Preclinical Evaluation of Amino Acid Prodrugs of Novel Antitumor 2-(4-Amino-3-Methylphenyl)Benzothiazoles[1]

Tracey D. Bradshaw,[2] Michael C. Bibby, John A. Double, Iduna Fichtner, Patricia A. Cooper, Michael C. Alley, Susan Donohue, Sherman F. Stinson, Joseph E. Tomaszewjski, Edward A. Sausville, and Malcolm F. G. Stevens

Cancer Research Laboratories, School of Pharmaceutical Sciences, University of Nottingham, Nottingham NG7 2RD, United Kingdom [T. D. B., M. F. G. S.]; Max-Delbrűck-Centrum fűr Molekulare Medizin, 13122 Berlin, Germany [I. F.]; Cancer Research Unit, School of Life Sciences, University of Bradford, Bradford BD7 1DP, United Kingdom [M. C. B., J. A. D., P. A. C.]; and Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, NIH, Frederick, Maryland 21702-1201 [M. C. A., S. D., S. F. S., J. E. T., E. A. S.]

Abstract

Novel 2-(4-aminophenyl)benzothiazoles (*e.g.,* compounds 1 and 2) possess highly selective, potent antitumor properties *in vitro* and *in vivo*. Elucidation of the mechanism of action of this structurally simple class of compounds has occurred in parallel with selection of a candidate clinical agent. Antitumor benzothiazoles induce and are biotransformed by cytochrome P 450 1A1 to putative active, as well as inactive metabolites. Metabolic inactivation of the molecule has been thwarted by isosteric replacement of hydrogen with fluorine atoms at positions around the benzothiazole nucleus. Amino acid conjugation to the exocyclic primary amine function of 2-(4-aminophenyl)benzothiazoles has been used to overcome limitations posed by drug lipophilicity. Water soluble, chemically stable prodrugs rapidly and quantitatively revert to their parent amine in mice, rats, and dogs *in vivo*. Plasma concentrations of 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (2) regenerated from the lysylamide prodrug (2b), sufficient to elicit cytocidal activity against ZR-75–1 and T47D human mammary carcinoma cell lines persist >6 h. The growth of breast (MCF-7) and ovarian (IGROV-1) xenograft tumors is significantly retarded by 2b. Manageable toxic side effects are reported from preclinically efficacious doses of 2b. Cytochrome P 450 1A1 protein expression, selectively induced in sensitive carcinoma cells, was detected in MCF-7 and IGROV-1 tumors 24 h after treatment of mice with 2b (20 mg/kg). The lysyl amide prodrug of 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole is potentially suitable for clinical evaluation.

Introduction

Novel 2-(4-aminophenyl)benzothiazoles (Fig. 1) possess potent and intriguing antitumor properties (1, 2). In the NCI[3] *in vitro* anticancer drug screen, remarkable selectivity is demonstrated. Mean graph patterns are highly characteristic of this class of compounds only (2, 3), which represent a mechanistic class distinct from clinically used chemotherapeutic agents. *In vivo,* superior antitumor activity was exhibited by 2-(4-amino-3-methylphenyl)benzothiazole 1 in breast, colon, and ovarian xenograft studies when compared with the 3′-halogeno congeners.

In vitro, induction of CYP1A1-catalyzed biotransformation of compound 1 is critical for execution of antitumor activity (4–6). Covalent binding, detected between 1 and recombinant CYP1A1, requires metabolism and is significantly reduced by glutathione (4). This suggests formation of an electrophilic, reactive intermediate species. Paradoxically, the major *C*-6 oxidation biotransformation product, liberated into nutrient media, is devoid of antitumor activity (7), possessing mitogenic properties at μM concentrations (8). Moreover, this metabolite antagonizes cellular uptake of 1, covalent binding between CYP1A1 and 1, CYP1A1 activity, and growth inhibition induced by 1. Thus, biotransformation to hydroxy derivatives is likely to effect the unique biphasic dose-response relationship characteristic of growth inhibition *in vitro*.

Ab initio Frontier Molecular Orbital Calculations have been used to predict the presence or absence of exportable hydroxy metabolites of 2-(4-amino-3-methylphenyl)fluorobenzothiazoles (9), and 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (2) has been synthesized, which successfully blocks *C*-6 oxidation (8). Thus, compound 2 is the favored analogue for clinical consideration, demonstrating enhanced efficacy *in vitro* and superior potency *in vivo* against human breast and ovarian tumor xenografts implanted in nude mice. Lipophilicity, however, poses a pharmaceutical challenge, as an aqueous i.v. formulation is desired to minimize the possibility of first pass deactivating metabolism and improve drug bioavailability.

The primary aromatic amine, dapsone, has been derivatized as amino acid amides (10). Similarly, the exocyclic primary amine function of 2-(4-amino-3-methylphenyl)benzothiazoles has been conjugated successfully to alanine and lysine residues as mono- and dihydrochloride salts, respec-

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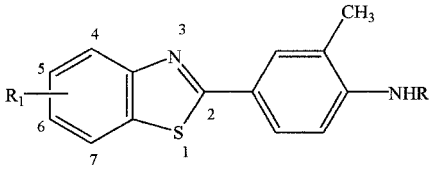
[2] To whom requests for reprints should be addressed, at the Cancer Research Laboratories, School of Pharmaceutical Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, United Kingdom.

[3] The abbreviations used are: NCI, National Cancer Institute; CYP1A1, cytochrome P 450 1A1; HPLC, high-performance liquid chromatography; AhR, aryl hydrocarbon receptor; GI_{50}, drug concentration which inhibits cell growth by 50%; TGI, total growth inhibition; civ, continuous i.v. infusion; MTD, maximum tolerated dose.



compound	R[b]	R_1
1	H	H
1a	L-alanyl	H
1b	L-lysyl	H
2	H	5-F
2a	L-alanyl	5-F
2b	L-lysyl	5-F
3	H	6-F
3a	L-alanyl	6-F
3b	L-lysyl	6-F

Fig. 1. Structure of 2-(4-aminophenyl)benzothiazoles and their amino acid prodrugs.

[b] L-alanyl: R = CO-CH(CH_3)-NH_2.HCl

L-lysyl: R = CO-CH($(CH_2)_4$-NH_2)-NH_2.2HCl

tively (11), fulfilling the criteria for a suitable prodrug: water solubility and chemical stability. *In vitro,* selective antitumor activity is retained as parent amine is rapidly and quantitatively regenerated in the presence of carcinoma cells.[4] Herein, we compare the pharmacokinetic properties of alanyl and lysyl prodrugs in mice, rats, and dogs and demonstrate retention of selective antitumor activity *in vivo*. Putative sites of toxicity have been identified as the mechanism of action of this class of compound is considered. Finally, their suitability as clinical candidates is discussed.

Materials and Methods

Pharmacokinetic Studies in Mice. Objectives of initial murine pharmacokinetic studies with 1a and 1b were to characterize the disposition of the compounds to allow optimization of dosing regimens for *in vivo* efficacy studies. Doses of 25–30 mg/kg were selected MTDs for repeated daily injection in mice. To approximate the doses of the initial studies, equimolar target doses (70 μM/kg) of prodrugs of fluorinated derivatives were selected. Male CD2F1 mice were given i.v. doses of 26.5 mg/kg 1a, 31 mg/kg 1b, 25.6 mg/kg 2a and 3a, or 32.2 mg/kg 2b and 3b as a 30-s infusion in the tail vein, using a vehicle of sterile water and a dose volume of 1 μl/gram body weight. The equivalent molar concentration of compound 2 (18 mg/kg) was similarly administered in a vehicle of DMSO. Groups of three mice were exsanguinated via the suborbital sinus at intervals from 2 min to 6 h. Plasma proteins were precipitated by the addition of 150 μl of acetonitrile to 50 μl of plasma. Samples were vortexed for 1 min and centrifuged for 5 min (14,000 $\times$ *g*). Supernatant (150 μl) was removed and mixed with 100 μl of 10% acetonitrile in 1% acetic acid. HPLC separations were effected on a J'Sphere ODS-AQ analytical column (4.6 $\times$ 150 mm; YMC, Inc., Wilmington, NC). The column was eluted at a flow rate of 1 ml/min with sequential linear gradients. The mobile phase was formed by increasing the acetonitrile concentration in 1% acetic acid over 20 min by mixing acetonitrile-water-acetic acid solutions: (10:90:1, volume for volume and 80:20:1, volume for volume). Samples (230 μl) were injected into the system and concurrently analyzed for concentrations of prodrug and parent amine. Column eluant was monitored at 324 nm using HP 1050 series UV diode array detector.

Pharmacokinetic Studies in Rats. In an initial study, rats received 25 mg/kg 1a and 1b i.v. HPLC methods were developed to allow simultaneous detection of 1, 1a, and 1b in addition to resolution of degradation products and metabolites. Separation was effected at room temperature on a Primesphere 5C-18 HC110 column (250 $\times$ 3.2 mm) using a mobile phase of 40% acetonitrile in 5 mM potassium dihydrogen phosphate (pH 7) delivered at a flow rate of 0.8 ml/min. Compounds were detected at 320 nm.

A single male Sprague Dawley rat received either 25 mg/kg 1a or 31 mg/kg 1b in sterile water as a 30-s infusion in a catheter implanted in the jugular vein (dose volume 1 μl/gram body weight). Blood samples were taken through the catheter at selected intervals from 3 min up to 6 h. Plasma samples were concurrently monitored for concentrations of 1, 1a, and 1b by HPLC analysis.

Pharmacokinetic Studies in Dogs. Compound 2b (2, 4, and 14.3 mg/kg) was administered to one male and one

[4] T. D. Bradshaw, M-S. Chua, H.L. Browne, V. Trapani, E.A. Sausville, and M.F.G. Stevens. *In vitro* evaluation of amino acid prodrugs of novel antitumour 2-(4-amino-3-methylphenyl)benzothiazoles, submitted for publication.

female beagle dog per treatment group as a 1-h civ. This concentration (14.3 mg/kg 2) is the molar equivalent of 7.5 mg/kg 1, used previously in mouse efficacy studies performed at the NCI.[5] Plasma samples were collected 5 min before completion of the infusion and at six scheduled time points, 2 min through 8 h after termination of the infusion. Plasma concentrations of 2 and 2b were concurrently assayed by HPLC.

Pharmacokinetic Data Analysis. Plots of plasma concentration against time were constructed. Polyexponential, nonlinear regression analysis was performed on the plasma concentration-time profiles using an iterative computer-assisted curve-fitting program (Table Curve; Jandel Scientific, San Rafael, CA). Computer analyses of parameters, including slopes and intercepts of the regression lines, allowed calculation of secondary pharmacokinetic parameters, such as area under the concentration-time profile and efficiency of prodrug conversion to parent amine.

***In Vivo* Studies.** Female B6D2F1 nude mice bearing s.c. MCF-7 (ER+) and MT-1 (ER−) breast xenograft tumors were treated with 1a and 1b administered i.v. in a vehicle of saline/Tween 80. The target schedule of three times once weekly with doses ranging from 5 to 25 mg/kg/injection, guided by previous studies (1), enabled MTD determination.

IGROV-1 ovarian and MCF-7 breast xenografts were transplanted s.c. into flanks of NCR-*Nu* female nude mice. Animals were treated i.v. with 2b in saline according to the following schedules: 20 mg/kg single dose, 20 mg/kg days 0 and 7, 4 mg/kg days 0 and 1–4. Drinking water of mice bearing MCF-7 xenografts was supplemented with 60-day release 17β-estradiol pellets (0.72 mg/pellet; Innovative Research), maintaining blood levels of 300–400 pg/ml. Antitumor activity in MCF-7 xenografts of prodrug 2b was compared with parent amine 2. To examine the selective nature of growth inhibition *in vivo,* mice bearing MCF-7 and MDA-MB-435 tumors in opposite flanks were treated with 5 mg/kg compound 2 in DMSO (10%) plus oil, administered i.p. on 4 consecutive days (days 0–3).

Toxicity Study in Dogs. A male and one female dog received one of the following treatments: vehicle alone (5% dextrose in water), 1-h civ 2 mg/kg compound 2b, 1-h civ 4 mg/kg 2b, and 24-h civ 14.3 mg/kg 2b. Hematology and clinical chemistry parameters were measured, and histopathology evaluation was conducted on one dog that became moribund.

Western Blot Protocol. Tissues were recovered from mice 24 h after treatment with 2b (20 mg/kg, i.p.) or vehicle alone (DMSO + oil) and snap frozen. Tumors, lungs, and livers were homogenized in buffer containing 20 mM Tris-HCl (pH 7.4), 2 mM EDTA (pH 7.4), 2 mM EGTA (pH 7.4), 6 mM β-mercaptoethanol, 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 1% NP40. Homogenates were sonicated (MSE Soniprep 150 3 $\times$ 10 s, amplitude 20 μm) and prepared for examination of CYP1A1 protein expression. After protein determination ($n = 3$; Ref. 12) and addition of sample buffer, samples were boiled at 95°C for 5 min, and solubilized proteins (50 μg) were separated by SDS polyacrylamide gel (10%) electrophoresis. Proteins were electroblotted to polyvinylidene difluoride membranes and probed for CYP1A1 protein with polyclonal antiserum specific for human CYP1A1/1A2 (Gentest Corp.). Secondary antibody was conjugated to alkaline phosphatase, and CYP1A1 was detected after brief (<10 min) incubation with bromochloroindolyl phosphate and nitroblue tetrazolium in alkaline phosphatase buffer. Molecular weight markers and a positive control of recombinant CYP1A1 (Gentest Corp.), included in blots, confirmed detection of M_r 52,000 CYP1A1 protein.

Results

Pharmacokinetic Results. A summary of the pharmacokinetic results obtained after prodrug administration to mice, rats, and dogs is given in Table 1.

Mice. Plasma concentrations of 1 achieved after injection of 1a exceeded those reached after injection of 1b. The area under the plasma concentration-time profile was greater by >40% and efficiency of conversion, or bioavailable fraction, of prodrug to parent was 24% with 1a and 18% with 1b.

Concentrations of 2b and 2 in plasma, after i.v. administration of 32.2 mg/kg 2b are shown in Fig. 2*A*. Plasma concentrations of 2b declined in a biexponential manner with elimination half-lives ($t_{1/2}\beta$) for the initial and terminal phases of 2 and 43 min, respectively. The total rate of plasma clearance was 191 ml/min/kg. Plasma levels of 2 rapidly increased, maintaining concentrations between 2 and 4 μM between 13 min and 120 min after administration of 2b (Table 1, duration of maximum amine plasma concentrations). A peak concentration of 4.11 μM 2 was achieved after 1 h and remained above 1 μM for 4 h after treatment, thereafter decreasing with $t_{1/2}\beta$ of 110 min. The efficiency of conversion, or bioavailable fraction, of prodrug to parent compound 2 was 42% with 2a and 45% with 2b. By comparison, the pharmacokinetic properties of 2 have been determined after equimolar i.v. administration. Plasma concentrations declined in a biexponential manner with initial and terminal $t_{1/2}\beta$ of 19 and 99 min, respectively. Total plasma clearance rate was 41 ml/min/kg, indicating a more rapid rate of elimination of 2 than for compound 1 ($t_{1/2}\beta$ 187 min, plasma clearance rate 22 ml/min/kg; results not shown).

Both plasma concentration and area under the plasma concentration-time profile of 3, after injection of 3b, was substantially higher than after injection of 3a. However, the area under the plasma concentration-time profile of 2, after administration of equimolar doses of 2a and 2b, exceeded that for 3.

Rats. Although 1a and 1b were stable in rat plasma *in vitro,* both these compounds were readily removed from plasma and converted to parent amine 1 when either prodrug was given to rats i.v. at 25 mg/kg; compound 1 was detectable in plasma as early as 3 min postadministration. Regenerated 1 was further *N*-acetylated, this being the main metabolic route of 1 in rats (13), forming the inactive *N*-acetyl derivative 9 min after dosing. *N*-acetylation was extensive, such that negligible liberated 1 could be detected after 360 min (result not shown).

[5] S. Donohue, NCI, NIH, Frederick, MD 21702-1201, personal communication.

Table 1 A summary of the pharmacokinetic data generated after i.v. administration of prodrugs to mice, rats, and dogs

Species	Dose (mg/kg: i.v.)	Prodrug	Prodrug ($t_{½}$ β: min)	Total rate plasma clearance: ml/min/kg	Max. plasma amine conc; μM	Duration: min	Amine ($t_{½}$ β: min)
Mouse	26.5	1a	3 75	241	3–6	7–90	79
	31.3	1b	1 14 88	160	3–5	10–90	67
	25.6	2a	2.4 115	190	2–3	9–120	101
	32.2	2b[a]	2 43	191	2–4	13–120	110
	26.6	3a	4 70	261	2	60	123
	32.2	3b	1.4 30	177	3–4	15–90	66
Rat	25	1a	2 29 249	77	5–6	30–45	143
	31	1b	3 28 97	188	6–12	10–90	83
Dog							
Male	14.3	2b[b]	17	17.7	2–5	55–300	127
Female	14.3	2b	30	28.0	2–5	62–180	91

[a,b] These data are represented graphically in Figs. 2, *A* and *B*, respectively.

In a separate study, summarized in Table 1, the efficiency of conversion, or bioavailable fraction of prodrug to parent, was 87% with 1a and 106% with 1b, far greater than observed in mice.

Dogs. Prodrug 2b (14.3 mg/kg) was rapidly converted to parent amine 2 with $t_{1/2}\beta$ of 17 and 30 min in one male and one female dog (Fig. 2*B*), respectively. Plasma concentrations of 2 were between 2 and 5 μM between 60 and 300 min after the start of the infusion. At doses of 2 and 4 mg/kg 2b, peak plasma concentrations of 2 were 0.5 and 0.9 μM, respectively.

Toxicity Results. Gastrointestinal toxicity (emesis, diarrhea, and low food consumption) and lethargy were exhibited by two dogs for 5–7 days after treatment with 14.3 mg/kg 2b, and by day 7, the dogs had lost between 9 and 14% body weight. Increased levels of serum bile acids, γ glutamyltransferase, aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase were measured. On day 5, the male dog that received a 24-h infusion exhibited labored respiration and was sacrificed because of moribund condition. Histopathology examination revealed depletion/atrophy in the bone marrow, lymphoid tissues and gut-associated lymphoid tissue, intestinal lesions, and extensive acute inflammation in the alveolar spaces with edema and hemorrhage; pulmonary toxicity was dose limiting. At doses of 2 and 4 mg/kg 2b (1-h civ), emesis and soft stool occurred on the day of dosing, but during days 1–5, no effects on clinical pathology parameters and no lesions in lung or liver tissues were observed. Moreover, plasma concentrations of 2 reached a peak of 0.9 μM, an efficacious concentration *in vitro* against sensitive breast cancer cell lines (MCF-7, ZR 75, T47D; 8).

In vivo antitumor activity MTDs of 1a and 1b, established in female B6D2F1 mice after i.v. injection, was 25 mg/kg and

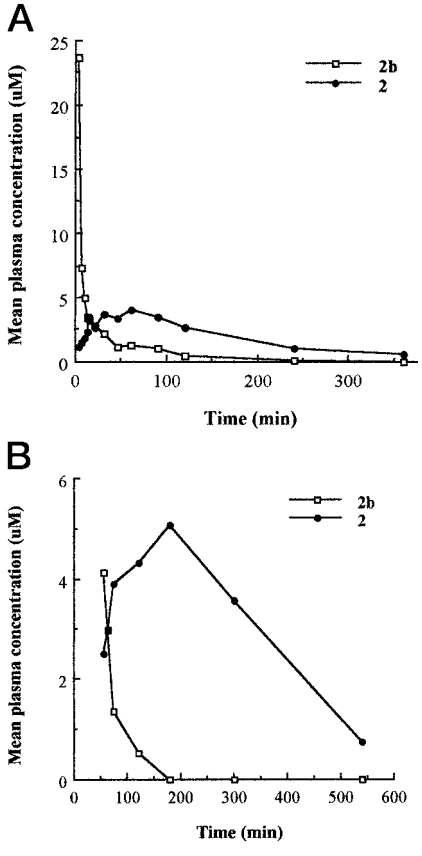


Fig. 2. Plasma concentrations of 2b (□) and 2 (●) after i.v. infusion of 32.2 mg/kg 2b in mice (*A*) and 14.3 mg/kg 2b in a male dog (*B*).

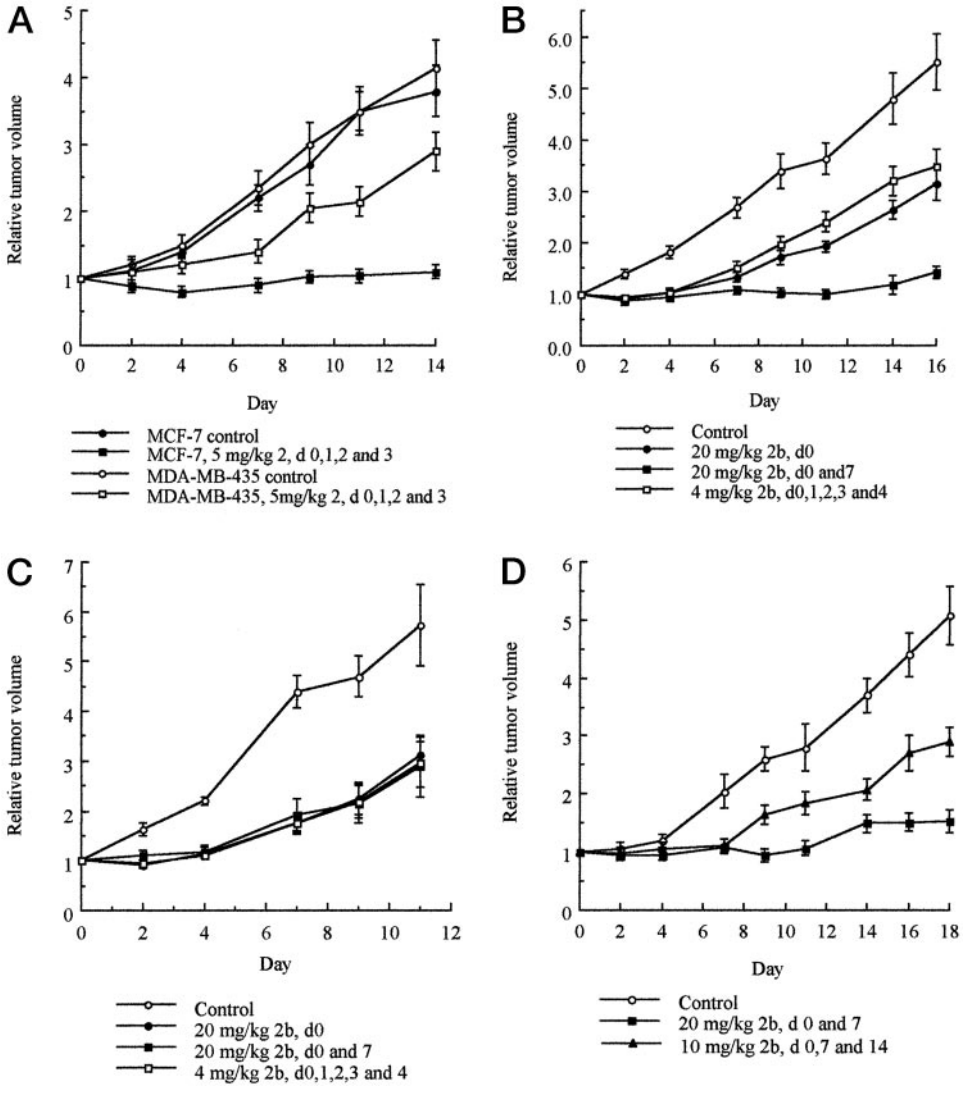


Fig. 3. *A,* effect of amine 2 on the growth of MCF-7 and MDA-MB-435 tumor xenografts transplanted in opposite flanks of the same mouse. Effect of 2b on the growth of IGROV-1 ovarian (*B*) and MCF-7 breast tumor xenografts (*C* and *D*).

resulted in catalepsia and convulsions. MTD for three times once weekly repeat treatments of 1a was 15 mg/kg/i.v. injection and for 1b was between 10 and 15 mg/kg/i.v. injection. Antitumor activity of 1a and 1b was examined in MCF-7 (ER+) and MT-1 (ER−) breast xenografts implanted s.c. into the flanks of nude mice. Significant antitumor effects were elicited by both 1a and 1b in MT-1 carcinoma xenografts adopting a three times once weekly schedule (percentage of treated *versus* control growth $< 50\%$). In MCF-7 xenografts, tumor growth retardation was not as prominent; however, at a dose of 12.5 mg/kg, 1a caused greater growth retardation than 1b. Negligible hematological effects were observed; however, body weight reduction and toxicity accompanied significant tumor growth inhibition (84%) after treatment with 25 mg/kg 1a.

The selective nature of growth inhibition of 2-(4-aminophenyl)benzothiazoles *in vitro* has been documented previously (1–3). GI_{50} values < 10 nM were obtained in sensitive cells (*e.g.,* MCF-7), whereas GI_{50} values exceeding 100 μM were encountered in inherently resistant cell lines (*e.g.,* MDA-MB-435 mammary carcinoma cells). Mechanisms underlying such stark selectivity have been studied (7, 4). The selective nature of growth inhibition *in vivo* has now been demonstrated unequivocally in a study where MCF-7 and MDA-MB-435 xenografts were transplanted in opposite flanks of the same mouse. The growth of MCF-7 tumors was only significantly retarded after i.p. treatment with compound 2 (Fig. 3*A*). Antitumor activity of amine 2 was not compromised after amino acid derivatization: significant *in vivo* tumor growth inhibition was encountered in nude mice bearing MCF-7 breast and IGROV-1 ovarian xenografts after treatment with 2b.

To optimize the dose, three schedules were compared in the IGROV-1 model and were found to be equiactive (Fig. 3*B*). In contrast, 20 mg/kg 2b administered on days 0 and 7 elicited superior antitumor activity in MCF-7 xenografts when compared with a single dose of 20 mg/kg, 4 mg/kg administered daily for 5 consecutive days (Fig. 3*C*), or 10 mg/kg administered three times once weekly (Fig. 3*D*).

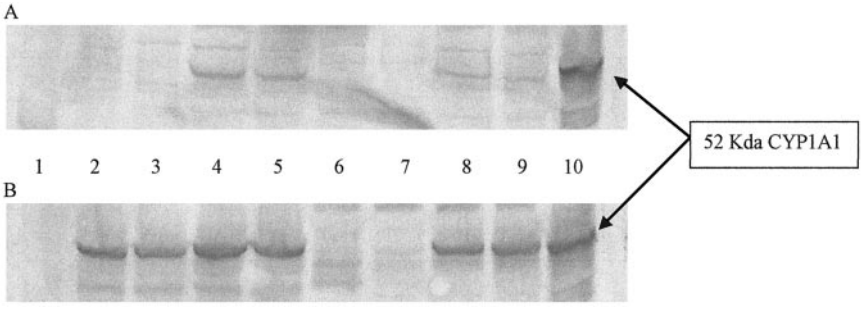


Fig. 4. Western Blot analyses of CYP1A1 expression in IGROV-1 (*A, Lanes 2–5*) and MCF-7 tumor (*Lanes 6–9*), liver (*B, Lanes 2–5*), and lung homogenates (*Lanes 6–9*) from four untreated mice (*Lanes 2, 3, 6,* and *7*; *A* and *B*) and four mice treated with 20 mg/kg 2b (*Lanes 4, 5, 8,* and *9*; *A* and *B*). Positive controls (*Lane 10*) included 5 μg of microsomes expressing recombinant CYP1A1.

Mechanism of Action. We have observed previously the selective induction of CYP1A1 protein expression in cell lines sensitive to growth inhibitory properties of 1 and reported NADPH-dependent covalent binding between [^{14}C]1 and recombinant CYP1A1 (4). During this study, we examined CYP1A1 protein expression in human tumor xenografts. Fig. 4*A* demonstrates induction of CYP1A1 protein expression in homogenates of IGROV-1 and MCF-7 tumors, 24 h after treatment of mice with prodrug 2b (20 mg/kg, i.p.). No CYP1A1 protein was detected in homogenates of IGROV-1 and MCF-7 tumors recovered from untreated mice. In MDA-MB-435 xenografts, CYP1A1 protein expression was neither constitutive nor induced by treatment of mice with 20 mg/kg 2b.

Lung and liver tissues were recovered from animals 24 h after treatment (20 mg/kg 2b). Fig. 4*B* compares CYP1A1 protein expression in these tissues with expression in lungs and livers of untreated mice. CYP1A1 protein was clearly induced in the lungs of mice treated with 2b; constitutive expression was not detected. In livers, CYP1A1 protein was expressed constitutively and after treatment of mice with 2b.

Discussion

Data have been presented describing the biological properties of alanyl and lysyl amide derivatives of 2-(4-aminophenyl)benzothiazoles *in vivo*. Prodrugs undergo rapid and quantitative reversion to parent species[4] (*e.g.,* 2b, Fig. 2). Plasma concentrations of 2, 2a, and 2b in mice have been compared with the concentrations required to elicit GI_{50}, TGI, and 50% lethality in MCF-7, T47D, and ZR-75 breast cell lines after 45-min exposure to 2, 2a, or 2b.[6] Potentially therapeutically favorable pharmacokinetic properties have been achieved. Plasma levels of 2 exceeding five times the concentrations required to achieve GI_{50}, TGI, and cytocidal activity in T47D- and ZR-75-sensitive breast cell lines and GI_{50} and TGI activity in all three cell lines persisted for 4 h and $>$6 h after a single i.v. infusion of 2a or 2b, respectively.

The remarkably potent and selective antitumor activity characteristic of compound 2 *in vitro* (8) is retained *in vivo* (Fig. 3*A*) and after prodrug modification *in vitro*[4] *and in vivo* (Fig. 3, *B–D*); 2b suppresses significantly the growth of MCF-7 breast and IGROV-1 ovarian xenografts *in vivo*.

An overview of our comprehension of the mechanisms of action of this class of agent is illustrated in Fig. 5. Planar, hydrophobic 2-(4-aminophenyl)benzothiazoles fulfill structural requirements for binding to the AhR and are potent AhR agonists (14). Enhanced CYP1A1 transcription in sensitive cells (5) is accompanied by translocation of AhR to the nucleus, increase in xenobiotic response element (XRE)-driven luciferase activity, and induced formation of protein-DNA complexes on the XRE sequence of the CYP1A1 promoter. Highly specific induction of CYP1A1 activity and protein expression in sensitive cell lines only follows exposure of cells to 2-(4-aminophenyl)benzothiazoles (4), including IGROV-1 and MCF-7 cells. *In vivo,* only tumor xenografts whose growth was inhibited by 2b (Fig. 3, *B–D*) demonstrated inducible CYP1A1 protein (Fig. 4). This observation raises the possibility that monitoring CYP1A1 expression in human tumors is achievable and may provide a biomarker for the identification of sensitive tumor phenotypes.

CYP1A1-mediated metabolism of aminophenylbenzothiazoles generates putative reactive electrophilic species and benzothiazole-derived covalent binding to CYP1A1 (4), which leads to the formation of significant DNA adducts,[7] DNA damage (15) and recognition (6), engagement of apoptotic machinery, and, ultimately, selective cell death.

Thus, in those organs possessing inducible CYP1A1 (*e.g.,* liver and lung), benzothiazole-induced hepato or pulmonary toxicity must be anticipated. 2b is able to induce CYP1A1 protein expression in the lungs of treated mice (20 mg/kg, Fig. 4*B*). Indeed, dose-limiting pulmonary lesions were detected in one dog receiving 14.3 mg/kg 2b over 24 h; in addition, increased levels of serum bile acids, γ glutamyltransferase, aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase were consistent with liver damage. However, doses of 2 and 4 mg/kg were well tolerated and yielded efficacious plasma concentrations of amine 2, indicating that a therapeutic window may be achievable. Preliminary studies have revealed the presence of benzothiazole-derived DNA adducts within the livers but not the lungs of mice receiving a therapeutically effective concentration of 2b (20 mg/kg).[7] However, in human hepatocyte

[6] M. Alley, NCI, NIH, Frederick, MD 21702-1201, personal communication.

[7] C-O. Leong, M. Gaskell, R. Heydon, E.A. Martin, P.B. Farmer, M.C. Bibby, J. A. Double, P.A. Cooper, T.D. Bradshaw, and M.F.G. Stevens. Potent, selective antitumor 2-(4-aminophenyl)benzothiazoles generate DNA adducts in sensitive tumors, submitted for publication.

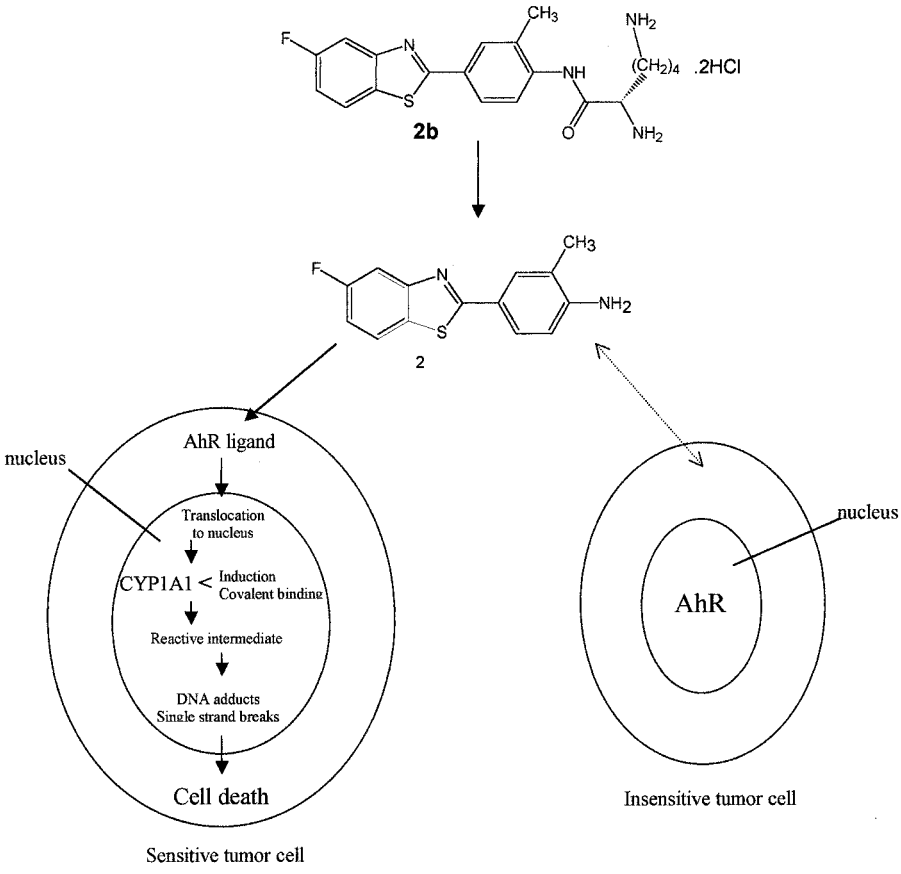


Fig. 5. Proposed mode of action of clinical candidate 2b. Prodrug (*2b*) breaks down in the presence of cells to release 2, a potent agonist for the cytosolic AhR. Thus, 2 is selectively sequestered by sensitive tumor cells only and after translocation to the nucleus induces CYP1A1 transcription, protein expression, and activity. Subsequent formation of reactive metabolites effects lethal DNA damage. Adducts are detected before cell death.

culture, viability remained > 65% after exposure of hepatocytes to 1 or 10 μM compound 2 (96 h). Future experiments will investigate the potential for DNA repair in murine liver.

To conclude, alanyl- and lysyl-amide hydrochloride salts may be conjugated to the exocyclic primary amine function of 2-(4-aminophenyl)benzothiazoles (11), rendering these prodrugs chemically stable, water soluble, and suitable for parenteral delivery. Of the prodrug candidates examined, 2b presents superior pharmacokinetic properties, with manageable toxicity in dogs at concentrations liberating plasma levels of 2, which are potentially efficacious. *In vivo* antitumor tests revealed selectivity and efficacy; in addition, CYP1A1 protein expression is induced in sensitive xenograft models after treatment of tumor-bearing mice with 2b. In view of the encouraging preclinical properties expounded by the lysyl-amide dihydrochloride salt, 2b, combined with the superior efficacy and retained selectivity of compound 2, prodrug 2b has been selected to undergo Phase I clinical evaluation in 2002, under the auspices of the Cancer Research Campaign, United Kingdom.

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References

1. Shi, D-F., Bradshaw, T. D., Wrigley, S., McCall, C. J., Lelieveld, P., Fichtner, I., and Stevens, M. F. G. Antitumor benzothiazoles. 3. Synthesis of 2-(4-aminophenyl)benzothiazoles and evaluation of their activities against breast cancer cell lines *in vitro* and *in vivo*. J. Med. Chem., *39:* 3375–3384, 1996.

2. Bradshaw, T. D., Wrigley, S., Shi, D-F., Schultz, R. J., Paull, K. D., and Stevens, M. F. G. 2-(4-Aminophenyl)benzothiazoles: novel agents with selective profiles of *in vitro* antitumour activity. Br. J. Cancer, *77:* 745–752, 1998.

3. Bradshaw, T. D., Shi, D-F., Schultz, R. J., Paull, K. D., Kelland, L., Wilson, A., Garner, C., Fiebig, H. H., Wrigley, S., and Stevens, M. F. G. Influence of 2-(4-aminophenyl)benzothiazoles on growth of human ovarian carcinoma cell lines *in vitro* and *in vivo*. Br. J. Cancer, *78:* 421–429, 1998.

4. Chua, M-S., Kashiyama, E., Bradshaw, T. D., Stinson, S. F., Brantley, E., Sausville, E. A., and Stevens, M. F. G. Role of CYP1A1 in modulation of antitumor properties of the novel agent 2-(4-amino-3-methylphenyl)-benzothiazole (DF 203, NSC 674495) in human breast cancer cells. Cancer Res., *60:* 5196–5203, 2000.

5. Hose, C., Riviera, M., Sausville, E., and Monks, A. Induction of cytochrome P450 CYP1A1 and cytochrome P450 CYP1B1 by 2-(4-amino-3-methylphenyl)benzothiazole (BZ) in 60 human tumor cell lines: correlation with BZ toxicity. Proc. Am. Assoc. Cancer Res., *42:* 511, 2001.

6. Monks, A., Rivera, M. I., Harris, E., Hose, C., and Sausville, E. A. Gene expression changes induced by a novel antitumor agent, 2-(4-amino-3-methylphenyl)benzothiazole, in MCF-7, a sensitive breast cancer cell line, using cDNA microarrays. Proc. Am. Assoc. Cancer Res., *42:* 635, 2001.

7. Kashiyama, E., Hutchinson, I., Chua, M-S., Stinson, S. F., Phillips, L. R., Kaur, G., Sausville, E. A., Bradshaw, T. D., Westwell, A. D., and Stevens, M. F. G. Antitumor benzothiazoles. 8. Synthesis, metabolic formation, and biological properties of the *C*- and *N*-oxidation products of antitumour 2-(4-aminophenyl)benzothiazoles. J. Med. Chem., *42:* 4172–4184, 1999.

8. Hutchinson, I., Chua, M-S., Browne, H. L., Trapani, V., Fichtner, I., Bradshaw, T. D., Westwell, A. D., and Stevens, M. F. G. Antitumor benzothiazoles. 14. The synthesis and unique antitumour properties of fluorinated 2-(4-aminophenyl)benzothiazoles. J. Med. Chem., *44:* 1446–1455, 2001.

9. O'Brien, S. E., Browne, H. L., Bradshaw, T. D., Westwell, A. D., Laughton, C. A., and Stevens, M. F. G. CYP1A1 mediated activation of novel antitumour agents: Frontier Molecular Orbital methods rationalise and predict metabolites. 12th International Conference on Cytochrome P450: 115, 2001.

10. Pochopin, N. L., Charman, W. N., and Stella, V. Y. Amino acid derivatives of dapsone as water soluble prodrugs. Int. J. Pharm., *121:* 157–167, 1995.

11. Hutchinson, I., Jennings, S. A., Vishnuvajjala, B. R., Westwell, A. D., and Stevens, M. F. G. Antitumor benzothiazoles. 16. Synthesis and pharmaceutical properties of antitumor 2-(4-aminophenyl)benzothiazole amino acid prodrugs. J. Med. Chem., *45:* 744–747, 2002.

12. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., *72:* 248–254, 1976.

13. Chua, M-S., Shi, D-F., Wrigley, S., Bradshaw, T. D., Hutchinson, I., Shaw, P. N., Barrett, D. A., Stanley, L. A., and Stevens, M. F. G. Antitumour benzothiazoles. 7. Synthesis of 2-(4-acylaminophenyl)benzothiazoles and investigations into the role of acetylation in the antitumour activities of the parent amines. J. Med. Chem., *42:* 381–392, 1999.

14. Loaiza-Perez, A. I., Trapani, V., Hose, C., Singh, S. S., Trepel, J., Stevens, M. F. G., Bradshaw, T. D., and Sausville, E. A. The AhR mediates sensitivity of MCF-7 breast cancer cells to the antitumor agent 2-(4-amino-3-methylphenyl)benzothiazole. Mol. Pharmacol., *61:* 13–19, 2002.

15. Bradshaw, T. D., Chua, M-S., Matthews, C. S., and Stevens, M. F. G. Mechanisms of acquired resistance to 2-(4-aminophenyl)benzothiazole (CJM 126, NSC 34445). Br. J. Cancer, *83:* 270–277, 2000.