Antitumor Benzothiazoles. 7. Synthesis of 2-(4-Acylaminophenyl)benzothiazoles and Investigations into the Role of Acetylation in the Antitumor Activities of the Parent Amines

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Received August 28, 1998

2-(4-Aminophenyl)benzothiazoles display potent and selective antitumor activity against inter alia breast, ovarian, colon, and renal cell lines, but their mechanism of action, though yet to be defined, may be novel. Metabolism is suspected to play a central role in the mode of action of these benzothiazoles since drug uptake and biotransformation were observed in sensitive cell lines (e.g., breast MCF-7 and MDA 468 cells) in vitro, whereas insensitive cell lines (e.g., prostate PC 3 cells) showed negligible uptake and biotransformation. *N*-Acyl derivatives of the arylamines have been synthesized, and in vitro studies confirm *N*-acetylation and oxidation as the main metabolic transformations of 2-(4-aminophenyl)benzothiazoles, with the predominant process being dictated by the nature of the 3′-substituent. The prototype amine **3** underwent mainly *N*-acetylation in vitro, while 3′-substituted analogues **4** and **5** were primarily oxidized. *N*-Acetylation of **4** to **11** exerts a drastic dyschemotherapeutic effect in vitro, but acetylation of the halogeno congeners **5**−**7** gave acetylamines **12**−**14** which substantially retain selective antitumor activity. In vivo pharmacokinetic studies in rats confirmed rapid and exclusive *N*-acetylation of the 3′-methyl analogue **4**, but less acetylation with the 3′-chloro analogue **5**. Distinct expression patterns of *N*-acetyltransferase NAT1 and NAT2 have been demonstrated in our panel of cell lines.

Introduction

2-(4-Aminophenyl)benzothiazoles, represented by structures **3**−**7**, are a novel class of potent and selective antitumor agents. We have previously reported on their discovery, unique biphasic dose−response profiles, structure−activity relationships in vitro and in vivo particularly in sensitive breast cell lines,[1] and resistance mechanisms.[2] Despite structural similarities between the 2-phenylbenzothiazole nucleus and molecules possessing known anti-estrogenic (e.g., 2-phenylindoles, 2-phenylbenzo[*b*]thiophenes, and 2-phenylbenzo[*b*]furans)[3] or anti-tyrosine kinase (e.g., genistein and quercetin) activity,[4,5] these agents form a distinct mechanistic class.[6] In the National Cancer Institute (NCI) in vitro anticancer drug screen utilizing a panel of 60 diverse human tumor cell lines, 2-(4-aminophenyl)benzothiazoles display a characteristic profile of cytotoxicity response across the cell lines; sensitive cell lines show GI_{50} values $<10^{-8}$ M and insensitive cell lines $>10^{-4}$ M. This response fingerprint is COMPARE negative[7] (Pearson correlation coefficient < 0.7) to all other test agents (except related benzothiazoles), including mutagenic/carcinogenic arylamines, and clinically used chemotherapeutic agents, suggesting a novel mechanism of action. Our efforts to define a pharmacological mechanism of action have been thwarted, thus far. However, the pharmacological properties of the benzothiazole series do show features in common with some structurally related 5-amino-2-(4-aminophenyl)-4*H*-1-benzopyran-4-ones.[8]

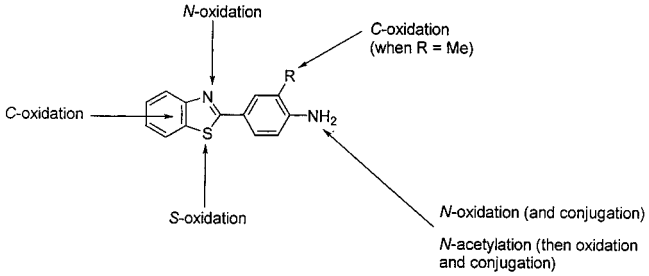

Figure 1. Potential sites for metabolic transformation of antitumor 2-(4-aminophenyl)benzothiazoles.

The suggestion that metabolism might play a prominent role in the mechanism of action of these benzothiazoles is indicated considering their arylamine structure. Common metabolic steps in arylamine metabolism include *N*-acetylation, *C*-oxidation, and *N*-oxidation. While *N*-acetylation and *C*-oxidation are typically deactivating[9] processes, *N*-oxidation can lead to the generation of genotoxic nitrenium species responsible for the carcinogenic properties of certain arylamines.[10] In the present examples oxidation at the thiazole ring heteroatoms is also possible (Figure 1), but unlikely, given the availability of alternative sites. Biotransformation studies are therefore critical to identify potential active entities: the relative compositions and tumor-specific expressions of xenobiotic-metabolizing enzymes may underlie the observed activity and selectivity patterns.

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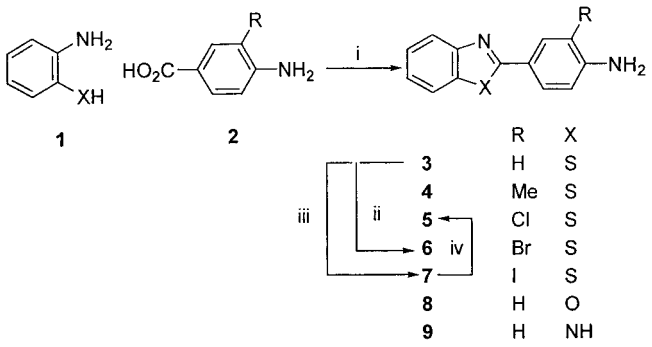
10.1021/jm981076x CCC: $18.00
Published on Web 01/23/1999

Scheme 1[a]



[a] Reagents: (i) polyphosphoric acid, 220 °C; (ii) Br_2, CH_2Cl_2, −5 °C; (iii) ICl, AcOH, 25 °C; (iv) $Cu^{I}Cl$, DMF, reflux.

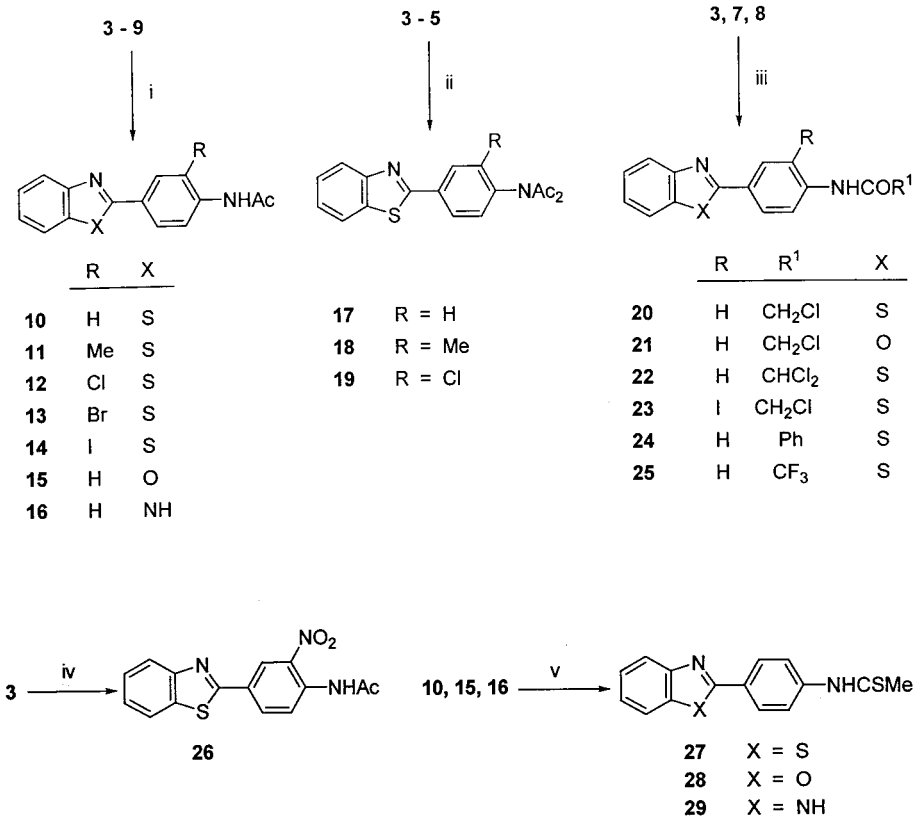
We have now synthesized *N*-acetylamino derivatives of a series of antitumor 2-(4-aminophenyl)benzothiazoles, and related structures, to investigate the influence of these modifications on antitumor activity. Significantly, *N*-acetyl metabolites derived from the arylamines have been identified in both in vitro and in vivo biotransformation studies. We have also shown the presence of *N*-acetyltransferases (NATs) in our panel of human tumor cell lines.

Chemistry

We reported recently a simple and efficient synthesis of 2-(4-aminophenyl)benzothiazoles **3** and **4** by the reaction of 2-aminothiophenol (**1**; X = S) with 4-aminobenzoic acids (**2**; R = H, Me) in polyphosphoric acid at 220 °C: the related benzoxazole **8** and benzimidazole **9** were prepared similarly from 4-aminobenzoic acid and 2-aminophenol (**1**; X = O) or *o*-phenylenediamine (**1**; X = NH), respectively.[2] The bromo- and iodo-substituted benzothiazoles **6** and **7** could be prepared by direct halogenation of the precursor **3** and the chloro analogue **5** from **7** by trans-halogenation with cuprous chloride in DMF (Scheme 1).

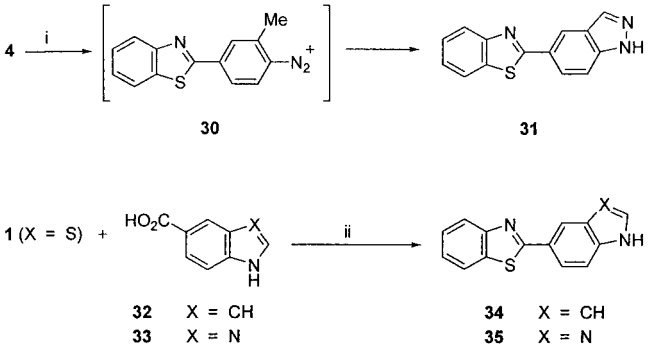
In the present work, attempted condensation of 4-acetylaminobenzoic acid and 2-aminothiophenol failed to yield the required *N*-acetylamine **10**. As an alternative approach amines **3**−**9** were monoacetylated by treatment with acetic anhydride in refluxing benzene, pyridine, or chloroform, for 4 h at 80 °C to give derivatives **10**−**16** in excellent yield (Scheme 2). Diacetylation of **3**−**5** was achieved by treatment of the amines with excess acetic anhydride in refluxing pyridine to yield compounds **17**−**19**. Reaction of the amines with either chloroacetyl, dichloroacetyl, or benzoyl chlorides in refluxing pyridine or dry benzene gave the corresponding acyl derivatives **20**−**24**, isolated in some cases initially as hydrochloride salts. In the case of the 3′-iodo derivative **23**, the base-weakening influence of the halogen substituent promoted dissociation of the salt and the isolation of the free base only. When amine **3** was acylated with trifluoroacetic anhydride, a trifluoroacetate salt of 2-(4-trifluoroacetamidophenyl)benzothiazole (**25**) was isolated initially. Surprisingly, interaction of amine **3** with copper(II) nitrate in acetic anhydride−benzene gave 2-(4-acetamido-3-nitrophenyl)benzothiazole (**26**) (33%), the product of both nitration and acetylation. The conversion of compounds **10**, **15**, and **16** into their corresponding thioacetamido derivatives

Scheme 2[a]



[a] Reagents: (i) Ac_2O or AcCl, benzene or $CHCl_3$, reflux; (ii) Ac_2O, in refluxing benzene or pyridine; (iii) acid chloride or acid anhydride, benzene, reflux; (iv) Cu^{II}nitrate, Ac_2O, benzene; (v) Lawesson's reagent, HMPA, 100 °C.

Scheme 3[a]



[a] Reagents: (i) isoamyl nitrite, $CHCl_3$, AcOH; (ii) polyphosphoric acid, 200 °C.

27−**29** was effected with Lawesson's reagent in HMPA (Scheme 2).

To explore the biological effects of constraining the primary arylamine group of 2-(4-aminophenyl)benzothiazoles within a heterocyclic ring, the indazolylbenzothiazole **31** was formed by a modification of the venerable Bamberger and von Goldberger toluidine diazotization route to indazoles.[11,12] Thus nitrosation of **4** with isoamyl nitrite in dichloromethane containing a catalytic amount of acetic acid gave a precipitate of the required indazole **31** (24%) presumably via the diazonium species **30**, or possibly via a diazomethylene intermediate following deprotonation of **30** (Scheme 3). In a different approach the indole- (**32**) and benzimidazole-5-carboxylic acid (**33**) were heated with 2-aminothiophenol in hot polyphosphoric acid. In the former case a black intractable tar was formed and none of the required 2-(indol-5-yl)benzothiazole **34** was isolated; in contrast the benzimidazolylbenzothiazole **35** was formed in 61% yield (Scheme 3).

The arylamines **3** and **4** are stable over 28 days in 0.1 M HCl and 0.1 M NaOH at 50 °C as measured by HPLC; the iodoarylamine **7** is stable in 0.1 M NaOH under these conditions but undergoes slow deiodination in 0.1 M HCl to the unsubstituted amine **3**. The acetylamine **10** is hydrolyzed to amine **3** in 0.1 M HCl and NaOH at 50 °C with $t_{1/2}$ values of approximately 20 h and 2 days, respectively.

Biological Results and Discussion

In Vitro Studies. We have reported previously on the unique biphasic dose−response relationship elicited by 2-(4-aminophenyl)benzothiazole **3** and its potency (IC_{50} values < 0.01 μM) against a panel of breast cancer cell lines, irrespective of their receptor status (ER+/−, EGFR+/−, erbB3+/−).[1] In contrast, a range of prostate, colon, ovarian, rhabdomyosarcoma, melanoma, and bladder lines were insensitive to the agent (IC_{50} > 30 μM).

We have ranked the activity of this structurally simple lead compound and a range of arylamine analogues **4**−**9** and their *N*-acyl and *N*-thioacyl derivatives against MCF-7 (ER^+) and MDA 468 (ER^-) mammary carcinoma cells in a 7-day MTT assay (Table 1). The MCF-7 cell line was slightly more sensitive than the MDA 468 cell line to the range of compounds tested. Those amines with a methyl or halogen substituent adjacent to the amine group (**4**−**7**) were more cytotoxic toward both cell lines than the unsubstituted amine **3**. The nature of the heteroatom in the azole fragment had an important influence, with potency in the order 2-(4-aminophenyl)benzothiazole **3** > benzoxazole **8** > benzimidazole **9**. Of the *N*-acetylaminobenzothiazole series bearing a 3′-substituent in the phenyl moiety, overall potency was in the order **12** and **13** > **10** and **14** ≫**11** with a remarkable 5 log M range of activity between the most and least active compounds. In addition to its lack of activity, the presence of compound **11** inhibited the activity of its parent amine **4** in these tests. The diacetylated amines **17**−**19** were less active than their monoacetylated counterparts: this suggests the requirement for an exocyclic primary or secondary amine function to exert potent antitumor activity, possibly by participation in hydrogen bond interactions with critical biological molecules. When the arylamine function is adapted by inclusion of the nitrogen atom within a heterocyclic system, such as in compounds **31** and **35**, activity was similarly impaired. Against the two breast cell lines the most potent of the haloacetyl compounds was 2-(4-chloroacetylaminophenyl)benzothiazole (**20**).

Table 1. In Vitro Activity of 2-(4-Aminophenyl)benzazoles and *N*-Acyl and *N*-Thioacyl Derivatives Against MCF-7 and MDA 468 Mammary Carcinoma Cell Lines

log IC_{50} (M)	MCF-7	MDA 468
<−9.0	**4**, **5**, **6**, **7**	**4**, **5**, **6**, **7**
>−9.0 to −8.0	**3**,[a] **12**, **13**, **20**, **23**, **25**	**3**,[a] **23**
>−8.0 to −7.0	**8**, **10**,[a] **14**, **17**,[a] **22**, **27**	**10**,[a] **12**, **13**, **14**, **17**,[a] **20**, **22**, **25**, **27**
>−7.0 to −6.0	**21**, **24**, **26**	**8**, **9**, **19**, **21**, **24**, **26**
>−6.0 to −5.0	**9**, **16**, **19**	
>−5.0	**11**, **15**, **16**, **18**, **31**	**11**, **15**, **16**, **18**, **31**

[a] Biphasic dose−response relationship.

In the NCI in vitro anticancer drug screen, novel structures are routinely tested against a panel of 60 diverse human tumor cell lines. Emerging from this screen is an information-intensive profile of cellular response for each test agent, and computed in the form of mean graphs, the potency and selectivity of test agents can be analyzed.[7]

In the 2-day NCI assay, 2-(4-aminophenyl)benzothiazoles show selective potency against certain tumor cell lines (Table 2). Thus, although the mean GI_{50} values[13] in the full panel are only modest (range 20−47 μM for compounds **4**−**7**), the screen revealed a consistent fingerprint of highly sensitive lung, colon, ovarian, renal, melanoma, and breast cancer cell lines (defined as having GI_{50} values > 2 log M lower than mean values). Although less active overall, this pattern extended to the 3′-halogenated *N*-acetyl compounds **12**−**14** but not the 3′-methyl analogue **11**, corroborating the ranking results of the MCF-7 and MDA 468 study (Table 1). In the 2-day assay the chloroacetylamine **20** proved to be the most cytotoxic compound overall but displayed no distinctive fingerprint of cell type selectivity.

In a limited number of 6-day drug exposure assays employing a modified 43-cell-line panel, the remarkable selectivity of the 3′-substituted 2-(4-aminophenyl)benzothiazoles against colon (COLO 205, HCC-2998, HCT-116), melanoma (UACC-257, UACC-62), ovarian (IGROV1, OVCAR-5), renal (A498, TK-10), and breast (MDA-MB-231/ATCC) cell lines was revealed (Table 2). This is exemplified starkly (Figure 2) in the GI_{50}

Table 2. In Vitro Activity of 2-(4-Aminophenyl)benzazoles and *N*-Acyl Derivatives in the NCI 2- and 6-Day Assays[a]

	2-day assay		6-day assay	
compd	mean log GI_{50} (M)	sensitive cell lines[b]	mean log GI_{50} (M)	sensitive cell lines[b]
3	−4.76	none	nt	
4	−4.61	Hop-92, NCC-2998, IGROV1, OVCAR-5, TK-10, MCF-7, T-47D	−5.05	IGROV1, OVCAR-5, TK-10, MCF-7
5	−4.75	NCI-H266, NCI-H460, HCT-116, IGROV1, OVCAR-3, OVCAR-4, OVCAR-5, A498, TK-10, MCF-7, T-17D	−5.26	COLO 205, HCC-2998, HCT-116, KM-12, UACC-62, UACC-257, SK-MEL-5, IGROV1, OVCAR-5, TK-10, MDA-MB-2331/ATCC
6	−4.67	NCI-H460, HCT-116, IGROV1, OVCAR-3, OVCAR-4, OVCAR-5, A498, TK-10, MCF-7, T-47D	−5.01	COLO 205, HCC-2998, HCT-116, UACC-62, UACC-257, TK-10, IGROV1, OVCAR-5, MDA-MB-231/ATCC
7	−4.75	NCI-H226, NCI-H460, HCT-116, UACC-6662, IGROV1, OVCAR-3, OVCAR-4, TK-10, MCF-7, T-47D	−5.12	COLO 205, HCC-2998, HCT-116, UACC-62, UACC-257, IGROV1, OVCAR-5, TK-10, MDA-MB-231/ATCC
8	−4.26	none	nt	
10	−4.40	none	nt	
11	−4.36	none	nt	
12	−4.54	NCI-H266, NCI-H460, IGROV1, TK-10, MCF-7, T-47D	nt	
13	−4.52	HCC-2998, IGROV1, OVCAR-5	nt	
14	−4.70	IGROV1, OVCAR-5, TK-10, MCF-7	nt	
15	−4.27	none	nt	
20	−5.7	none	nt	

[a] For protocols, see ref 13. [b] GI_{50} values > 2 log M lower than mean value; nt, not tested.

Table 3. Clonal Growth Inhibition by 2-(4-Aminophenyl)benzothiazoles **3**, **4**, and **7** and 2-(4-Chloroacetylaminophenyl)benzothiazole (**20**) in Human Tumor Xenografts in Vitro

	active[a]/total at drug concn (μg/mL)			mean IC_{70}[b]
compd	0.1	1.0	10	(μg/mL)
3	0/22	0/22	5/22	10
4	2/22[c]	2/22	2/22	6.9
7	2/22[d]	2/22	8/22	6.1
20	0/22	3/22	18/22	3.5

[a] Active, T/C <30%. [b] Drug concentration to reduce colony formation to 30% of control. [c] Sensitive cell lines were lung adenocarcinoma 289 and mammary MCF-7. [d] Sensitive cell lines were colorectal carcinoma 280 and mammary MCF-7.

antitumor fingerprint for the bromo analogue **6**, which is typical of this intriguing series of compounds.

The arylamines **3**, **4**, and **7** and the chloroacetylamine **20** were tested in in vitro clonogenic assays from 22 human tumor xenografts at three dose levels (0.1, 1.0, 10 μg/mL). Compounds effecting colony inhibition (T/C < 30%) were considered active. The most active arylamine **7** achieved significant growth inhibition in 2/22 tumors at 1 μg/mL and 8/22 tumors at 10 μg/mL (Table 3). Compound **20** was active in 18/22 tumors at 10 μg/mL with a mean IC_{70} concentration of 3.5 μg/mL (11.6 μM), a value consistent with NCI in vitro results.

Uptake of 2-(4-Aminophenyl)benzothiazoles into Tumor Cells. Uptake of selected compounds from growth media into sensitive and resistant cells was monitored by HPLC and confocal laser scanning microscopy. Sensitive breast cancer cell lines, MCF-7 and MDA 468, sequester compound **3** extensively from the media. For example 65% of drug was sequestered by MCF-7 cells in 24 h and 84% after 72 h with negligible amounts of compound **3** remaining in the growth media after 7 days of drug exposure (Figure 3). Variant MCF-7 cells resistant to adriamycin (MCF-7/Adr) showed only minor (<15%) uptake of drug after 72-h exposure; similarly, insensitive prostate cells, PC 3 and DU 145, showed minimal drug uptake (data not shown).

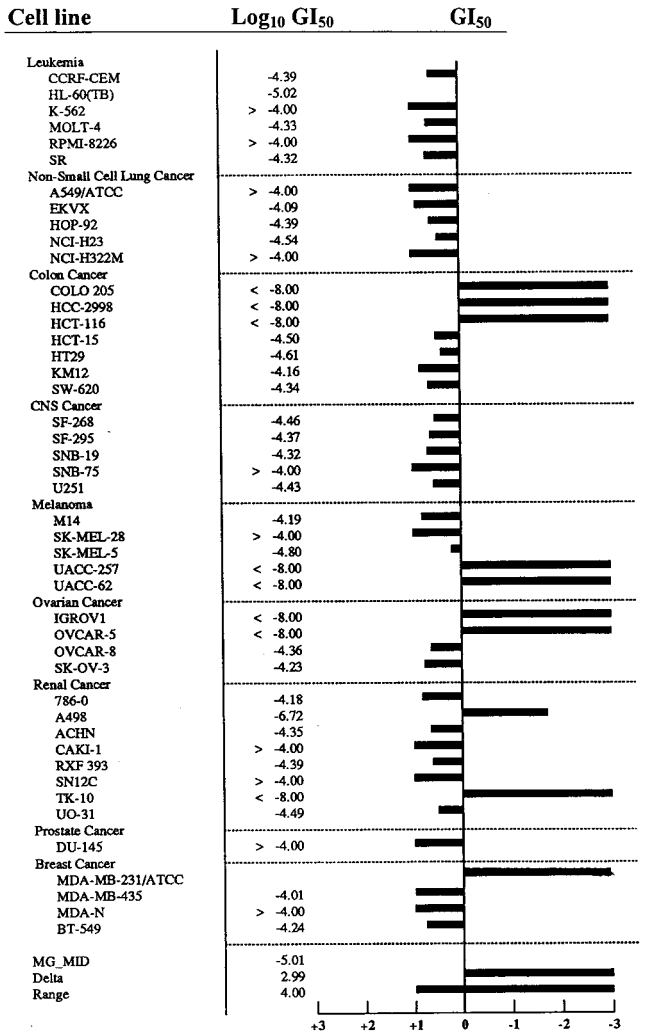


Figure 2. NCI mean graph (log GI_{50} values) for a 6-day in vitro assay on 2-(4-amino-3-bromophenyl)benzothiazole (**6**).

Two acquired resistant cell lines, MCF-7/LT 10 nM and MCF-7/LT 10 μM, have been derived from parent MCF-7 cells following extended exposure to 10 nM and

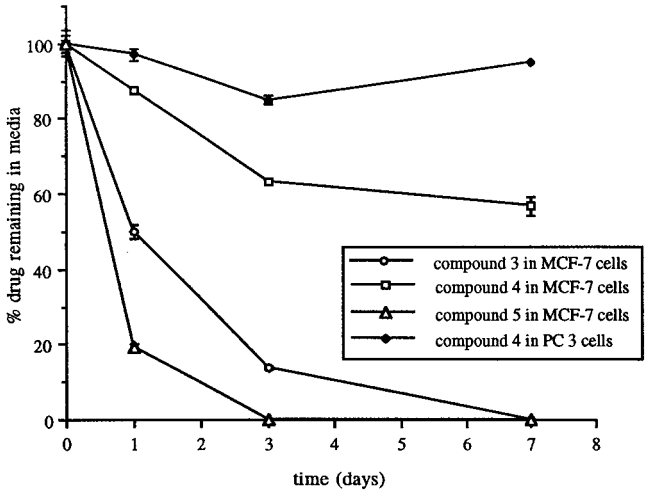


Figure 3. Differential uptake of 2-(4-aminophenyl)benzothiazoles **3**−**5** by MCF-7 and PC 3 cells. Cells were treated with 30 μM compound **3**, **4**, or **5**, and media samples were removed at indicated time intervals for HPLC analysis.

10 μM compound **3**, respectively.[2] The mechanism of acquired resistance is not known. Although the LT 10 μM cells take up compound **3** to a similar extent as their parent cells, the LT 10 nM variant showed greatly reduced uptake, with only 32% of the compound extracted from the media following 72-h drug exposure (data not shown). Reduced drug uptake and accumulation is one of the many mechanisms that may lead to acquired resistance, and this may be a contributing factor to the resistance displayed by the LT 10 nM variant. Apparently, two distinct mechanisms of resistance have evolved to equip each of these two variant cell lines with their own defense against drug toxicity.

The uptake of amine **4** into sensitive MCF-7 and MDA 468 cell lines was slower than that of **3** (Figure 3), and residual amounts (43% and 35%, respectively) of drug remained in the medium after 7 days of incubation. In contrast, analogue **5** with a 3′-chloro substituent was completely removed from the media by these cell lines in 3 days (Figure 3), possibly as a result of the enhanced lipophilicity conferred on the molecule by the halogen atom. Consistent with the aforementioned observations, PC 3 prostate cells showed no evidence of uptake of compound **4** (Figure 3); similarly, chemoresistant prostate DU 145, together with insensitive breast cells HBL 100, did not remove **4** from culture media (data not shown).

The ease of cellular entry of these small, lipophilic and fluorescent molecules into sensitive cell lines was confirmed by confocal microscopy. Within 24-h drug exposure, nuclear accumulation of compound **3** was visible in MCF-7 cells (Figure 4a), although it was localized to the perinuclear area within the cytoplasm of MDA 468 cells (Figure 4b). Analogues bearing 3′-substituents (**4**−**7**) showed enhanced cellular entry, gaining ready access to the nuclei of both MCF-7 and MDA 468 cells within 1 h of drug exposure. Compound **4** in particular was concentrated densely in the nuclei of both sensitive cell lines after 72-h incubation with drug. Altered cell morphology, such as membrane blebbing and disrupted cell division suggestive of apoptosis, was apparent.

Differences between the acquired resistant cell lines were also observable by confocal microscopy. Whereas MCF-7/LT 10 μM cells retained compounds **3** and **4** within their nuclei, these compounds were concentrated within the cytoplasm of MCF-7/LT 10 nM cells, again suggesting divergent mechanisms to acquire resistance.

The *N*-acetylated compounds **11** and **12** had impaired abilities to enter sensitive cells compared to their nonacetylated congeners **4** and **5**: this was especially so with compound **11** (data not shown) and may be one factor contributing to its lack of activity in the cell lines sensitive to its parent amine **3**.

In Vitro Biotransformation Studies. HPLC was utilized for the detection and quantification of biotransformation products isolated from the media following incubation of sensitive and insensitive cell lines with selected amines and their *N*-acetyl derivatives. Structural identification of detected metabolites was confirmed by comparisons with synthetic standards by LC−MS. All the significant amines **3**−**7** were stable in tissue culture media over a period of 7 days at 37 °C in the absence of cells.

Sensitive breast cancer cell lines, including MCF-7, MDA 468, and SKBR3, convert 2-(4-aminophenyl)-benzothiazole **3** to the *N*-acetylated compound **10** as the major biotransformation route. This occurred to the greatest extent in MCF-7 cells (Figure 5a). Insensitive cell lines such as MCF-7/Adr and PC 3 showed only minimal (<5%) degrees of *N*-acetylation. Interestingly, cell lines MCF-7/LT 10 nM and MCF-7/LT 10 μM with acquired resistance to 2-(4-aminophenyl)benzothiazoles retained *N*-acetylation capability, with the MCF-7/LT 10 nM variant demonstrating enhanced acetylation efficacy compared to parental MCF-7 cells (data not shown). Two additional products of increased polarity were detected after 48−72 h of drug exposure in sensitive cell lines only. These were tentatively identified by LC−MS as hydroxylated derivatives of compound **10** (*m*/*z* 284). Minor biotransformation routes of compound **3** also include sulfation and glucuronidation, to form highly polar metabolites in the presence of MCF-7, MDA 468, MCF-7/LT 10 nM, and MCF-7/LT 10 μM cells.

Whereas *N*-acetylation was the major metabolic process of compound **3**, this was only a minor pathway from compound **4**: only trace amounts of its *N*-acetylated metabolite could be detected from MCF-7, MDA 468, and SKBR3 cells, and usually only after 6 days of incubation with drug. Compound **4** predominantly underwent oxidation in sensitive breast cancer cell lines such as MCF-7 and MDA 468 (Figure 5b); SKBR3 and T-47D cells similarly preferentially oxidized the drug (data not shown). Oxidation of **4** was also the dominant metabolic process in the acquired resistant variants MCF-7/LT 10 nM and especially MCF-7/LT 10 μM.[2] Preliminary in vitro screening results suggest that phenolic products oxidized in the benzothiazole moiety, *N*-oxidation products, and secondary metabolites are *detoxification* products. The synthesis, antitumor properties, and metabolic formation of oxidation products of 2-(4-aminophenyl)benzothiazoles will form the topic of a future paper in this series.

Interconversion between acetylated and deacetylated states also occurred when the acetylamines **10**−**12** were

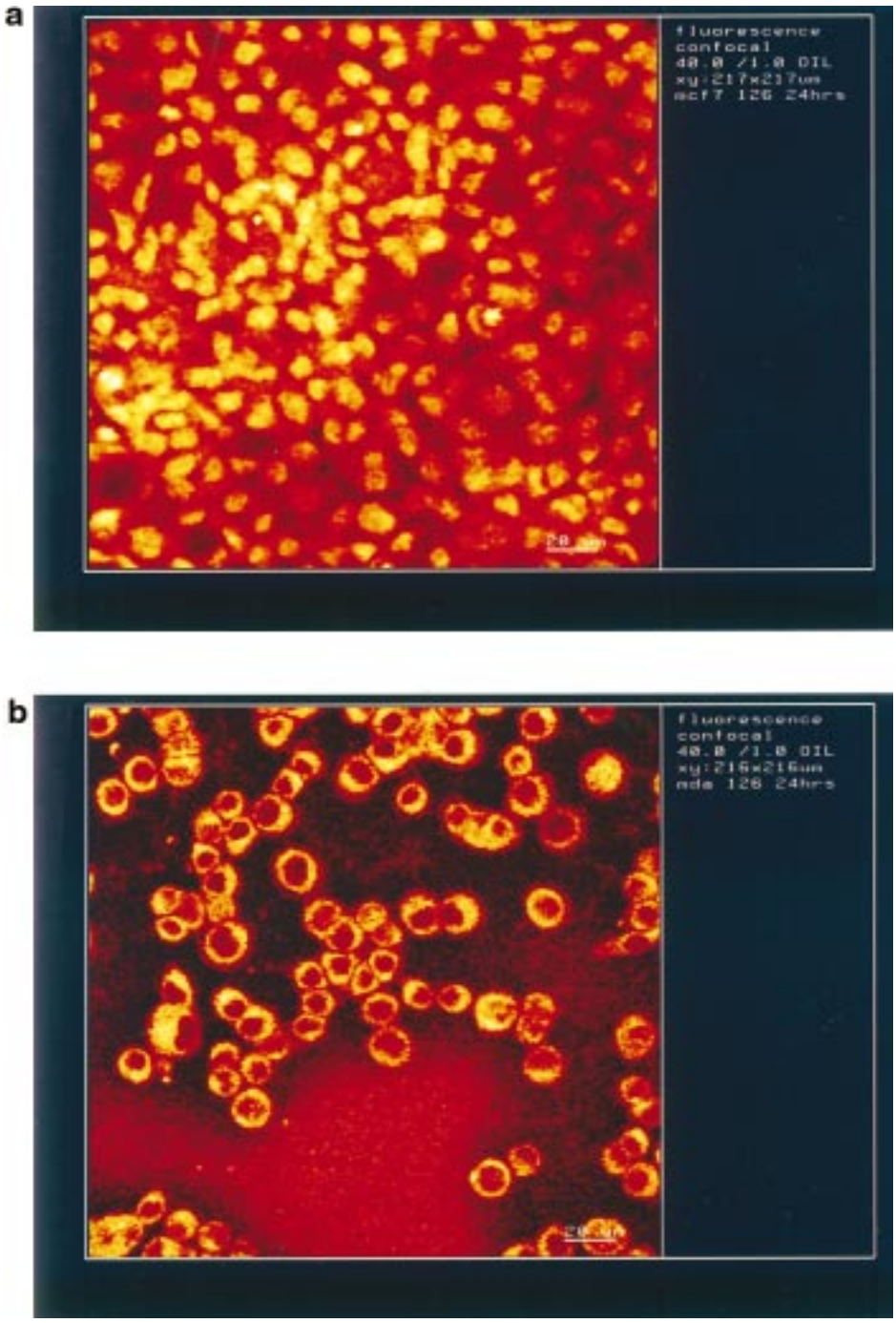


Figure 4. Confocal microscopy photographs showing different intracellular localizations of compound **3** in (a) MCF-7 cells and (b) MDA 468 cells after 24-h exposure to 30 μM compound **3**.

incubated with both sensitive (MCF-7 and MDA 468) and inherently resistant (HBL 100 and PC 3) cell lines. This is presumably a biochemical, rather than purely chemical, process since these acetylamines were stable in culture media alone at 37 °C for 7 days. The nature of the substituent in the 3′-position of the phenyl group had a strong influence on the extent of deacetylation in the presence of tumor cells. In contrast to negligible uptake of amines **4** and **5** by HBL 100 and PC 3 cells, their respective *N*-acetyl derivatives **11** and **12** were extensively taken up by these cells, rapidly regenerating amines **4** and **5** (Figure 6). No subsequent metabolism of these regenerated amines was detected. MCF-7 and MDA 468 cells take up the 3′-chloroacetylamine **12** more slowly than amine **5**, with significant deacetylation to liberate **5** which then underwent metabolism to oxidation products. Similarly, the 3′-methylacetylamine **11** was taken up and deacetylated by these sensitive cell lines, though at a slower rate than **12**. However, amine **4** regenerated from **11** was neither taken up nor metabolized, and this may be a factor contributing to its lack of activity in the cell lines sensitive to its parent amine **4**. We hypothesize that only sensitive cell lines possess a bioactivating sequestering molecule to which **11** also binds and saturates, thereby inhibiting the activity of its liberated amine **4**. Presumably, **12** has reduced binding affinity to this sequestering molecule (if such binding requires an amido proton), thereby

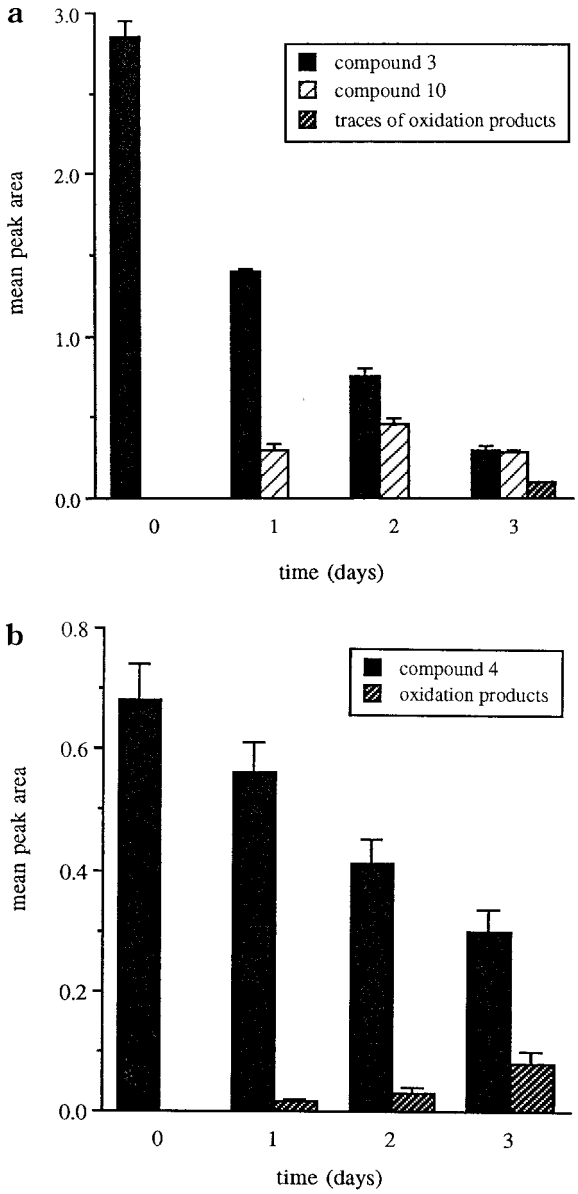


Figure 5. Uptake and in vitro biotransformation of (a) compound **3** by MCF-7 cells and (b) compound **4** by MDA 468 cells. Parent amine and metabolites were detected by HPLC analysis of media samples from cells incubated with 30 μM compound **3** or **4**.

allowing bioactivation of its liberated amine and accounting for the enhanced chemoselectivity of **12** compared to **11** (Tables 1 and 2).

Expression of *N*-Acetyltransferases (NATs) in Test Cell Lines. NATs are key enzymes catalyzing *N*-acetylation, although they also possess the ability for *O*-acetylation and the transfer of an acetyl group from *N* to *O*. There are two main classes of these enzymes, NAT1 and NAT2,[14] distinguishable by their substrate specificities, tissue-specific expressions, and specific reactions catalyzed. Large interindividual differences in acetylation capacity arise from the polymorphic nature of genes encoding these enzymes, and this is a contributing factor in the susceptibility of individuals to the chemotherapeutic/carcinogenic effects of many chemicals and drugs.[15]

Using specific primary antibodies raised against NAT1 and NAT2, we demonstrated the presence of NAT1 in our panel of tested cell lines, regardless of

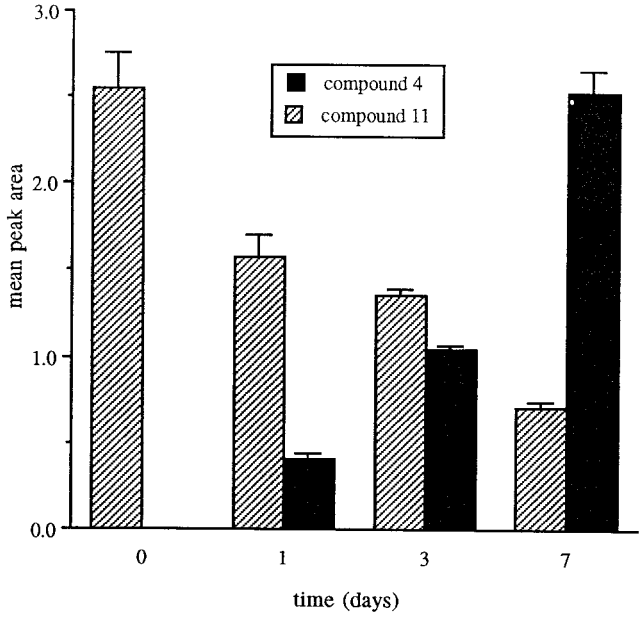


Figure 6. Uptake and deacetylation of compound **11** to its amine **4** by MCF-7 cells, monitored by HPLC analysis of media samples of MCF-7 cells incubated with 30 μM compound **11**.

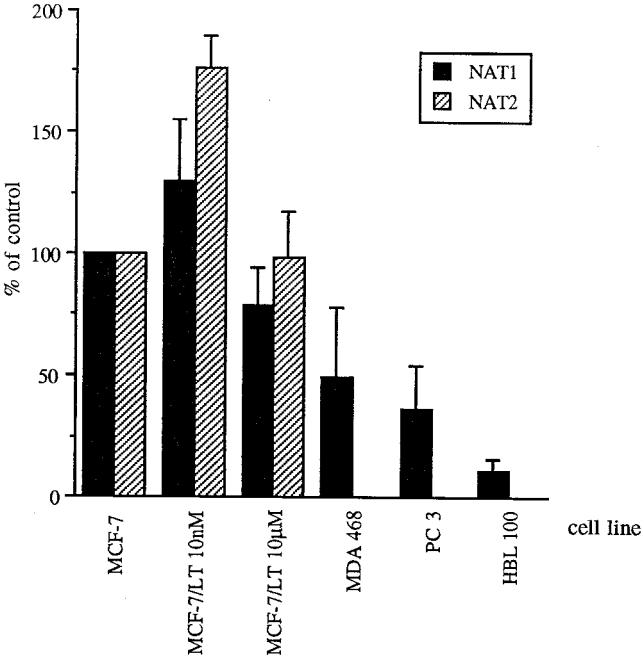


Figure 7. Expression of NAT1 and NAT2 in sensitive (MCF-7, MDA 468) and resistant (MCF-7/LT 10 nM, MCF-7/LT 10 μM, PC 3, HBL 100) cell lines.

sensitivity to 2-(4-aminophenyl)benzothiazoles (Figure 7). Three inherently resistant cell lines, MCF-7/Adr, HBL 100, and PC 3, which only poorly acetylate aminophenylbenzothiazoles, express the lowest levels of NAT1. Conversely, the level of expression of NAT1 is highest in MCF-7/LT 10 nM, consistent with the efficient *N*-acetylation capability of this acquired resistant cell line.

The expression of NAT2, however, is limited to MCF-7 and its derived resistant variants, MCF-7/LT 10 nM and MCF-7/LT 10 μM cells. As with NAT1, the highest level of NAT2 expression was found in MCF-7/LT 10 nM cells; MCF-7/LT 10 μM cells have similar levels of NAT1 and NAT2 to parental MCF-7 cells. Interestingly, the sensi-

tive MDA 468 cell line resembles insensitive cell lines in not expressing detectable levels of NAT2 (Figure 6).

Treatment of MCF-7 cells with 50 μM compound **3** for 72 h did not significantly alter the levels of NAT1 or NAT2.

Biotransformation of 2-(4-Aminophenyl)benzothiazoles in Primary Rat Hepatocyte Culture. Substantial biotransformation of arylamine **3** occurred in primary rat hepatocytes, to form the acetylated derivative **10** as the major metabolite. An oxidized derivative of compound **10** (*m*/*z* 284) could also be detected by LC−MS, together with other uncharacterized more polar metabolites, including glucuronide conjugates. Biotransformation pathways of compound **3** are therefore similar in both primary rat hepatocytes and sensitive breast cancer cell lines.

In Vivo Biotransformation Studies. Preliminary in vivo biotransformation studies of compound **4** in rats have been conducted. Absorption of compound **4** occurred following oral administration at doses of 10 mg/kg with the highest plasma concentrations being reached at 30 min postdosing. However, oral bioavailability of **4** was low since absorption of drug was accompanied by rapid metabolism to high levels of a single metabolite identified as the *N*-acetyl derivative **11** from UV analysis (λ_{max} 212 and 314 nm) and mass spectral analysis (*m*/*z* 284), as well as by comparison with a synthetic standard. This metabolite, which could be detected at 15 min postdosing, achieved a maximum plasma concentration after 2 h, with significant levels persisting after 4 h. At its peak, the concentration of metabolite **11** was 10-fold greater than that of its parent amine **4** (Figure 8a). Following iv administration of 0.5 mg/kg compound **4** to rats, only one metabolite, the *N*-acetyl derivative **11**, was identified by cochromatography with an authentic sample (data not shown).

A similar study on the chlorophenylbenzothiazole **5** has been carried out in rats. Following oral dosing at 10 mg/kg, this amino compound could be detected in the plasma at 30 min, with maximum concentrations achieved at 30−60 min postdosing. Rapid metabolism was evident with the formation of a single *N*-acetyl derivative **12**. Peak concentrations of this metabolite were reached at 30 min postdosing, representing 40−50% of the parent amine **5** (Figure 8b). *N*-Acetylation of compound **5** may be less detrimental to putative in vivo efficacy, since metabolism is less extensive and the *N*-acetyl derivative retains much of the potency of the parent amine (Table 2).

In related studies conducted at the NCI (unpublished), the in vivo metabolism of compound **4** has been shown to be highly species specific. There was no evidence of metabolic *N*-acetylation after iv administration of 50 mg/kg (or oral administration of 150 mg/kg) compound **4** to mice.

The available evidence suggests that humans may behave more like the mouse than the rat in the manner in which they metabolize benzothiazole **4**. Thus, pooled human liver S9 fractions (which support both phase I and phase II metabolism), in the presence of acetyl coenzyme A, gave only minor amounts of the *N*-acetyl metabolite **11**, together with traces of oxidized products. More significantly, incubation of compound **4** with human lymphoblast-expressed P450 isoforms CYP1A1 and CYP1B1 gave an oxidized metabolite which has been identified as 2-(4-amino-3-methylphenyl)-6-hydroxybenzothiazole. The complexities of oxidative metabolism of compound **4** and the involvement of specific P450 isoforms will form the subject of a separate paper.

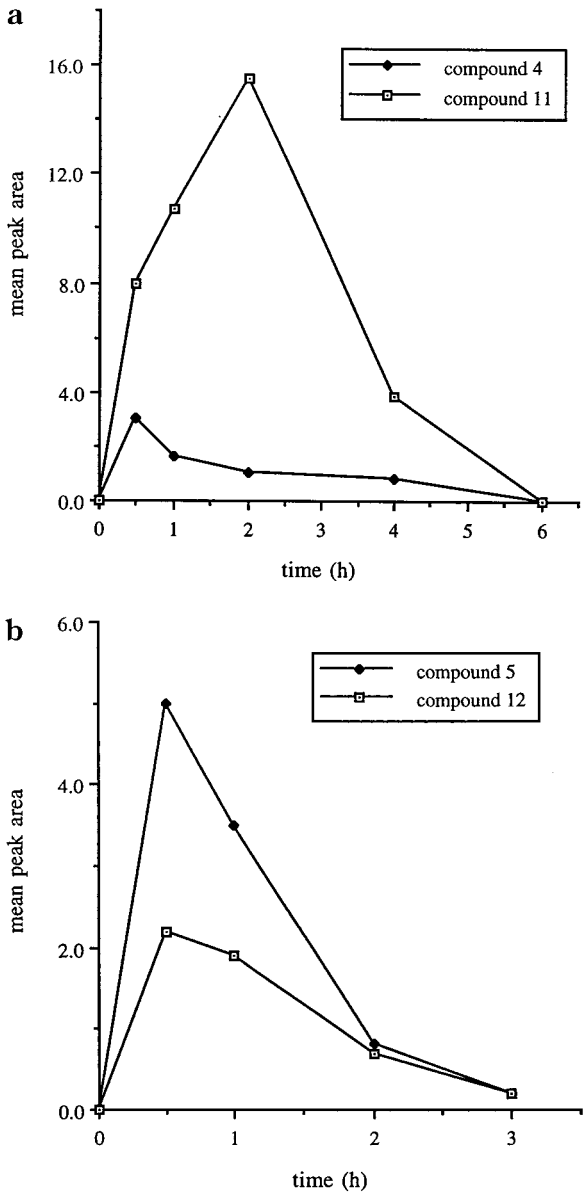


Figure 8. Plasma profiles of (a) compound **4** and (b) compound **5** after oral administration of each compound (10 mg/kg) to rats.

Conclusion

The novel structures reported here exert their remarkable activity through a mechanism that is not understood, but selective metabolism in target cells may be an important determinant in the mode of action. Drug uptake and biotransformation were evident only in sensitive cell lines in vitro, occurring to negligible extents in unresponsive cell lines. SAR studies of compounds with structural modifications at the exocyclic amine function emphasized the stringent requirement of a primary amine for selective activity.

The nature of the 3′-substituent in the arylamine fragment exerts a profound influence on the predominant metabolic process, the extent of metabolism, and

the bioactivity of *N*-acetyl metabolites. Compound **4** was mainly oxidized in sensitive human cell lines in vitro but exclusively *N*-acetylated by isolated rat hepatocytes and in vivo in rats to inactive product **11**; in mice, on the other hand, there was no evidence of conversion of arylamine **4** to the *N*-acetylated product **11**.

Confounding the results in the present work, the in vivo antitumor activity of compound **4** against a panel of human breast,[1] ovarian,[16] and colon tumor[17] xenografts is consistently superior to that of the halogenated counterparts **5**−**7**, and amine **4** remains the favored clinical candidate to date. Finally, the relative compositions and tissue/tumor-specific expression of potentially activating (e.g., CYP1A1 and CYP1B1) and deactivating (e.g., NAT1 and NAT2) enzymes may explain the selective antitumor actions of 2-(4-aminophenyl)benzothiazoles. This is an area of current investigation which may be useful in predicting the response to treatment of both the tumor and the patient.

Experimental Section

Chemistry. All new compounds were characterized by elemental microanalysis (C, H, and N values ± 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 Bruker AC250 or ARX250 spectrometers. IR spectra were determined in KBr on a Mattson 2020 GALAXY series FT-IR spectrophotometer. Mass spectra were recorded on an AEI MS-902 or a VG Micromass 7070E spectrometer. TLC systems for routine monitoring of reaction mixtures and confirming the homogeneity of analytical samples employed Kieselgel 60F_{254} (0.25 mm) silica gel TLC plastic sheets. Sorbsil silica gel C 60-H (40−60 μm) was used for flash chromatographic separations.

2-(4-Acetamidophenyl)benzothiazole (10). A solution of compound **3** (0.5 g, 2.21 mmol) in benzene (30 mL) and acetic anhydride (0.5 mL) was stirred under reflux for 4 h and cooled. The precipitate was filtered off and washed with benzene and hot ethyl acetate to give a white powder (0.52 g, 88%): mp 227−229 °C (lit.[18] mp 225−227 °C); IR 3281, 3247, 1163, 1598, 1537, 1477, 1434, 1317, 1262, 849, 753, 730 cm^{-1}; δ_H (DMSO-d_6) 10.30 (s, 1H, NH), 8.14−8.02 (m, 4H, H-4, H-7, H-2′, H-6′), 7.81 (d, 2H, J = 8.7 Hz, H-3′, H-5′), 7.54 (dt, 1H, J = 1.2, 7.5 Hz, H-5), 7.44 (dt, 1H, J = 1.2, 7.5 Hz, H-6), 2.12 (s, 3H, COCH_3); δ_C (DMSO-d_6) 169.0 (C), 167.2 (C), 153.9 (C), 142.4 (C), 134.5 (C), 128.2 (2 × CH), 127.6 (C), 126.8 (CH), 125.4 (CH), 122.5 (CH), 119.3 (2 × CH), 24.3 (CH_3).

2-(4-Acetamido-3-methylphenyl)benzothiazole (11). A solution of compound **4** (0.5 g, 2.08 mmol) and acetic anhydride (0.5 mL, 5.3 mmol) in benzene (30 mL) was heated at 80 °C for 4 h and allowed to cool overnight. After evaporation of solvent under reduced presure, the residue was recrystallized from ethanol−water to give white microcrystals (0.51 g, 82%): mp 207−208 °C; IR 3473, 3266, 1657, 1583, 1522, 1480, 1385, 1287, 760 cm^{-1}; δ_H (DMSO-d_6) 9.44 (s, 1H, NH), 8.14 (d, 1H, J = 7.5 Hz, H-4), 8.04 (d, 1H, J = 7.8 Hz, H-7), 7.96 (d, 1H, J = 1.3 Hz, H-2′), 7.89 (dd, 1H, J = 2.0, 8.5 Hz, H-6′), 7.79 (d, 1H, J = 8.4 Hz, H-5′), 7.54 (dt, 1H, J = 1.5, 7.8 Hz, H-5), 7.45 (dt, 1H, J = 1.3, 7.6 Hz, H-6), 2.35 (s, 3H, COCH_3), 2.14 (s, 3H, CH_3); δ_C (DMSO-d_6) 169.4 (C), 167.9 (C), 154.5 (C), 140.5 (C), 135.3 (C), 132.1 (C), 129.8 (CH), 129.7 (C), 126.2 (CH), 125.2 (CH), 123.5 (CH), 123.1 (CH), 24.5 (CH_3), 18.8 (CH_3); m/z 282 (M^+), 240 (M − C_2H_2O). Anal. ($C_{16}H_{14}N_2OS$) C, H, N.

2-(4-Acetamido-3-chlorophenyl)benzothiazole (12). A solution of compound **5** (0.1 g, 0.38 mmol) and acetic anhydride (0.1 mL, 1.06 mmol) in pyridine (1 mL) was refluxed for 30 min. The reaction was terminated by the addition of methanol (0.2 mL), and the mixture was neutralized to pH 7 with 1 M HCl. The product was extracted into ethyl acetate, solvent was removed by rotary evaporation, and the product was purified by column chromatography, eluting with EtOAc−hexane (2:5) to give a pale-orange powder (0.09 g, 80%): mp 165−166 °C; IR 3291, 3219, 1661, 1537, 1499, 1397, 1300, 754 cm^{-1}; δ_H ($CDCl_3$) 8.58 (d, 1H, J = 9 Hz, H-5′), 8.21 (d, 1H, J = 2 Hz, H-2′), 8.06 (dd, 1H, J = 2, 9 Hz, H-6′), 7.96−7.90 (m, 2H, H-4, H-7), 7.78 (s, 1H, NH), 7.48 (dt, 1H, J = 1.25 Hz, H-6), 7.46 (dt, 1H, J = 1.25, 7.25 Hz, H-5), 2.30 (s, 3H, COCH_3); m/z 302, 304 (M^+), 260, 262 (M − C_2H_2O). Anal. ($C_{15}H_{11}ClN_2OS$) C, H, N.

2-(4-Acetamido-3-bromophenyl)benzothiazole (13). Acetyl chloride (0.47 mL, 6.56 mmol) was added dropwise to a solution of **6** (1 g, 3.28 mmol) in chloroform (20 mL) containing triethylamine (0.5 mL, 3.6 mmol). The resulting solution was stirred at 25 °C for 30 min and quenched with water (10 mL). The organic layer was removed and washed with water (10 mL). The solvent was evaporated to leave a white solid which was recrystallized from ethanol to give white crystals (0.94 g, 83%): mp 195−197 °C; IR 3211, 1656, 1535, 1294, 752 cm^{-1}; δ_H ($CDCl_3$) 8.5 (d, 1H, J = 8.75 Hz, H-5′), 8.3 (d, 1H, J = 2 Hz, H-2′), 8.0 (dd, 1H, J = 1.25, 8.25 Hz, H-4), 7.9 (dd, 1H, J = 2, 8.75 Hz, H-6′), 7.85 (dd, 1H, J = 1.25, 8.25 Hz, H-7), 7.7 (br s, 1H, NH), 7.45 (dt, 1H, J = 1.25, 8.75 Hz, H-5), 7.36 (dt, 1H, J = 1.25, 8.25 Hz, H-6), 2.26 (s, 3H, CH_3); m/z 347, 349 (M^+ + 1). Anal. ($C_{15}H_{11}BrN_2OS$) C, H, N.

2-(4-Acetamido-3-iodophenyl)benzothiazole (14). Acetyl chloride (0.4 mL, 5.68 mmol) was added dropwise to a solution of **7** (1 g, 2.84 mmol) in chloroform (20 mL) containing triethylamine (0.32 g, 3.12 mmol). Product **14** was isolated and recrystallized as described (above) for **13** to give pale-cream crystals (1.0 g, 89%): mp 225−226 °C; IR 3202, 1653, 1491, 1290, 727 cm^{-1}; δ_H ($CDCl_3$) 8.55 (d, 1H, J = 2.25 Hz, H-2′), 8.38 (d, 1H, J = 8.75 Hz, H-5′), 8.02 (dd, 1H, J = 1.25, 8.25 Hz, H-4), 7.95 (dd, 1H, J = 2.25, 8.75 Hz, H-6′), 7.86 (dd, 1H, J = 1.25, 8.25 Hz, H-7), 7.56 (br s, 1H, NH), 7.45 (dt, 1H, J = 1.25, 8.25 Hz, H-5), 7.36 (dt, 1H, J = 1.25, 8.25 Hz, H-6), 2.26 (s, 3H, CH_3); m/z 395 (M^+ + 1). Anal. ($C_{15}H_{11}IN_2OS$) C, H, N.

2-(4-Acetamidophenyl)benzoxazole (15). Prepared from 2-(4-aminophenyl)benzoxazole (**8**) (0.1 g, 4.76 mmol) and acetic anhydride (0.5 mL) in benzene according to the method for the preparation of **10**, compound **15** (0.9 g, 75%): mp 214−215 °C (lit.[19] mp 212 °C); IR 3243, 3177, 3079, 3039, 1693, 1612, 1529, 1500, 1455, 1412, 1368, 1312, 1259, 1175, 1068, 1012, 849, 744 cm^{-1}; δ_H (DMSO-d_6) 10.32 (s, 1H, NH), 8.13 (d, 2H, J = 8.7 Hz, H-2′, H-6′), 7.82 (d, 2H, J = 8.7 Hz, H-3′, H-5′), 7.78−7.72 (m, 2H, H-4, H-7), 7.39−7.34 (m, 2H, H-5, H-6), 2.11 (s, 3H, CH_3); δ_C (DMSO-d_6) 169.7 (C), 163.1 (C), 151.0 (C), 143.5 (C), 142.5 (C), 129.1 (2 × CH), 125.9 (CH), 125.6 (CH), 121.5 (CH), 120.4 (CH), 120.0 (2 × CH), 111.6 (CH), 25.0 (CH_3); m/z 252 (M^+), 210 (M − C_2H_2O).

2-(4-Acetamidophenyl)benzimidazole (16). Prepared, as above, from 2-(4-aminophenyl)benzimidazole (**9**) (1.0 g, 4.76 mmol) and acetic anhydride (0.5 mL) in benzene, the benzimidazole was isolated as a gray powder (1.2 g, 95%): mp 308−309 °C (lit.[20] mp 309 °C); IR 3223, 3056, 1673, 1603, 1553, 1431, 1371, 1280, 839, 744 cm^{-1}; δ_H (DMSO-d_6) 12.82 (br s, 1H, NH), 10.19 (s, 1H, NH), 8.10 (d, 2H, J = 8.6 Hz, H-3′, H-5′), 7.76 (d, 2H, J = 8.6 Hz, H-2′, H-6′), 7.59−7.55 (m, 2H, H-4, H-7), 7.20−7.17 (m, 2H, H-5, H-6), 2.10 (s, 3H, CH_3); δ_C (DMSO-d_6) 169.5 (C), 152.0 (C), 141.7 (C), 128.0 (CH), 125.5 (C), 122.8 (CH), 120.0 (CH), 25.0 (CH_3); m/z 251 (M^+), 209 (M − C_2H_2O).

2-(4-Diacetamidophenyl)benzothiazole (17). A solution of compound **3** (0.2 g, 0.88 mmol) and acetic anhydride (0.4 mL, 4.24 mmol) in pyridine (2 mL) was refluxed overnight. The benzothiazole **17** was isolated as a pale-yellow powder (0.06 g, 22%): mp 128−129 °C; IR 3408, 1721, 1692, 1481, 1368, 1233, 970, 762 cm^{-1}; δ_H ($CDCl_3$) 8.20 (dd, 2H, J = 2.5, 8.5 Hz, H-2′, H-6′), 8.10 (d, 1H, J = 8 Hz, H-7), 7.93 (d, 1H, J = 8 Hz, H-4), 7.56−7.39 (m, 2H, H-5, H-6), 7.29 (dd, 2H, J = 2.5, 8.5 Hz, H-3′, H-5′), 2.33 (s, 6H, 2 × $COCH_3$). Anal. ($C_{17}H_{14}N_2O_2S$) C, H, N.

2-(4-Diacetamido-3-methylphenyl)benzothiazole (18). Prepared, from starting material **4** (0.59 g, 2.46 mmol) and acetic anhydride (5 mL), in refluxing benzene (10 mL) over-

night. After cooling, the precipitated product was collected and washed with benzene and diethyl ether to furnish **18** as a white powder (0.7 g, 88%): mp 147−149 °C; IR 1710, 1479, 1366, 1253, 1018, 757, 626, 600 cm^{-1}; δ_H ($CDCl_3$) 8.13−8.10 (m, 2H, H-2′, H-4), 8.01 (dd, 1H, J = 2.0, 8.1 Hz, H-6′), 7.94 (d, 1H, J = 7.8 Hz, H-7), 7.54 (dt, 1H, J = 1.3, 7.7 Hz, H-5), 7.43 (dt, 1H, J = 1.2, 7.6 Hz, H-6), 7.24 (d, 1H, J = 8.1 Hz, H-5′), 2.33 (s, 6H, 2 × $COCH_3$); δ_C ($CDCl_3$) 172.9 (2 × C), 167.1 (C), 154.5 (CH), 125.9 (CH), 123.8 (CH), 122.2 (CH), 27.1 (2 × $COCH_3$), 18.0 (CH_3). Anal. ($C_{18}H_{16}N_2O_2S$) C, H, N.

2-(4-Diacetamido-3-chlorophenyl)benzothiazole (19). Prepared from **5** (0.50 g, 1.92 mmol) and acetic anhydride (0.4 mL, 4.24 mmol) in refluxing pyridine (4.5 mL) overnight, the product **19** was isolated as a pale-yellow powder (0.11 g, 16%): mp 155−157 °C; IR 1728, 1478, 1370, 1242, 1017, 762, 598 cm^{-1}; δ_H ($CDCl_3$) 8.30 (d, 1H, J = 2 Hz, H-2′), 8.12−8.05 (m, 2H, H-4, H-7), 7.92 (dd, 1H, J = 2, 8.25 Hz, H-6′), 7.54 (dt, 1H, J = 2 Hz, H-6), 7.44 (dt, 1H, J = 2, 8.25 Hz, H-5), 7.37 (d, J = 8.25 Hz, H-5), 2.34 (s, 6H, 2 × $COCH_3$). Anal. ($C_{17}H_{13}ClN_2O_2S$) C, H, N.

2-(4-Chloroacetamidophenyl)benzothiazole (20). To a solution of **3** (0.8 g, 3.45 mmol) in refluxing benzene (40 mL) was added dropwise chloroacetyl chloride (0.8 mL), and the mixture was boiled for a further 30 min. The precipitate was collected and washed with diethyl ether to give 2-(4-chloroacetamidophenyl)benzothiazole hydrochloride as a yellow powder (1.08 g, 90%): mp 232−234 °C; IR 3160, 3082, 3025, 2435, 1703, 1598, 1535, 1446, 1384, 1342, 1191, 838, 754 cm^{-1}; δ_H (DMSO-d_6) 11.03 (s, 1H, NH), 9.45 (br s, 1H, N^+H), 8.13−8.01 (m, 4H, H-2′, H-6′, H-4, H-7), 7.85 (d, 2H, J = 8.6 Hz, H-3′, H-5′), 7.53 (t, 1H, J = 7.6 Hz, H-5), 7.43 (t, 1H, J = 7.5 Hz, H-6), 4.38 (s, 2H, CH_2Cl); δ_C (DMSO-d_6) 167.8 (C), 166.0 (C), 154.4 (C), 142.3 (C), 135.2 (C), 128.9 (2 × CH), 127.5 (CH), 126.2 (CH), 123.5 (CH), 123.1 (CH), 120.5 (2 × CH), 44.4 (CH_2).

Basification of the salt (0.8 g) with 10% aqueous Na_2CO_3 (40 mL) at 50 °C for 1 h gave the pale-yellow free base **20** (0.63 g, 88%): mp 214−215 °C; IR 3453, 3321, 1674, 1528, 1410, 1384, 828, 754 cm^{-1}; δ_H (DMSO-d_6) 10.66 (s, 1H, NH), 8.15−8.02 (m, 4H, H-2′, H-6′, H-4, H-7), 7.81 (d, 2H, J = 8.6 Hz, H-3′, H-5′), 7.54 (t, 1H, J = 7.5 Hz, H-5), 7.44 (t, 1H, J = 7.4 Hz, H-6), 4.33 (s, 2H, CH_2Cl); δ_C (DMSO-d_6) 167.7 (C), 165.9 (C), 154.5 (C), 142.1 (C), 135.2 (C), 129.0 (2 × CH), 127.5 (CH), 126.2 (CH), 123.5 (CH), 123.1 (CH), 120.5 (2 × CH), 44.5 (CH_2). Anal. ($C_{15}H_{11}ClN_2OS$) C, H, N.

2-(4-Chloroacetamidophenyl)benzoxazole (21). Similarly prepared, from 2-(4-aminophenyl)benzoxazole (**8**) (0.28 g, 1.33 mmol) and chloroacetyl chloride (0.5 mL) in benzene (15 mL), 2-(4-chloroacetamidophenyl)benzoxazole hydrochloride was formed (0.31 g, 72%): mp 211−215 °C; IR 3253, 3100, 3042, 2415, 1716, 1674, 1606, 1537, 1460, 1340, 1182, 933, 749 cm^{-1}; δ_H (DMSO-d_6) 10.93 (s, 1H, NH), 8.18 (d, 2H, J = 8.7 Hz, H-2′, H-6′), 7.87 (d, 2H, J = 8.7 Hz, H-3′, H-5′), 7.78 (m, 2 H, H-4, H-7), 7.40 (m, 2 H, H-5, H-6), 7.00 (br s, 1H, N^+H), 4.37 (s, 2H, CH_2Cl); δ_C (DMSO-d_6) 166.1 (C), 162.9 (C), 151.0 (C), 142.7 (C), 142.5 (C), 129.2 (2 × CH), 126.1 (CH), 125.7 (CH), 122.3 (C), 120.5 (CH), 120.3 (2 × CH), 111.7 (CH), 44.5 (CH_2).

The hydrochloride salt was basified with 10% aqueous Na_2CO_3 to form the free base of **21** as white crystals (0.18 g, 89%): mp 200−202 °C; IR 3262, 3122, 1670, 1618, 1542, 1501, 1405, 1343, 1249, 1061, 840, 744 cm^{-1}; δ_H (DMSO-d_6) 10.68 (s, 1H, NH), 8.19 (d, 2H, J = 8.7 Hz, H-2′, H-6′), 7.85 (d, 2H, J = 8.7 Hz, H-3′, H-5′), 7.81−7.76 (m, 2H, H-4, H-7), 7.45−7.37 (m, 2H, H-5, H-6), 4.33 (s, 2H, $CHCl_2$); δ_C (DMSO-d_6) 166.0 (C), 163.0 (C), 152.0 (C), 142.6 (C), 142.5 (C), 129.2 (2 × CH), 126.1 (CH), 125.7 (CH), 122.3 (C), 120.5 (CH), 120.4 (2 × CH), 111.7 (CH), 44.5 ($CHCl_2$). Anal. ($C_{15}H_{11}ClN_2O_2$) C, H, N.

2-(4-Dichloroacetamidophenyl)benzothiazole (22). Similarly prepared, from compound **3** (0.4 g, 1.77 mmol) and dichloroacetyl chloride (0.34 mL) in benzene according to the above procedure, the yellow hydrochloride salt of **22** was formed (0.56 g, 85%): mp 237−241 °C; IR 2998, 2426, 1708, 1594, 1534, 1500, 1444, 1344, 1248, 1187, 1165, 840, 753 cm^{-1}; δ_H (DMSO-d_6) 11.69 (s, 1H, NH), 10.06 (br s, 1H, N^+H), 8.11−8.08 (m, 3H, H-4, H-2′, H-6′), 8.03 (d, 1H, J = 7.5 Hz, H-7), 7.89 (d, 2H, J = 8.8 Hz, H-3′, H-5′), 7.52 (dt, 1H, J = 1.3, 7.6 Hz, H-5), 7.42 (dt, 1H, J = 1.2, 7.5 Hz, H-6), 6.98 (s, 1H, $CHCl_2$); δ_C (DMSO-d_6) 167.6 (C), 163.0 (C), 154.3 (C), 141.4 (C), 135.2 (C), 129.6 (C), 129.0 (2 × CH), 127.5 (CH), 126.2 (CH), 123.5 (CH), 123.2 (CH), 120.9 (2 × CH), 67.9 ($CHCl_2$). Anal. ($C_{15}H_{10}Cl_2N_2OS$·HCl) C, H, N.

The free base of **22** was liberated as a white powder (0.2 g, 89%): mp 223−225 °C; IR 3254, 3054, 1679, 1605, 1543, 1481, 1409, 1250, 968, 841, 806, 758, 729 cm^{-1}; δ_H (DMSO-d_6) 11.01 (br s, 1H, NH), 8.13 (d, 3H, J = 8.7 Hz, H-4, H-2′, H-6′), 8.05 (d, 1H, J = 7.9 Hz, H-7), 7.83 (d, 2H, J = 8.6 Hz, H-3′, H-5′), 7.55 (t, 1H, J = 7.4 Hz, H-5), 7.45 (t, 1H, J = 7.4 Hz, H-6), 6.66 (s, 1H, $CHCl_2$); δ_C (DMSO-d_6) 167.5 (C), 162.9 (C), 154.5 (C), 141.2 (C), 135.2 (C), 129.8 (C), 129.1 (2 × CH), 127.5 (CH), 126.3 (CH), 123.6 (CH), 123.2 (CH), 121.0 (2 × CH), 68.2 ($CHCl_2$).

2-(4-Chloroacetamido-3-iodophenyl)benzothiazole (23). To a solution of compound **7** (0.15 g, 0.426 mmol) in benzene (15 mL) was added dropwise chloroacetyl chloride (0.18 g) at room temperature. A yellow precipitate was formed, and the resulting mixture was stirred at 50 °C for 30 min and cooled in an ice bath. The solid was collected, washed with petroleum ether, and dried to give a yellow powder of a hydrate of **23** (0.13 g, 71%): mp 192−194 °C; IR 3441, 3312, 1695, 1569, 1534, 1511, 1484, 1384, 1308, 750 cm^{-1}; δ_H (DMSO-d_6) 9.88 (br s, 1H, NH), 8.58 (d, 1H, J = 1.9 Hz, H-2′), 8.18 (d, 1H, J = 7.9 Hz, H-4), 8.13−8.08 (m, 2H, H-7, H-6′), 7.77 (d, 1H, J = 8.4 Hz, H-5′), 7.58 (t, 1H, J = 7.7 Hz, H-5), 7.49 (t, 1H, J = 7.5 Hz, H-6), 4.47 (s, 2H, CH_2Cl); δ_C (DMSO-d_6) 166.1 (C), 165.8 (C), 154.3 (C), 142.1 (C), 137.9 (CH), 135.5 (C), 132.5 (C), 128.6 (CH), 127.7 (CH), 127.1 (CH), 126.6 (CH), 123.9 (CH), 123.3 (CH), 97.1 (C), 44.0 (CH_2). Anal. ($C_{15}H_{10}ClIN_2OS$·H_2O) C, H, N.

2-(4-Benzamidophenyl)benzothiazole (24). A mixture of compound **3** (0.3 g, 1.32 mmol) and benzoyl chloride (0.3 mL) in pyridine (8 mL) was stirred at reflux for 2 h, then cooled, and poured into water (100 mL). The precipitate was recrystallized from dichloromethane−methanol to give a white powder (0.36 g, 82%): mp 227−229 °C; IR 3456, 3364, 1657, 1530, 1480, 1404, 1385, 1316, 967, 829, 757, 712 cm^{-1}; δ_H (DMSO-d_6) 10.60 (s, 1H, NH), 8.17−7.99 (m, 8H), 7.62 (m, 5H); δ_C (DMSO-d_6) 167.9 (C), 166.8 (C), 154.5 (C), 143.0 (C), 135.5 (C), 135.2 (C), 132.7 (CH), 129.3 (2 × CH), 128.82 (C), 128.76 (2 × CH), 128.7 (2 × CH), 127.5 (CH), 126.2 (CH), 123.5 (CH), 123.2 (CH), 121.3 (2 × CH); m/z 330 (M^+), 105. Anal. ($C_{20}H_{14}N_2OS$) C, H, N.

2-(4-Trifluoroacetamidophenyl)benzothiazole (25). Compound **3** (0.49 g, 2.17 mmol) was treated with trifluoroacetic anhydride (1 g) in benzene under reflux for 5 h. The precipitate was collected by filtration and washed with benzene to give yellow microcrystals of 2-(4-trifluoroacetamidophenyl)benzothiazole trifluoroacetate salt (0.935 g, 99%): mp 140−143 °C; IR 3305, 1745, 1601, 1543, 1297, 1184, 1150, 707 cm^{-1}; δ_H (DMSO-d_6) 11.57 (s, 1H, NH), 9.73 (br s, 1H, N^+H), 8.17−8.13 (m, 3H, H-4, H-2′, H-6′), 8.06 (d, 1H, J = 8.0 Hz, H-7), 7.91 (d, 2H, J = 8.8 Hz, H-3′, H-5′), 7.55 (t, 1H, J = 7.6 Hz, H-5), 7.46 (t, 1H, J = 7.5 Hz, H-6); δ_C (DMSO-d_6) 167.3 (C), 159.2 (q, J = 38.4 Hz, $COCF_3$), 155.5 (q, J = 37.2 Hz, $NHCOCF_3$), 154.4 (C), 139.9 (C), 135.3 (C), 130.7 (C), 128.9 (2 × CH), 127.5 (CH), 126.4 (CH), 123.7 (CH), 123.2 (CH), 122.2 (2 × CH), 116.5 (q, J = 228.7 Hz, $NHCOCF_3$), 115.9 (q, J = 289.3 Hz, $COCF_3$).

Basification of the salt gave the free base of **25** as a white powder (0.23 g, 69%): mp 232−235 °C; IR 3345, 1705, 1596, 1537, 1482, 1415, 1287, 1249, 1156, 971, 908, 837, 760, 729 cm^{-1}; δ_H (DMSO-d_6) 11.57 (s, 1H, NH), 8.17−8.13 (m, 3H, H-4, H-2′, H-6′), 8.06 (d, 1H, J = 7.8 Hz, H-7), 7.91 (d, 2H, J = 8.8 Hz, H-3′, H-5′), 7.56 (dt, 1H, J = 1.2, 7.6 Hz, H-5), 7.47 (dt, 1H, J = 1.2, 7.6 Hz, H-6); δ_C (DMSO-d_6) 167.3 (C), 155.5 (q, J = 37.3 Hz, $NHCOCF_3$), 139.9 (C), 135.3 (C), 130.7 (C), 128.9 (2 × CH), 127.5 (CH), 126.4 (CH), 123.7 (CH), 123.2 (CH), 122.2 (2 × CH), 116.5 (q, J = 228.7 Hz, $NHCOCF_3$); m/z 322 (M^+). Anal. ($C_{15}H_9F_3N_2OS$) C, H, N.

2-(4-Acetamido-3-nitrophenyl)benzothiazole (26). To a solution of compound **3** (0.356 g, 1.57 mmol) in acetic anhydride (25 mL) and benzene (15 mL) was added copper(II) nitrate hydrate (0.31 g). The reaction mixture was stirred at room temperature overnight. Evaporation of the mixture under reduced pressure gave a residue which was neutralized with 10% aqueous sodium bicarbonate. After addition of water (100 mL) the organic layer was separated, washed with water (2 × 80 mL), and dried ($MgSO_4$). The solution was chromatographed using EtOAc−hexane (1:3, 1:1) as eluent to give **26** as a brown powder (0.16 g, 33%): mp 232−234 °C; IR 4362, 3360, 1710, 1578, 1484, 1384, 1341, 1228, 1146, 761 cm^{-1}; δ_H (DMSO-d_6) 10.57 (s, 1H, NH), 8.58 (d, 1H, J = 2.1 Hz, H-2′), 8.38 (dd, 1H, J = 2.1, 8.6 Hz, H-6′), 8.22 (d, 1H, J = 7.6 Hz, H-4), 8.13 (d, 1H, J = 7.6 Hz, H-7), 7.89 (d, 1H, J = 8.6 Hz, H-5′), 7.60 (dt, 1H, J = 1.4, 7.6 Hz, H-5), 7.52 (dt, 1H, J = 1.3, 7.7 Hz, H-6), 2.15 (s, 3H, CH_3); δ_C (DMSO-d_6) 169.6 (C), 165.5 (C), 154.2 (C), 142.9 (C), 135.5 (C), 134.3 (C), 133.1 (CH), 129.8 (C), 127.8 (CH), 126.8 (CH), 126.4 (CH), 124.0 (CH), 123.9 (CH), 123.4 (CH), 24.5 (CH_3); m/z 313 (M^+). Anal. ($C_{15}H_{11}N_3O_3$) C, H, N.

2-(4-Thioacetamidophenyl)benzothiazole (27). A mixture of compound **10** (0.4 g, 1.49 mmol) and Lawesson's reagent (0.37 g, 0.9 mmol) in HMPA (10 mL) was stirred at 100 °C for 6 h. The reaction mixture was poured into water and the precipitate filtered off, washed with water, and dried. The crude product was purified by column chromatography, eluting with EtOAc−hexane (5:6), to give pale-yellow crystals of the thioacetylbenzothiazole **27** (0.26 g, 61%): mp 222−223 °C; IR 3306, 1515, 1476, 1409, 1357, 1150, 970, 760, 621 cm^{-1}; δ_H (DMSO-d_6) 11.85 (s, 1H, NH), 8.18−8.06 (m, 6H, H-2′, H-3′, H-5′, H-6′, H-4, H-7), 7.60−7.45 (m, 2H, H-5, H-6), 2.67 (s, 3H, CH_3); δ_C ($CDCl_3$) 200.8 (CS), 167.5 (C), 154.5 (C), 143.0 (C), 135.4 (C), 130.8 (C), 128.4 (2 × CH), 127.6 (CH), 126.4 (CH), 124.0 (2 × CH), 123.7 (CH), 123.2 (CH), 36.5 (CH_3); m/z 284 (M^+). Anal. ($C_{15}H_{12}N_2S_2$) C, H, N.

2-(4-Thioacetamidophenyl)benzoxazole (28). Compound **15** (0.3 g, 1.19 mmol) was thionated as above. The crude product was purified by chromatography, eluting with EtOAc−hexane (2:1), to give small pale-orange crystals (0.19 g, 60%): mp 212−214 °C; IR 3449, 3267, 3021, 1618, 1500, 1454, 1359, 1245, 744 cm^{-1}; δ_H (DMSO-d_6) 11.87 (s, 1H, NH), 8.25−8.14 (m, 4H, H-2′, H-3′, H-5′, H-6′), 7.83−7.77 (m, 2H, H-4, H-7), 7.44−7.41 (m, 2H, H-5, H-6), 2.67 (s, 3H, CH_3); δ_C (DMSO-d_6) 201.0 (CS), 162.7 (C), 151.1 (C), 143.5 (C), 142.4 (C), 128.6 (2 × CH), 126.3 (CH), 125.8 (CH), 124.2 (C), 123.8 (2 × CH), 120.6 (CH), 111.8 (CH), 36.6 (CH_3); m/z 268 (M^+). Anal. ($C_{15}H_{12}N_2SO$) C, H, N.

2-(4-Thioacetamidophenyl)benzimidazole (29). Compound **16** (0.3 g, 1.19 mmol) was thionated with Lawesson's reagent, as above. The crude product was purified by chromatography, eluting with EtOAc−hexane (20:3), to give **29** (0.12 g, 38%): mp 234−236 °C; IR 3438, 3262, 3202, 2924, 1495, 1449, 1428, 1367, 1276, 1148, 837, 742 cm^{-1}; δ_H (DMSO-d_6) 12.99 (br s, 1H, NH), 11.78 (s, 1H, NHCS), 8.19 (d, 2H, J = 8.6 Hz, H-3′, H-5′), 8.05 (d, 2H, J = 8.6 Hz, H-2′, H-6′), 7.63−7.59 (m, 2H, H-4, H-7), 7.25−7.19 (m, 2H, H-5, H-6), 2.66 (s, 3H, CH_3); δ_C (DMSO-d_6) 200.4 (CS), 151.6 (C), 141.7 (C), 128.2 (C), 127.5 (2 × CH), 123.9 (2 × CH), 123.0 (CH), 36.4 (CH_3); m/z 265 (M^+). Anal. ($C_{15}H_{13}N_3S$) C, H, N.

2-(Indazol-5-yl)benzothiazole (31). Isoamyl nitrite (0.37 mL, 2.7 mmol) was added to a solution of **4** (0.5 g, 2.08 mmol) in chloroform (20 mL) containing acetic acid (0.03 g, 0.5 mmol). The resulting pale-yellow solution turned dark red over a period of 1 h, and TLC showed consumption of the starting amine. This solution was allowed to stand at 25 °C overnight, and the resulting precipitate was collected. Recrystallization from THF gave a pale-cream solid (0.128 g, 24%): mp 264−268 °C; IR 3134, 1620, 1433, 1346, 752 cm^{-1}; δ_H (DMSO-d_6) 12.8−13.8 (br s, 1H, NH), 8.3 (s, 1H, H-3), 8.0 (s, 1H, H-4′), 7.9 (d, 2H, J = 8.5 Hz, H-6′, H-7′), 7.8 (d, 1H, J = 7.75 Hz, H-7), 7.5 (d, 1H, J = 7.75 Hz, H-4), 7.3 (t, 1H, J = 7.25 Hz, H-5), 7.2 (t, 1H, J = 7.25 Hz, H-6); m/z 252 (M^+ + 1). Anal. ($C_{14}H_9N_3S$·0.5 H_2O) C, H, N.

2-(Benzimidazol-5-yl)benzothiazole (35). 2-Aminothiophenol (0.5 g, 4.0 mmol) was added to benzimidazole-5-carboxylic acid (1.0 g, 4.0 mmol) in polyphosphoric acid (10 g), and the temperature was maintained at 200 °C for 2 h. The cooled mixture was poured into water (100 mL), and the resulting precipitate was filtered off. Recrystallization from methanol gave a white solid (0.95 g, 61%): mp 231−233 °C; IR 3441, 1610, 1423, 1259, 956 cm^{-1}; δ_H (DMSO-d_6) 12.7 (br s, 1H, NH), 8.1 (s, 1H, H-2′), 8.3 (s, 1H, H-4′), 8.1 (dd, 1H, J = 0.75, 8.0 Hz, H-7), 8.0 (dd, 1H, J = 0.75, 8.0 Hz, H-4), 8.0 (d, 1H, J = 8.5 Hz, H-6′), 7.7 (d, 1H, J = 8.5 Hz, H-7′), 7.5 (2 × dt, 2H, J = 0.75, 8.0 Hz, H-5, H-6); m/z 252 (M^+ + 1). Anal. ($C_{14}H_9N_3S$) C, H, N.

In Vitro Cell Culture. Monolayer cells were cultured at 37 °C in an atmosphere of 5% CO_2 in RPMI 1640 medium containing 2 mM L-glutamine and supplemented with 10% fetal calf serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained in continuous logarithmic growth by routine subculturing twice weekly. Cell lines utilized include human breast carcinoma cell lines MCF-7, MCF-7/LT 10 nM, MCF-7/LT 10 μM, MCF-7/Adr, MDA 468, T-47D, SKBR3, ZR 75, ex vivo MCF-7B, and ex vivo MCF-7T; human prostate carcinoma cell lines PC 3 and DU 145; and nonmalignant human breast cell line HBL 100.

MTT Colorimetric Assays. Cells were seeded into 96-well microtiter plates at densities of 4000−5000 cells/well (for 3-day assays) or 250−300 cells/well (for 7-day) assays. Cells were allowed to adhere during a 24-h drug-free incubation period before drugs were added over a concentration range of 1 pM to 100 μM (n = 8). After drug exposures of 3 or 7 days, MTT was added to each well at a final concentration of 400 mg/mL. After a 4-h incubation, allowing metabolism of MTT by mitochondrial dehydrogenase to an insoluble formazan product, medium was aspirated and formazan solubilized by the addition of 125 μL of DMSO−glycine buffer (4:1). Cell viability was determined as absorbance at 550 nm, read on an Anthos Labtec systems plate reader. For 3-day assays, a baseline protein content was estimated at the time of drug treatment.

Test agents were stored as 10 mM stock solutions in DMSO at 4 °C protected from light, up to 4 weeks. Compounds were stable for >28 days. Serial dilutions were prepared in media prior to each assay, with final DMSO concentration < 0.25%.

NCI in Vitro Cytotoxicity Assays. The NCI uses the sulforhodamine B assay for assessing the cytotoxicity of test agents in their panel of 60 cell lines.[13] Briefly, cell lines were inoculated into a series of 96-well microtiter plates, with varied seeding densities depending on the growth characteristics of particular cell lines. Following a 24-h drug-free incubation, test agents were added routinely at five 10-fold dilutions with a maximum concentration of 10^{-4} M. After 2 or 6 days of drug exposure, the change in protein stain optical density allowed the inhibition of cell growth to be analyzed.

Stability Studies. Solutions of test compounds (30 μM) in growth media alone were incubated at 37 °C, and 1-mL samples were collected at various time points over 28 days. Samples were frozen at −20 °C until anaylsis. To 300 μL of sample was added 600 μL of acetonitrile, and samples were vortexed and microcentrifuged at 1300 rpm for 5 min. The supernatant (800 μL) was removed for analysis on a Beckman System Gold HPLC using a Shandon Hypersil ODS reversed-phase C18 column (100-mm × 4.6-mm internal diameter) fitted with a Hypersil ODS precolumn. Separation was effected using a mobile phase of 65% methanol−35% water, running at a flow rate of 1 mL/min in isocratic mode. Samples were injected in triplicates of 20 μL, with detection at 330 nm. Calibration curves were constructed for each compound investigated, and internal standards used were 2-(4-aminophenyl)-6-methylbenzothiazole and compound **3**.

In Vitro Uptake and Biotransformation Studies. Cells were seeded at appropriate densities ((1−2) × 10^5 cells) into 25-mm^2 tissue culture flasks and allowed to reach >70% confluent growth before treatment with appropriate agents at 30 μM each (n = 3). An untreated flask served as the control. Samples (1 mL) were removed from each flask at various time

intervals (routinely over 7 days). After centrifugation, supernatant was frozen at −20 °C until anaylsis. At the end of the sampling period, cells were washed twice with PBS, detached by trypsinization, resuspended in 1 mL of PBS, and sonicated for 10 s. Cell debris was pelleted by centrifugation and the supernatant collected for analysis of intracellular metabolites. Metabolites were extracted and analyzed as described above for stability studies.

For confirmation of the identity of glucuronide conjugates, 500 μL of sample was injected for HPLC analysis and the eluent collected over the first 2 min. To this was added 20 units of β-glucuronidase in sodium acetate buffer (0.1 M) maintained at 37 °C. Samples were analyzed after 24 h and 7 days. Controls included such eluents without β-glucuronidase, incubations of β-glucuronidase alone, and media (obtained from untreated sensitive cells) incubated with β-glucuronidase.

Electrospray LC−MS. A VG Organic Platform mass spectrometer equipped with electrospray and atmospheric pressure chemical ionizations was used. Data were analyzed using the Mass Lynx data system. HPLC conditions were as described above, although injection volumes were increased to 50−100 μL. Increasing cone voltages (20, 35, 50, and 90 V) were used to facilitate molecule fragmentation. Between samples, the column was washed with 100% methanol at 1 mL/min for 15 min.

Isolation of Rat Hepatocytes. Rat hepatocytes were isolated from adult male Wistar rats (6−8 weeks old) by the lobe perfusion method.[21] William's medium E was removed, and cells were washed once with Krebs-Ringer, after which Krebs-Ringer containing 10 mM test agent (prepared from 10 mM stock solutions in DMSO) was added. A control sample consisted of 0.1% v/v DMSO in Krebs-Ringer. Following 3-h incubation, cells were scraped and collected in 2 mL of fresh Krebs-Ringer solution. All samples were frozen at −20 °C until analysis. The supernatant was used for HPLC analysis.

Preliminary Pharmacokinetic Studies in Rats. For oral dosing, compounds **3** and **4** were formulated as 4 mg/mL suspensions in 10% v/v ethanol in an aqueous solution containing 10% w/v hydroxypropyl-β-cyclodextrin. In addition, a 0.2 mg/mL formulation of compound **3** was prepared in the same vehicle for iv dosing. Compounds **4** and **5** were orally dosed at 10 mg/kg to 8 and 2 male rats, respectively, and compound **4** was additionally given iv at 0.5 mg/kg to 8 male rats via the tail vein. Blood samples were collected at intervals over 6 h postadministration, prepared immediately, and frozen pending analysis.

SDS−PAGE and Immunoblotting. Cytosolic proteins were prepared,[22] separated on 12% polyacrylamide gels by SDS−PAGE, and immunoblotted with specific antibodies according to established procedures.[14]

Acknowledgment. This research was supported by a program grant from the Cancer Research Campaign, U.K. The authors thank members of the Screening and Pharmacology Group of the European Organisation for Research and Treatment of Cancer (EORTC) for many helpful discussions. We are grateful to Dr. E. A. Sausville, Dr. R. J. Schultz, Dr. V. L. Narayanan, (the late) Dr. K. D. Paull, and their colleagues of the NCI Developmental Therapeutics Program for their productive collaboration on this project. We thank Dr. Charmaine Quaterman, Aston Molecules Ltd., Birmingham, U.K., for HPLC analyses, Dr. C. A. Ortori for mass spectrometry, and Dr. A. H. Hammond for the rat hepatocyte work.

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JM981076X