Anti-Cancer Drugs 2001, **12,** pp. 213–220

Preclinical report

Preclinical antitumor activity of the azonafide series of anthracene-based DNA intercalators

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The azonafides are a series of anthracene-based DNA intercalators which inhibit tumor cell growth *in vitro* at low nanomolar concentrations and are not affected by the multidrug resistance phenomenon (MDR). Prior studies have described antitumor efficacy in murine tumor models including L-1210 and P-388 leukemias, and B-16 melanoma. The current results extend these cell line observations to human tumors tested in the NCI panel of 56 cell lines, in freshly isolated tumors tested in colony-forming assays in soft agar and in several animal models. In the NCI panel, the overall mean 50% cell kill (LC_{50}) for the unsubstituted azonafide, AMP-1, was $10^{-5.53}$ M, with some selectivity noted in melanomas ($10^{-6.22}$ M). The mean LC_{50} for the 6-ethoxy substituted analog, AMP-53, was $10^{-5.53}$ M, with some selectivity found in non-small cell lung cancer ($10^{-5.91}$) and renal cell carcinoma ($10^{-5.84}$). In freshly isolated human tumors tested in soft agar, there was marked activity (mean IC_{50} in μg/ml) for AMP-53 in four cell types: breast cancer (0.09), lung cancer (0.06), renal cell carcinomas (0.06) and multiple myeloma (0.03). These effects were superior to doxorubicin and to several other azonafides, including AMP-1, AMP-104 and the 6-hydroxyethoxy derivative, AMP-115. Compound AMP-1 was shown to be superior to amonafide in the mammary 16C breast cancer model in B6CF31 mice, but it had little activity in Colon-38 nor in M5076 ovarian sarcomas *in vivo*. Nine azonafides were evaluated in the Lewis lung cancer model in C57/bl mice, but only AMP-53 demonstrated significant efficacy with a treated/control $\times$ 100% (T/C) value of 30%. Because AMP-53 demonstrated the greatest breadth of activity, it was then evaluated in several human tumor cell lines growing in mice with severe combined immunodeficiency disease (SCID). Only three tumors were sensitive (T/C $<$ 42%), including HL-60 leukemia (T/C=39%), MCF-7 breast cancer (T/C=39%) and A549 non-small cell lung cancer (T/C=37%). Overall, these results demonstrate that the 6-ethoxy substituted azonafide, AMP-53, has consistent (*in vitro* and *in vivo*) experimental antitumor activity in human breast and lung cancer, and could be considered for clinical testing in patients with MDR tumors. [© 2001 Lippincott Williams & Wilkins.]

Key words: Anthracene, azonafide, DNA intercalator.

Introduction

The azonafide series of antitumor agents includes over 140 anthracene-based compounds which have demonstrated cytotoxic activity in preclinical systems.[1–4] These compounds have a chemical similarity with the naphthylene-based DNA intercalator, amonafide, which was evaluated for anticancer activity in patients with endometrial carcinoma[5] and advanced breast cancer.[6] Like amonafide, the azonafides intercalate into DNA and some, but not all, inhibit topoisomerase II enzymes at cytotoxic concentrations *in vitro*.[7] In cell culture studies, the azonafides demonstrate DNA binding and cytotoxic potency greater than that of amonafide and several other classes of DNA intercalating compounds, including doxorubicin and mitoxantrone.[1,8] In addition, the azonafides are consistently active *in vitro* in human and murine tumor cells which exhibit multidrug resistance (MDR) that is mediated by P-glycoprotein overexpression.[2] Azonafides were initially selected for testing in animal tumor models using the criteria of high potency in tumor cells, low cardiotoxic potency in neonatal rat heart cells and a lack of MDR-based cross-resistance.[1,3] These prior *in vitro* studies of antitumor efficacy were limited to a battery of 10 different tumor cell lines which included sensitive

Supported by Grant GM 49875 from the National Institutes of Health, National Cancer Institute, Bethesda, MD and by grant no. 1-013, Project no. 9721, from the Arizona Disease Control Research Commission, State of Arizona, Phoenix, AZ.

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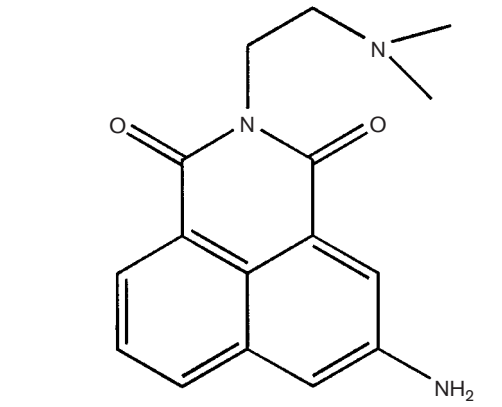
and MDR pairs of human hematologic cancers. The results showed that the 6-ethoxy-substituted azonafide, AMP-53, had the preferred characteristics of high potency in solid tumor cell lines such as MCF-7 breast cancer, a lack of cross-resistance in a MDR L-1210 cell line, with low potency in neonatal rat heart cells.[4]

Prior *in vivo* studies of the azonafide's preclinical antitumor activity were limited to murine tumor models such as P-388 leukemia[1] and B-16 melanoma,[2,3] with only a few studies in human tumors *in vivo*.[4] The effects of these agents in fresh human tumor cells *in vitro* and in MDR human tumors *in vivo* had not been assessed. In this report, we describe the results with selected azonafides in fresh human tumors tested in colony-forming assays and in human tumor cells growing in mice with the severe combined immunodeficiency disease (SCID) phenotype.

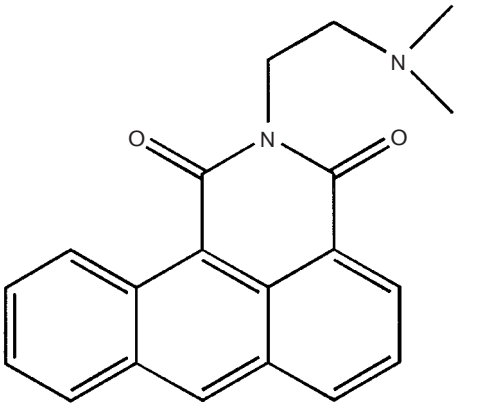
Materials and methods

Drugs

The azonafides were supplied by Dr William Remers following chemical syntheses using previously published methods.[1–3] The structures of the compounds selected for study are displayed in Figure 1. These compounds were selected for testing in the current experiments based on their activity in cell line screens from previously published studies.[1–4] For *in vitro* testing, the water-soluble compounds, doxorubicin (Adriamycin; Pharmacia and Upjohn, Peapack, NJ), and AMP-1, -35, and -53 were dissolved in 0.9% sodium chloride for injection. The other compounds (AMP-4, -60, -97, -115, -104, -127, -141, and -144) were initially solubilized in 99% (w/v) dimethylsulfoxide (DMSO), and then serially diluted in water to a final DMSO concentration $\leqslant 0.1\%$ (v/v). When tested *in vivo*, these same agents were initially dissolved in the same co-solvent system (containing ethanol, PEG 800 and polysorbate 80), used for the commercial anti-cancer agent etoposide (Vepesid).[9] Amonafide (nafidimide, benzylisoquinolinedione, NSC-308847) was supplied by the National Cancer Institute (NCI) in 100 mg vials of lyophilized powder.



Amonafide



Azonafides

AMP No.	Substitution
1	None
35	7-NH_2
53	6-O-CH_2-CH_3
60	10-C1
97	8-C1
104	6-O$(CH_2)_2$-OH
115	6-O$(CH_2)_2$-N-$(CH_3)_2$
127	4-NH_2 (tetrahydro)
141	6-SCH_3
144	6-S-CH_2-CH_3

Figure 1. Structures of amonafide (NSC-308847) and the azonafides.

Mice

Female C57BL/6 mice were obtained from the NCI, Biological Testing Branch (Frederick, MD). They were housed four per cage with access to food and water *ad libitum*. The mice, 19–22 g, were acclimatized for 2 weeks before tumor implantation. Male and female *scid* mice were obtained from a colony maintained at the University of Arizona (Tucson, AZ). They were originally developed from the original *scid* (C.B-17 *scid/scid*) and Balb/cByJSmn-*scid*/J mice obtained from the Jackson Laboratory (Bar Harbor, ME). All of the mice are housed in microisolator cages (Allentown Caging Equipment, Allentown, PA). The *scid* mice are further maintained under specific pathogen-free conditions using autoclaved food pellets and UV-irradiated water in a barrier facility.[10] All mouse experiments

involved four mice per group and were repeated once, for an n=8/treatment. All zero tumor measures were counted in the median calculations.

Tumor models

Tumor cell line panel. The 56 human tumor cell line study was performed by the NCI, Division of Cancer Treatment, NCI–Frederick Cancer Research and Development Center (Frederick, MD), according to published procedures.[11] Viability was assessed using the protein-staining dye, sulforhodamine B (SRB), following a 48-h exposure to the agent. The data was summarized using the COMPARE algorithm.[12]

Fresh human tumor cells. These were obtained from patients undergoing excisional biopsies who had given consent for *in vitro* drug testing of their tumors. The disaggregation procedures followed those of Salmon *et al.*[13] Viability was assessed in tumor colonies grown in soft agar, using the radiochemical ([^{3}H]thymidine) method of Kern *et al.*[14] A continuous (10 day) drug exposure and culture time were used, and agents were tested at concentrations up to a maximum of 10 μg/ml. Any tumors for which 50% inhibition could not be obtained at 10 μm/ml were determined to have an 'unachievable' IC_{50} value and the total number of achievable/tested specimens counted. The mean and standard deviation (SD) for each cell type with an achievable IC_{50} was calculated to allow for comparison with other cell types. A subset of nine different histologic tumor types comprising 99 individual specimens was simultaneously tested for sensitivity to two agents, AMP-53 and doxorubicin, allowing for a direct comparison between each agent.

Mouse tumor models

Lewis lung cancer cells were obtained from the NCI and evaluated in a s.c. growth model as described.[15] All drugs were administered by i.p. injection on days 1, 5 and 9 after s.c. tumor implantation in the front flank of anesthetized C57/bl male mice (weight 19.5–22 g). Tumors were measured 3 times weekly with a caliper until the tumor reached 2 000 mg or until the animal died. Solid tumor weights (mg) were estimated from the two widest tumor dimensions (in mm), according to the formula:

$$\text{Tumor weight} = \frac{\text{Length} \times \text{width}^2}{2}$$

The tumor growth inhibition (T/C value) was recorded using median tumor weight of the control (C) and treated (T) groups when the tumors in the control group reached 1500 mg of weight according to the formula:

$$\text{T/C (\%)} = \frac{\text{median tumor weight of the treated}}{\text{median tumor weight of the control}} \times 100$$

Antitumor efficacy was assessed according to NCI guidelines with the minimum level of significant activity defined at T/C $\leqslant$ 42% and high activity being T/C $\leqslant$ 10%.[16]

Colon-38 tumor cells were obtained from Professor Thomas Corbett (Wayne State University, Detroit, MI).[16] Tumors were implanted in the front flank on day 0, and drug treatments, AMP-1 or floxuridine (FUDR; Roche, Nutley, NJ), were administered beginning on day 3 by i.p. administration. The dose of AMP-1 was 15 mg/kg/day, on days 3 and 7, and 100 mg/kg/day $\times$ 9 days (days 3–11) for the positive control, FUDR. Tumor measurement and scoring were performed on day 25 (at 1.6 g mean control tumor weight) as outlined above.

Two other solid tumor models were used to compare amonafide with the unsubstituted azonafide, AMP-1, in studies performed under contract at the Southern Research Institute (Birmingham, AL). These models included the murine M5076 ovarian sarcoma tumor and mammary 16C adenocarcinoma, both tested in female B6C3F1 mice given s.c. tumor implants into the front flank on day 0. Each drug was dissolved in saline, and administered i.p. on days 1, 5 and 9 after tumor implantation. Amonafide was dosed at either 30 or 45 mg/g/injection and AMP-1 was dosed at 10, 15 or 20 mg/kg/injection. Tumor measurement and scoring were performed as detailed above. The T/C values were calculated on days 13 and 14 after tumor implantation (at approximately 1 g control tumor weight) for the breast and sarcoma models, respectively.

Results

NCI tumor cell line panel

Two azonafides were submitted to the NCI for screening in the 56 tumor cell line panel: the unsubstituted compound, AMP-1, and the 6-ethoxy derivative, AMP-53. Table 1 displays the summary results for these two compounds, listing only the $\log_{10}$ 50% cell killing concentration, (LC_{50}) (M), for each agent in the nine different types. Both agents exhibited similar LC_{50} values of about $10^{-5.5}$ M in the 56 different human tumor cell lines. For AMP-1, there was some evidence of selectivity in the subset of six melanoma cell lines, wherein the mean LC_{50} was nearly 1 $\log_{10}$ lower at

Table 1. Results with AMP-1 and -53 in the NCI human tumor cell line panel

Tumor type (no.)	Mean (SD) 50% lethal concentration ($-\text{Log}_{10}$ M)	
	AMP-1	AMP-53
Leukemia (4)	5.00 (0.01)	5.16 (0.023)
Lung cancer (9)	4.95 (0.97)	5.61 (0.44)
Colon cancer (7)	5.33 (0.42)	5.69 (0.32)
CNS cancer (6)	5.43 (0.52)	5.56 (0.33)
Melanoma (6)	6.22 (0.09)	5.44 (0.14)
Ovary cancer (6)	5.23 (0.39)	5.49 (0.35)
Renal cancer (8)	5.36 (0.56)	5.84 (0.45)
Prostate cancer (2)	5.31	5.2
Breast cancer (8)	5.48 (0.47)	5.32 (0.19)
Overall mean (range)	5.45 (1.34)	5.53 (1.29)

$10^{-6.22}$ M. Breast cancer cell lines did not appear to be selectively sensitive to AMP-1 and there was partial cross-resistance in the multidrug resistant MCF-7/ADR-RES cell line (LC_{50} of $10^{-5.84}$ M) compared to the parental MCF-7 cell line (LC_{50} of $10^{-5.0}$ M).

The 6-ethoxy substituted compound, AMP-53, produced similar inhibitory effects at mean drug concentrations of $10^{-5.53}$ M for 50% cell killing. Among the nine histologic classes of tumors tested, no tumor type displayed a significant (>1 $\log_{10}$) increase in sensitivity to AMP-53. However, the graphical display format (not shown) suggested that there was slightly enhanced sensitivity in five out of eight renal carcinoma cell lines. In this instance, the degree of enhanced inhibitory potency amounted to about a 0.5 $\log_{10}$ increase in sensitivity. Another interesting finding with AMP-53 was the complete lack of cross-resistance in the MCF-7/ADR-RES breast cancer cell line. In this case, the $\log_{10}$ LC_{50} values were virtually identical at $10^{-5.53}$ M for the parental MCF-7 cells and $10^{-5.33}$ M for the MCF-7/ADR-RES cells.

Human tumor colony-forming assays

Five azonafides were tested in fresh human tumors and each agent demonstrated some activity in the seven solid tumor and two hematologic tumor cell panel (Table 2). The largest number of specimens were evaluated with the 6-ethoxyazonafide, AMP-53, which was directly compared with doxorubicin in Table 3. A comparison of the mean IC_{50} values for all five azonafides and doxorubicin yielded the following rank order (from highest to lowest potency): AMP-115 > AMP-53 > AMP-104 > doxorubicin > AMP-1. This analysis shows that each of the substituted azonafides had greater potency in fresh human tumors than the unsubstituted compound, AMP-1. There was also evidence for tumor selectivity of specific compounds

Table 2. Human tumor colony-forming assay results with selected AMPs *in vitro*

Tumor type	No. IC_{50} achieved/no. tested (mean IC_{50})[a]		
	AMP-1	AMP-104	AMP-115
Breast	7/11 (80.1)	2/2 (0.06)	10/10 (0.03)
Colon	2/7 (30.9)	ND	ND[b]
Lung	5/11 (10.1)	ND	3/3 (0.2)
Non-Hodgkin's Lymphoma	ND	1/1 (10.9)	ND
Melanoma	7/11 (5.7)	2/2 (34.5)	10/10 (0.15)
Myeloma	ND	6/9 (3.38)	11/11 (0.0006)
Ovary	5/8 (11.5)	4/5 (0.0003)	11/11 (0.2)
Renal	3/4 (31.1)	1/2 (1.43)	8/8 (0.03)
Sarcoma	5/7 (9.7)	2/3 (0.19)	7/8 (0.006)
Mean (SD)	28.9 (270)	7.50 (12.5)	0.08 (0.08)

[a] μg/ml continuous drug exposure (IC_{50} in μg/ml) for 10 days with [^{3}H]thymidine uptake endpoint.
[b] Not done.

such as AMP-115 in myeloma and sarcoma, and AMP-104 in ovarian cancers. In each of these tumors, the IC_{50} values for each agent were several logs lower than in all of the other tumor types (Table 2). Compound AMP-53 demonstrated similar selectivity for breast, lung and myeloma specimens (Table 3).

When compared directly to doxorubicin, AMP-53 demonstrated improved growth inhibitory potency: the overall mean IC_{50} in 100 different specimens representing nine fresh human tumor types was 10-fold lower for AMP-53 (Table 3). For example, the IC_{50} value for AMP-53 was 17-fold lower than doxorubicin in breast cancer and 10-fold lower in lung cancer. Furthermore, an IC_{50} could be obtained in 88% of AMP-53-treated tumors, compared to 67% of those treated with doxorubicin (p=0.014 by ANOVA). Conversely, doxorubicin was superior (lower IC_{50}) in renal cell cancer and especially in myeloma specimens. These results have importance since doxorubicin is known to have significant clinical activity in breast and lung cancers, two tumor types in which AMP-53 was more active in this head to head, fresh tumor comparison. In contrast, there was roughly comparable potency for both agents in lymphoma and sarcoma specimens (Table 3). Finally, although the NCI cell line panel data (Table 1) had suggested that AMP-53 might have slight selectivity for renal cell carcinoma, the set of 12 renal cell cancer specimens did not confirm this selectivity in the colony-forming assays (Table 3). In this case, AMP-53 appeared to be decidedly less potent than doxorubicn and much higher AMP-53 drug concentrations were required to inhibit renal cell colony formation than in all of the other eight cell types tested (Table 3).

Table 3. Direct comparison of AMP-53 to doxorubicin in fresh human tumor cells using a colony-forming assay

Tumor type	AMP-53		Doxorubicin	
	No. achieved/no. tested	Median IC_{50}[a]	No. achieved/no. tested	Median IC_{50}[a]
Breast	10/11	0.09	5/9	1.5
Colon	4/4	0.12	1/2	93.7
Kidney	10/12	5.5	3/3	0.03
Lung	9/11	0.06	6/11	0.6
Lymphoma	7/9	0.2	6/9	0.07
Melanoma	11/12	0.1	NT[b]	NT[b]
Myeloma	12/13	0.03	12/13	7×10^{-4}
Ovary	9/11	0.1	NT[b]	NT[b]
Sarcoma	16/17	0.2	10/17	0.1
Total (%)	88/100 (88%)		43/64 (67%)	
Mean (SD)		1.3 (3.6)		13.7 (35.3)

[a]Tested using continuous exposure (IC_{50} in μg/ml).
[b]Not tested.

Antitumor activity in mouse tumor models

The parent azonafide, AMP-1, demonstrated dose-dependent antitumor activity in the 16C mammary carcinoma model and in the M5076 ovarian sarcoma model (Table 4). However, a significant T/C value ($\leqslant$42%) was only achieved at the highest AMP-1 dose level tested (20 mg/kg/day) in the 16C mammary carcinoma model. Amonafide also produced dose-dependent effects in each of the breast and sarcoma models, but did not reduce the T/C value to $\leqslant$42% in either and was not tested in the Colon-38 model. In the colon model, AMP-1 was not active (T/C=71%), whereas the positive control, FUDR, showed highly significant activity with a T/C of only 4% (Table 4).

Several azonafides were evaluated in the Lewis lung cancer model in C57/bl mice to follow up on the *in vitro* data that suggested that human lung cancers might be sensitive to these agents. Nine different azonafides were evaluated in the Lewis lung cancer model at i.p. doses ranging from 1 to 4 mg/kg/injection, administered on 3 days (1, 5 and 9) after s.c. tumor implantation. These schedules had been previously validated in pilot experiments in mice bearing B-16 melanomas.[4] The results, summarized in Table 5, show that significant antitumor activity (T/C $<$42% of control) was achieved by only one azonafide, AMP-53, at doses of 3 and 4 mg/kg/day. Each of the other eight azonafides did not produce tumor inhibition at doses which were near their LD_{10} for this 3-day schedule.

Table 4. Azonafide (AMP-1) antitumor activity in murine breast, colon and sarcoma tumors

Agent	T/C (%) values			
	mg/kg[a]	Breast	Colon-38	Sarcoma
Amonafide	30	91.5	NT[b]	74.3
	45	66	NT[b]	72.7
AMP-1	10	76.4	NT[b]	85.5
	15	55.9	71	68.6
	20	34.9	NT[b]	54.4
FUDR	100	NT[b]	4	NT[b]

[a]Doses per injection (see Methods for different schedules).
[b]Not tested.

Table 5. Antitumor activity of selected azonafides in Lewis lung cancer in C57/B1 mice

AMP no.	Dose (mg/kg/day)[a]	%T/C
35	3.0	52.0
53	3.0	39.2[b]
	4.0	20.0[b]
54	1.5	88.2
	2.0	88.2
	3.0	59.1
60	3.0	63.8
97	3.0	54.4
115	3.0	81.9
127	3.0	50.1
141	1.0	98.6
	2.0	88.2
144	1.0	95.5
	2.0	94.1
	3.0	95.5

[a]Intraperitoneal dose given days 1, 5 and 9 after 10^5 tumor cell implantation.
[b]%T/C$\leqslant$42% considered significant per NCI standards.[16]

Antitumor activity of AMP-53 in human tumors growing in SCID mice

Previous experiments had detected activity for AMP-53 in SCID mice.[4] In addition, some new experiments were performed to follow up on the activity of AMP-53 in sensitive and multidrug-resistant 8226 myeloma cells, and in A549 non-small cell lung cancer cells. The

prior data[4] were also re-analyzed in this report to consolidate the data presentation using the % T/C analysis method. The results, presented in Table 6, show that AMP-53 is equally effective in an 8226 myeloma cell line that exhibits MDR to DNA intercalators after prolonged exposure to doxorubicin.[18] In both the parental 8226 cells and in the 8226_{DOX40} myeloma cells, AMP-53 produced comparable tumor growth inhibition with % T/C values of 31–34% (Table 6). Furthermore, as the other tumor data shows, AMP-53 produced greater antitumor activity than mitoxantrone given at its maximal tolerated dose in the same schedule (Table 6). Importantly, this data confirms the experimental antitumor efficacy of AMP-53 in human lung cancer *in vivo* and the 8226 data validates the lack of MDR cross-resistance seen in MCF-7 breast cancer cells in the NCI tumor panel study.

Discussion

The azonafides represent an entirely new class of anthracene-based antitumor agents that were originally designed by computer-modeling of DNA binding.[1–3] The resultant 140+ compounds includes a diverse group of substituted compounds which appear to have unique mechanistic properties when compared with existing classes of DNA intercalators. Specifically, not all of the azonafides localize in the nucleus and some, such as AMP-53, localize in the cytoplasm in a punctuate type of distribution pattern.[19] In contrast, the unsubstituted azonafide AMP-1 was shown to distribute into cells in a perinuclear pattern. Other differences with existing DNA intercalators, such as mitoxantrone, include the lack of inhibition of topoisomerase II enzymes at equi-cytotoxic concentrations of AMP-53.[2] This difference was not seen with several other azonafides such as AMP-47 and -104, both of which strongly concentrated in the nucleus and inhibited topoisomerase II at cytotoxic drug concentrations. However, in the current results with AMP-1 and -53, in the NCI tumor cell line, there were nearly identical LC_{50} values for the two agents, despite the in marked differences in subcellular distribution,[19] and topoisomerase II inhibition.[7] The mechanistic differences between azonafides and other intercalators are likely not due to azonafide metabolism, which has been shown to primarily involve *N*-demethylation at the (dimethylamino)ethyl side chain, resulting in secondary or primary amine derivatives with markedly reduced cytotoxic activity.[20] This metabolic pattern differs substantially from that of amonafide which exhibited variable toxicity due to acetylation at its primary amino group at the 4-position of the naphthylene ring system.[21] The variability in amonafide acetylation produced much greater myelosuppression in patients who were rapid acetylators, which typically involves about one-third of a mixed population in the US. Nonetheless, despite this variability in metabolism, amonafide did demonstrate clinical antitumor activity. For example, amonafide produced an 18% response rate in patients with previously untreated advanced breast cancer[6] and a 6% response rate in patients with advanced metastatic or recurrent endometrial cancer.[5]

The activity of the azonafides reported herein is compatible with previous azonafide studies in other experimental preclinical settings. These include activity in several human tumor cell lines, and in murine tumors models including P-388 and L-1210 leukemias, and in B-16 melanoma.[4] The current results expand these murine tumor observations into the mammary 16C breast and Lewis lung cancer models, and into other human tumor systems. In addition, the azonafides were active in three different human tumor cell models, including (1) a standard panel of 56 human tumor cell lines, (2) colony-forming assays freshly

Table 6. Antitumor activity of AMP-53 for human tumors in SCID mice

Human tumor cell line	Agent	Dose (mg/kg/day)	%T/C	Week after implantation
8226 myeloma	AMP-53	4	31.3[a]	6
8226_{DOX40} myeloma	AMP-53	4	34.0[a]	6
HL-60 leukemia	AMP-53	4	39.2[b]	5
MCF-7 breast	AMP-53	4	39.4[b]	4
	mitoxantrone	0.45	55.3[b]	4
768-0 renal	AMP-53	4	105[b]	4
	mitoxantrone	0.45	103[b]	4
A549 non-small cell lung	AMP-53	3	36.6[a]	6
	mitoxantrone	0.45	47.9[a]	6

[a]New data.
[b]%T/C calculated from original data reported in Remers *et al.*[4]

isolated human tumor cells *in vitro* and (3) human tumor cell lines growing in SCID mice. In each of these models, there was consistent evidence of inhibition of human tumor cell growth, with the greatest activity noted for the 6-ethoxy-substituted azonafide, AMP-53. The superiority of AMP-53 in the direct comparison to doxorubicin in the colony-forming assays is important since this assay has been successfully used to identify second-generation anthracyclines with activity in human breast cancer.[22] These results also show much greater activity for azonafide than was seen with amonafide.[23] Amonafide was active against only 12% of primary human tumors,[23] compared to 88% of fresh tumors for which an IC_{50} could be obtained with AMP-53 (Table 3). In addition, the colony-forming assays also correctly excluded any specificity for AMP-53 in renal cell cancer, a finding that was substantiated by the lack of activity for AMP-53 in the 786-0 human renal cell cancer in SCID mice. The apparent specificity of AMP-1 for melanomas in the NCI tumor panel was not evaluated further, but should be investigated in SCID mice since the LC_{50} was >1 $\log_{10}$ lower in melanoma cell lines treated with this agent.

Nonetheless, AMP-53 demonstrated the greatest breadth of antitumor activity. It was singularly active in the Lewis lung cancer. It was also active in two human tumors growing in SCID mice: A549 non-small cell lung cancer and MCF-7 breast cancer. These results suggest that the spectrum of activity of AMP-53 is similar to that of doxorubicin and raises the question of what differentiates the two classes of agents. One major difference, reinforced in the current findings, is that AMP-53 maintains its activity in MDR tumors. In this case, AMP-53 remained active in a doxorubicin-resistant human MCF-7/ADR-RES breast cancer cell line *in vitro*, and in 8226_{DOX40} human myeloma cells growing in SCID mice. Furthermore, AMP-53 exhibited greater inhibitory potency than mitoxantrone against several human tumors growing in the SCID mouse model. Another advantage is reduced cardiotoxicity. Prior *in vitro* studies have shown markedly less cardiotoxicity *in vitro* for the azonafides compared to other classes of DNA intercalators.[2–4] These combined features, the lack of cross-resistance in human tumor cells over-expressing P-glycoprotein and greater selectivity for human cancer cells over cardiac myocytes *in vitro*, suggest that the azonafides, and particularly AMP-53, have important preclinical advantages over existing classes of DNA intercalators. These experimental findings suggest that AMP-53 should be considered for clinical evaluation in human breast and non-small cell lung cancer.

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(Received 12 December 2000; accepted 5 January 2001)