

Antiangiogenic Scheduling of Chemotherapy Improves Efficacy against Experimental Drug-resistant Cancer¹

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ABSTRACT

To reveal the antiangiogenic capability of cancer chemotherapy, we developed an alternative antiangiogenic schedule for administration of cyclophosphamide. We show here that this antiangiogenic schedule avoided drug resistance and eradicated Lewis lung carcinoma and L1210 leukemia, an outcome not possible with the conventional schedule. When Lewis lung carcinoma and EMT-6 breast cancer were made drug resistant before therapy, the antiangiogenic schedule suppressed tumor growth 3-fold more effectively than the conventional schedule. When another angiogenesis inhibitor, TNP-470, was added to the antiangiogenic schedule of cyclophosphamide, drug-resistant Lewis lung carcinomas were eradicated. Each dose of the antiangiogenic schedule of cyclophosphamide induced the apoptosis of endothelial cells within tumors, and endothelial cell apoptosis preceded the apoptosis of drug-resistant tumor cells. This antiangiogenic effect was more pronounced in p53-null mice in which the apoptosis of p53-null endothelial cells induced by cyclophosphamide was so vigorous that drug-resistant tumors comprising 4.5% of body weight were eradicated. Thus, by using a dosing schedule of cyclophosphamide that provided more sustained apoptosis of endothelial cells within the vascular bed of a tumor, we show that a chemotherapeutic agent can more effectively control tumor growth in mice, regardless of whether the tumor cells are drug resistant.

INTRODUCTION

Since anticancer cytotoxic chemotherapy was first introduced over 50 years ago (1), the repertoire of drugs directed against tumor cells has greatly increased. Despite these advances, the genetic instability and high mutation rate of neoplastic cells ensure that chemotherapy directed mainly or solely at the neoplastic cell still carries a high risk of selection for drug resistance (2). Preclinical studies of experimental cancer in mice conducted in the 1960s determined that one of several chemotherapy schedules tested, the maximum tolerated dose, yielded a higher percentage cure rate (3). This schedule, which consisted of the highest survivable (minimum lethal) dose, was chosen for the conventional administration of chemotherapy to cancer patients. However, such high, up-front doses required an extended treatment-free period to permit recovery of normal host cells, *e.g.*, rapidly growing hematopoietic progenitors (4). Similar to hematopoietic progenitors, the vascular endothelial cells in the tumor bed might also resume growth during this treatment-free period. We hypothesized that endothelial cell recovery occurring during this treatment-free period could support regrowth of tumor cells. This could increase the risk of the emergence of drug-resistant tumor cells.

Although tumor cells readily acquire resistance to cytotoxic chemotherapy, this would not be expected for vascular endothelial cells (5,

6). To more effectively suppress the proliferating endothelial cells in the tumor bed, a dosing schedule was developed that administered cyclophosphamide at shorter intervals without interruption. This antiangiogenic schedule of cyclophosphamide: (a) increased apoptosis of endothelial cells within the tumor bed; (b) secondarily increased apoptosis of cyclophosphamide-resistant tumor cells; (c) demonstrated long-term suppression of the growth of cyclophosphamide-resistant Lewis lung carcinoma and EMT-6/CTX breast carcinoma (7), a significant improvement over the conventional schedule; (d) eradicated drug-sensitive Lewis lung carcinoma (8) and L1210 leukemia (9) tumors by avoiding acquired drug resistance, an outcome not possible with the conventional schedule; and (e) eradicated the majority of drug-resistant Lewis lung carcinomas when combined with another angiogenesis inhibitor, TNP-470 (10).

MATERIALS AND METHODS

Mouse Experiments. After the eighth cycle of selection for drug resistance as detailed in "Results," drug-resistant Lewis lung carcinoma was explanted into tissue culture as described for the cyclophosphamide-resistant breast cancer cell line EMT-6/CTX (7). The EMT-6/CTX breast cancer cell line (7) was obtained as a generous gift from Dr. Beverly Teicher (Eli Lilly, Indianapolis, IN), and the drug-sensitive L1210 leukemia cell line (9) was obtained from the American Type Culture Collection (Manassas, VA). All cancer cell lines, including the original, drug-sensitive Lewis Lung carcinoma (8), were screened for mouse hepatitis virus and other pathogens and frozen in aliquots in liquid nitrogen. For tumor studies with Lewis lung carcinoma, cells were thawed and passaged once in C57Bl6/J mice (Jackson Laboratories, Bar Harbor, ME). When tumor volumes reached 200 mm³ (7.5 mm in diameter), mice harboring drug-resistant Lewis lung carcinoma received cyclophosphamide (170 mg/kg) *s.c.* every 6 days for two cycles, and then the tumor was allowed to grow for transfer. Tumor brei of drug-sensitive or drug-resistant Lewis lung carcinoma (10⁶ cells/0.1 ml) was inoculated *s.c.* and dorsally between the scapulae in 28–30-g adult male C57Bl6/J or p53^{-/-} C57Bl6/J mice (Jackson Laboratories). Therapy was initiated 2–4 days after inoculation, just as tumor volumes reached 100 mm³ (6 mm in diameter). Drug-resistant EMT-6/CTX maintains *in vivo* drug resistance after up to 6 months of *in vitro* culture (7). EMT-6/CTX cells expanded in culture for less than 2 weeks were similarly injected (10⁶ cells/0.1 ml) into male 28–30-g CByD2F1/J mice (Jackson Laboratories), and treatment was also initiated as tumors reached 100 mm³ (6 mm in diameter). L1210 cells from *in vitro* culture (3 × 10⁵ cells/0.1 ml) were implanted into the right posterior lateral flank of 28–30-g male B6D2F1/J mice (Jackson Laboratories) because tumor growth in the midline dorsum frequently resulted in early paraplegia. In separate experiments, treatment of L1210 tumors was initiated as tumor volumes reached 100 (6 mm in diameter), 200 (7.5 mm in diameter), 500 (10 mm in diameter), and 1000 mm³ (12.5 mm in diameter), respectively. Mice harboring drug-sensitive and drug-resistant Lewis lung carcinoma received ondansetron (3 mg/kg) and dexamethasone (1 mg/kg) *s.c.* 30 min before cyclophosphamide to ameliorate gastrointestinal dysfunction (11) and chronic weight loss. This therapy was omitted in the CByD2F1/J mice harboring EMT-6/CTX because of a lethal idiosyncratic toxicity and in therapy of L1210 leukemia because of a possible direct antileukemic effect. Preparation of cyclophosphamide and measurement of tumors were performed as described previously (6). For combination experiments with TNP-470, all drugs were administered *s.c.* Mice in these experiments were fed a "Western-type" diet with 42% of calories from fat (TD

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88137; Harlan Teklad, Madison, WI) to ameliorate weight loss. These food pellets were placed on the floor of the cage. TNP-470 (a gift from TAP Holdings, Inc., Deerfield, IL) was obtained as a lyophilized powder of 100 mg of drug and 726 mg of G2- β -cyclodextrin. Lyophilized powder (10.3 mg) was reconstituted with 0.95 ml of sterile 5% glucose in water just before administration. TNP-470 was injected s.c. at 0.01 ml/g body weight on the opposite flank 30 min after cyclophosphamide. After seven cycles of cyclophosphamide on the antiangiogenic schedule with TNP-470, approximately 70% of treated mice received 1–2 ml, twice-daily, s.c. administration of dextrose/saline (10% glucose in 75 mM NaCl at 37°C) for 3–5 days because during this brief time period, the mice ate and drank poorly. All cages were changed twice each week, and all experiments were carried out in the animal facilities at Children's Hospital in accordance with approved protocols.

In Vitro Experiments. For proliferation, 12,500 bovine adrenal capillary endothelial cells (12) in DMEM and 10% bovine calf serum were plated onto gelatinized (8) 24-well plates in quadruplicate. For apoptosis and cell cycle determinations, 2×10^6 cells were similarly split into T150 flasks, and 16 h later, the media were aspirated and replaced with DMEM and 5% bovine calf serum with or without 5 ng/ml bFGF³ (Scios Nova, Mountain View, CA) as indicated. Freshly reconstituted 4-hydroperoxycyclophosphamide (Omicron Biochemicals, San Antonio, TX), which spontaneously converts to 4-HC in aqueous solution, was added at the concentrations indicated, and 18 h later, the cells were trypsinized and enumerated for proliferation as described previously (8) or washed with PBS and incubated with annexin-fluorescein as per the ApoAlert Annexin V apoptosis detection kit (Clontech, Palo Alto, CA). Cells were then washed in cold PBS, fixed by drop-wise dispersion while vortexing into cold 80% ethanol, and incubated for 30 min on ice. Cells were again washed in cold PBS. Propidium iodide (Sigma, St. Louis, MO) and RNase A (Boehringer Mannheim, Indianapolis, IN) were added to give a concentration of 2.5 and 50 μ g/ml, respectively. Samples were incubated for 30 min at 37°C and analyzed by flow cytometry. For migration studies, bovine capillary endothelial cells were exposed to 4-HC as described above. Migration was performed as described previously (13) without adding additional 4-HC.

Corneal Angiogenesis Assay. The antiangiogenic efficacy of different schedules of cyclophosphamide and other chemotherapeutic agents was screened using the 6-day mouse corneal angiogenesis assay (8). Cyclophosphamide was administered as described previously (6) on schedules detailed in "Results" and in Fig. 2, *b* and *c*. 5-Fluorouracil (Roche Laboratories, Nutley, NJ) or 6-mercaptopurine ribose phosphate (Sigma) was administered as daily bolus injections of 50 mg/kg/day \times 5 days (conventional schedule) or as 50 mg/kg/day continuous infusions (antiangiogenic schedule) via Alzet osmotic minipumps (#2001; ALZA Pharmaceuticals, Palo Alto, CA). Pumps were surgically implanted in the peritoneal cavity of large (30–35-g) C57Bl6/J mice on the day before corneal pellet implantation. Inhibition was determined as described in the legend to Fig. 2 on day 6 ($n = 4$ mice/group; repeated twice with similar results). Doxorubicin hydrochloride (Gensia Laboratories, Irvine, CA) or the pegylated liposomal formulation (Doxil, Sequus Pharmaceuticals, Menlo Park, CA) was administered in 5% dextrose in water at 2.5 mg/kg (doxorubicin equivalent dose) once by tail vein injection in severe combined immunodeficient (SCID) mice (Massachusetts General Hospital, Boston, MA) 24 h after pellet implant. Inhibition was similarly determined ($n = 6$ mice/group; experiment repeated twice with similar results).

Tumor Cell and Endothelial Cell Turnover. Mice harboring drug-resistant Lewis lung carcinoma received injections of 0.5 ml of 10 mM BrdUrd (Boehringer Mannheim, Indianapolis, IN) in PBS i.p. 1 h before being euthanized with Metofane (Mallinckrodt Veterinary, Mundelein, IL), followed by cervical subluxation. For mice on the conventional schedule, drug-resistant Lewis lung carcinomas were analyzed on days 1, 3, 5, 7, 10, 13, 17, and 21. For p53^{+/+} mice on the antiangiogenic schedule, tumors were analyzed on days 1, 2, 4, 6, 6.5, 7, 8, 10, 12, 14, 16, 19, and 21. For p53^{-/-} mice on the antiangiogenic schedule, tumors were analyzed on days 1, 2, 4, and after the second dose of cyclophosphamide on day 6 at 10, 20, and 180 min and days 7, 13, 19, and 25. Day 0 reflects the analysis of two control tumors, each of p53^{+/+} or p53^{-/-} mice harvested at 100–200 mm³. Tumors were resected, fixed immediately in cold buffered formalin, incubated overnight at 4°C,

changed into cold PBS, and paraffin-embedded within 24 h of excision. Tumor sections of 5 μ m were deparaffinized. Antigen retrieval included 10 mM EDTA (pH 6.0) at 70°C for 5 min, which was allowed to cool to room temperature for 45 min, followed by digestion with 10 μ g/ml proteinase K (Boehringer Mannheim) in 0.1 M Tris (pH 7.4) at 37°C for 20 min. TUNEL assay was performed according to the fluorescein ApopTag kit (Oncor, Gaithersburg, MD). Slides were incubated with rabbit antihuman von Willebrand factor polyclonal antibody (DAKO, Carpinteria, CA) at 1:500 overnight at 4°C. Biotinylated antirabbit secondary antibody was added, followed by Texas Red-avidin and anti-digoxigenin-fluorescein. Sections were costained with Hoechst 33258 (Sigma). Slides were photographed using an Axiophot photomicroscope equipped with a Texas Red and fluorescein double filter (Zeiss, Oberkochen, Germany). The same field was then photographed using the Hoechst filter. Total endothelial cell apoptosis (*yellow nuclei*) per microvessel count (*red segments*) was tabulated per $157 \times$ field from projected 35-mm slides. Total tumor cell apoptosis was determined by counting tumor cell apoptotic nuclei (*green*) per total Hoechst staining nuclei (*blue*) for each slide pair. Results were plotted as the mean of over 25 separate fields for each day \pm the SE. Two independent observers obtained similar results.

RESULTS

Determination of an Optimum Antiangiogenic Dosing Schedule for Cyclophosphamide

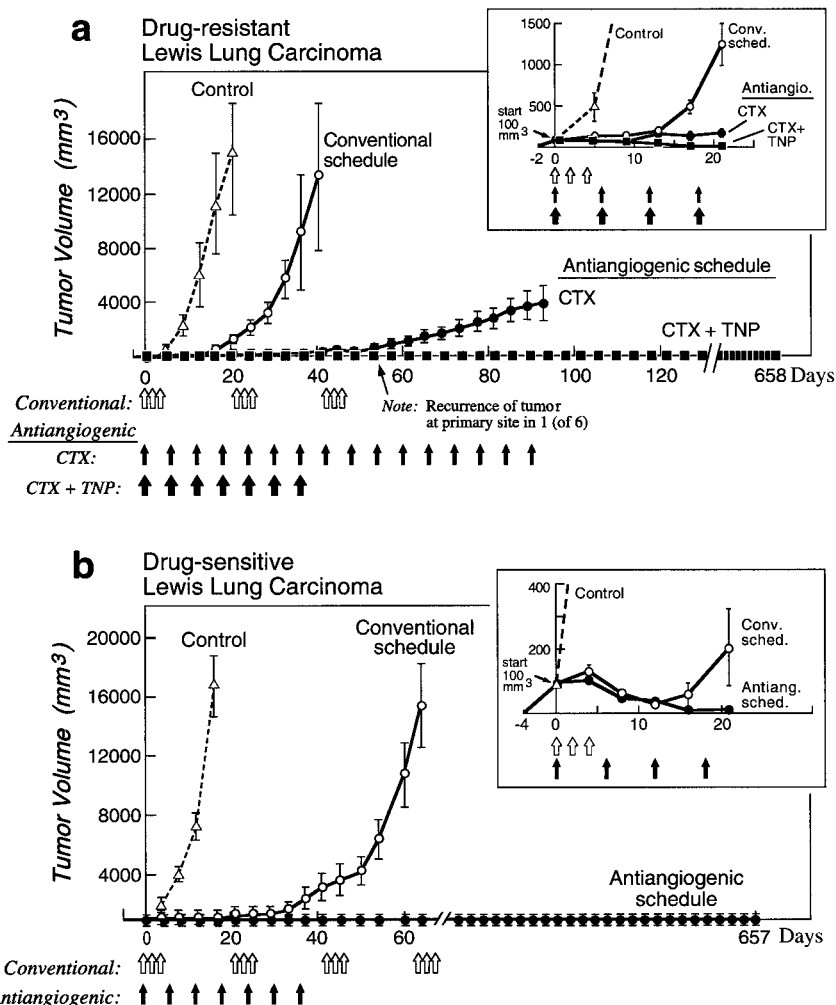
Lewis lung carcinoma is the most refractory murine tumor used by the National Cancer Institute for screening effective chemotherapy (14). We reasoned that by rendering this tumor highly drug resistant, any dosing schedule that improved tumor control was likely to be the direct result of optimized antiangiogenic activity. We then followed a method similar to that of Teicher *et al.* (7) and selected for drug-resistant Lewis lung carcinoma by treatment of tumor-bearing mice with a supra-lethal dose of cyclophosphamide (500 mg/kg). After 24 h, the tumor was passaged into syngeneic mice, and selection was continued over eight cycles of passage and retreatment. The relative resistance of this drug-resistant Lewis lung carcinoma was then compared to the original, drug-sensitive tumor by treatment of tumor-bearing mice with cyclophosphamide (500 mg/kg) *in vivo*. After 24 h, tumor tissue was isolated, digested with collagenase, and plated *in vitro*. The drug-resistant Lewis lung carcinoma yielded 25-fold more colonies of Lewis lung carcinoma cells than the drug-sensitive tumor (data not shown).

Cyclophosphamide was then administered daily or every 3, 4, 5, 6, 7, or 8 days to mice bearing drug-resistant Lewis lung carcinoma. Each of our dosing schedules used higher doses and was more sustained than similar nonconventional schedules (15) reported previously for Lewis lung carcinoma (16, 17) yet resulted in no more than 5% weight loss over the duration of the experiment. Cyclophosphamide (170 mg/kg) every 6 days proved more effective in controlling tumor growth than other cyclophosphamide schedules tested [including schedules with a higher dose intensity (*e.g.*, 135 mg/kg every 4 days; data not shown)].

In Fig. 1*a*, the growth of drug-resistant Lewis lung carcinoma in mice treated with cyclophosphamide on a conventional schedule of the maximum tolerated dose (Refs. 4 and 18; 150 mg/kg every other day for three doses given every 21 days = 450 mg/kg every 21 days) is compared to tumor growth on our antiangiogenic schedule (170 mg/kg every 6 days). On the conventional schedule, drug-resistant tumors escaped by day 13 and grew rapidly (Fig. 1*a*, *inset*). In addition, these mice lost 21% of body weight, which was regained before the next treatment cycle. In contrast, on the antiangiogenic schedule, there was no net tumor growth for 36 days, and weight loss was less than 5%. After the first seven cycles (36 days) of therapy on the antiangiogenic schedule, tumor growth occurred at a slow rate. This partial escape from complete suppression of a drug-resistant

³ The abbreviations used are: bFGF, basic fibroblast growth factor; 4-HC, 4-hydroxycyclophosphamide; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; BrdUrd, bromodeoxyuridine.

Fig. 1. *a*, antiangiogenic versus conventional scheduling of cyclophosphamide for drug-resistant Lewis lung carcinoma. Δ , control saline; \circ , conventional schedule [150 mg/kg every other day for three doses (white arrows, total 450 mg/kg) every 21 days]; \bullet , antiangiogenic schedule (170 mg/kg every 6 days, CTX, thin black arrows); \blacksquare , antiangiogenic schedule of cyclophosphamide and TNP-470 (170 mg/kg cyclophosphamide and 12.5 mg/kg TNP-470 administered on the same day of the 6-day cycle for seven cycles, CTX + TNP, thick black arrows). The inset (top right) has magnified axes for the first 21 days of therapy ($n = 6$ mice/group). All control and conventional schedule-treated mice died with large tumor burdens. Therapy was discontinued on the antiangiogenic schedule of cyclophosphamide alone after two of six mice died with pulmonary inflammation, accompanied by high peripheral leukocyte counts. No mouse on either schedule had visibly detectable pulmonary metastases at time of death. Therapy was discontinued on the antiangiogenic schedule of cyclophosphamide plus TNP-470 after seven cycles, three cycles beyond the point at which tumors were no longer visible. This graph depicts the long-term survival of five of six mice treated with the antiangiogenic schedule of cyclophosphamide and TNP-470 in one of five separate experiments. The arrow and Note on the graph reflect the recurrence of one of six drug-resistant tumors at 18 days off therapy in this experiment. *b*, antiangiogenic versus conventional scheduling of cyclophosphamide for drug-sensitive Lewis lung carcinoma. Δ , control saline; \circ , conventional schedule [150 mg/kg every other day for 3 doses (white arrows, total 450 mg/kg) every 21 days]; \bullet , antiangiogenic schedule (170 mg/kg every 6 days, thin black arrows). The inset (top right) reveals magnified axes for the first 21 days of therapy ($n = 6$ mice/group). Therapy on the antiangiogenic schedule was discontinued after seven cycles, three cycles beyond the point at which tumors were no longer visible. Three separate experiments produced similar results. Two mice from two separate experiments were reinoculated with Lewis lung carcinoma >500 days after the eradication of the original tumor. The growth of these second Lewis lung carcinomas was similar to that of tumors in untreated mice, an outcome not consistent with immune-mediated regression of the primary Lewis lung tumor by cyclophosphamide.



tumor may have resulted from the known induction by cyclophosphamide of its own metabolism (19). Similar results were obtained with drug-resistant EMT-6/CTX (7) breast carcinomas in a different mouse strain (Fig. 5a). We therefore sought to determine whether cyclophosphamide on this schedule was in fact antiangiogenic and, in particular, whether antiangiogenesis explains the improved control of tumor growth in drug-resistant Lewis lung carcinoma.

Evidence that Cyclophosphamide Controls Drug-resistant Lewis Lung Carcinoma through Endothelial Cell Inhibition

Endothelial Cell Inhibition *in Vitro*. Cyclophosphamide is a pro-drug that requires *in vivo* activation by hepatic mixed function oxidases to 4-HC (4). Capillary endothelial cells (12) were exposed for 16 h to 4-HC *in vitro* at concentrations similar to those obtained *in vivo* (20). 4-HC induced a concentration-dependent cell cycle arrest and apoptosis of bFGF-stimulated capillary endothelial cells (Fig. 2a). The majority of endothelial cells at high concentrations of 4-HC (10 μ g/ml) arrested in G₁ and showed increased apoptosis. Lower concentrations (0.1 μ g/ml 4-HC) were cytostatic and were associated with a prolongation of S phase. Importantly, when endothelial cell migration is stimulated *in vitro* by bFGF, even these lower concentrations (0.1 μ g/ml 4HC) caused a 45% decrease in migration (Fig. 2a) without affecting the protein levels of three integrins (data not shown).

Angiogenesis Inhibition *in Vivo*. To determine the extent of angiogenesis inhibition caused by either schedule of cyclophosphamide

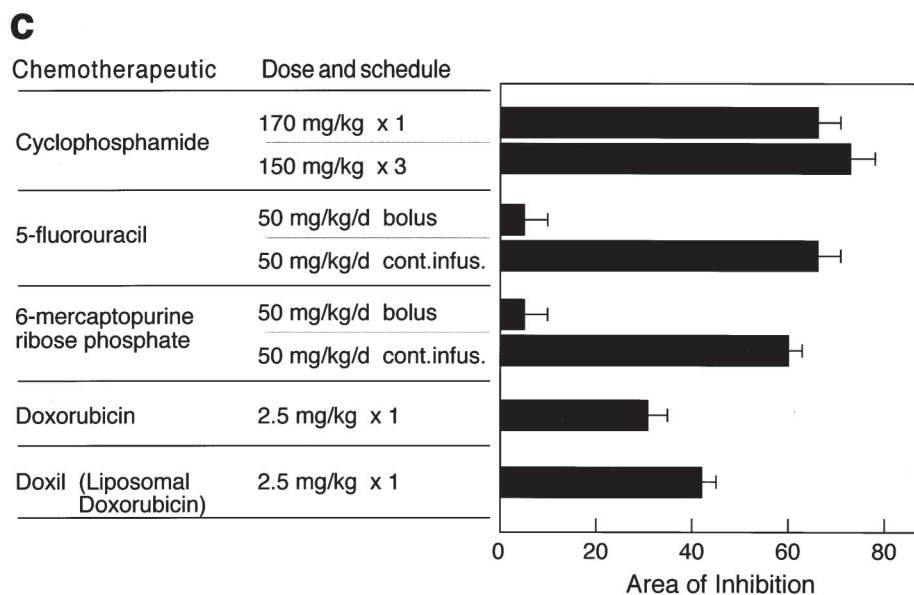
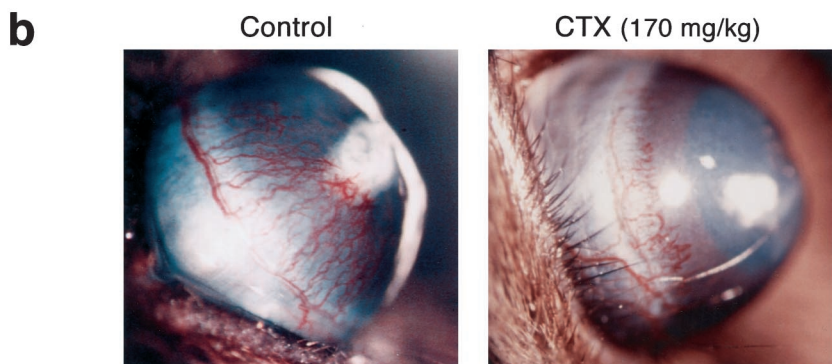
in vivo, mouse corneas were implanted with bFGF pellets that stimulated corneal neovascularization over 6 days (8). Therapy with cyclophosphamide was initiated 24 h after pellet implantation, when limbal dilation and vascular sprouts first appear. Cyclophosphamide administration equivalent to one cycle of the antiangiogenic schedule (170 mg/kg \times 1 at 24 h) inhibited the area of new vessel growth by $66 \pm 5\%$ (Fig. 2b). Treatment with the entire conventional schedule of cyclophosphamide, *i.e.*, three doses of 150 mg/kg at 24, 72, and 120 h, resulted in $73 \pm 5\%$ inhibition (Fig. 2c). Whereas inhibition of corneal angiogenesis did not differ statistically between the two schedules, valid comparison of the level of inhibition is limited to 6 days because the bFGF stimulus fades. However, in a tumor-bearing mouse, this antiangiogenic effect would occur 3.5 times on the 6-day antiangiogenic schedule in contrast to 1 time on the 21-day conventional schedule.

***In Vivo* Apoptosis of Endothelial Cells followed by Apoptosis of Drug-resistant Tumor Cells.** To determine whether cyclophosphamide induced endothelial cell apoptosis in the tumor bed, we analyzed cell turnover in drug-resistant tumors. Whereas BrdUrd incorporation of tumor cells was similar in control and cyclophosphamide-treated mice, endothelial cell and tumor cell apoptosis revealed marked differences between treatment groups (Fig. 3). Untreated drug-resistant Lewis lung carcinomas showed a tumor cell labeling index of 37%, a low tumor cell apoptotic rate of 1.9%, and minimal detectable (0.2%) endothelial cell apoptosis. The conventional schedule of cyclophosphamide generated one broad peak of tumor cell apoptosis that

a

	bFGF	4HC conc.	Relative migration	Relative cell number	% Apoptosis	Cell cycle (%)		
						G ₁	S	G ₂ -M
Control	—		0	10510 ± 149	1.06	81.71	13.76	4.59
	+		791 ± 34	13005 ± 212	0.51	20.53	74.16	5.32
4HC	+	0.1 μg/ml	437 ± 21	9255 ± 326	0.56	11.03	87.02	1.95
	+	1.0 μg/ml	182 ± 12	6780 ± 208	0.65	46.17	53.83	0
	+	10 μg/ml	ND	2050 ± 159	19.92	78.99	13.77	7.24

Fig. 2. *a*, *in vitro* antiendothelial effects of activated cyclophosphamide on bovine capillary endothelial cell migration, survival and proliferation, apoptosis, and cell cycle distribution. 4-Hydroperoxycyclophosphamide, which spontaneously converts to 4-HC in aqueous solution, was added at the indicated concentrations (*conc.*). Values shown are the mean ± SE. Relative cell number refers to the remaining, adherent endothelial cells (>600 fl) from an initial plating of 12,500 cells. Apoptosis was determined as a percentage of 10,000 intact (gated) cells by fluorescence flow cytometry. Cell cycle analysis was determined similarly using ModFit LT software. Without the stimulation of S phase (14% to 74%) produced by bFGF, activated cyclophosphamide (4-HC) was 1.5-fold less inhibitory of proliferation and caused 3-fold less induction of apoptosis (data not shown). *b*, antiangiogenic activity of systemic cyclophosphamide against bFGF-induced corneal neovascularization. Treatment was initiated 24 h after pellet implantation, when limbal dilation and vascular sprouts first appear. *Left panel*, saline-treated control; *right panel*, 170 mg/kg cyclophosphamide administered once 24 h after pellet implantation in a 6-day assay (8). The area of inhibition was calculated in *c* using the formula $0.2 \times \pi \times \text{neovessel length} \times \text{clock hours of neovessels}$. The percentage of inhibition was calculated by normalizing the area of neovascularization in treated mice to the area of neovascularization in saline-treated controls ($n = 8$ mice/group). The experiment was repeated three times with similar results. *c*, relative inhibition of the area of corneal neovascularization and schedule-dependent antiangiogenic efficacy of cyclophosphamide and other chemotherapeutic agents as detailed in "Materials and Methods." Continuous infusion of the antimetabolites (5-fluorouracil and 6-mercaptopurine ribose phosphate) demonstrated superior inhibition of growth factor-induced angiogenesis when compared with equivalent bolus injections. Likewise, the pegylated liposomal formulation of doxorubicin enhanced the antiangiogenic efficacy of an equivalent dose of doxorubicin (see "Discussion").



fell to background levels from day 13 through day 21 after the start of treatment (Fig. 3*a*). In contrast, the antiangiogenic schedule generated four peaks of tumor cell apoptosis over the 21-day period (Fig. 3*b*). Double immunofluorescence (von Willebrand factor antibody and TUNEL assay) was used to discriminate endothelial cell apoptosis from tumor cell apoptosis (Fig. 3). On both schedules, endothelial cell apoptosis from cyclophosphamide therapy preceded the apoptosis of drug-resistant tumor cells. When doses of cyclophosphamide were spaced 6 days apart on the antiangiogenic schedule, endothelial cell apoptosis preceded a significant increase in tumor cell apoptosis by

3.5 days, suggesting that the antiendothelial effect of cyclophosphamide is primary and causative. Because the half-life of cyclophosphamide in mice is less than 30 min (21, 22), and the BrdUrd incorporation rate of tumor cells on the antiangiogenic schedule remained at 35% (similar to untreated controls), the apoptosis of drug-resistant tumor cells on both schedules most likely resulted from endothelial cell suppression and not from delayed tumor penetration of activated cyclophosphamide. Furthermore, these data demonstrate that tumor growth, which occurred after the first 13 days on the conventional schedule (see Fig. 1*a*, *inset*), was prevented on the antiangiogenic

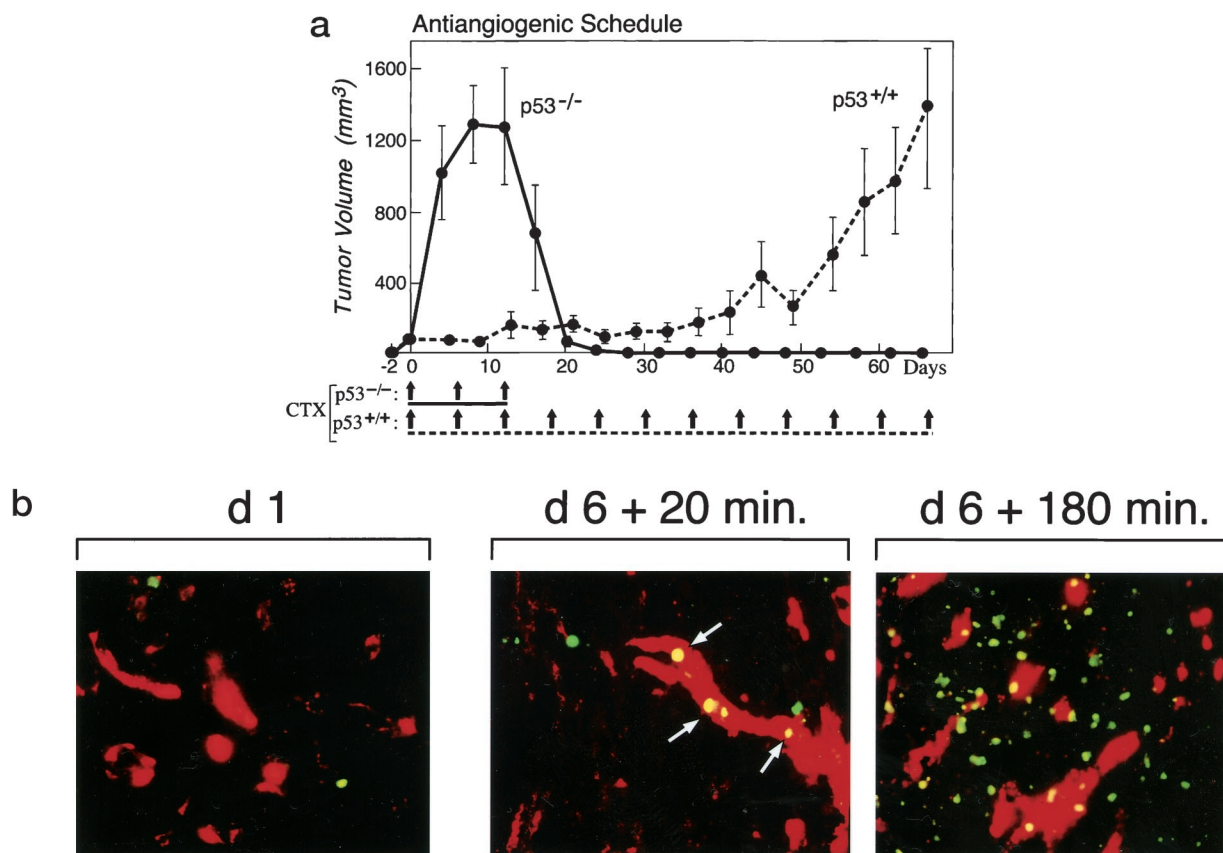


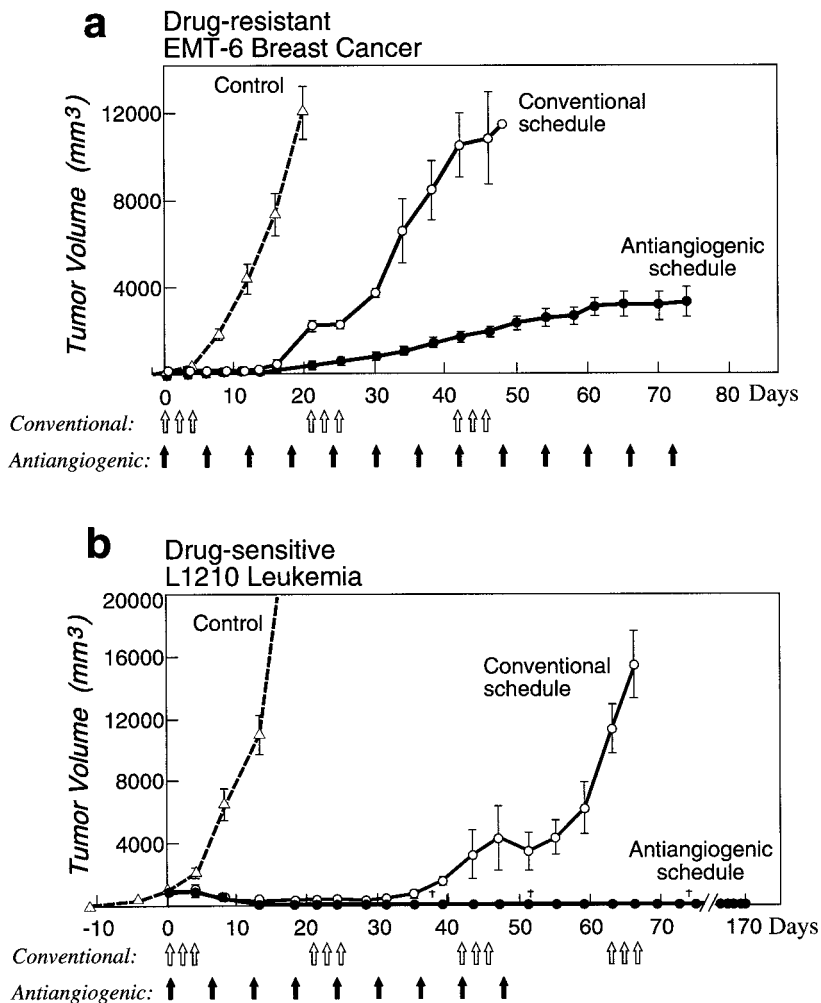
Fig. 4. *a*, growth of drug-resistant Lewis lung carcinoma in p53^{+/+} (dashed line) versus p53^{-/-} (solid line) C57Bl6/J mice treated on the antiangiogenic schedule of cyclophosphamide (170 mg/kg every 6 days, black arrows). Mice were treated as described in the Fig. 1 legend, except that therapy was discontinued after three cycles in the p53^{-/-} mice to prevent the development of cardiopulmonary toxicity. The p53^{-/-} mice showed no evidence of primary Lewis lung carcinoma regrowth or pulmonary metastases and died of spontaneous tumors (expected in p53^{-/-} mice; Ref. 23) within 2 months after therapy. Four separate experiments produced similar results. In the fourth experiment, mice were re inoculated with drug-resistant Lewis lung carcinoma 2 days after the third dose on the antiangiogenic schedule of cyclophosphamide. The first (cyclophosphamide-treated) drug-resistant Lewis lung carcinoma regressed completely, whereas these second (untreated) tumors grew in four of four mice (data not shown). This outcome is not compatible with an anamnestic response from immune-mediated regression of the primary tumor in these congenic p53^{-/-} C57Bl6/J mice. *b*, immunofluorescence (von Willebrand factor/TUNEL) of drug-resistant Lewis lung carcinoma in p53^{-/-} mice treated with the antiangiogenic schedule of cyclophosphamide. *Left panel*, a representative field of the growing tumor on day 1 after cyclophosphamide administration, which remained unchanged (*e.g.*, days 0, 1, 2, and 4) until the second dose of cyclophosphamide was administered on day 6. *Middle panel*, within 20 min after the second dose of cyclophosphamide on day 6, extensive endothelial cell apoptosis was manifested, without an increase in tumor cell apoptosis. The three white arrows mark apoptotic endothelial cell nuclei (yellow). By 180 min after the second dose of cyclophosphamide on day 6, 70–90% of this cyclophosphamide-resistant tumor underwent massive, central necrosis. Only a thin, cortical rim of identifiable tumor tissue (approximately 330- μ m thick) remained on H&E-stained sections. *Right panel*, a representative area of this rim of identifiable tumor tissue that displayed massive endothelial cell and tumor cell apoptosis (described in the Fig. 3 legend).

treated on the antiangiogenic schedule of cyclophosphamide. The growth of drug-resistant tumors in p53^{+/+} mice was completely suppressed for at least six 6-day cycles of cyclophosphamide. In contrast, the growth of drug-resistant tumors in p53^{-/-} mice was similar to that of untreated control tumors after the first dose of cyclophosphamide. Tumor cell proliferation and tumor and endothelial cell apoptosis of these growing tumors in p53^{-/-} mice also remained equivalent to untreated control tumors until day 6 (Fig. 4*b*, *left panel*). Within 10–20 min after the second dose of cyclophosphamide on day 6, marked apoptosis of p53^{-/-} endothelial cells occurred without an increase in the tumor cell apoptotic rate (Fig. 4*b*, *middle panel*). By 180 min after this second dose, 70–90% of the central mass of these large, cyclophosphamide-resistant tumors had become necrotic. In the remaining viable cortical rim, endothelial cell apoptosis and tumor cell apoptosis were progressing nearly synchronously (Fig. 4*b*, *right panel*). After a third dose of cyclophosphamide, tumors in the p53^{-/-} mice were eradicated and did not recur off therapy (Fig. 4*a*).

Because the tumor was cyclophosphamide-resistant in both sets of mice, our interpretation of these results is that drug-resistant tumor cells did not die until the endothelial cells within the tumor died from therapy with cyclophosphamide. In p53^{+/+} mice, cyclophosphamide

would inhibit endothelial cell migration (see Fig. 2, *a* and *b*), elicit an arrest of endothelial cell cycle (see Fig. 2*a*), and induce a level of endothelial cell apoptosis (see Figs. 2*a* and 3*b*) that results in a balance of tumor cell proliferation and apoptosis (see Figs. 1*a* and 3*b*) during the initial 36 days. In contrast, p53^{-/-} endothelial cells would not undergo an arrest of cell cycle and did not undergo detectable apoptosis after the first dose of cyclophosphamide (Fig. 4*b*, *left panel*). This resulted in growth of the tumor (Fig. 4*a*) and documents the drug resistance of the tumor. After the second dose of cyclophosphamide in p53^{-/-} mice, p53-independent apoptosis of endothelial cells rapidly engulfed most of the tumor bed (Fig. 4*b*), presumably reflecting the attainment of cumulative and lethal DNA damage. Thereafter, despite drug resistance, tumor cells could not evade death resulting from this extensive endothelial cell apoptosis. The difference in magnitude (partial versus complete) and timing (4 days versus 180 min after cyclophosphamide) of drug-resistant tumor cell apoptosis appeared to be based predominantly on the endothelial cellular response to cyclophosphamide mediated through p53 (Fig. 4). Thus, the exquisite control of tumor growth exerted by endothelial cells is revealed by the ability of cyclophosphamide to elicit a differential level of endothelial cell apoptosis. In p53^{+/+} mice, sporadic endothelial cell apoptosis induced by the antiangiogenic schedule of cy-

Fig. 5. *a*, antiangiogenic versus conventional scheduling of cyclophosphamide for the drug-resistant breast cancer EMT-6/CTX (7). Δ , control saline; \circ , conventional schedule [150 mg/kg every other day for three doses (white arrows, total 450 mg/kg) every 21 days]; \bullet , antiangiogenic schedule (170 mg/kg every 6 days, CTX, black arrows); $n = 12$ in two experiments. All control and conventional schedule-treated mice died with large tumor burdens. Therapy was discontinued on the antiangiogenic schedule of cyclophosphamide alone after two of six mice died similarly to mice described in the Fig. 1a legend. *b*, antiangiogenic versus conventional scheduling of cyclophosphamide for the drug-sensitive leukemia cell line L1210 (9). Δ , control saline; \circ , conventional schedule [150 mg/kg every other day for three doses (white arrows, total 450 mg/kg) every 21 days]; \bullet , antiangiogenic schedule (170 mg/kg every 6 days, black arrows); $n = 10$ in two experiments. Therapy on the antiangiogenic schedule was discontinued after nine cycles, five cycles beyond the point at which tumors were no longer visible. Three of 10 mice developed recurrent tumor toward the end or immediately after the discontinuation of therapy on the antiangiogenic schedule (small crosses). Seven of 10 mice (70%) are long-term, tumor-free survivors 170 days after the initiation of therapy at this writing.



cyclophosphamide is sufficient to enable drug-resistant tumor growth suppression. In p53^{-/-} mice, cyclophosphamide causes a total involution of the vascular bed that leads to the eradication of drug-resistant tumors comprising 4.5% of body weight.

Eradication of Drug-sensitive Lewis Lung Carcinoma and L1210 Leukemia by the Antiangiogenic Schedule of Cyclophosphamide

Because repetitive waves of tumor cell apoptosis occurred on the antiangiogenic schedule in drug-resistant Lewis lung carcinoma (see Fig. 3b), a similar effect could interfere with the generation of acquired drug resistance in a drug-sensitive tumor. We therefore treated the original, drug-sensitive Lewis lung carcinoma with either the antiangiogenic or conventional schedule of cyclophosphamide. The antiangiogenic schedule was not only more effective when compared with the conventional schedule, but therapy could be discontinued with long-term tumor-free survival (Fig. 1b). Similar initial tumor burdens of Lewis lung carcinoma that here and historically (4) acquired drug resistance on the conventional schedule did not do so on the antiangiogenic schedule. We confirmed these observations with a more inherently cyclophosphamide-sensitive tumor, L1210 leukemia (9). Both the conventional and antiangiogenic schedules of cyclophosphamide were curative of L1210 leukemia when therapy was initiated at tumor volumes of 100, 200, and 500 mm³ (data not shown). An advantage of the antiangiogenic schedule of cyclophosphamide was revealed when therapy of L1210 tumors was initiated at 1000 mm³

(Fig. 5b). Whereas 10 of 10 mice harboring 1000 mm³ L1210 tumors developed acquired drug resistance and died of tumor on the conventional schedule of cyclophosphamide, 7 of 10 mice (70%) treated with the antiangiogenic schedule of cyclophosphamide are long-term, tumor-free survivors off therapy. We interpret this eradication of drug-sensitive Lewis lung carcinoma and L1210 leukemia as resulting from two actions of cyclophosphamide: (a) the direct cell kill of drug-sensitive tumor cells; and (b) the direct cell kill of endothelial cells, leading to the apoptosis of both drug-sensitive and, more importantly, drug-resistant tumor cells.

Eradication of Drug-resistant Lewis Lung Carcinoma by Adding a Second Angiogenesis Inhibitor (TNP-470) to the Antiangiogenic Schedule of Cyclophosphamide

The angiogenesis inhibitor TNP-470 has been reported to slow the growth of drug-sensitive Lewis lung carcinoma (27) but not to regress it. Subsequently, Teicher *et al.* (18) reported that TNP-470 combined with cyclophosphamide, identical to our conventional schedule (plus minocycline), yielded a 40–50% cure rate. However, in our study, the antiangiogenic schedule of cyclophosphamide alone eradicated similar burdens of drug-sensitive Lewis lung carcinoma in 100% of mice. To eradicate drug-resistant tumors, it was necessary to augment the antiendothelial activity of cyclophosphamide by adding TNP-470. The dose of TNP-470 was lowered to one-seventh of the dose used by Teicher *et al.* (18) to avoid severe weight loss when combined with the antiangiogenic schedule of cyclophosphamide. This lower dose,

12.5 mg/kg TNP-470 every 6 days, was administered on the same day or on day 1, 2, or 4 after 170 mg/kg cyclophosphamide. The combination of cyclophosphamide and TNP-470 on the same day of the 6-day cycle proved most efficacious (data not shown). After seven cycles of combination antiangiogenic therapy in five experiments, drug-resistant Lewis lung carcinoma was eradicated in 32 of 38 (84%) mice (Fig. 1a). All mice had complete regression of drug-resistant Lewis lung carcinoma, and only 3 of 38 mice (8%) developed recurrent primary tumor 14–18 days after completion of therapy. Another 3 of 38 mice (8%) died of toxicity within 10 days of the completion of therapy. These mice showed no evidence of tumor recurrence; nevertheless, they had severe ataxia and died despite the administration of parenteral fluids. These ataxic mice were unevaluable for tumor recurrence and were considered treatment failures in the total of 38 mice. No tumor relapses occurred later than 18 days after therapy was completed. However, sterilization of cages, food, and water by autoclaving was found to be critical. In two experiments performed without autoclaving, tumor eradication occurred in 20 of 20 mice, yet 14 of 20 mice developed pulmonary inflammation resulting in premature death 50 ± 6 days after therapy was completed on day 36. Because no tumors recurred later than 18 days after the completion of therapy, and these mice had no evidence of primary or metastatic tumor at the time of death, the drug-resistant tumors in these 14 of 20 mice were considered eradicated. We assume that these late deaths were in part due to pulmonary endothelial cell damage (20) and immunosuppression (4) by cyclophosphamide complicated by an acquired infection because late deaths did not occur in the other three experiments in which we presterilized the food, water, and cages.

DISCUSSION

These results show that a standard anticancer chemotherapeutic agent, cyclophosphamide, also has an antiangiogenic component. By scheduling cyclophosphamide to provide more sustained apoptosis of vascular endothelial cells within the tumor bed, the full therapeutic advantage of this antiangiogenic strategy is revealed. Redirection of cyclophosphamide against the still-sensitive endothelial cell compartment of a solid tumor results in increased apoptosis of tumor cells, regardless of whether or not the tumor cells are drug resistant. Thus, by using a new logic for an old drug, this antiangiogenic schedule of cyclophosphamide reduced the risk of acquired drug resistance in Lewis lung carcinoma and L1210 leukemia and enabled tumor eradication. In mice bearing drug-resistant Lewis lung carcinoma, TNP-470 potentiated the prolonged suppression of tumor growth by the antiangiogenic schedule of cyclophosphamide so that even cyclophosphamide-resistant tumors could be eradicated.

Angiogenesis, the process of pathological vascular in-growth critical for tumor expansion, was first proposed as a target for anticancer therapy in 1971 (28). Evidence that a chemotherapeutic agent directly causes cytotoxicity to the vasculature in a drug-resistant solid tumor followed in 1991 (29). In this report, Baguley *et al.* demonstrated that vinblastine led to greater than 90% necrosis of drug-resistant solid tumors within hours but had no effect when the same cells were grown as ascites (29). However, because the maximum tolerated dose of vinblastine was administered, these authors were unable to continue on an antiangiogenic schedule and thus demonstrate long-term suppression of drug-resistant tumor growth. In contrast, our strategy was to optimize the schedule for continued cytotoxic pressure on the endothelial cells within the vascular bed of the tumor. Optimized antiangiogenesis renders cyclophosphamide indirectly and repeatedly capable of killing drug-resistant tumor cells, limits the expression of clinical resistance, and improves tumor response. Using this closely cycled dosing schedule, we did not observe the rapid, widespread

vascular collapse and extensive necrosis in wild-type mice seen by Baguley *et al.* (29) with vinblastine and by Denekamp (30, 31) using other therapies. Further, the 3.5-day interval between the onset of endothelial cell apoptosis and maximum drug-resistant tumor cell apoptosis is inconsistent with vascular necrosis (see Fig. 3b). However, the rapid and nearly synchronous apoptosis of endothelial cells observed in p53^{-/-} mice harboring drug-resistant tumor treated with the antiangiogenic schedule of cyclophosphamide may have had an undetected component of ischemic or hemorrhagic vascular necrosis, as described by Baguley *et al.* (29) and Denekamp (30, 31).

Our antiangiogenic schedule also bears a distant resemblance to “optimal dose” schedules (15) used in therapy of mouse leukemias and solid tumors reported over 30 years ago (3, 15–17, 32, 33) and to schedules predicted from *in vivo* tumor cell cycle kinetics (34, 35).

Since the report by Baguley *et al.* (29), there have been numerous reports relating the short-term effects of cytotoxic chemotherapy on vascular endothelial cells. Antiendothelial effects have been demonstrated *in vitro* for cyclophosphamide (20), 5-fluorouracil (36), and mitomycin C (36, 37), and short-term antiangiogenic effects have been demonstrated *in vivo* for vincristine (38), vinblastine (29, 38, 39), doxorubicin (38), mitoxantrone (38), etoposide (38), paclitaxel (40–42), 6-methylmercaptopurine (43), tegafur (44), 9-amino-20(S)-camptothecin (45), topotecan (45), camptosar (45), and combretastatin A-4 (46, 47). However, our data with cyclophosphamide lead us to conclude that demonstration of antiangiogenic efficacy in short-term assays must now be followed by determination of a schedule that allows this effect to be sustainable. Certain agents, as described here for cyclophosphamide, readily lead to antiangiogenic effects within tumors on different schedules, and one need only determine the most effective antiangiogenic schedule. Other agents, as described here for 5-fluorouracil and 6-mercaptopurine (see Fig. 2), are nearly devoid of antiangiogenic efficacy when given as bolus injections but reveal a potent antiangiogenic effect as continuous infusions. At least one chemotherapeutic, methotrexate, did not possess significant antiangiogenic efficacy on any schedule that we tested (data not shown; Ref. 38), possibly because endothelial cells are reliant on the salvage pathway for nucleic acids (48). We speculate that certain other chemotherapeutic agents will be demonstrated to possess an enhanced antiangiogenic capability after schedule modifications that are dose-dense and range from continuous infusion to weekly therapy delivered without interruption. Thus, other cytotoxic chemotherapies, delivered on an antiangiogenic schedule specific for that agent, may more readily suppress tumor growth in mice as described here for cyclophosphamide and, by inference from previous reports (49–51), also for weekly Doxil (see Fig. 2c).

Because conventional schedules of combination chemotherapy have led to a profound increase in the survival of children with cancer and have improved the survival of adults with certain types of cancer, we do not believe that these clinical protocols should be changed for the sake of increasing the antiangiogenic efficacy of any given drug. Furthermore, it can be argued that our results, in part, may reflect a higher fraction of new, immature vessels present in the rapidly growing, recently established transplantable tumor system used. However, our results in mice may help to explain why some patients who are receiving long-term maintenance or even palliative chemotherapy continue to have stable disease beyond the time that the tumor cells would have been expected to develop drug resistance. Moreover, a closer approximation to antiangiogenic scheduling may explain the improved outcome of empiric treatment of “slower growing” human cancer using continuous infusion 5-fluorouracil (52–54), weekly paclitaxel (55–57), and daily oral etoposide (58–60). If this hypothesis proves generalizable, it may suggest which agents and on which schedules chemotherapy may be best combined with more specific

angiogenesis inhibitors for improved antiangiogenic and anticancer efficacy.

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