Antitumor Benzothiazoles. 16.[1] Synthesis and Pharmaceutical Properties of Antitumor 2-(4-Aminophenyl)benzothiazole Amino Acid Prodrugs

Ian Hutchinson,[†] Sharon A. Jennings,[†] B. Rao Vishnuvajjala,[‡] Andrew D. Westwell,[†] and Malcolm F. G. Stevens*[,†]

Cancer Research Laboratories, School of Pharmaceutical Sciences, University of Nottingham, Nottingham NG7 2RD, U.K., and Pharmaceutical Resources Branch, Division of Cancer Treatment and Diagnosis, National Cancer Institute, 6130 Executive Boulevard, Executive Plaza North, Room 8052, Rockville, Maryland 20852-7446

Received August 20, 2001

A series of water-soluble L-lysyl- and L-alanyl-amide prodrugs of the lipophilic antitumor 2-(4-aminophenyl)benzothiazoles has been synthesized to address formulation and bioavailability issues related to the desired parenteral administration of the chosen clinical candidate. The prodrugs exhibit the required pharmaceutical properties of good water solubility (in weak acid) and stability at ambient temperature and degradation to free base in vivo. The lysyl-amide of 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (NSC 710305, **6d**) has been selected for phase 1 clinical evaluation.

Introduction

Small and simple heterocyclic structures often have surprisingly complex biological properties. Antitumor 2-(4-aminophenyl)benzothiazoles are a case in point: their development from humble beginnings as synthetic intermediates in a program searching for tyrosine kinase inhibitors to their present status as agents in advanced preclinical development is a remarkable one. Structure−activity relationship studies based on the initial lead compound 2-(4-aminophenyl)benzothiazole (**1a**) established that certain substituents (Me, Cl, Br, I) in the 3′-position of the phenyl group (Figure 1) produced novel agents with potent activity in certain breast, ovarian, renal, colon, and lung cell lines in vitro.[2−4] Particularly noteworthy features of this series were their unique in vitro selectivity fingerprint (COMPARE[5] negative with other known clinical classes of antitumor agent in the NCI Developmental Therapeutics 60 cell line screen) and highly unusual biphasic dose−response relationship. On the basis of superior in vivo activity, 2-(4-amino-3-methylphenyl)benzothiazole (DF 203; NSC 674495; **1b**) was initially selected as the lead compound for further study.

Mechanistic studies have established the crucial role of metabolism[6,7] in mediating the antitumor effects of this class of agent. The major metabolite of compound **1b** in vitro was found to be the corresponding 6-hydroxy analogue (6OH 203; NSC 703785; **2**)[7] (Figure 1) and the enzyme responsible for this biotransformation to be the P450 isoform CYP1A1.[8,9] The identification of the 6-hydroxy metabolite, however, presented problems in terms of potential preclinical advancement of the project. Compound **2** was found to be both inactive in cell lines sensitive to parent compound **1b** and to antagonize the CYP1A1 activation step crucial to the antitumor activity of **1b** (thus accounting, at least in part, for the biphasic dose−response relationship). Our medicinal chemistry approach to circumvent this deactivating metabolism centered on the synthesis of various fluorinated analogues of **1b**, from which, surprisingly, 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (5F 203; NSC 703786; **1d**) emerged as the most potent analogue in in vitro evaluations.[10,11] Intriguingly this agent, unlike the corresponding 6-fluoro isomer (6F 203, **1e**), abolished the biphasic dose−response relationship seen in vitro, presumably by inhibiting the formation of inactive exportable hydroxylated metabolites.[11]

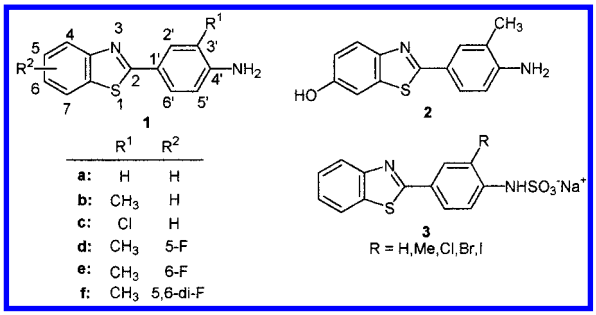


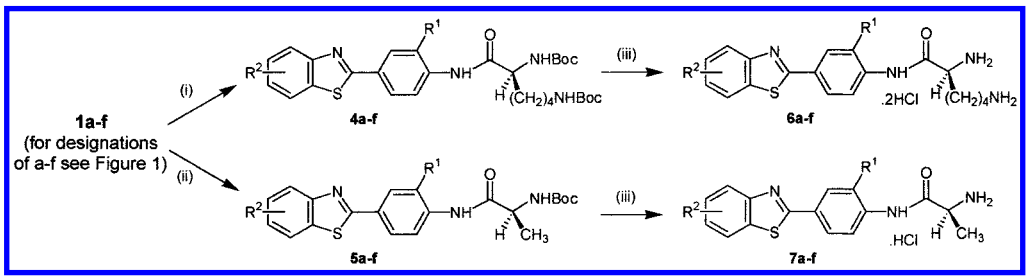
Figure 1. Chemical structures of antitumor 2-(4-aminophenyl)benzothiazoles **1a**−**f** with numbering scheme, 6-hydroxy DF 203 metabolite **2**, and sulfamate salt prodrugs **3**.

To minimize the possibility of first pass deactivating metabolism and/or potential hepatotoxicity of a clinical candidate following induction of P450 1A1 in the liver, a parenteral formulation of the chosen drug was desired. As is common at this stage of a development program, the lipophilicities of this series of agents severely limited drug formulation options. (For compound **1b**: log *P* in octanol:buffer at pH 7.4 = 3.96; log *P* in cyclohexane: buffer at pH 7.4 = 2.51; Δlog *P* = 1.45.) Also, their weakly basic properties (pK_a values ≤ 3.0),[12] militated against simple addition salts being considered since they dissociated to insoluble free bases at physiologically acceptable pH values.

* To whom correspondence should be addressed. Tel: (115)9513414. Fax: (115)9513412. E-mail: malcolm.stevens@nottingham.ac.uk.
[†] University of Nottingham.
[‡] National Cancer Institute.

10.1021/jm011025r CCC: $22.00
Published on Web 12/20/2001

Scheme 1[a]



[a] Reagents: (i) BocLys(Boc)OH, WSC·HCl, HOBt, CH_2Cl_2; (ii) BocAlaOH, WSC·HCl, HOBt, CH_2Cl_2; (iii) HCl (g), CH_2Cl_2.

Our first attempt to address these formulation and bioavailability issues through a study of the synthesis and physicochemical properties of novel sulfamate salt derivatives **3** (Figure 1) as potential prodrugs of 2-(4-aminophenyl)benzothiazoles was reported recently.[1] Although the salts were found to be sparingly soluble under aqueous conditions (pH 4−9), significant degradation to the active free amine occurred only under acidic conditions (pH 4) at 50 °C. Clearly a new approach to prodrug design was required which would allow release of the active free amine under physiological conditions.

We now report the synthesis of L-lysyl- and L-alanyl-amide prodrugs of representative 2-(4-aminophenyl)-benzothiazole antitumor agents. This strategy is similar to the one used by Pochopin et al.[13] in preparing water-soluble prodrugs of Dapsone.

Subsequent physicochemical evaluations focused on the chosen clinical candidate, the prodrug lysyl-amide dihydrochloride (NSC 710305; **6d**)[14] (Scheme 1). Moreover, analysis of the plasma concentration−time profiles following i.v. infusion of **6d** in mice and dogs has shown desired accumulation of active free amine **1d** accompanied by depletion of prodrug in plasma within 1 h of treatment.[15] A comparison of the mouse pharmacokinetics of prodrugs **6d** and **7d** revealed a higher sustained plasma concentration of active drug **1d** in the case of the lysyl derivative **6d** at levels known to elicit cytocidal activity in vitro in sensitive cell lines.[15]

Chemistry

We have reported previously on the synthesis of 3′-substituted derivatives of 2-(4-aminophenyl)benzothiazoles **1a**−**f** which were used as the starting point for our current investigations.[2,11] These were coupled to Boc protected lysine and Boc protected alanine using the water-soluble carbodiimide 1-ethyl-3-[3′-(dimethylamino)-propyl]carbodiimide·HCl (WSC·HCl) to give the derivatized amino acids **4a**−**f** and **5a**−**f**, respectively (Scheme 1). Removal of the Boc protecting groups was accomplished by bubbling HCl gas through dichloromethane solutions of the coupled benzothiazoles. In an earlier NMR study we have shown that simple 2-(4-aminophenyl)benzothiazoles can undergo additional protonation on the benzothiazole ring nitrogen in strong acids.[12] Accordingly, the products were dissolved in water to dissociate any salts formed at these weakly basic sites, and the dihydrochloride salts of the resulting deprotected lysines **6a**−**f** and the monohydrochloride salts of the alanines **7a**−**f** were isolated as yellow solids following recrystallization from methanol/acetone.

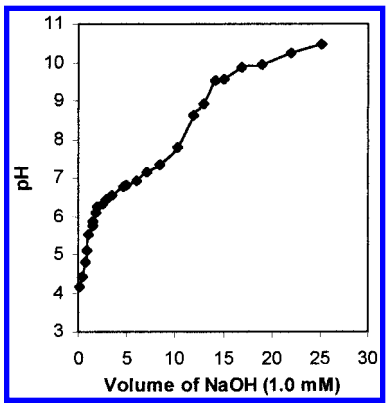


Figure 2. Titration of **6d** (NSC 710305) with aqueous sodium hydroxide to determine pK_{a1} and pK_{a2}.

Table 1. pH-Solubility Profile of **6d** (NSC 710305)

pH	solubility (mg/mL)
5.0	>53.2
6.3	7.00
7.4	0.39
8.5	0.075
9.6	0.02
10.5	0.010

Pharmaceutical Properties of the Lysyl-Amide Dihydrochloride (NSC 710305, 6d)

The synthesis of the prodrug **6d** from the amine precursor **1d**[11] using the above method has been scaled up[16] to provide 100 g batches for formulation, toxicology, and clinical work. The HPLC impurity profile[17] of a typical batch of the drug substance indicates >99.00% purity accompanied by several minor impurities (data not shown). Titration of **6d** with aqueous sodium hydroxide was used to determine the pK_{a1} and pK_{a2} (Figure 2). Results confirm that pK_{a1} is in the region 7.5 (representing dissociation of the dihydrochloride to monohydrochloride) and pK_{a2} at 10.2 (monohydrochloride to free base). These observations clearly impact on the pH-solubility profile of **6d** (Table 1): the prodrug has appreciable solubility (>53.2 mg/mL) at pH 5, where it is essentially fully diprotonated, but solubility diminishes from 0.39 mg/mL at pH 7.4 to a meagre 0.010 mg/mL at pH 10.5 where it is >50% in the neutral species form.[18]

Accelerated stability studies of **6d** are shown in Figure 3.[17] The prodrug was found to be particularly stable at 25 °C (pH 4.5) with no loss of product after 45 days; a 10% loss of product was observed at 25 °C (pH 7.4). Final design of the clinical dosage form will be determined at the conclusion of these studies.

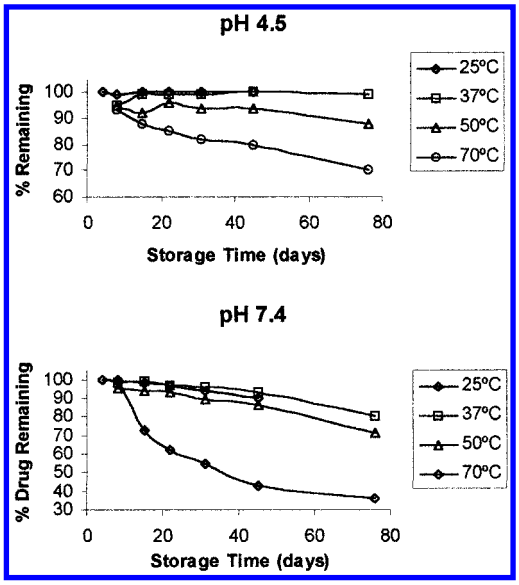


Figure 3. Long-term stability studies of **6d** (NSC D710305) at different temperatures.

Conclusion

L-Lysyl- and L-alanyl-amide prodrugs of the antitumor 2-(4-aminophenyl)benzothiazoles (both fluorinated and nonfluorinated) have been synthesized in two steps via Boc protected precursors, from which the salt of **6d** has been selected as the clinical candidate. Prodrug **6d** possesses an attractive solubility and stability profile amenable for design of a parenteral dosage form. The results of pharmacokinetic antitumor efficacy and toxicological studies on these agents will be published elsewhere.

Experimental Section

All new fluorinated benzothiazoles were characterized by elemental microanalysis (C, H, and N values within 0.4% of theoretical values). Melting points were determined with a Gallenkamp melting point apparatus and are reported uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker ARX250 spectrometer. IR spectra (as KBr disks) were determined on a Mattson 2020 GALAXY series FT-IR spectrometer. Mass spectra were recorded on an AEI MS-902 or a VG Micromass 7070E spectrometer. TLC systems for routine monitoring of reaction samples and confirming the homogeneity of analytical samples, used Kieselgel 60F254 (0.25 mm) silica gel TLC aluminum sheets. Sorbsil silica gel C 60-H (40−60 μm) was used for flash chromatographic separations. Analytical data for compounds **6d** and **7d** are presented here; data for the remaining compounds are presented as Supporting Information.

General Method for the Synthesis of Amino Acid Salt Derivatives of 2-(4-Aminophenyl)benzothiazoles (6a−f, 7a−f). 2-(4-Aminophenyl)benzothiazoles **(1a−f)** (7.75 mmol) were dissolved in dichloromethane (100 mL) and stirred at room temperature. To this solution was added 1-ethyl-3-(3′-(dimethylamino)-propyl)carbodiimide·HCl (WSC·HCl) (2.3 mmol), 1-hydroxybenzotriazole (HOBt) (2.3 mmol), and Boc protected amino acid (2.3 mmol). After the mixture was stirred for 24 h, a further 2.3 mmol of each reactant was added, and stirring continued for a further 24 h. This procedure was repeated twice more and stirring continued for a further 3 days, until a clear solution resulted. The solvent was removed under reduced pressure and the resulting oil purified by column chromatography (2% methanol/dichloromethane). Recrystallization from ethanol gave a white solid.

The Boc protected amino acid derivative (**4**, **5**) (3.5 mmol) was dissolved in dichloromethane (20 mL). Dry HCl gas was bubbled through the solution to saturate it, and then the reaction mixture was stirred for a further 2 h at 25 °C. The precipitate was filtered and washed with dichloromethane (10 mL) to leave a bright yellow crystalline solid. Recrystallization, if required, was carried out using methanol/acetone.

(*S*)-2,6-Diaminohexanoic acid [4-(5-fluorobenzothiazol-2-yl)-2-methylphenyl]amide (6d): from **4d**, yield 72%; mp 290−294 °C; IR 3441 (NH), 1664 (C═O) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 10.48 (1H, brs, NH), 8.63 (3H, brs, NH_3^+), 8.22 (1H, dd, *J* 5.0, 7.5 Hz, H-7), 8.07 (3H, brs, NH_3^+), 8.02 (1H, d, *J* 2.0 Hz, H-2′), 7.89−8.00 (2H, m, H-4, H-6′), 7.80 (1H, d, *J* 7.5 Hz, H-5′), 7.39 (1H, dt, *J* 2.0, 7.5 Hz, H-6), 4.28 (1H, m, CH), 2.82 (2H, m, CH_2), 2.43 (3H, s, CH_3), 2.10−1.41 (6H, m, $CH_2CH_2CH_2$); MS (CI) *m*/*z* 387.4 (M+1); Acc. mass (ES) *m*/*z* 387.1690 (calc. mass for $C_{20}H_{24}N_4OSF$ 387.1655); Anal. ($C_{20}H_{23}N_4OSF$·2HCl) C, H, N.

(*S*)-2-Amino-*N*-[4-(5-fluorobenzothiazol-2-yl)-2-methylphenyl]propionamide (7d): from **5d**, yield 90%; mp 280−284 °C; IR 3405 (NH), 1791 (C═O) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 10.31 (1H, brs, NH), 8.43 (3H, brs, NH_3^+), 8.23 (1H, dd, *J* 5.0, 7.5 Hz, H-7), 8.19 (1H, d, *J* 2.3 Hz, H-2′), 7.99 (2H, m, H-4, H-6′), 7.72 (1H, d, *J* 7.5 Hz, H-5′), 7.37 (1H, dt, *J* 2.5, 7.5 Hz, H-6), 4.28 (1H, m, CH), 2.40 (3H, s, CH_3), 1.55 (3H, d, *J* 7.0 Hz, CH_3); MS (CI) *m*/*z* 330.3 (M+1); Anal. ($C_{17}H_{16}N_3OSF$·HCl) C, H, N.

Acknowledgment. The authors thank the Cancer Research Campaign, U.K., for support to the CRC Experimental Cancer Chemotherapy Research Group.

Supporting Information Available: Physical and spectral data for compounds **4a**−**f**, **5a**−**f**, **6a**−**c**,**e**,**f**, and **7a**−**c**,**e**,**f**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Part 15 of this series: Shi, D.-F.; Bradshaw, T. D.; Chua, M.-S.; Westwell, A. D.; Stevens, M. F. G. Antitumour benzothiazoles. Part 15. The synthesis and physicochemical properties of 2-(4-aminophenyl)benzothiazole sulphamate salt derivatives. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1093−1095.

(2) Shi, D.-F.; Bradshaw, T. D.; Wrigley, S.; McCall, C. J.; Lelieveld, P.; Fichtner, I.; 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.* **1996**, *39*, 3375−3384.

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

(4) Bradshaw, T. D.; Shi, D.-F.; Schultz, R. J.; Paull, K. D.; Kelland, L.; Wilson, A.; Garner, C.; Fiebig, H. H.; Wrigley, S.; Stevens, M. F. G. Influence of 2-(4-aminophenyl)benzothiazoles on growth of human ovarian carcinoma cells in vitro and in vivo. *Br. J. Cancer* **1998**, *77*, 421−429.

(5) Weinstein, J. N.; Myers, T. G.; O'Connor, P. M.; Friend, S. H.; Fornace, A. J.; Kohn, K. W.; Fojo, T.; Bates, S. E.; Rubinstein, L. V.; Anderson, N. L.; Buolamwini, J. K.; van Osdol, W. W.; Monks, A. P.; Scudiero, D. A.; Sausville, E. A.; Zaharevitz, D. W.; Bunow, B.; Viswanadhan, V. N.; Johnson, G. S.; Wittes, R. E.; Paull, K. D. An information-intensive approach to the molecular pharmacology of cancer. *Science* **1997**, *275*, 343−349.

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

(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.; Stevens, M. F. G. Antitumor benzothiazoles. 8. Synthesis, metabolic formation, and biological properties of the *C*- and *N*-oxidation products of antitumor 2-(4-aminophenyl)-benzothiazoles. *J. Med. Chem.* **1999**, *42*, 4172−4184.

(8) Chua, M.-S.; Kashiyama, E.; Bradshaw, T. D.; Stinson, S. F.; Brantley, E.; Sausville, E. A.; 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.* **2000**, *60*, 5196−5203.

(9) The development of the 2-(4-aminophenyl)benzothiazole series to this point has recently been reviewed: Bradshaw, T. D.; Stevens, M. F. G.; Westwell, A. D. The discovery of the potent and selective antitumour agent 2-(4-amino-3-methylphenyl)-benzothiazole (DF 203) and related compounds. *Curr. Med. Chem.* **2001**, *8*, 203−210.
(10) Hutchinson, I.; Stevens, M. F. G.; Westwell, A. D. The regiospecific synthesis of 5- and 7-monosubstituted and 5,6-disubstituted 2-arylbenzothiazoles. *Tetrahedron Lett.* **2000**, *41*, 425−428.
(11) Hutchinson, I.; Chua, M.-S.; Browne, H. L.; Trapani, V.; Bradshaw, T. D.; Westwell, A. D.; Stevens, M. F. G. Antitumor benzothiazoles. 14. Synthesis and in vitro biological properties of fluorinated 2-(4-aminophenyl)benzothiazoles. *J. Med. Chem.* **2001**, *44*, 1446−1455.
(12) Wheelhouse, R. T.; Shi, D.-F.; Wilman, D. E. V.; Stevens, M. F. G. Antitumour benzothiazoles. Part 4.[1] An NMR study of the sites of protonation of 2-(4-aminophenyl)benzothiazoles. *J. Chem. Soc., Perkin Trans. 2* **1996**, 1271−1274.
(13) Pochopin, N. L.; Charman, W. N.; Stella, V. J. Amino acid derivatives of Dapsone as water soluble prodrugs. *Int. J. Pharm.* **1995**, *121*, 157−167.
(14) Stevens, M. F. G.; Poole, T. D.; Westwell, A. D.; Hutchinson, I.; Chua, M.-S. Substituted 2-arylbenzazole compounds and their use as antitumour agents. International Patent Number WO 01/14354 A1 (Publication Date: March 1, 2001). Accepted for Phase 1 clinical evaluation by the Cancer Research Campaign, U.K.; also given the name "Phortress".
(15) Bradshaw, T. D.; Browne, H. L.; Trapani, V.; Chua, M.-S.; Fichtner, I.; Bibby, M. J.; Cooper, P. A.; Alley, M. C.; Donohue, S. J.; Sausville, E. A.; Stevens, M. F. G. Preclinical evaluation of amino acid prodrugs of novel antitumor 2-(4-amino-3-methylphenyl)benzothiazoles. *Mol. Cancer Ther.*, in press.
(16) The scale-up synthesis was performed by Starks Associates Inc., Buffalo, NY, under an NCI contract: P. I., Dr. Jack Parsons.
(17) Data generated by Midwest Research Institute, Kansas City, MO, under an NCI contract: P. I., Dr. Gregory Turner.
(18) Data generated by University of Kansas, Lawrence, KS, under an NCI Contract: P. I., Dr. Valentino J. Stella.

JM011025R