Fig. 3).

Reduction in copper levels with ATN-224. Comparison between total plasma copper levels and ceruloplasmin levels for each dose level showed a close correlation and confirmed the validity of ceruloplasmin as a surrogate marker of copper

status [dose level 5: mean total copper day -7 versus 28, 17.5 μmol/L (range, 12.6-20.1) versus 5.57 μmol/L (range, 3.0-10.0); $P = 0.0017$, paired t test; correlation to ceruloplasmin, $r = 0.92$; $P < 0.0001$].

Pharmacokinetic analysis. After a single dose of 60 to 210 mg ATN-224 orally, in the majority of patients, total and free molybdenum were detected out to 72 h (Fig. 3). In 5 patients who were not coadministered PPI, total and free molybdenum, area under the curve, and C_{max} (maximum plasma concentration) were significantly lower than in patients who were coadministered PPIs (total molybdenum doses 1-4: no PPI C_{max}, 1.1 μmol/L; PPI C_{max}, 4.4 μmol/L). In patients on PPIs, there was a dose-dependent increase in C_{max} and area under the curve values for both total and free molybdenum. Normalized to an ATN-224 dose of 180 mg, total molybdenum mean C_{max} was 5.7 ± 1.4 μmol/L/180 mg and t_{max} (time of maximum concentration) occurred at 5.7 ± 1.8 h, elimination half-life was 34.3 ± 6.6 h, and mean residence time 48.8 ± 9.5 h. The free molybdenum fraction was 15.5 ± 4.9%, suggesting that ~85% of the molybdenum species in plasma are protein bound.

With chronic dosing from 90 to 300 mg/d ATN-224 out to days 15 and 22 of cycle 1, the mean plasma molybdenum concentration was 3.2 ± 1.2 μmol/L (range, 1.2-6.8 μmol/L).

Antitumor activity. Patients received a scan after 7 weeks on ATN-224 therapy regardless of whether copper depletion had been obtained. Two patients left the study before day 21 and 3 patients did not achieve target ceruloplasmin levels by the time of their scan. For the remaining 13 patients, 2 (11% of all patients) achieved stable disease for at least 6 months. One patient with metastatic endometrial adenocarcinoma had stable disease for >2.5 years with small reductions in the measurements of abdominal lymphadenopathy.

SOD-1 activity. Blood cell SOD-1 activity was significantly decreased in all patients assayed (patient 5 onwards) after 14 and 28 days of treatment with ATN-224 ($P < 0.0001$, paired t test), and this reduction in SOD-1 activity remained significant at 56 days. The percentage inhibition of SOD-1 activity exceeded the percentage reduction in ceruloplasmin level and increased with drug dose level (mean SOD-1 activity, 32.7% of baseline at 28 days for dose level 4 and 6.8% baseline at dose level 5; Fig. 4). With a few exceptions, plasma SOD-1

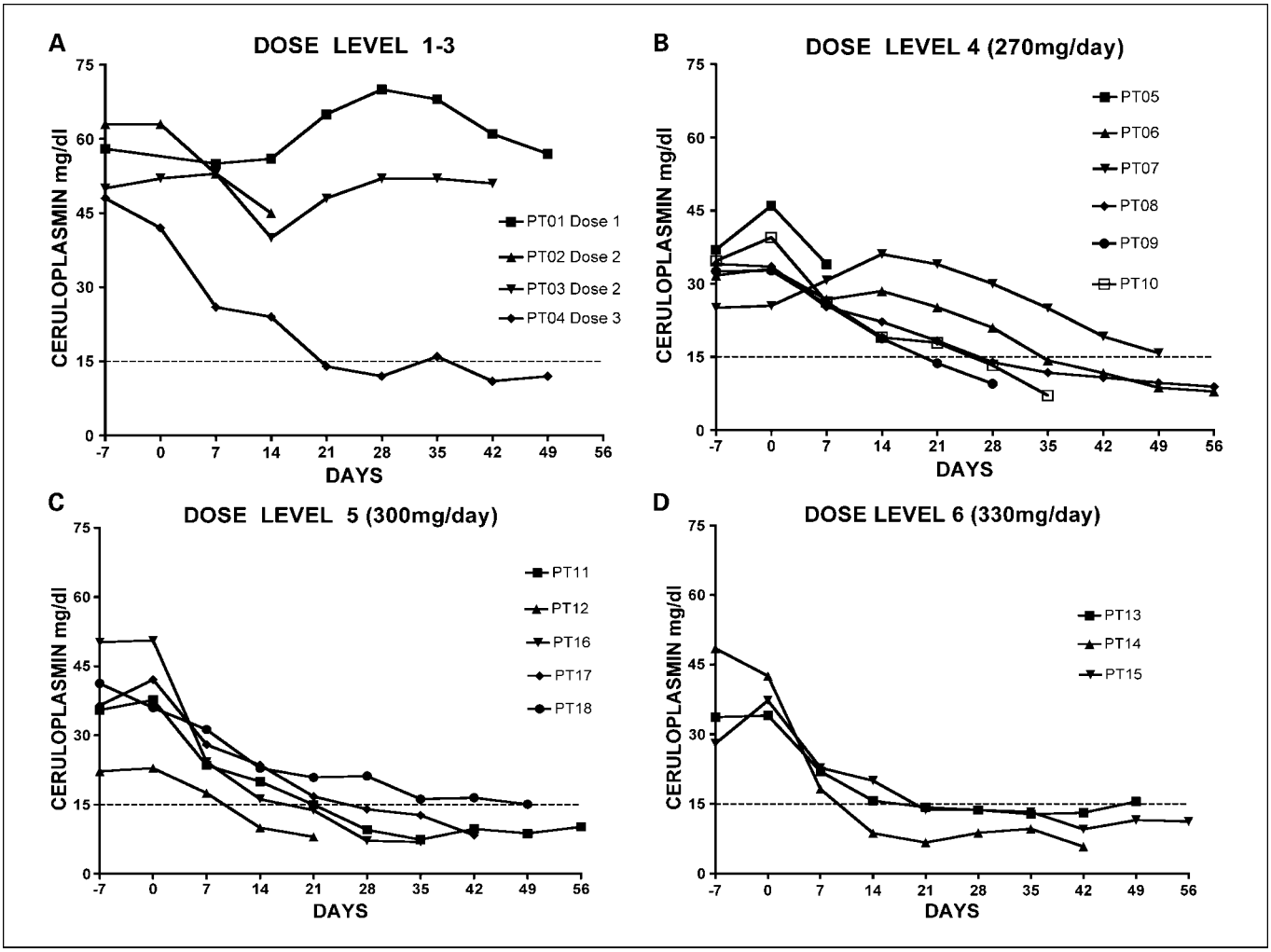

Fig. 2. Decrease in plasma ceruloplasmin with ATN-224 dose level. *A,* dose levels 1 to 3. *B,* dose level 4 (270 mg/d). *C,* dose level 5 (300 mg/d). *D,* dose level 6 (330 mg/d). *Dotted line,* target ceruloplasmin of 15 mg/dL. *CP,* ceruloplasmin. Patients received a pharmacokinetic dose of ATN-224 on day -7 (after ceruloplasmin measurement) and loading with ATN-224 commenced on day 0.

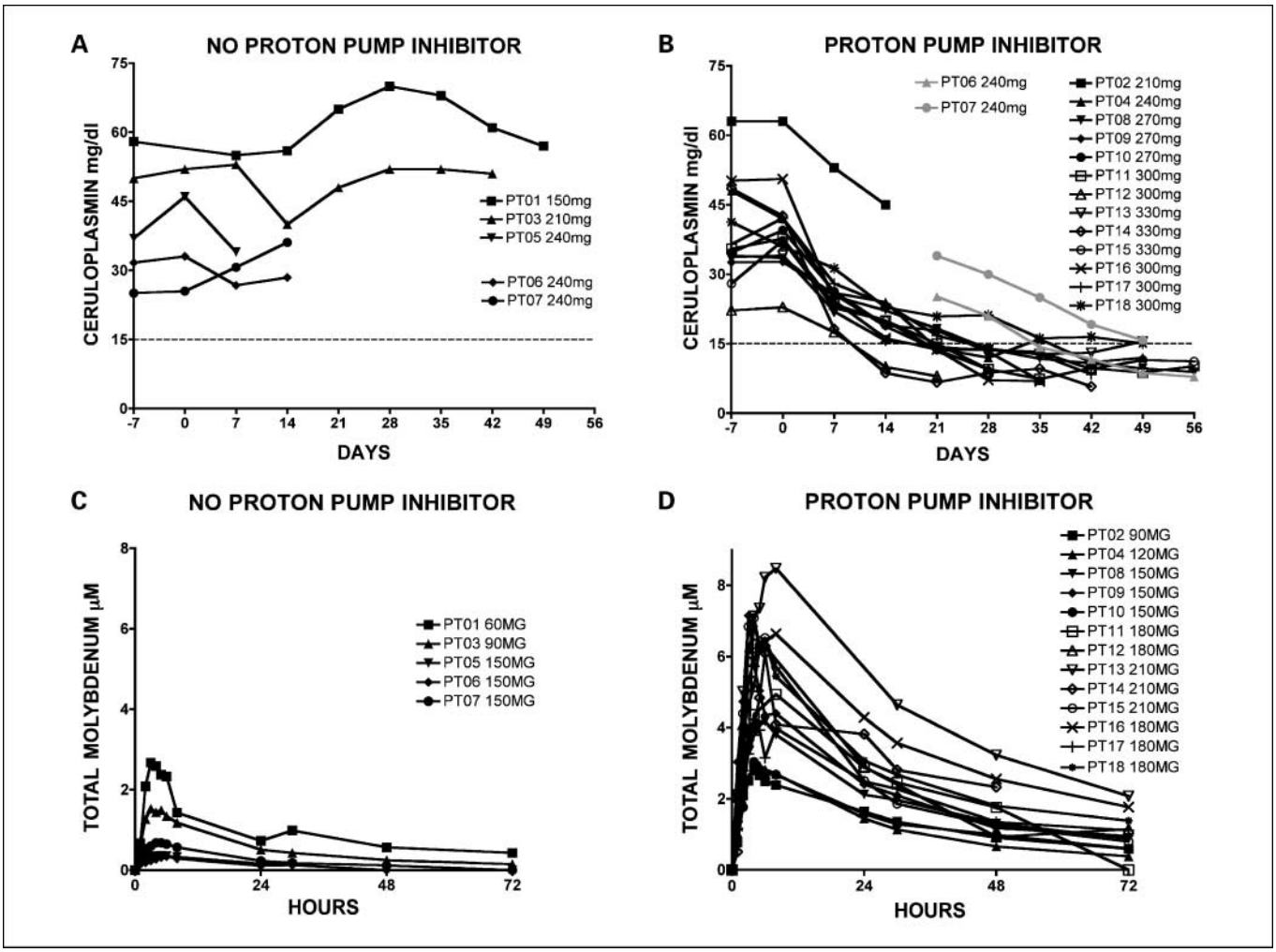

Fig. 3. Effect of PPI on plasma ceruloplasmin and molybdenum levels with ATN-224. *A,* reduction in ceruloplasmin in patients not on PPI. *B,* reduction in ceruloplasmin in patients on PPI. Patients 6 and 7 commenced PPI on day 21; therefore, their data are plotted up to day 14 on Fig. 1A and from day 21 on Fig. 1B. *Dotted line,* target ceruloplasmin of 15 mg/dL. The dose shown is the daily loading dose for each patient. *C,* total plasma molybdenum after a single dose of ATN-224 in patients not taking PPI. *D,* total plasma molybdenum after a single dose of ATN-224 in patients taking PPI. The dose shown is the single pharmacokinetic dose given to each patient.

activity at baseline was below the lower limit of quantification for the assay.

There was an increase in mean ceruloplasmin at dose level 5 on day 56 and at dose level 6 at day 49 accompanied by an increase in blood cell SOD-1 activity, but these increases were not statistically significant (Fig. 4). These were due to reductions in ATN-224 dose on day 28 at dose level 5 due to anemia and neutropenia in some patients, which lead to a rise in ceruloplasmin a few weeks later. This was more marked rise in SOD-1 activity on day 49 at dose level 6. This was due to the 3 patients on dose level 6 being required to have large dose reductions in the second cycle due to DLT. The median dose prescribed on day 29 at dose level 4 was 240 mg (range, 210-270 mg), dose level 5 was 90 mg (range, 60-180 mg), and dose level 6 was 90 mg (range, 60-120 mg). However, as documented in Fig. 1, ceruloplasmin still remained within the target range for those patients where the target range had been achieved.

Circulating endothelial cells. There were significant interpatient variations in the pretreatment CEC and EPC numbers as shown by the differences between the baseline values for patients at the different dose levels (baseline CEC, 182 ± 135, 258 ± 256, and 135 ± 71 cells/mL for dose levels 4-6, respectively; baseline EPC, 71 ± 81, 250 ± 297, and 88 ± 68 cells/mL for dose levels 4-6, respectively). Analysis of mature CECs and EPCs, however, showed a significant reduction in both types of cell in plasma after 14 days on ATN-224 for patients at dose levels 4 to 6 combined and this reduction was maintained at 28 days (mean EPC at baseline, days 14 and 28 were 127, 37, and 34 cells/mL, respectively; days -14 versus 14, $P = 0.029$, and versus day 28, $P = 0.043$, paired t test; mean CEC at baseline, days 14 and 28 were 199, 103, and 92 cells/mL, respectively; days -14 versus 14, $P = 0.042$, and versus day 28, $P = 0.037$, paired t test).

Circulating cytokines. The mean baseline circulating VEGF concentration for all patients was 67 pg/mL (range, 18-251) and after 28 days treatment with ATN-224 was 56 pg/mL (range, 20-172; nonsignificant). There was a trend to reduction in circulating VEGF levels at all dose levels, but these did not reach significance. No dose-dependent effects were observed on the other cytokines assayed (fibroblast growth factor-2, interleukin-6, and interleukin-8).

Discussion

Previous studies of copper chelation therapy in oncology have shown decreased plasma cytokines and extended periods of stable disease in some patients but have been limited by the time taken to reduce plasma copper levels and gastrointestinal side effects (13, 16). This study has shown that, by using a loading dose of 300 mg/d followed by a titrated maintenance dose, ATN-224 can reduce serum copper levels in a mean of 21 days with twice daily dosing and an acceptable side effect profile. This is in comparison with tetrathiomolybdate, which, in a phase II study of 180 mg/d (which contains equivalent amounts of active moiety as 290 mg ATN-224), leads to a reduction of ceruloplasmin to target levels in a median of 35 days (16). In one study of tetrathiomolybdate, the dose was not reduced when the target range was reached but rather when the hematocrit fell by >20% or the patient experienced grade 3/4 toxicity. Ceruloplasmin has a half-life of 5 days, leading to a lag between degree of ceruloplasmin inhibition and the corresponding ceruloplasmin plasma levels. By using our regimen, and reducing the ATN-224 dose as soon as the ceruloplasmin reached the upper limit of the target range, it was possible to achieve the target ceruloplasmin without overshooting. We chose to give ATN-224 after meals to reduce sulfur burps caused by the breakdown of ATN-224 in the stomach. This was further reduced by gastric acid suppression that also leads to increased plasma levels of ATN-224, which is likely due to increased gastric pH leading to decreased drug decomposition in the stomach.

Previous studies have suggested that ceruloplasmin levels above 5 mg/dL are not associated with significant toxicity, except mild anemia (13). However, in this study, high doses of ATN-224 (330 mg/d) lead to grade 3 fatigue after 14 days of therapy and grade 2 to 4 neutropenia after 28 days of therapy, even in those patients whose ceruloplasmin was maintained above 10 mg/dL throughout. Fatigue was the DLT with ATN-224 and has not been described previously with copper chelation therapy. It occurred in all 3 patients who received 330 mg/d and presented 10 to 14 days after commencing therapy. The fatigue appeared to be related to the dose of ATN-224 and rate of reduction of ceruloplasmin, rather than the absolute level of ceruloplasmin, which was above 5 mg/dL in all 3 patients. However, ceruloplasmin has a long half-life and at these high loading doses ceruloplasmin may not be an

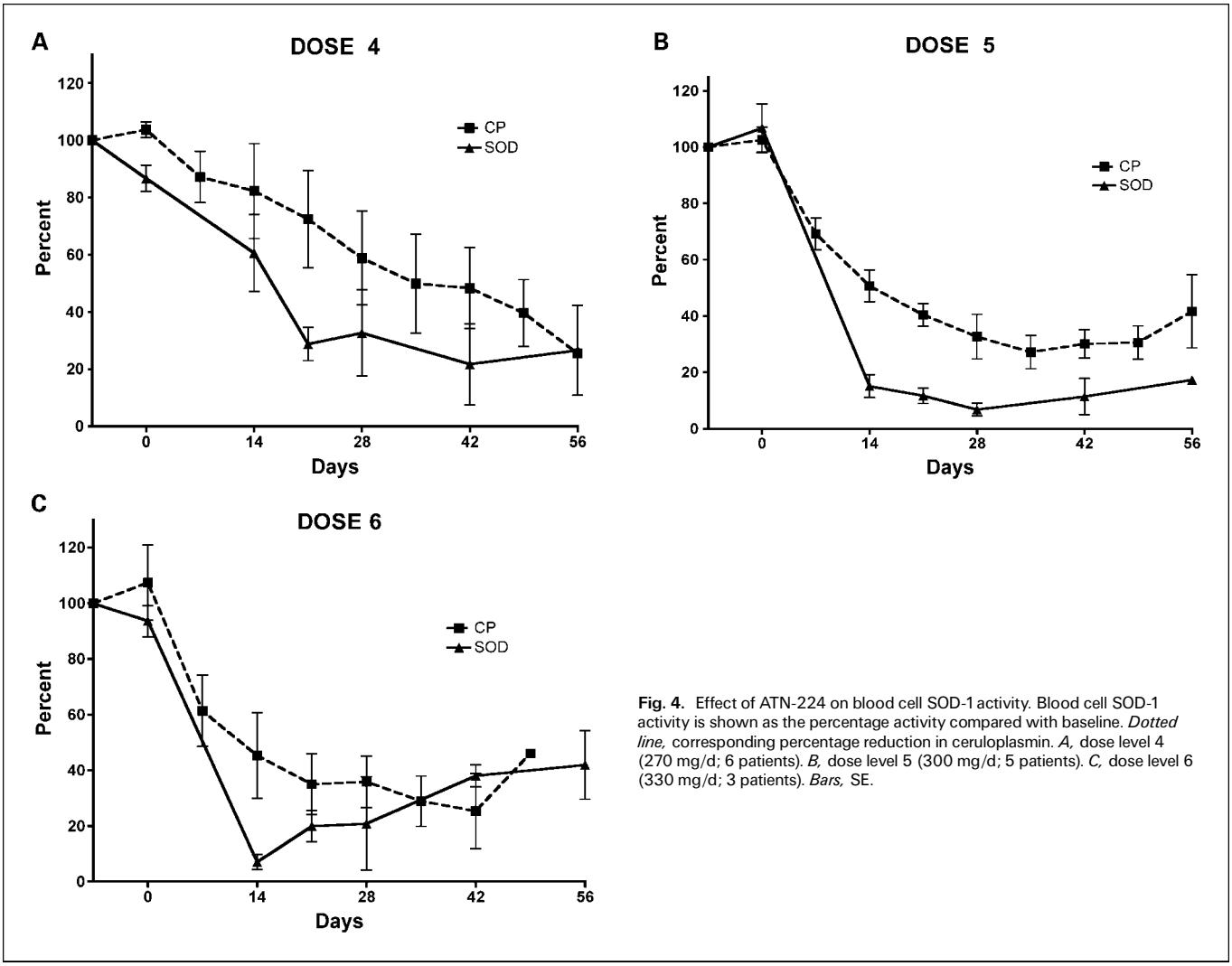

Fig. 4. Effect of ATN-224 on blood cell SOD-1 activity. Blood cell SOD-1 activity is shown as the percentage activity compared with baseline. *Dotted line,* corresponding percentage reduction in ceruloplasmin. *A,* dose level 4 (270 mg/d; 6 patients). *B,* dose level 5 (300 mg/d; 5 patients). *C,* dose level 6 (330 mg/d; 3 patients). *Bars,* SE.

adequate indicator of free copper levels. At 330 mg/d ATN-224, there was a 93% reduction in blood cell SOD-1 activity compared with a 55% reduction in serum ceruloplasmin after 14 days, and this may be a more sensitive indicator of depletion of copper from copper-dependent enzymes, such as cytochrome *c* oxidase, and other cellular processes. All 3 patients who experienced grade 3 fatigue at the highest dose level had coexistent grade 2 anemia, which is likely to have contributed to the symptom.

Neutropenia has been described previously in copper deficiency (18) and may be due to arrested maturation of granulocytes (19). Grade 3 to 4 neutropenia was also seen in the phase II study of tetrathiomolybdate (16). Grade 1 to 2 anemia was seen in the majority of patients on ATN-224 and grade 3 anemia was seen in 2 patients at the MTD. It is well recognized that copper is essential for iron metabolism and erythropoiesis, and copper-deficient animals and humans develop iron deficiency anemia (20, 21). These toxicities diminished during the second cycle of treatment and on maintenance therapy.

No cardiac toxicity was documented in patients on ATN-224 including one female patient age 70 years who has been on study for >2.5 years. This is important because cardiomyopathy and cardiac failure have been observed in animals fed a copper-deficient diet (22, 23) and copper deficiency has suggested to be linked to ischemic heart disease in humans (24).

Pharmacokinetic analysis showed that, at the MTD, twice daily dosing with 150 mg ATN-224 with concomitant lansoprazole leads to plasma molybdenum levels of 4 to 8 μmol/L, and chronic dosing with a maintenance dose of 90 to 150 mg/d leads to plasma drug levels greater than 1 μmol/L. These plasma concentrations are above those at which the inhibitory effects of ATN-224 are seen on assays of angiogenesis in the laboratory (15). ATN-224 had a half-life greater than 30 h; therefore, once daily dosing is possible. We chose to use twice daily dosing during the loading phase to decrease gastrointestinal side effects, but once daily dosing in the maintenance phase is acceptable and well tolerated.

In this phase I study, tumor response was limited to stable disease in 2 patients. This is lower than reported in other phase I and II studies of tetrathiomolybdate (13, 16). However, computed tomography scans were done at baseline 2 to 3 weeks before commencing ATN-224 and repeated after 7 to 8 weeks of therapy regardless of whether copper depletion had been obtained. This is in contrast to the tetrathiomolybdate studies in which the baseline scan was not done until copper depletion was obtained; therefore, there cannot be a direct comparison of the studies in terms of tumor response.

There was biomarker evidence of antiangiogenic function of ATN-224 in humans. CECs are a marker of angiogenic activity in tumors and are reduced by surgery and chemotherapy (25). Circulating EPCs are also raised in malignancy and correlate with response to treatment (26). Both types of endothelial cells have therefore been proposed as a surrogate marker for monitoring antiangiogenic therapy (27). There was a significant reduction in mature CECs and EPCs after 14 days of ATN-224 treatment and this was sustained at 28 days and beyond in patients remaining on treatment. There was, however, considerable interpatient and intrapatient variability in the baseline levels of CECs and EPCs and their response to ATN-224 and they will continue to be investigated as potential biomarkers of ant-angiogenic activity in subsequent phase II studies.

SOD-1 activity has been linked previously to angiogenesis (28, 29) and preclinical studies have shown that ATN-224 inhibits SOD-1 activity *in vitro* (15). In this study, we found that ATN-224 potently inhibits blood cell SOD-1 activity in patients and this inhibition occurs earlier and to a greater extent than the reduction in ceruloplasmin. Because inhibition of SOD-1 activity may represent the primary action of ATN-224, this assay may potentially be used to monitor and titrate copper chelation therapy and will be followed in future studies.

The toxicities seen with ATN-224 diminished during the second cycle of treatment and on maintenance therapy. Further to the data presented here, a 2-week loading dose of 300 mg/d followed by a titrated maintenance dose of 90 to 150 mg/d ATN-224 given with a PPI is recommended for ongoing investigation in a phase II study. A limit of 14 days loading is suggested to reduce the incidence of neutropenia seen in the second cycle and thereby to enable the investigation of combination therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Folkman J. Fundamental concepts of the angiogenic process. Curr Mol Med 2003;3:643–51.
2. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 2004; 350:2335–42.
3. Shim H, Harris ZL. Genetic defects in copper metabolism. J Nutr 2003;133:1527–31S.
4. Hu GF. Copper stimulates proliferation of human endothelial cells under culture. J Cell Biochem 1998;69: 326–35.
5. Parke A, Bhattacherjee P, Palmer RM, Lazarus NR. Characterization and quantification of copper sulfate-induced vascularization of the rabbit cornea. Am J Pathol 1988;130:173–8.
6. Brem SS, Zagzag D, Tsanaclis AM, Gately S, Elkouby MP, Brien SE. Inhibition of angiogenesis and tumor growth in the brain. Suppression of endothelial cell turnover by penicillamine and the depletion of copper, an angiogenic cofactor. Am J Pathol 1990;137:1121–42.
7. Pan Q, Kleer CG, van Golen KL, et al. Copper deficiency induced by tetrathiomolybdate suppresses tumor growth and angiogenesis. Cancer Res 2002;62: 4854–9.
8. Landriscina M, Bagala C, Mandinova A, et al. Copper induces the assembly of a multiprotein aggregate implicated in the release of fibroblast growth factor 1 in response to stress. J Biol Chem 2001;276: 25549–57.
9. Patstone G, Maher P. Copper and calcium binding motifs in the extracellular domains of fibroblast growth factor receptors. J Biol Chem 1996;271:3343–6.
10. Soncin F, Guitton JD, Cartwright T, Badet J. Interaction of human angiogenin with copper modulates angiogenin binding to endothelial cells. Biochem Biophys Res Commun 1997;236:604–10.
11. Cox C, Teknos TN, Barrios M, Brewer GJ, Dick RD, Merajver SD. The role of copper suppression as an antiangiogenic strategy in head and neck squamous cell carcinoma. Laryngoscope 2001;111:696–701.
12. Khan MK, Miller MW, Taylor J, et al. Radiotherapy and antiangiogenic TM in lung cancer. Neoplasia 2002;4:164–70.
13. Brewer GJ, Dick RD, Grover DK, et al. Treatment of metastatic cancer with tetrathiomolybdate, an anticopper, antiangiogenic agent: phase I study. Clin Cancer Res 2000;6:1–10.
14. Brewer GJ. Copper control as an antiangiogenic anticancer therapy: lessons from treating Wilson's disease. Exp Biol Med (Maywood) 2001;226:665–73.
15. Juarez JC, Betancourt O, Jr., Pirie-Shepherd SR, et al. Copper binding by tetrathiomolybdate attenuates angiogenesis and tumor cell proliferation through the inhibition of superoxide dismutase 1. Clin Cancer Res 2006;12:4974–82.
16. Redman BG, Esper P, Pan Q, et al. Phase II trial of tetrathiomolybdate in patients with advanced kidney cancer. Clin Cancer Res 2003;9:1666–72.
17. Gitlin JD, Schroeder JJ, Lee-Ambrose LM, Cousins RJ. Mechanisms of caeruloplasmin biosynthesis in normal and copper-deficient rats. Biochem J 1992; 282:835–9.

18. Nagano T, Toyoda T, Tanabe H, et al. Clinical features of hematological disorders caused by copper deficiency during long-term enteral nutrition. Intern Med 2005;44:554–9.
19. Karimbakas J, Langkamp-Henken B, Percival SS. Arrested maturation of granulocytes in copper deficient mice. J Nutr 1998;128:1855–60.
20. Lee GR, Nacht S, Lukens JN, Cartwright GE. Iron metabolism in copper-deficient swine. J Clin Invest 1968;47:2058–69.
21. Danks DM. Copper deficiency in humans. Ciba Found Symp 1980;79:209–25.
22. Li Y, Wang L, Schuschke DA, Zhou Z, Saari JT, Kang YJ. Marginal dietary copper restriction induces cardiomyopathy in rats. J Nutr 2005;135:2130–6.
23. Elsherif L, Ortines RV, Saari JT, Kang YJ. Congestive heart failure in copper-deficient mice. Exp Biol Med (Maywood) 2003;228:811–7.
24. Klevay LM. Cardiovascular disease from copper deficiency—a history. J Nutr 2000;130:489–92S.
25. Mancuso P, Calleri A, Cassi C, et al. Circulating endothelial cells as a novel marker of angiogenesis. Adv Exp Med Biol 2003;522:83–97.
26. Igreja C, Courinha M, Cachaco AS, et al. Characterization and clinical relevance of circulating and biopsy-derived endothelial progenitor cells in lymphoma patients. Haematologica 2007;92:469–77.
27. Bertolini F, Shaked Y, Mancuso P, Kerbel RS. The multifaceted circulating endothelial cell in cancer: towards marker and target identification. Nat Rev Cancer 2006;6:835–45.
28. Marikovsky M, Nevo N, Vadai E, Harris-Cerruti C. Cu/Zn superoxide dismutase plays a role in angiogenesis. Int J Cancer 2002;97:34–41.
29. Marikovsky M. Thiram inhibits angiogenesis and slows the development of experimental tumours in mice. Br J Cancer 2002;86:779–87.