Antitumor Benzothiazoles. 8.[1] Synthesis, Metabolic Formation, and Biological Properties of the *C*- and *N*-Oxidation Products of Antitumor 2-(4-Aminophenyl)-benzothiazoles▽

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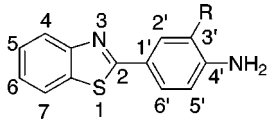
2-(4-Aminophenyl)benzothiazoles **1** and their *N*-acetylated forms have been converted to *C*- and *N*-hydroxylated derivatives to investigate the role of metabolic oxidation in the mode of action of this series of compounds. 2-(4-Amino-3-methylphenyl)benzothiazole (**1a**, DF 203, NSC 674495) is a novel and potent antitumor agent with selective growth inhibitory properties against human cancer cell lines. Very low IC_{50} values (<0.1 μM) were encountered in the most sensitive breast cancer cell lines, MCF-7 and T-47D, whereas renal cell line TK-10 was weakly inhibited by **1a**. Cell lines from the same tissue origin, MDA-MB-435 (breast), CAKI-1 (renal), and A498 (renal), were insensitive to **1a**. Accumulation and metabolism of **1a** were observed in sensitive cell lines only, with the highest rate of metabolism occurring in the most sensitive MCF-7 and T-47D cells. Thus, differential uptake and metabolism of **1a** by cancer cell lines may underlie its selective profile of anticancer activity. A major metabolite in these sensitive cell lines has been identified as 2-(4-amino-3-methylphenyl)-6-hydroxybenzothiazole (**6c**). Hydroxylation of **1a** was not detected in the homogenate of previously untreated MCF-7, T-47D, and TK-10 cells but was readily observed in homogenates of sensitive cells that were pretreated with **1a**. Accumulation and covalent binding of [^{14}C]**1a** derived radioactivity was observed in the sensitive MCF-7 cell line but not in the insensitive MDA-MB-435 cell line. The mechanism of growth inhibition by **1a**, which is unknown, may be dependent on the differential metabolism of the drug to an activated form by sensitive cell lines only and its covalent binding to an intracellular protein. However, the 6-hydroxy derivative **6c** is not the 'active' metabolite since, like all other *C*- and *N*-hydroxylated benzothiazoles examined in this study, it is devoid of antitumor properties in vitro.

Introduction

2-(4-Aminophenyl)benzothiazoles **1a**−**e** (see Figure 1 for structures) comprise a novel mechanistic class of antitumor agents. Their unusual activity was first recognized from the distinctive biphasic dose−response relationship shown in in vitro assays against sensitive breast tumor cell lines, e.g. MCF-7 and MDA 468: potency against these breast lines, and others, was independent of the estrogen or growth factor receptor status of the cells.[2] Introduction of a methyl or halogen substituent into the 3′-position of the 2-phenyl group enhances potency and extends the spectrum of action to certain colon, lung, melanoma, renal, and ovarian cell lines.[3,4] The 3′-methyl analogue **1a** (DF 203, NSC 674495) has consistently out-performed the 3′-halogeno derivatives in in vivo studies against breast,[2] ovarian,[4] and colon xenografts,[5] and this compound, readily available from a one-step synthesis,[2] is the current focus of preclinical interest.



1a: R = Me (DF 203; NSC 674495)
b: R = H
c: R = Cl
d: R = I
e: R = Br

Figure 1. Chemical structures of antitumor 2-(4-aminophenyl)benzothiazoles **1a**−**e** and numbering scheme.

The mechanism of action of these structurally simple compounds is not understood: their unique activity fingerprint in the National Cancer Institute (NCI) 60-cell line panel does not COMPARE[6] with other known mechanistic classes of chemotherapeutic agent or with carcinogenic aromatic amines, to which they bear a structural resemblance (e.g. 4-aminobiphenyl and 2-aminofluorene). However, apparent similarities in biological

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▽ Abbreviations: CYP, cytochrome P450; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; PBS, phosphate-buffered saline, pH 7.4; HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide; PMSF, phenylmethanesulfonyl fluoride; TCA, trichloroacetic acid.

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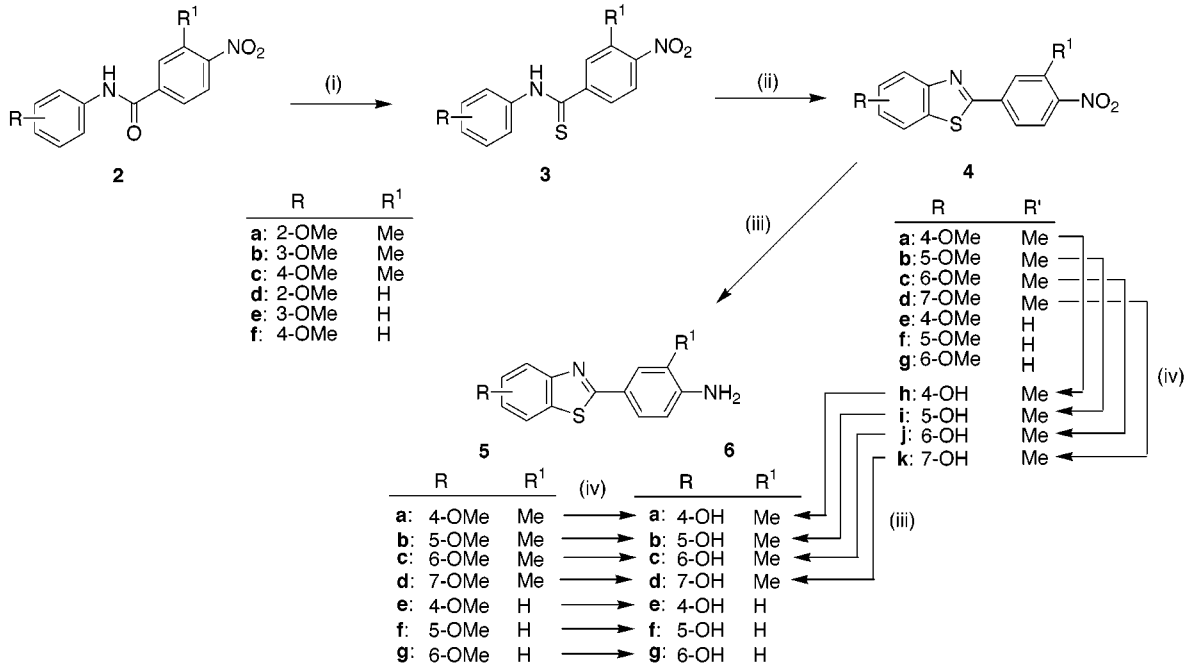
Scheme 1[a]



[a] Reagents: (i) Lawesson's reagent, HMPA, 100 °C; (ii) $K_3Fe(CN)_6$, aq NaOH, 90 °C; (iii) $SnCl_2 \cdot H_2O$, EtOH, reflux; (iv) BBr_3, CH_2Cl_2, 25 °C.

properties exist between these new benzothiazoles and a series of 5-amino-2-(4-aminophenyl)-4*H*-1-benzopyran-4-ones.[7]

Metabolism studies of novel structures are essential in helping to clarify their mode of action, as well as to predict the response to treatment of both the tumor and the patient. The ability to biotransform these lipophilic and metabolically labile benzothiazoles distinguishes sensitive from insensitive cell lines. The nature of the 3′-substituent exerts a profound influence on the predominant biotransformation pathway. The prototypic amine **1b** is significantly *N*-acetylated in sensitive human breast cancer cell lines (MCF-7, MDA 468) in vitro but not in unresponsive prostate PC 3 cells: in vivo pharmacokinetic studies in rats confirmed rapid and exclusive *N*-acetylation of **1b** but less acetylation of the 3′-chloro analogue **1c**. Although *N*-acetylation abolishes the excellent potency of **1a**, the activities of **1b**−**e** were only slightly reduced by *N*-acetylation probably because they can undergo cycling between the acetylated and nonacetylated states.[1]

2-(4-Aminophenyl)benzothiazoles harbor many alternative sites for metabolism. In this article we report on our work on the synthesis of *C*- and exocyclic *N*-oxidized aminobenzothiazoles: these compounds were required to define the contribution of oxidative metabolism to the mode of action of this group of intriguing and selectively potent antitumor agents.

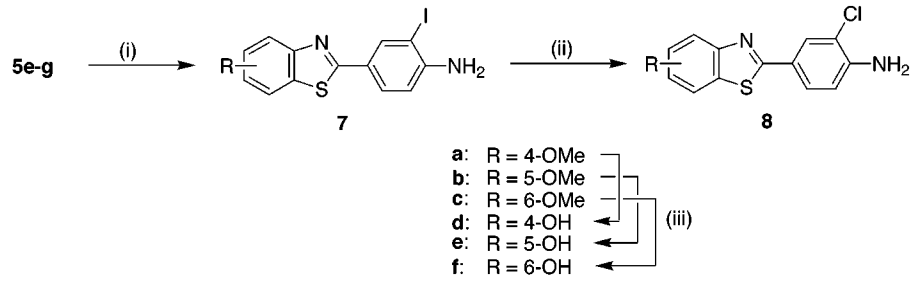
Chemistry

Synthesis of Compounds *C*-Hydroxylated on the Benzothiazole Ring. We have reported previously on the synthesis of the 6-hydroxy, 5,7-dihydroxy, and 5-hydroxy-7-methoxy derivatives of 2-(4-aminophenyl)-benzothiazole (**1b**) which were prepared by demethylation of the precursor methoxy compounds.[2] We have adapted this route to secure the synthesis of ring-hydroxylated benzothiazoles **6a**−**g** as outlined in Scheme 1. The variously methoxy-substituted nitrobenzanilides **2a**−**f** were prepared by the interaction of 3-methyl-4-nitrobenzoyl chloride or 4-nitrobenzoyl chloride with the corresponding anisidine (see Experimental Section, method A). The benzanilides were converted to thiobenzanilides **3a**−**f** with Lawesson's reagent in HMPA (method B) and then cyclized by the Jacobson synthesis to the methoxy-substituted nitrobenzothiazoles **4a**−**g** using potassium ferricyanide in aqueous NaOH (method C). Whereas the 2- and 4-methoxythiobenzanilides **3a**,**c** gave only a single benzothiazole **4a**,**c**, respectively, in the case of 3′-methyl-3-methoxythiobenzanilide (**3b**), a mixture of 5- and 7-substituted nitrobenzothiazoles **4b** (51%) and **4d** (20%), respectively, was formed; these isomers were separated by silica gel chromatography. Reduction of the nitrobenzothiazoles to their corresponding arylamines **5a**−**g** was carried out with tin(II) chloride dihydrate in refluxing ethanol (method D). Finally, demethylation of the methoxyarylamines was accomplished with excess boron tribromide in CH_2Cl_2 to yield the required hydroxylated derivatives **6a**−**g**, respectively (method E). Alternatively, demethylation of the ethers **4a**−**d** could be achieved at the nitrophenyl stage to give the corresponding phenols **4h**−**k** followed by reduction of nitro groups to **6a**−**d**, but this was a less efficient route to the required hydroxy-substituted 2-(4-aminophenyl)benzothiazoles.

Synthesis of the hydroxylated 2-(4-amino-3-halogenophenyl)benzothiazoles is outlined in Scheme 2. Iodination of the methoxyarylamines **5e**−**g** using iodine monochloride in acetic acid (method F) afforded the 3′-iodo series **7a**−**c**, and then displacement of the iodo group with copper(I) chloride in boiling DMF gave the 3′-chloro derivatives **8a**−**c** (method G). Boron tribromide demethylation then afforded the hydroxylated benzothiazoles **8d**−**f**.

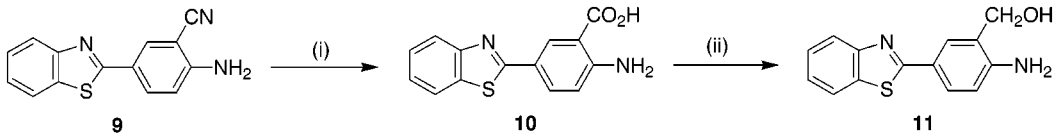
2-(4-Amino-3-cyanophenyl)benzothiazole (**9**)[2] was the starting point for the synthesis of the hydroxymethyl

Scheme 2[a]



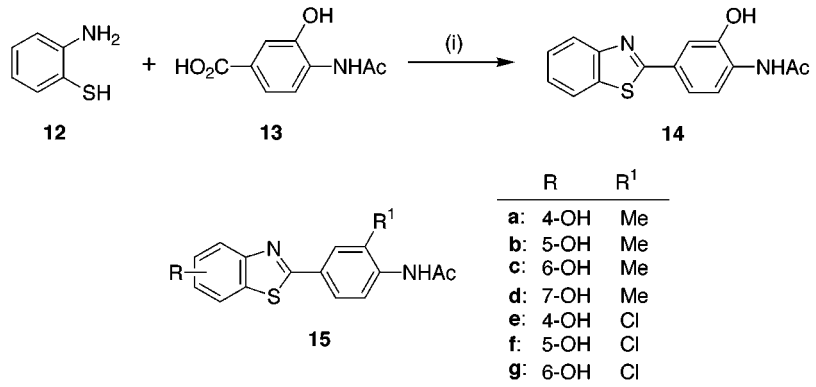
[a] Reagents: (i) ICl, AcOH, 25 °C; (ii) CuCl, DMF, reflux; (iii) BBr_3, CH_2Cl_2, 25 °C.

Scheme 3[a]



[a] Reagents: (i) 80% H_2SO_4, 100 °C; (ii) $LiAlH_4$, THF, 25 °C.

Scheme 4[a]



[a] Reagent: (i) Polyphosphoric acid, 150 °C.

compound **11** (Scheme 3). The nitrile was readily hydrolyzed to the acid **10** in hot 80% sulfuric acid; reduction with $LiAlH_4$ in THF then gave the required hydroxymethyl derivative **11**.

We have previously reported the synthesis of the *N*-acetylated analogues of amines **1a**−**e**.[1] Additional examples of hydroxylated *N*-acetylated congeners were prepared by several routes. Condensation of 2-aminothiophenol (**12**) and 4-acetylamino-3-hydroxybenzoic acid (**13**) in polyphosphoric acid at 150 °C gave a direct route to the 3′-hydroxyacetylamine **14** (Scheme 4). Acetylation of arylamine substrates with hydroxy groups protected as methyl ethers, followed by boron tribromide demethylation, gave the products **15a**−**g** unequivocally; however, a more efficient modification involved diacetylation at both the amino and phenolic groups of hydroxylated aminobenzothiazole substrates with acetyl chloride in CH_2Cl_2, followed by basic aqueous workup with sodium carbonate (method H), which hydrolyzed the acetoxy function, to give exclusively the required acetylamines **15a**−**g**.

Structures, yields, and physical characteristics of all nitrobenzanilides and thiobenzanilides are given in Table 1; 2-(4-nitrophenyl)- and 2-(4-aminophenyl)benzothiazoles bearing alkoxy substituents are listed in Table 2. Full experimental details of the synthesis and physical characterization of all *C*-hydroxylated benzothiazoles are given in the Experimental Section.

Table 1. Synthetic Methods, Yields, and Physical Characteristics of Methoxy-Substituted Nitrobenzanilides **2** and Nitrothiobenzanilides **3**

compd	synthetic method[a]	yield (%)	mp (°C)	formula	MW[b]
2a	A	93	120−122	$C_{15}H_{14}N_2O_4$	286
2b	A	97	117−119	$C_{15}H_{14}N_2O_4$	286
2c	A	89	167−169	$C_{15}H_{14}N_2O_4$	286
2d	A	80	143−145	$C_{14}H_{12}N_2O_4$	272
2e	A	97	193−194	$C_{14}H_{12}N_2O_4$	272
2f	A	81	200−202[c]	$C_{14}H_{12}N_2O_4$	272
3a	B	80	114−116	$C_{15}H_{14}N_2O_3S$	302
3b	B	78	125−126	$C_{15}H_{14}N_2O_3S$	302
3c	B	85	177−179	$C_{15}H_{14}N_2O_3S$	302
3d	B	68	138−140	$C_{14}H_{12}N_2O_3S$	288
3e	B	97	162−164	$C_{14}H_{12}N_2O_3S$	288
3f	B	80	183−185	$C_{14}H_{12}N_2O_3S$	288

[a] See the Experimental Section. [b] Confirmed by CI-MS. The compounds were also fully characterized by ^1H and ^{13}C NMR and IR spectroscopy. [c] Lit.[2] mp 199−202 °C.

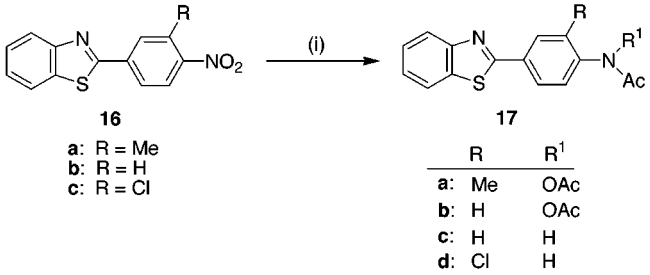
Synthesis of Compounds *N*-Hydroxylated on the Anilino Nitrogen. Metabolic *N*-oxidation is a route whereby aromatic amines are activated to mutagenic and carcinogenic nitrenium electrophiles.[8] In other work we have been able to generate reactive nitrenium species from 2-(4-aminophenyl)benzothiazoles, albeit under nonbiological conditions, by decomposition of the 4-azidophenyl analogues in trifluoromethanesulfonic acid. The reactive species can be trapped by nucleophiles.[9] Thus, although the in vitro activities of our aminophenylbenzothiazoles do not match with those of

Table 2. Synthetic Methods, Yields, and Physical Characteristics of Methoxy-Substituted 2-(4-Nitrophenyl)benzothiazoles **4** and 2-(4-Aminophenyl)benzothiazoles **5**, **7**, and **8**

compd	synthetic method[a]	yield (%)	mp (°C)	formula	MW[b]
4a	C	45	176−179	$C_{15}H_{12}N_2O_3S$	300
4b	C	51	*c*	$C_{15}H_{12}N_2O_3S$	300
4c	C	30	195−197	$C_{15}H_{12}N_2O_3S$	300
4d	C	20	*c*	$C_{15}H_{12}N_2O_3S$	300
4e	C	68	200−202	$C_{14}H_{10}N_2O_3S$	286
4f	C	60	*d*	$C_{14}H_{10}N_2O_3S$	286
4g	C	30	215−218	$C_{14}H_{10}N_2O_3S$	286
5a	D	89	123−126	$C_{15}H_{14}N_2OS$	270
5b	D	*e*	*e*	$C_{15}H_{14}N_2OS$	270
5c	D	84	166−168	$C_{15}H_{14}N_2OS$	270
5d	D	*e*	*e*	$C_{15}H_{14}N_2OS$	270
5e	D	72	154−157	$C_{14}H_{12}N_2OS$	256
5f	D	89	168−169	$C_{14}H_{12}N_2OS$	256
5g	D	98	191−194	$C_{14}H_{12}N_2OS$	256
7a	F	30	151−152	$C_{14}H_{11}IN_2OS$	381.9
7b	F	34	131−133	$C_{14}H_{11}IN_2OS$	381.9
7c	F	43	123−125	$C_{14}H_{11}IN_2OS$	381.9
8a	G	34	169−171	$C_{14}H_{11}ClN_2OS$	290.5
8b	G	71	159−161	$C_{14}H_{11}ClN_2OS$	290.5
8c	G	72	150−152	$C_{14}H_{11}ClN_2OS$	290.5

[a] See the Experimental Section. [b] Confirmed by CI-MS. The compounds were also fully characterized by ^1H and ^{13}C NMR and IR spectroscopy. [c] Isolated as a mixture from the Jacobson cyclization of compound **3e**. [d] A mixture containing some 7-methoxy-2-(4-nitrophenyl)benzothiazole. [e] A mixture of **5b**,**d** which was not separated.

Scheme 5[a]



16

a: R = Me
b: R = H
c: R = Cl

17

	R	R^1
a:	Me	OAc
b:	H	OAc
c:	H	H
d:	Cl	H

[a] Reagents: (i) Zn, Ac_2O, $(NH_4)_2SO_4$, DME, reflux.

the xenobiotic amines, it was of interest to prepare examples of *N*-oxidized 2-(4-aminophenyl)benzothiazoles which are hitherto unknown.

The starting points for the synthesis of acetylated hydroxylamine derivatives **17** were the 2-(4-nitrophenyl)benzothiazoles **16a**−**c**. Compounds **16a**,**b** were synthesized by condensation of 2-aminothiophenol with 3-methyl-4-nitrobenzoyl chloride or 4-nitrobenzoyl chloride in hot pyridine, respectively. Compound **16c** was prepared via the sodium perborate oxidation[10] of 2-(4-amino-3-chlorophenyl)benzothiazole (**1c**)[2] in acetic acid. Reductive acetylation of 2-(4-nitrophenyl)benzothiazoles **16a**−**c** using zinc and excess acetic anhydride under neutral conditions (ammonium sulfate) in boiling dimethoxyethane (method I)[8] gave products, the nature of which varied according to the influence of the substituent (Scheme 5). Nitro compound **16a** gave exclusively the diacetylated hydroxylamine **17a** (53%), whereas reduction of **16b** gave an approximately equimolar mixture of the acetylated hydroxylamine derivative **17b** (36%) accompanied by the further reduced acetamido derivative **17c** (42%).[1] Under the same conditions, however, **16c** gave only the fully reduced acetamido derivative **17d** (67%).

It should be noted that the thermal *ortho* rearrangement of a range of *N*,*O*-diacetyl-*N*-arylhydroxylamines to 1,2-diacetylated *o*-aminophenols (related to compound **14**) has been reported.[11] No such rearrangements were observed under the conditions of reductive acetylation of **16a**−**c**.

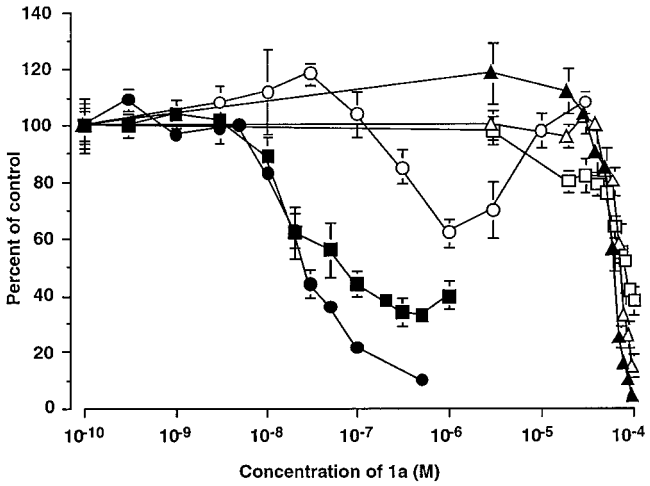


Figure 2. Growth inhibitory activity of **1a** against human cancer cell lines. Cells were plated at seeding densities of 2000/well except for MDA-MB-435 (1500/well) and incubated with **1a** for 6 days. Cell growth was assessed by MTT assay. Mean and standard deviation are given for one representative experiment in which $n = 6$. Experiments were performed at least three times. Symbols represent MCF-7 (closed circle), T-47D (closed square), MDA-MB-435 (closed triangle), TK-10 (open circle), A498 (open square), and CAKI-1 (open triangle).

Biological Results and Discussion

Differential Activity of 2-(4-Amino-3-methylphenyl)benzothiazole 1a Against Cancer Cell Lines. On the basis of the patterns of selectivity elicited by compounds of the benzothiazole class in the NCI 60-cell line panel,[3] three breast cancer cell lines (MCF-7, T-47D, and MDA-MB-435) and three renal cancer cell lines (TK-10, A498, and CAKI-1) were selected for a detailed investigation of the effect of compound **1a** on cell growth following 6-day exposure (Figure 2). The exquisitely sensitive breast cell lines MCF-7 and T-47D were growth-inhibited by **1a** at low IC_{50} values (concentration causing 50% growth inhibition) of 26 and 70 nM, respectively. The growth of TK-10 cells was suppressed with increasing drug concentration from 30 nM to 1 μM, but 50% growth inhibition was not achieved. As with the MCF-7 and T-47D cell lines, cell growth was recovered at high drug concentrations (>1 μM), reconfirming the unique biphasic dose−response relationship characteristic of the effects of **1a** against sensitive cell lines.[2,3] Compound **1a** did not inhibit the growth of MDA-MB-435, A498, and CAKI-1 cells, the decline in cell growth above 10 μM being due to DMSO toxicity (data not shown). Thus, results of MTT assays corroborate those of sulfurhodamine B assays used in the NCI screen: MCF-7 and T-47D cells are most sensitive to **1a**, TK-10 cells have intermediate sensitivity, whereas MDA-MB-435, A498, and CAKI-1 cells are completely insensitive.

Sequestration of 1a from Culture Media. Concentrations of **1a** in culture media were measured under the same conditions as the cell growth inhibition assay. Regardless of initial treatment range (0.01, 0.1, 1 μM), concentrations of **1a** rapidly decreased in the culture

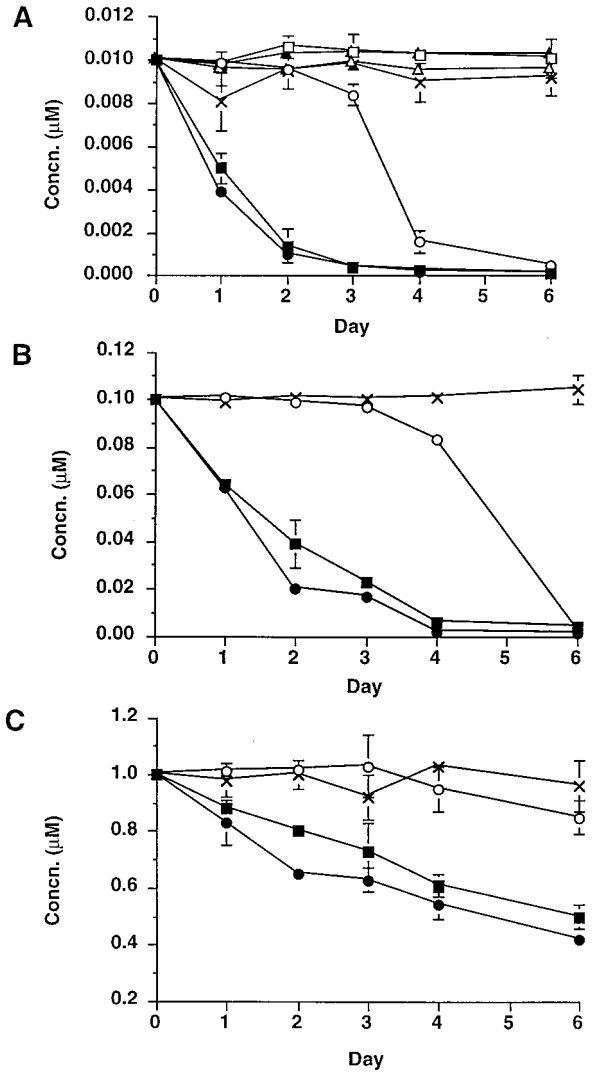


Figure 3. Disappearance of **1a** from cell medium. Cells were incubated with **1a** at a concentration of 0.01 μM (A), 0.1 μM (B), and 1 μM (C). At selected time intervals, an aliquot of the medium was analyzed for levels of **1a** using HPLC with fluorescence detection as described in the Experimental Section. Data are mean ± standard deviation (n = 3). Symbols represent MCF-7 (closed circle), T-47D (closed square), MDA-MB-435 (closed triangle), TK-10 (open circle), A498 (open square), CAKI-1 (open triangle), and control (only medium, X).

media sustaining MCF-7 and T-47D cells (Figure 3). Despite a time lag, **1a** similarly disappeared from culture media of TK-10 cells. However, **1a** remained in culture media nurturing insensitive cells MDA-MB-435, A498, and CAKI-1 throughout the incubation period of 6 days. Thus, the disappearance of drug from cell culture media correlates with the sensitivity of the tested cell lines to growth inhibition. Either accumulation or metabolism of drug within the sensitive cells may account for the disappearance of **1a** from culture media.

Metabolism of 1a in Cell Culture. The six tested cell lines were incubated with 0.1 μM **1a** for 3 days, and culture media were analyzed by HPLC with fluorescence detection. A major metabolite, subsequently identified as 2-(4-amino-3-methylphenyl)-6-hydroxybenzothiazole (**6c**) (see later), with a retention time of 21 min, was detected in culture media of sensitive cell lines (MCF-7, T-47D, and TK-10) only (Figure 4). In the time-

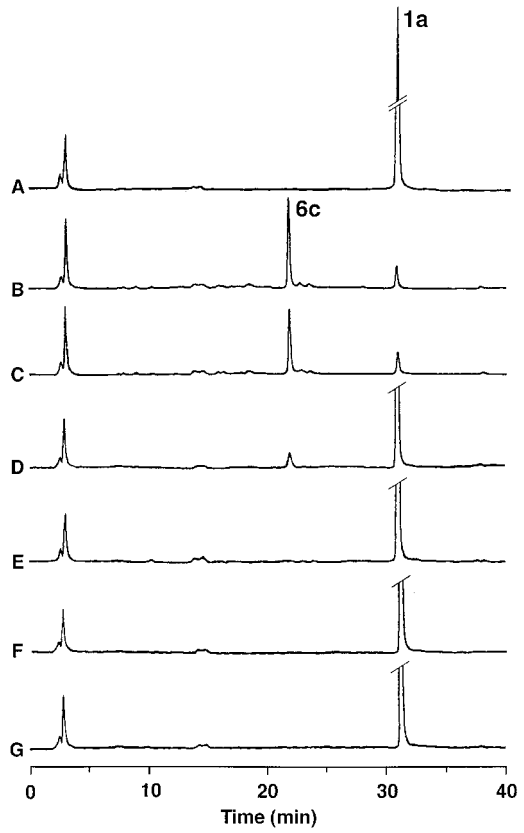


Figure 4. Differential metabolism of **1a** by six cancer cell lines tested. Cells were seeded as in the MTT assay and incubated with 0.1 μM **1a** for 3 days. Aliquots of media were analyzed by HPLC with fluorescence detection. The scale of fluorescence intensity is the same in all chromatograms shown: medium only (A), MCF-7 (B), T-47D (C), TK-10 (D), MDA-MB-435 (E), A498 (F), and CAKI-1 (G).

dependent analysis of **1a**-treated MCF-7 cells, metabolite **6c** was detected as early as 1 day after drug exposure, but its concentration declined with increasing incubation time, during which other minor more polar metabolites were produced (Figure 5). This metabolic profile was similarly observed with T-47D and TK-10 cells (data not shown), but no metabolite peaks were detected from insensitive cell lines, MDA-MB-435, A498, and CAKI-1.

A sample of [^{14}C]**1a**, radiolabeled at the 2-position of the benzothiazole ring, was prepared for detailed studies of its metabolism by sensitive cell lines. The time course of metabolite formation was investigated in MCF-7 cells (Figure 6). After 1 day of incubation, radioactivity was eluted in two main fractions: at 30−32 min, representing unchanged drug; and 20−22 min, representing metabolite **6c**. Radioactivity in these two fractions diminished with prolonged drug exposure of up to 6 days and was distributed among numerous minor fractions. This is consistent with HPLC analysis, confirming **6c** as the major metabolite of **1a** in sensitive MCF-7 cells, and that other minor metabolites were formed after long incubation periods. Total radioactivity in culture media remained constant throughout the drug incubation periods (data not shown), whereas nonradiolabeled **1a** disappeared readily from culture media. There was little net retention of unchanged drug within the cells.

In a separate study the unsubstituted arylamine **1b** was shown to be predominantly *N*-acetylated in sensi-

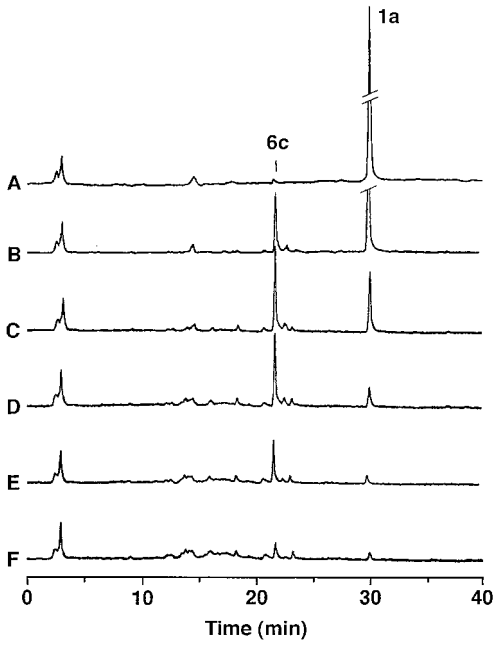


Figure 5. Time course of metabolite formation by MCF-7 cells. Cells were seeded as in the MTT assay and incubated with 0.1 μM **1a** for the desired time periods, and media were sampled for analysis by HPLC with fluorescence detection. The scale of fluorescence intensity is the same in all chromatograms shown: 0 time (A) and after incubation for 1 day (B), 2 days (C), 3 days (D), 4 days (E), and 6 days (F).

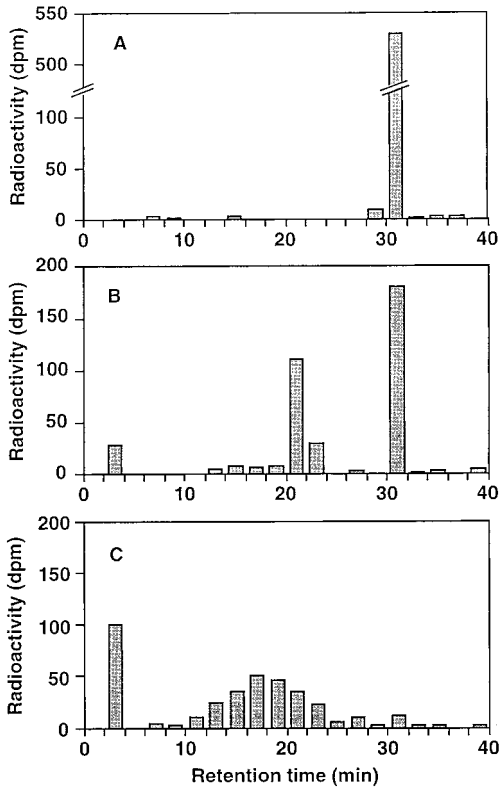


Figure 6. Profile of **1a** metabolism in MCF-7 cells. MCF-7 cells were seeded as in the MTT assay and incubated with 0.1 μM [^{14}C]**1a**. An aliquot of the media was analyzed by HPLC and the eluant collected in 2-min fractions. The radioactivity in each collected fraction was then measured. Histograms represent the metabolism profile at 0 time (A) and after 1 day (B) and 6 days (C) of incubation.

tive cell lines (MCF-7, MDA 468, T-47D, SKBR 3).[1] However three *C*-oxidation products were consistently observed after 72-h drug incubation with sensitive cell lines only: one (*m*/*z* 242) is hydroxylated in the benzothiazole nucleus, presumably at the 6-position, and two others (*m*/*z* 284) are hydroxylated derivatives of the *N*-acetylated metabolite. Consistent with the experience with amine **1a**, negligible metabolism of **1b** was observed in intrinsically resistant PC 3, HBL 100, and MCF-7/Adr cells.

Structural Characterization of 2-(4-Amino-3-methylphenyl)-6-hydroxybenzothiazole (6c) as the Major Metabolite of 2-(4-Amino-3-methylphenyl)-benzothiazole (1a) in Sensitive Cell Lines. The major metabolite with a retention time of 21 min was purified from the culture media of **1a**-treated MCF-7 cells for structural determination by mass and ^{1}H NMR spectrometry.

The mass spectrum of the metabolite showed a molecular ion at *m*/*z* 256, and an accurate mass measurement was within 0.001 amu of the calculated value for a molecular formula of $C_{14}H_{12}N_2SO$, corresponding to the addition of an oxygen atom to the parent drug.

Simple inspection of the aromatic portion of the ^{1}H NMR spectrum of the oxidized metabolite revealed the structure to have one less aromatic proton than **1a**. The remaining three protons on the benzothiazole of the metabolite gave rise to coupling patterns which can be rationalized only by the hydroxyl group being located at the 5- or 6-position in the benzothiazole nucleus. With the availability of authentic synthesized samples of all the *C*-hydroxylated derivatives of **1a** (see earlier) this major metabolite was identified unequivocally as 2-(4-amino-3-methylphenyl)-6-hydroxybenzothiazole (**6c**). A minor metabolite identified in a separate analysis was detected as 2-(4-amino-3-hydroxymethylphenyl)benzothiazole (**11**), and a further metabolite did not cochromatograph with any of the standard samples (data not shown).

Metabolism of 1a by Cell Homogenates. To identify the enzymes that may be involved in the biotransformation of **1a**, we investigated its metabolism by homogenates of the six tested cell lines. None of the homogenates, not even those of sensitive MCF-7 and T-47D cells, metabolized **1a**. Significantly, the homogenate of the MCF-7 cells that had been preincubated with **1a** produced 2-(4-amino-3-methylphenyl)-6-hydroxybenzothiazole (**6c**) (Figure 7). This activity required the cofactor NADPH and was inhibited to the extent of 70% by SKF-525A, a typical broad-spectrum inhibitor of the main oxidizing cytochromes. This suggests that the enzymes catalyzing 6-hydroxylation of **1a** in MCF-7 cells were induced by the drug and that the induced enzymes are cytochrome P450s (CYPs). Certain derivatives of 2-phenylbenzothiazole, e.g. 2-(4-cyanophenyl)benzothiazole, are known to induce benzo[*a*]pyrene hydroxylase activity[12] which is mediated by CYP1A1 in rats;[13] also, 2-(4-chlorophenyl)benzothiazole induces CYP1A1 in human choriocarcinoma cells.[14] We will report on the specificity of activation of compound **1a** by different CYP isoforms in a separate paper in this series.

Uptake and Covalent Binding by Cells of ^{14}C-Labeled 1a. Radioactivity within MCF-7 and MDA-MB-435 cells after incubation with 0.1 and 1 μM labeled

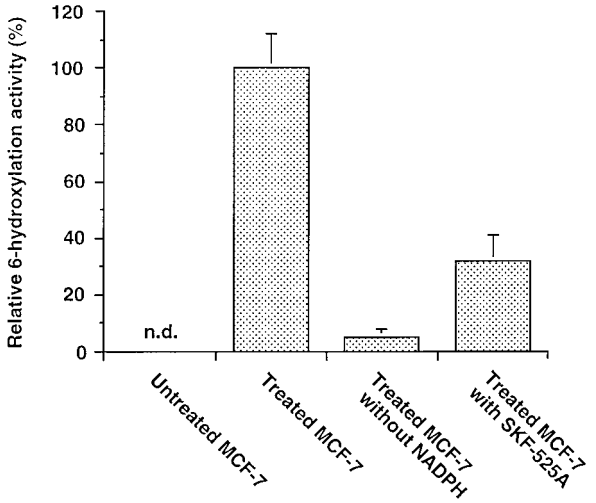


Figure 7. 6-Hydroxylation activity in the homogenates of untreated and **1a**-treated MCF-7 cells. Homogenates were prepared from MCF-7 cells incubated in the absence or presence of 1 μM **1a** for 24 h, and the production of 2-(4-amino-3-methylphenyl)-6-hydroxybenzothiazole (**6c**) by homogenates was measured by HPLC. Activity in homogenate of **1a**-treated cells was also measured without NADPH or with SKF-525A. Data represent the mean ± standard deviation of 3 experiments (n.d., not detectable).

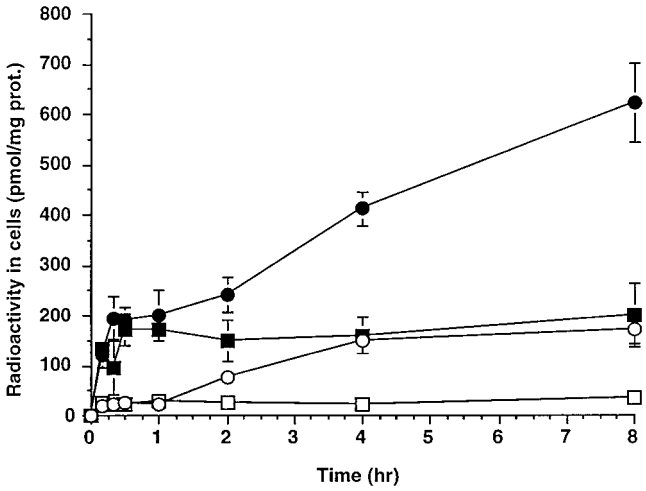


Figure 8. Accumulation of [^{14}C]**1a**-derived radioactivity in sensitive and insensitive cell lines. MCF-7 (circle) and MDA-MB-435 (square) cells were exposed to 0.1 μM (open symbol) or 1 μM (closed symbol) [^{14}C]**1a** for specific times. Cells were washed with cold PBS, solubilized in 1 N NaOH, and measured for protein content and radioactivity. Each point represents mean ± standard deviation (n = 3).

1a was measured (Figure 8). The levels of radioactivity within both cell lines were similar after 1-h incubation. However, after 2-h incubation, radioactivity accumulated in sensitive MCF-7 cells, whereas the level of radioactivity remained unchanged in resistant MDA-MB-435 cells.

The amount of covalently bound radioactivity after incubation with 0.1 and 1 μM labeled **1a** was inversely correlated to the concentration of **1a** remaining in media. Covalent binding of radioactivity in MCF-7 cells was both time- and concentration-dependent. Maximum covalent binding was achieved after 8-h incubation with 0.1 μM drug and after 24-h incubation with 1 μM; after these time points, covalent binding gradually decreased (Figure 9A). No unchanged drug remained in culture

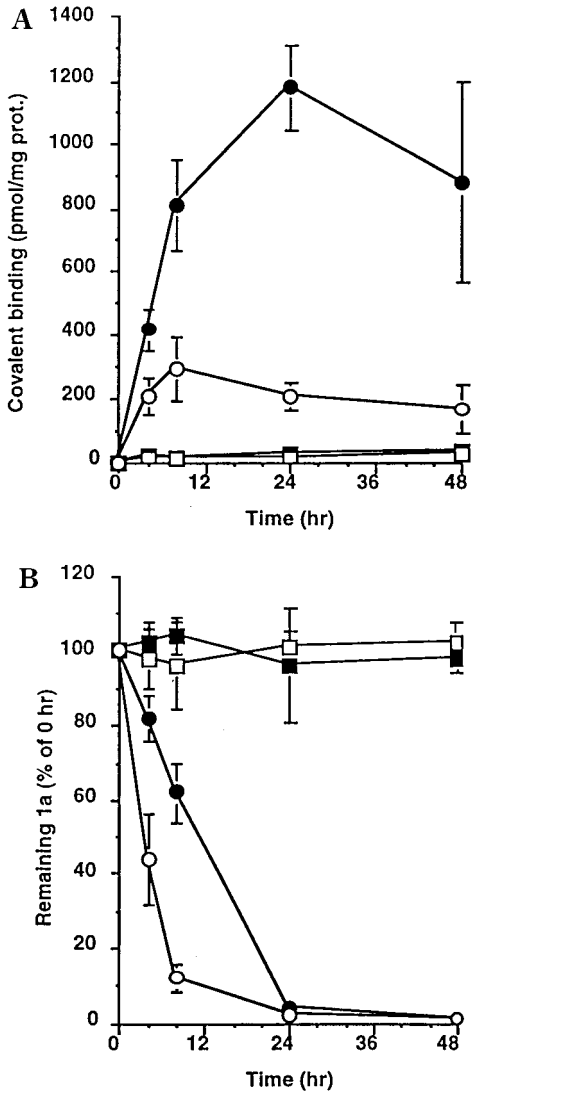
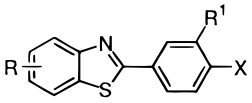


Figure 9. Intracellular covalent binding of [^{14}C]**1a**-derived radioactivity in sensitive and insensitive cell lines (A) and percentage of unchanged **1a** remaining in the media (B) at selected times during incubation of cell lines with [^{14}C]**1a**. MCF-7 (circle) and MDA-MB-435 (square) cells were exposed to 0.1 μM (open symbol) or 1 μM (closed symbol) [^{14}C]**1a** for specific times. Media were removed for HPLC analysis, and covalent binding was determined in the precipitated cellular proteins as described in the Experimental Section. Each point represents mean ± standard deviation (n = 3).

media after 24-h incubation with 0.1 and 1 μM labeled **1a** (Figure 9B). No covalently bound radioactivity was detected in the insensitive cell line, MDA-MB-435, consistent with the lack of drug accumulation in these cells. This suggests that differential uptake and retention of radioactivity may result from covalent binding of reactive intermediates, other than hydroxylated benzothiazoles, to intracellular components specific to sensitive cell lines. This proposal is supported by the observation that culture media containing only metabolites of **1a** (formed after 48-h incubation of MCF-7 and MDA-MB-435 cells with 1 μM drug) failed to inhibit the growth of MCF-7 and MDA-MB-435 cells (data not shown). Active metabolites (or reactive intermediates) must be retained within the cells, and only inactive hydroxylated metabolites (see later) are exported back into the culture media.

Table 3. In Vitro Cytotoxicities of Hydroxylated Benzothiazoles Against MCF-7 Human Mammary Carcinoma Cells



compd	R	R^1	X	IC_{50} (M)[a]
1a	H	Me	NH_2	<0.001
1b	H	H	NH_2	<0.001
1c	H	Cl	NH_2	<0.001
1d	H	I	NH_2	<0.001
6a	4-OH	Me	NH_2	>100
6b	5-OH	Me	NH_2	78.7
6c	6-OH	Me	NH_2	>100
6d	7-OH	Me	NH_2	90.8
6e	4-OH	H	NH_2	>100
6f	5-OH	H	NH_2	>100
6g	6-OH	H	NH_2	21.6
8d	4-OH	Cl	NH_2	61.4
8e	5-OH	Cl	NH_2	60.6
8f	6-OH	Cl	NH_2	54.5
11	H	CH_2OH	NH_2	0.006
14	H	OH	NHAc	9.0
15a	4-OH	Me	NHAc	33.0
15b	5-OH	Me	NHAc	72.5
15c	6-OH	Me	NHAc	46.1
15d	7-OH	Me	NHAc	48.2
15e	4-OH	Cl	NHAc	56.8
15f	5-OH	Cl	NHAc	31.0
15g	6-OH	Cl	NHAc	71.2
17a	H	Me	N(OAc)Ac	0.055
17b	H	H	N(OAc)Ac	<0.001

[a] 3-Day assay. All IC_{50} values are the mean of at least 3 determinations.

In Vitro Cytotoxicities of Hydroxylated Benzothiazoles. *C*-Hydroxylated compounds representative of 2-(4-aminophenyl)- and 2-(4-amino-3-methylphenyl)- (**6**), 2-(4-amino-3-iodophenyl)- (**7**), and 2-(4-amino-3-chlorophenyl)benzothiazole (**8**) series were initially evaluated against sensitive MCF-7 cells in 3-day MTT assays and their activities compared with those of their unoxidized counterparts (Table 3). All oxidized compounds gave IC_{50} values in the 20−100 μM range confirming that *C*-oxidation causes profound loss of activity. Similarly, the *N*-acetylated phenol **14** and representative *C*-hydroxylated *N*-acetylamino compounds **15** were all of low potency (IC_{50} 30−100 μM) compared to the nanomolar cytotoxicity of the precursor amines **1a**−**e**.[2,3] An exception was 2-(4-amino-3-hydroxymethylphenyl)benzothiazole (**11**), which showed the same overall activity against MCF-7 cells (IC_{50} 0.006 μM) as other 3′-substituted amines against this cell line. However it is unlikely that the 3′-hydroxymethylbenzothiazole **11** (or the carboxylic acid **10**) is an intermediate leading to covalent interaction with a cellular macromolecule since the corresponding 3′-halo compounds **1c**−**e** display the same characteristic signature of in vitro activity. Clearly, the 3′-hydroxymethyl substituent of compound **11** merely extends the range of groups consistent with good activity at the 3′-position of the 2-(4-aminophenyl)benzothiazole pharmacophore.

These results were corroborated by a detailed investigation of the hydroxylated derivatives of the lead compound **1a** in the NCI 60-cell line in vitro anticancer drug screen. The mean GI_{50} values for the 4-, 5-, 6-, and 7-hydroxylated derivatives **6a**−**d** and the 3′-hydroxymethyl dervative **11** in the 2-day assay were 26.3, 30.2, 41.7, 25.1, and 39.8 μM, respectively; the comparable value for amine **1a** was 28.2 μM. However, this apparent uniformity disguises crucial differences in the response fingerprint. Thus the profile of **1a** (Figure 10A) is typical of compounds of this class, showing certain cell lines to be 1000-fold more sensitive than the mean; the profile of 2-(4-amino-3-methylphenyl)-6-hydroxybenzothiazole (**6c**), the main metabolite of **1a** (Figure 10B), shows no selectivity; that of 2-(4-amino-3-hydroxymethylphenyl)-benzothiazole (**11**), a minor metabolite, shows a pattern broadly comparable to that of the parent amine (data not shown).

The GI_{50} mean graph of the diacetylated hydroxylamine **17a** also resembled that of the parent amine **1a** but showed reduced potency toward sensitive cell lines. The acetylated hydroxylamine **17b** also showed the same selectivity profile as its parent amine **1b**, but greatly reduced potency in the sensitive cell lines, e.g. MCF-7, IGROV1, A498, and TK-10 (data not shown). These results suggest that *N*-oxidation followed by acetylation to an *N*-acetoxy derivative is not an activating metabolic step in the 2-(4-aminophenyl)benzothiazole series (cf. carcinogenic arylamines). Rather these unstable moieties are probably degrading to the active amines **1a**,**b** under the conditions of the bioassays.

Conclusion

The synthesis of a range of *C*- and *N*-hydroxylated 2-(4-aminophenyl)benzothiazoles has been achieved to investigate the role of metabolic oxidation in the mode of antitumor activity of this enigmatic series. The main metabolite of 2-(4-amino-3-methylphenyl)benzothiazole (**1a**) formed in sensitive cell lines is 2-(4-amino-3-methylphenyl)-6-hydroxybenzothiazole (**6c**), but this product is devoid of selective antitumor effects in vitro. The oxidation step is catalyzed by enzymes induced by **1a** in MCF-7 cells. These induced enzymes required NADPH as cofactor, and their activity was inhibited by the broad-spectrum CYP inhibitor, SKF-525A. We have evidence that CYP1A1 is the main enzyme induced by **1a** in sensitive cells (to be published separately).

The levels of radioactivity derived from ^{14}C-labeled **1a** in both sensitive and insensitive cell lines were similar in the first hour of drug exposure, suggesting that uptake of **1a** occurred at a similar rate in both groups of cell lines. However, accumulation of radioactivity within the cells was observed only in sensitive cell lines. This increase in intracellular radioactivity ceased at the time point when **1a** was no longer detectable in culture media. Thus, intracellular retention within sensitive cell lines only was due to covalent binding of **1a** or a **1a**-derived product to an intracellular macromolecule, which may in turn be related to its cell-line-specific metabolism. A 50-kDa protein, tentatively identified as the principal covalently labeled macromolecule (Chua and Kashiyama, unpublished results), may hold the key to the unique mechanism of action of the 2-(4-aminophenyl)benzothiazole class of antitumor agent, and current efforts are focused on its identification.

Experimental Section

All new hydroxylated 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. ^1H 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 spectrophotometer. Mass spectra were recorded on an AEI MS-902 or a VG Micromass 7070E spectrometer. TLC systems for routine

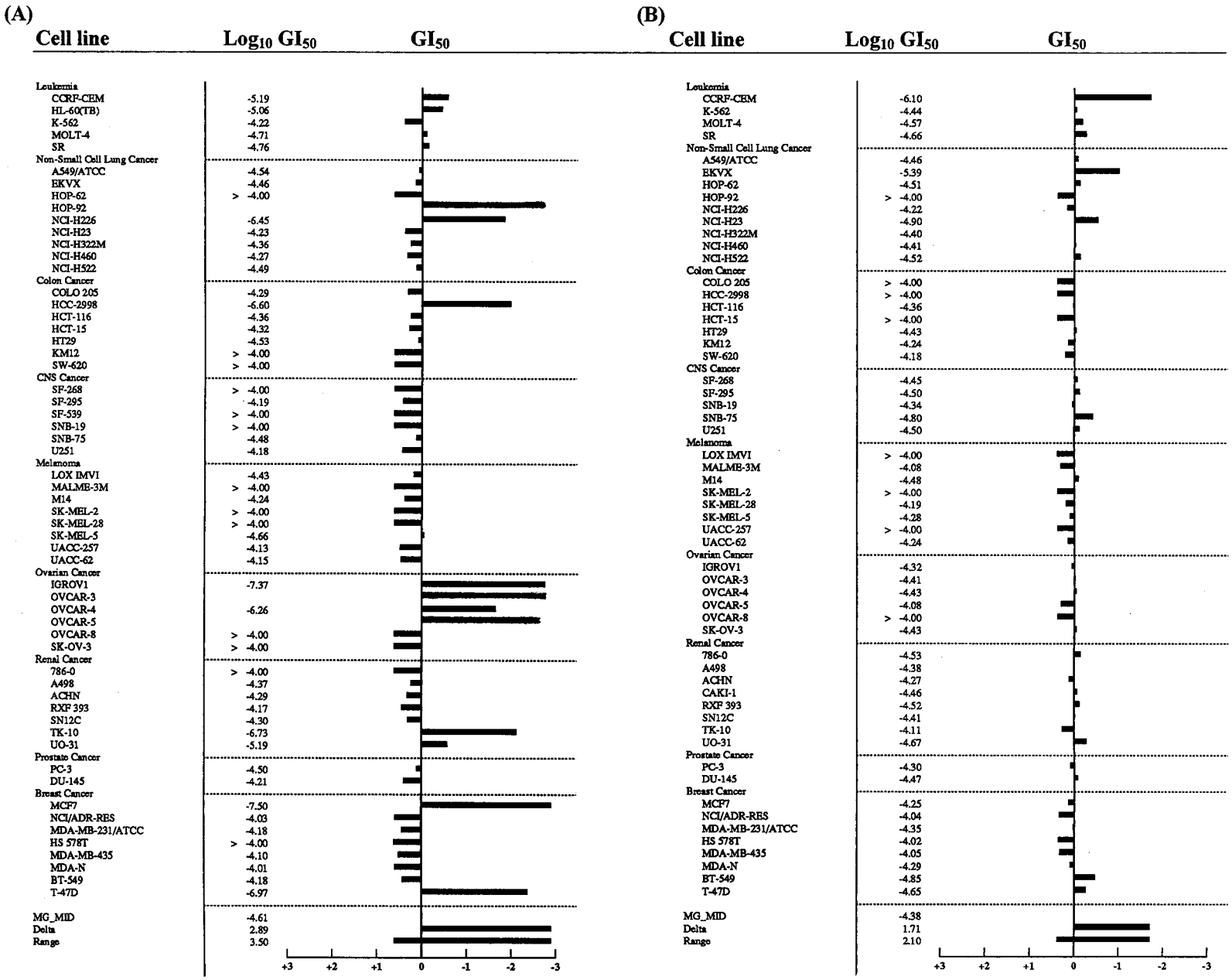


Figure 10. NCI mean graphs (log GI_{50} values) for 2-day in vitro assays on (A) 2-(4-amino-3-methylphenyl)benzothiazole (**1a**) (NSC 674495) and (B) 2-(4-amino-3-methylphenyl)-6-hydroxybenzothiazole (**6c**) (NSC 703785).

monitoring of reaction mixtures, and confirming the homogeneity of analytical samples, used Kieselgel 60F_{254} (0.25 mm) silica gel TLC aluminum sheets. Sorbsil silica gel C 60-H (40−60 μm) was used for flash chromatographic separations.

General Method for the Synthesis of Methoxy-Substituted Nitrobenzanilides 2. Method A. A mixture of the substituted anisidine (0.02 mol) and the appropriate nitrobenzoyl chloride (0.02 mol) in pyridine (20 mL) was stirred under reflux (2 h) and poured into water. The precipitate was collected and washed with water, followed by ice-cold methanol. Yields and physical properties of methoxy-substituted benzanilides are listed in Table 1.

General Method for the Synthesis of Methoxy-Substituted Nitrothiobenzanilides 3. Method B. A mixture of the methoxy-substituted nitrobenzanilide (0.01 mol) and Lawesson's reagent (0.6 mol equiv) in HMPA (30 mL) was stirred at 100 °C for 6 h and poured into water. The precipitate was collected and washed with water and then ice-cold methanol. Yields and physical characteristics are listed in Table 1.

General Method for the Jacobson Synthesis of Methoxy-Substituted 2-(4-Nitrophenyl)benzothiazoles 4. Method C. The methoxy-substituted nitrothiobenzanilides **3** (0.1 mol) were dissolved in a solution of NaOH (1 mol) in water (40 mL) and ethanol (4 mL). The mixture was added dropwise to a solution of potassium ferricyanide (0.4 mol) in water (20 mL) at 90 °C, stirred for 30 min, then allowed to cool. The precipitate was collected, washed with water (50 mL), and purified by chromatography using chloroform−hexane (1:1) as eluant. Details of yields and physical characteristics of products are given in Table 2.

General Method for the Synthesis of Methoxy-Substituted 2-(4-Aminophenyl)benzothiazoles 5. Method D. A mixture of the appropriate methoxy-substituted 2-(4-nitrophenyl)benzothiazole **4** (0.015 mol) and tin(II) chloride dihydrate (0.075 mol) in boiling ethanol (50 mL) was stirred for 2 h. Ethanol was removed by vacuum evaporation, the residue was extracted into ethyl acetate (3 × 100 mL), and the combined organic fractions were shaken with 2 M aqueous sodium hydroxide solution (3 × 100 mL) followed by water (2 × 100 mL). The organic layer was evaporated to leave a residue of the amine which was purified by crystallization (from methanol). Yields and physical characteristics of products are listed in Table 2.

General Method for the Demethylation of Methoxy-Substituted 2-(4-Nitrophenyl)benzothiazoles 4 and 2-(4-Aminophenyl)benzothiazoles 5. Method E. To a stirred suspension of the methoxy-substituted benzothiazole (0.01 mol) in dry dichloromethane (25 mL) at 25 °C was added a 1 M solution of boron tribromide (5 mol equiv) dropwise over 10 min. The reaction mixture was stirred for 1 h and quenched by the dropwise addition of methanol until no further reaction occurred. The mixture was poured into concentrated aqueous ammonia (50 mL). The aqueous phase was separated, neutralized to pH 7 with 5 M hydrochloric acid, and extracted with ethyl acetate (3 × 50 mL). The combined organic layers were dried ($MgSO_4$) and evaporated to yield hydroxy-substituted 2-(4-nitrophenyl)- or 2-(4-aminophenyl)benzothiazoles. The crude products were purified by flash column chromatography using chloroform as solvent.

The following hydroxy-substituted 2-(3-methyl-4-nitrophenyl)benzothiazoles were prepared by method E.

4-Hydroxy-2-(3-methyl-4-nitrophenyl)benzothiazole (4h): yield 45%; mp 210−212 °C; IR 3313 (OH), 1579 (C═N), 1530 (NO_2) cm^{-1}; MS (CI) *m/z* 287 (M + 1). Anal. ($C_{14}H_{10}N_2O_3S$) C,H,N.

5-Hydroxy-2-(3-methyl-4-nitrophenyl)benzothiazole (4i): yield 69%; mp 215−217 °C; IR 3392 (OH), 1601 (C═N), 1530 (NO_2) cm^{-1}; MS (CI) *m/z* 287 (M + 1). Anal. ($C_{14}H_{10}N_2O_3S$) C,H,N.

6-Hydroxy-2-(3-methyl-4-nitrophenyl)benzothiazole (4j): yield 70%; mp 234−237 °C; IR 3447 (OH), 1606 (C═N), 1530 (NO_2) cm^{-1}; MS (CI) *m/z* 287 (M + 1). Anal. ($C_{14}H_{10}N_2O_3S$) C,H,N.

7-Hydroxy-2-(3-methyl-4-nitrophenyl)benzothiazole (4k): yield 20%; mp 220−224 °C; IR 3441 (OH), 1592 (C═N), 1530 (NO_2) cm^{-1}; MS (CI) *m/z* 287 (M + 1). Anal. ($C_{14}H_{10}N_2O_3S$) C,H,N.

The following hydroxy-substituted 2-(4-aminophenyl)benzothiazoles were prepared by method E.

2-(4-Amino-3-methylphenyl)-4-hydroxybenzothiazole (6a): yield 74%; mp 208−210 °C; ^{1}H NMR (DMSO-d_6) δ 10.48 (1H, brs, OH), 7.73 (1H, d, *J* 2.0 Hz, H-2′), 7.70 (1H, dd, *J* 2.0, 8.3 Hz, H-6′), 7.43 (1H, dd, *J* 1, 8.0 Hz, H-7), 7.32 (1H, t, *J* 8.0 Hz, H-6), 6.84 (1H, dd, *J* 1.0, 8.0 Hz, H-5), 6.75 (1H, d, *J* 8.3 Hz, H-5′), 5.70 (2H, brs, NH_2), 2.20 (3H, s, CH_3); IR 3341, 3402 cm^{-1}; MS (CI) *m/z* 257 (M + 1). Anal. ($C_{14}H_{12}N_2OS$) C,H,N.

2-(4-Amino-3-methylphenyl)-5-hydroxybenzothiazole (6b): yield 84%; mp 238−241 °C; ^{1}H NMR (DMSO-d_6) δ 9.92 (1H, brs, OH), 7.72 (1H, d, *J* 2.0 Hz, H-2′), 7.68 (1H, dd, *J* 2.5, 8.3 Hz, H-6′), 7.10 (1H, d, *J* 8.5 Hz, H-7), 7.06 (1H, d, *J* 2.3 Hz, H-4), 6.63 (1H, d, *J* 8.3 Hz, H-5′), 6.50 (1H, dd, *J* 2.3, 8.5 Hz, H-6), 5.48 (2H, brs, NH_2), 2.12 (3H, s, CH_3); IR 3421, 3304 cm^{-1}; MS (CI) *m/z* 257 (M + 1). Anal. ($C_{14}H_{12}N_2OS$) C,H,N.

2-(4-Amino-3-methylphenyl)-6-hydroxybenzothiazole (6c): yield 81%; mp 256−259 °C; ^{1}H NMR (DMSO-d_6) δ 9.76 (1H, brs, OH), 7.71 (1H, d, *J* 8.8 Hz, H-4), 7.60 (1H, d, *J* 2.0 Hz, H-2′), 7.56 (1H, dd, *J* 2.0, 8.3 Hz, H-6′), 7.33 (1H, d, *J* 2.3 Hz, H-7), 6.93 (1H, dd, *J* 2.3, 8.8 Hz, H-5), 6.68 (1H, d, *J* 8.3 Hz, H-5′), 5.57 (2H, brs, NH_2), 2.15 (3H, s, CH_3); IR 3489, 3352 cm^{-1}; MS (CI) *m/z* 257 (M + 1). Anal. ($C_{14}H_{12}N_2OS$) C,H,N.

2-(4-Amino-3-methylphenyl)-7-hydroxybenzothiazole (6d): yield 42%; mp 254−257 °C; ^{1}H NMR (DMSO-d_6) δ 9.95 (1H, brs, OH), 7.69 (1H, d, *J* 2.0 Hz, H-2′), 7.66 (1H, dd, *J* 2.0, 8.3 Hz, H-6′), 7.45 (1H, dd, *J* 1.0, 8.0 Hz, H-4), 7.20 (1H, t, *J* 8.0 Hz, H-5), 6.98 (1H, dd, *J* 1.0, 8.0 Hz, H-6), 6.77 (1H, d, *J* 8.3 Hz, H-5′), 5.66 (2H, brs, NH_2), 2.20 (3H, s, CH_3); IR 3402, 3361 cm^{-1}; MS (CI) *m/z* 257 (M + 1). Anal. ($C_{14}H_{12}N_2OS$) C,H,N.

2-(4-Aminophenyl)-4-hydroxybenzothiazole (6e): yield 76%; mp 192−194 °C; ^{1}H NMR (DMSO-d_6) δ 9.95 (1H, brs, OH), 7.75 (2H, d, *J* 8.5 Hz, H-2′, H-6′), 7.40 (1H, dd, *J* 1.0, 8.0 Hz, H-7), 7.16 (1H, t, *J* 8.0 Hz, H-6), 6.84 (1H, dd, *J* 1.0, 8.0 Hz, H-5), 6.67 (2H, d, *J* 8.5 Hz, H-3′, H-5′), 5.87 (2H, brs, NH_2); IR 3379, 3296 cm^{-1}; MS (CI) *m/z* 243 (M + 1). Anal. ($C_{13}H_{10}N_2OS$) C,H,N.

2-(4-Aminophenyl)-5-hydroxybenzothiazole (6f): yield 76%; mp 285−287 °C; ^{1}H NMR (DMSO-d_6) δ 9.64 (1H, brs, OH), 7.80 (1H, d, *J* 8.75 Hz, H-7), 7.75 (2H, d, *J* 8.75 Hz, H-2′, H-6′), 7.25 (1H, d, *J* 2.25 Hz, H-4), 6.85 (1H, dd, *J* 2.25, 8.75 Hz, H-6), 6.66 (2H, d, *J* 8.75 Hz, H-3′, H-5′), 5.89 (2H, brs, NH_2); IR 3443, 3362 cm^{-1}; MS (CI) *m/z* 243 (M + 1). Anal. ($C_{13}H_{10}N_2OS$) C,H,N.

2-(4-Aminophenyl)-6-hydroxybenzothiazole (6g): yield 69%; mp 241−244 °C; ^{1}H NMR (DMSO-d_6) δ 9.71 (1H, brs, OH), 7.72 (1H, d, *J* 8.75 Hz, H-4), 7.68 (2H, d, *J* 8.75 Hz, H-2′, H-6′), 7.33 (1H, d, *J* 2.25 Hz, H-7), 6.92 (1H, dd, *J* 2.25, 8.75 Hz, H-5), 6.66 (2H, d, *J* 8.75 Hz, H-3′, H-5′), 5.82 (2H, brs, NH_2); IR 3487, 3387 cm^{-1}; MS (CI) *m/z* 243 (M + 1). Anal. ($C_{13}H_{10}N_2OS$) C,H,N.

General Method for the Synthesis of Methoxy-Substituted 2-(4-Amino-3-iodophenyl)benzothiazoles 7a−c. Method F. To a solution of a methoxy-substituted 2-(4-aminophenyl)benzothiazole **5e−g** (0.013 mol) in acetic acid (35 mL) was added (dropwise over 10 min at 25 °C) a solution of iodine monochloride (0.017 mol) in acetic acid (35 mL). The mixture was stirred at 25 °C for a further 2 h and excess acetic acid was removed by vacuum evaporation. Products were purified as previously described.[2] Physical properties of 2-(4-amino-3-iodophenyl)-4-methoxybenzothiazole (**7a**), 2-(4-amino-3-iodophenyl)-5-methoxybenzothiazole (**7b**), and 2-(4-amino-3-iodophenyl)-6-methoxybenzothiazole (**7c**) are shown in Table 2.

General Method for the Synthesis of Methoxy-Substituted 2-(4-Amino-3-chlorophenyl)benzothiazoles 8a−

c. Method G. A mixture of a methoxy-substituted 2-(4-amino-3-iodophenyl)benzothiazole **7a**−**c** (0.01 mol) and copper(I) chloride (0.03 mol) was boiled in DMF (20 mL) for 15 h. The reaction mixture was concentrated by vacuum evaporation and the residue extracted into ethyl acetate. The organic layer was washed with water, dried, and evaporated to yield crude methoxy-substituted 2-(4-amino-3-chlorophenyl)benzothiazoles which were purified by column chromatography (dichloromethane). Physical properties of 2-(4-amino-3-chlorophenyl)-4-methoxybenzothiazole (**8a**), 2-(4-amino-3-chlorophenyl)-5-methoxybenzothiazole (**8b**), and 2-(4-amino-3-chlorophenyl)-6-methoxybenzothiazole (**8c**) are shown in Table 2.

The following hydroxy-substituted 2-(4-amino-3-chlorophenyl)benzothiazoles **8d**−**f** were prepared by demethylation of methoxy-substituted 2-(4-amino-3-chlorophenyl)benzothiazoles (above) according to general method E.

2-(4-Amino-3-chlorophenyl)-4-hydroxybenzothiazole (8d): yield 69%; mp 176−178 °C; ^{1}H NMR ($CDCl_3$) δ 7.98 (1H, d, *J* 2.3 Hz, H-2′), 7.74 (1H, dd, *J* 2.3, 8.5 Hz, H-6′), 7.33 (1H, dd, *J* 1.0, 8.3 Hz, H-7), 7.23 (1H, t, *J* 8.3 Hz, H-6), 6.94 (1H, dd, *J* 1.0, 8.3 Hz, H-5), 6.79 (1H, d, *J* 8.5 Hz, H-5′), 4.40 (2H, brs, NH_2); IR 3379 cm^{-1}; MS (CI) *m*/*z* 277/279 (M + 1). Anal. ($C_{13}H_9ClN_2OS$) C,H,N.

2-(4-Amino-3-chlorophenyl)-5-hydroxybenzothiazole (8e): yield 50%; mp 220−222 °C; ^{1}H NMR (DMSO-d_6) δ 9.89 (1H, brs, OH), 7.88 (1H, d, *J* 2.3 Hz, H-2′), 7.68 (1H, dd, *J* 2.3, 8.5 Hz, H-6′), 7.62 (1H, d, *J* 2.3 Hz, H-4), 7.23 (1H, d, *J* 8.5 Hz, H-7), 7.12 (1H, dd, *J* 2.3, 8.5 Hz, H-6), 6.81 (1H, d, *J* 8.5 Hz, H-5′), 6.04 (2H, brs, NH_2); IR 3489 (NH), 3401 (OH) cm^{-1}; MS (CI) *m*/*z* 277/279 (M + 1). Anal. ($C_{13}H_9ClN_2OS$) C,H,N.

2-(4-Amino-3-chlorophenyl)-6-hydroxybenzothiazole (8f): yield 87%; mp 258−260 °C; ^{1}H NMR ($CDCl_3$) δ 9.81 (1H, brs, OH), 7.83 (1H, d, *J* 2.0 Hz, H-2′), 7.75 (1H, d, *J* 8.8 Hz, H-4), 7.67 (1H, dd, *J* 2.0, 8.5 Hz, H-6′), 7.37 (1H, d, *J* 2.3 Hz, H-7), 6.95 (1H, dd, *J* 2.3, 8.8 Hz, H-5), 6.90 (1H, d, *J* 8.5 Hz, H-5′), 6.07 (2H, brs, NH_2); IR 3481, 3377 cm^{-1}; MS (CI) *m*/*z* 277/279 (M + 1). Anal. ($C_{13}H_9ClN_2OS$) C,H,N.

2-(4-Amino-3-hydroxymethylphenyl)benzothiazole (11). 2-(4-Amino-3-cyanophenyl)benzothiazole[2] (0.2 g, 0.8 mmol) was dissolved in 80% H_2SO_4 (5 mL) and the resulting solution heated at 100 °C for 2 h. After cooling the pH was adjusted to 7.5 using 50% aqueous NaOH. The precipitate was filtered from solution to give a yellow solid of the intermediate carboxylic acid **10** which was dried over P_2O_5. The carboxylic acid was dissolved in THF (5 mL) and added dropwise to a suspension of $LiAlH_4$ (0.11 g, 2.8 mmol) in THF (5 mL). After the mixture stirred at room temperature for 1 h, water (10 mL) was added to destroy the excess hydride. The product was extracted using ethyl acetate (2 × 20 mL) and the combined organic layers washed with water (10 mL). Concentration in vacuo gave crude product as a gum which was purified by column chromatography using ethyl acetate−chloroform (1:4) as eluant. Recrystallization from ethanol gave the hydroxymethylphenylbenzothiazole **11** (0.11 g, 54%) as cream-colored needles: mp 196−197 °C; ^{1}H NMR (DMSO-d_6) δ 8.05 (1H, dd, *J* 0.8, 8.0 Hz, H-4), 7.93 (1H, dd, *J* 0.8, 8.0 Hz, H-7), 7.88 (1H, d, *J* 2.3 Hz, H-2′), 7.72 (1H, dd, *J* 2.3, 8.3 Hz, H-6′), 7.50 (1H, dt, *J* 0.8, 8.0 Hz, H-5), 7.39 (1H, dt, *J* 0.8, 8.0 Hz, H-6), 6.73 (1H, d, *J* 8.3 Hz, H-5′), 5.72 (2H, brs, NH_2), 5.25 (1H, t, *J* 5.5 Hz, OH), 4.70 (2H, d, *J* 5.5 Hz, CH_2); IR 3329, 3225 cm^{-1}; MS (CI) *m*/*z* 257 (M + 1). Anal. ($C_{14}H_{12}N_2OS$) C,H,N.

2-(4-Acetylamino-3-hydroxyphenyl)benzothiazole (14). A mixture of 4-acetamido-3-hydroxybenzoic acid (1.33 g, 6.81 mmol) and polyphosphoric acid (20 mL) was heated to 50−60 °C with stirring; then 2-aminothiophenol (0.73 mL, 6.81 mmol) added dropwise. The mixture was heated at 150 °C for 2 h with stirring then poured onto ice. After neutralizing with concentrated aqueous ammonia solution, the crude product was collected by filtration. Purification by column chromatography (ethyl acetate) gave the benzothiazole **14** (1.74 g, 61%) as a white solid: mp 236−238 °C; ^{1}H NMR (DMSO-d_6) δ 10.47 (1H, s, OH), 9.43 (1H, s, NH), 8.14 (2H, d, *J* 7.5 Hz, ArH), 8.04 (1H, d, *J* 7.5 Hz, H-5′), 7.65 (1H, d, *J* 2.5 Hz, H-2′), 7.50 (3H, m, ArH), 2.17 (3H, s, CH_3CO); ^{13}C NMR (DMSO-d_6) δ 169.34 (C), 167.27 (C), 153.82 (C), 147.67 (C), 134.48 (C), 130.04 (C), 128.50 (C), 126.74 (CH), 125.43 (CH), 122.80 (CH), 122.43 (CH), 121.74 (CH), 118.80 (CH), 113.36 (CH), 24.18 (CH_3); IR 3405, 1661 (C=O), 1605, 1523, 1413, 752 cm^{-1}; MS (EI+) 266 (M − 16), 135. Anal. ($C_{15}H_{12}N_2O_2S$) C, H, N.

General Procedure for the Synthesis of Hydroxy-Substituted 2-(4-Acetamidophenyl)benzothiazoles 15. Method H. Acetyl chloride (0.3 mol) was added slowly to a solution of the appropriate hydroxy-substituted 2-(4-aminophenyl)benzothiazole (0.10 mol) and triethylamine (0.20 mol) in dichloromethane (200 mL) at 5 °C with stirring. The resulting solution was stirred at 5 °C for 1 h then concentrated in vacuo. The residue was dissolved in methanol (200 mL), 1 M aqueous Na_2CO_3 (150 mL) was added, and the resulting mixture was stirred at ambient temperature for 2 h. After neutralization using 2 M HCl, the product was extracted using ethyl acetate (4 × 100 mL), dried ($MgSO_4$), and concentrated in vacuo. The resulting crude product was recrystallized from methanol.

The following hydroxy-substituted 2-(4-acetamidophenyl)-benzothiazoles were prepared.

2-(4-Acetamido-3-methylphenyl)-4-hydroxybenzothiazole (15a): yield 86%; mp 208−210 °C; ^{1}H NMR (DMSO-d_6) δ 10.18 (1H, brs, OH), 9.42 (1H, brs, NH), 7.92 (1H, d, *J* 2.5 Hz, H-2′), 7.85 (1H, dd, *J* 2.5, 7.5 Hz, H-6′), 7.74 (1H, d, *J* 7.5 Hz, H-5′), 7.50 (1H, dd, *J* 2.5, 7.5 Hz, H-7), 7.24 (1H, t, *J* 7.5 Hz, H-6), 6.90 (1H, dd, *J* 2.5, 7.5 Hz, H-5), 2.33 (3H, s, CH_3CO), 2.12 (3H, s, CH_3); IR 3435 (NH), 3207 (OH), 1680 (C=O) cm^{-1}; MS (CI) *m*/*z* 299 (M + 1). Anal. ($C_{16}H_{14}N_2O_2S$) C,H,N.

2-(4-Acetamido-3-methylphenyl)-5-hydroxybenzothiazole (15b): yield 52%; mp 304−306 °C; ^{1}H NMR (DMSO-d_6) δ 9.78 (1H, brs, OH), 9.42 (1H, brs, NH), 7.90 (1H, d, *J* 2.5 Hz, H-2′), 7.89 (1H, d, *J* 8.5 Hz, H-7), 7.86 (1H, dd, *J* 2.5, 8.5 Hz, H-6′), 7.74 (1H, d, *J* 8.5 Hz, H-5′), 7.36 (1H, d, *J* 2.3 Hz, H-4), 6.95 (1H, dd, *J* 2.3, 8.5 Hz, H-6), 2.34 (3H, s, CH_3CO), 2.10 (3H, s, CH_3); IR 3252, 1655 (C=O) cm^{-1}; MS (CI) *m*/*z* 299 (M + 1). Anal. ($C_{16}H_{14}N_2O_2S$) C,H,N.

2-(4-Acetamido-3-methylphenyl)-6-hydroxybenzothiazole (15c): yield 57%; mp 263−266 °C; ^{1}H NMR (DMSO-d_6) δ 9.88 (1H, brs, OH), 9.41 (1H, brs, NH), 7.95 (1H, d, *J* 2.3 Hz, H-2′), 7.88 (1H, d, *J* 8.8 Hz, H-4), 7.80 (1H, dd, *J* 2.3, 8.5 Hz, H-6′), 7.73 (1H, d, *J* 8.5 Hz, H-5′), 7.41 (1H, d, *J* 2.5 Hz, H-7), 7.00 (1H, dd, *J* 2.5, 8.8 Hz, H-5), 2.33 (3H, s, CH_3CO), 2.13 (3H, s, CH_3); IR 3423 (NH), 3298 (OH), 1655 (C=O) cm^{-1}; MS (CI) *m*/*z* 299 (M + 1). Anal. ($C_{16}H_{14}N_2O_2S$) C,H,N.

2-(4-Acetamido-3-methylphenyl)-7-hydroxybenzothiazole (15d): yield 78%; mp 238−241 °C; ^{1}H NMR (DMSO-d_6) δ 10.61 (1H, brs, OH), 9.42 (1H, brs, NH), 7.94 (1H, d, *J* 2.5 Hz, H-2′), 7.85 (1H, dd, *J* 2.5, 7.5 Hz, H-6′), 7.75 (1H, d, *J* 7.5 Hz, H-5′), 7.50 (1H, dd, *J* 1.0, 7.5 Hz, H-4), 7.34 (1H, t, *J* 7.5 Hz, H-5), 6.86 (1H, dd, *J* 1.0, 7.5 Hz, H-6), 2.33 (3H, s, CH_3CO), 2.12 (3H, s, CH_3); IR 3217, 1637 (C=O) cm^{-1}; MS (CI) *m*/*z* 299 (M + 1). Anal. ($C_{16}H_{14}N_2O_2S$) C,H,N.

2-(4-Acetamido-3-chlorophenyl)-4-hydroxybenzothiazole (15e): yield 87%; mp 220−222 °C; ^{1}H NMR (DMSO-d_6) δ 10.13 (1H, brs, OH), 9.58 (1H, brs, NH), 8.04 (1H, d, *J* 2.5 Hz, H-2′), 7.92 (1H, d, *J* 8.5 Hz, H-5′), 7.83 (1H, dd, *J* 2.5, 8.5 Hz, H-6′), 7.38 (1H, dd, *J* 1.0, 8.8 Hz, H-7), 7.15 (1H, t, *J* 8.8 Hz, H-6), 6.78 (1H, dd, *J* 1.0, 8.8 Hz, H-5), 2.05 (3H, s, CH_3CO); IR 3410 (NH), 3321 (OH), 1687 (C=O) cm^{-1}; MS (CI) *m*/*z* 319/321 (M + 1). Anal. ($C_{15}H_{11}ClN_2O_2S$) C,H,N.

2-(4-Acetamido-3-chlorophenyl)-5-hydroxybenzothiazole (15f): yield 83%; mp 260−264 °C; ^{1}H NMR (DMSO-d_6) δ 9.83 (1H, brs, OH), 9.80 (1H, brs, NH), 8.12 (1H, d, *J* 2.0 Hz, H-2′), 8.03 (1H, d, *J* 9.0 Hz, H-5′), 7.96 (1H, dd, *J* 2.0, 9.0 Hz, H-6′), 7.90 (1H, d, *J* 8.5 Hz, H-7), 7.37 (1H, d, *J* 2.3 Hz, H-4), 6.96 (1H, dd, *J* 2.3, 8.5 Hz, H-6), 2.09 (3H, s, CH_3); IR 3402 (NH), 3361 (OH), 1678 (C=O) cm^{-1}; MS (CI) *m*/*z* 319/321 (M + 1). Anal. ($C_{15}H_{11}ClN_2O_2S$) C,H,N.

2-(4-Acetamido-3-chlorophenyl)-6-hydroxybenzothiazole (15g): yield 74%; mp 238−241 °C; ^{1}H NMR (DMSO-d_6) δ 9.96 (1H, brs, OH), 9.68 (1H, brs, NH), 8.06 (1H, d, *J* 2.0 Hz, H-2′), 8.00 (1H, d, *J* 8.5 Hz, H-5′), 7.96 (1H, dd, *J* 2.0, 8.5 Hz,

H-6′), 7.84 (1H, d, *J* 8.5 Hz, H-4), 7.42 (1H, d, *J* 2.3 Hz, H-7), 6.99 (1H, dd, *J* 2.3, 8.5 Hz, H-5), 2.16 (3H, s, CH_3); IR 3275, 1660 (C═O) cm^{-1}; MS (CI) *m/z* 319/321 (M + 1). Anal. ($C_{15}H_{11}ClN_2O_2S$) C,H,N.

Synthesis of 2-(4-Nitrophenyl)benzothiazoles 16. 2-(3-Methyl-4-nitrophenyl)benzothiazole (16a). 2-Aminothiophenol (3.0 mL, 28 mmol) was added dropwise to a solution of 3-methyl-4-nitrobenzoyl chloride (5.15 g, 28 mmol) in pyridine (25 mL) with stirring. The mixture was heated under reflux for 30 min, then allowed to cool to 50−60 °C, and poured into ice−water (150 mL). The yellow precipitate was collected and crystallized from ethanol to give the nitrophenylbenzothiazole **16a** in 91% yield: mp 174−176 °C; ^{1}H NMR ($CDCl_3$) δ 8.12 (3H, m, ArH), 8.03 (1H, d, *J* 8.5 Hz, ArH), 7.96 (1H, d, *J* 8.0 Hz, ArH), 7.56 (1H, dt, *J* 1.5, 7.7 Hz, H-5), 7.46 (1H, dt, *J* 1.5, 7.7 Hz, H-6), 2.72 (3H, s, CH_3); ^{13}C NMR ($CDCl_3$) δ 165.02 (C), 153.93 (C), 150.02 (C), 135.29 (C), 134.59 (C), 131.38 (CH), 126.80 (CH), 126.03 (CH), 125.74 (CH), 125.48 (CH), 123.71 (CH), 121.76 (CH), 20.55 (CH_3); IR 1584, 1514 (NO_2), 1339 (NO_2), 1311, 764 cm^{-1}; MS (EI) *m/z* 270 (M^+), 223, 209, 69. Anal. ($C_{14}H_{10}N_2O_2S$) C,H,N.

Similarly prepared, from 4-nitrobenzoyl chloride, was 2-(4-nitrophenyl)benzothiazole (**16b**).[2]

2-(3-Chloro-4-nitrophenyl)benzothiazole (16c). A slurry of 2-(4-amino-3-chlorophenyl)benzothiazole (2.13 g, 8.2 mmol)[2] and acetic acid (20 mL) was added slowly to a stirred suspension of sodium perborate (6.28 g, 40.8 mmol) in acetic acid (20 mL) at 60 °C. The reaction mixture was stirred at 60 °C for a further 1 h then cooled, and chloroform (200 mL) and water (150 mL) were added. The aqueous layer was neutralized using concentrated aqueous ammonia and the chloroform layer separated and washed with saturated sodium thiosulfate solution (150 mL). The organic layer was dried ($MgSO_4$), and concentrated in vacuo to give crude product which was purified by column chromatography (25% ethyl acetate−hexane) to yield **16c** (0.52 g, 22%) as an off-white powder: mp 143−146 °C; ^{1}H NMR ($CDCl_3$) δ 8.32 (1H, d, *J* 0.5 Hz, H-2′), 8.13 (1H, d, *J* 8.3 Hz, H-7), 8.09 (1H, d, *J* 8.3 Hz, H-4), 8.01 (1H, d, *J* 8.3 Hz, H-5′), 7.96 (1H, dd, *J* 1.0, 8.3 Hz, H-6′), 7.57 (1H, dt, *J* 1.3, 8.0 Hz, H-5), 7.51 (1H, dd, *J* 1.3, 8.0 Hz, H-6); ^{13}C NMR ($CDCl_3$) δ 163.28 (C), 153.83 (C), 148.60 (C), 138.06 (C), 135.34 (C), 130.27 (CH), 128.07 (C), 126.99 (CH), 126.38 (CH), 126.26 (CH), 126.08 (CH), 123.92 (CH), 121.81 (CH); IR 3425, 1578, 1526 (NO_2), 1331 (NO_2), 766 cm^{-1}; MS (EI+) 290 (M − 1), 260. Anal. ($C_{14}H_{10}N_2O_2S$) C,H,N.

General Method for the Synthesis of Acetylated Hydroxamic Acid Derivatives 17. Method I. Powdered zinc was stirred in acetic acid for 10 min, washed with water, dried by suction filtration, and then activated by a brief heating under a stream of nitrogen. A mixture of the appropriate 2-(4-nitrophenyl)benzothiazole (0.67 mmol) and ammonium sulfate (0.133 g, 1.01 mmol) was partially dissolved in degassed dimethoxyethane (5 mL). A slurry of zinc (0.219 g, 3.34 mmol) in dimethoxyethane (10 mL) then acetic anhydride (0.19 mL, 2.01 mmol) were added with stirring under nitrogen and the mixture was heated under reflux for 2 h. The reaction mixture was cooled and filtered through Celite. Water (30 mL) was added to the filtrate and the aqueous layer was extracted with ethyl acetate (2 × 50 mL). The organic layers were then dried (Na_2SO_4) and concentrated in vacuo to give crude product which was purified by column chromatography (40% ethyl acetate−hexane) to gave the appropriate acetylated *N*-arylhydroxamic acid derivative.

The following hydroxamic acid derivatives were prepared by method I.

2-[4-(*N*-Acetoxy-*N*-acetylamino)-3-methylphenyl]benzothiazole (17a): yield 53%; mp 144−146 °C; ^{1}H NMR ($CDCl_3$) δ 8.10 (2H, d, *J* 8.3 Hz, ArH), 7.94 (2H, t, *J* 7.8 Hz, ArH), 7.60 (1H, broad s, ArH), 7.53 (1H, t, *J* 7.6 Hz, H-5), 7.42 (1H, t, *J* 7.6 Hz, H-6), 2.48 (3H, s, CH_3Ar), 2.20 (3H, s, CH_3COO), 1.99 (3H, s, CH_3CON); ^{13}C NMR ($CDCl_3$) δ 167.27 (C), 165.93 (C), 165.91 (C), 153.74 (C), 139.76 (C), 138.45 (CH), 134.92 (CH), 130.51 (CH), 129.75 (CH), 126.27 (CH), 125.86 (CH), 125.32 (CH), 123.13 (CH), 121.46 (CH), 20.82 (CH_3Ar), 18.05 (CH_3COO), 17.58 (CH_3CON); IR 1780 (C═O, ester), 1678 (C═O, amide), 1370, 1181, 775 cm^{-1}; MS (EI) *m/z* 341 (M^+), 267, 240. Anal. ($C_{18}H_{16}N_2O_3S$) C,H,N,S.

2-[4-(*N*-Acetoxy-*N*-acetylamino)phenyl]benzothiazole (17b): yield 36%; mp 105−107 °C; ^{1}H NMR ($CDCl_3$) δ 8.11 (2H, dd, *J* 1.0, 8.5 Hz, H-2′, H-6′), 8.06 (1H, d, *J* 8.0 Hz, H-7), 7.88 (1H, d, *J* 8.3 Hz, H-4), 7.58 (2H, dd, *J* 1.0, 8.5 Hz, H-3′, H-5′), 7.48 (1H, dt, *J* 1.0, 7.6 Hz, H-5), 7.37 (1H, dt, *J* 1.0, 7.3 Hz, H-6), 2.25 (3H, s, CH_3COO), 2.14 (3H, s, CH_3CON); ^{13}C NMR ($CDCl_3$) δ 167.83 (C═O), 166.44 (C═O), 153.74 (C), 141.27 (C), 134.94 (C), 128.77 (CH), 128.36 (CH), 126.54 (CH), 125.52 (CH), 123.24 (CH), 121.64 (CH), 21.59 (CH_3COO), 18.31 (CH_3CON); IR 1790 (C═O, ester), 1690 (C═O, amide), 1483, 1371, 1343, 1182, 968, 758 cm^{-1}; MS (electrospray) *m/z* 327 (M + 1), 285, 253. Anal. ($C_{17}H_{14}N_2O_3S$) C,H,N,S.

Other Drugs and Reagents. [^{14}C]**1a** (^{14}C at the C-2 position of the thiazole ring, 26.2 mCi/mmol) was synthesized in the Research Triangle Institute (Research Triangle Park, NC). Compound **1a** was dissolved in DMSO as a 50 mM stock solution and stored at −80 °C. Cell culture reagents were purchased from Quality Biological Inc. (Gaithersburg, MD) except for heat-inactivated fetal bovine serum, which was purchased from Hyclone Lab., Inc. (Logan, UT). HPLC-grade acetonitrile was purchased from Baker (Phillipsburg, NJ). All other reagents were the highest grade commercially available.

Cell Culture. Culture medium and supplements were purchased from GIBCO-BRL Life Technologies, Inc. (Gaithersburg, MD) and Costar Corp. (Cambridge, MA). All human tumor cell lines (obtained from the repository of the National Cancer Institute) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (heat-inactivated), 2 mM glutamine, 100 μg/mL streptomycin, and 100 U/mL penicillin, in a humidified atmosphere of 5% CO_2, at 37 °C.

Cell Growth Inhibitory Assay. Growth of breast and renal cancer cells was quantitated by the ability of living cells to reduce the yellow MTT to a purple formazan product.[15] Cells were plated at 1500−2000 cells/well in 100 μL of medium in 96-well plates. After 24 h, 100 μL of drug solutions (prepared at twice the final concentration by serial dilution with culture medium) were added. The formazan product of MTT reduction was dissolved in DMSO, and absorbance at 570 nm was measured using a MR5000 multiplate reader (Dynatech Laboratories Corp., Chantilly, VA). Absorbance of untreated cells was used as a control in calculating the dose−response.

In experiments to rank the in vitro cytotoxicities of oxidized benzothiazoles and their precursors in MTT assays against MCF-7 cells, cells were seeded into 96-well microtiter plates at a density of 5×10^3/well and allowed to adhere overnight. Cells were treated with test agent at a final concentration range between 10 pM and 100 μM ($n = 8$). The total drug exposure was 72 h, and growth and viability were assessed by MTT reduction (see above) with formazan solubilized by addition of DMSO (100 μL) and glycine (25 μL) buffer. Absorbance was read at 550 nm on an Anthos Labtec Instruments plate reader.

Analysis of 1a and Its Metabolites. Culture medium and cell homogenates were mixed with 3-fold volumes of acetonitrile to precipitate protein. After centrifugation at 14 000 rpm for 10 min, the supernatant was analyzed by HPLC.

The HPLC system consisted of a Hewlett-Packard 1050 series module (solvent delivery pump, autosampler, and multiple wavelength detector; Hewlett-Packard, Palo Alto, CA, and a Hewlett-Packard 1046A fluorescence detector. Separation of the compounds was achieved at room temperature on a C18 reversed-phase column (YMC-ODS-AQ, 150 × 4.6-mm i.d., S-5 μm; YMC Inc., Wilmington, NC). Compounds were eluted with a gradient run in which the mobile phase composition was changed linearly during 40 min from 10:90:1 (acetonitrile:water:acetic acid) to 80:20:1 (acetonitrile:water:acetic acid). The mobile phase was continuously degassed with nitrogen, and flow rate through the column was 1 mL/min. Compounds were detected at 338 nm with UV detection and with fluorescence detection (excitation, 344 nm; emission, 434 nm). In experiments using [^{14}C]**1a**, fractions of each 2 min were

collected after injection into HPLC and radioactivity in the collected samples was determined.

Identification of the Major Metabolite of 1a. After incubation of MCF-7 cells with 1 μM **1a** for 24 h, media were collected and extracted by *tert*-butylmethyl ether. The organic layer was concentrated to dryness and the residue reconstituted in acetonitrile for HPLC analysis as described above. The major metabolite eluted after 21 min and was purified by two HPLC separations: using 50% aqueous acetonitrile as mobile phase in the first isolation step, followed by 30% acetonitrile in the second step.

Elucidation of the chemical structure was accomplished by application of mass spectrometry, nuclear magnetic resonance spectroscopy, and subsequently HPLC comparison with authentic material. Briefly, gas chromatography/mass spectrometry was performed using a 15-m DB-1 capillary column with mass scanning detection electron ionization 70 eV. Accurate mass determinations were performed with a forward-geometry magnetic sector mass spectrometer using perfluorokerosene as an internal standard. One- and two-dimensional NMR spectra (^{1}H and ^{1}H$-^1$H correlations) were recorded at 500 MHz using CD_3CN as both solvent and internal standard.

Metabolism of 1a by Cell Homogenates. Cells were grown in 150-mm tissue culture Petri dishes to 50−70% confluency. Where required, cells were treated with 1 μM **1a** for 24 h, after which media were removed and cells rinsed with cold PBS (pH 7.4). Cells were collected by scraping and resuspended in a hypotonic buffer containing 5 mM Tris-HCl buffer (pH 7.4), 10 mM KCl, 1.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 0.5 mM PMSF. Homogenates were prepared using a Potter-Elvehjem homogenizer.

A typical reaction mixture consisted of 100 μM **1a**, 100 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$, 1 mM NADPH, and cell homogenate (1 mg/mL) in a final volume of 0.2 mL. Where required, 100 μM SKF-525A (Research Biochemicals International, Natick, MA) was added to the reaction mixture. **1a** was dissolved in DMSO and added to the incubation mixture (final DMSO concentration of 1%). The reaction mixture was preincubated at 37 °C for 5 min and initiated by addition of NADPH. After 2 h incubation at 37 °C, reaction was terminated by the addition of 0.6 mL of ice-cold acetonitrile and centrifuged at 14 000 rpm for 10 min, and supernatant (100 μL) was analyzed by the above HPLC system.

Uptake of Radiolabeled [^{14}C]1a into Cells. Cells were grown in 50-mm tissue culture Petri dishes to 50−70% confluency and treated with 0.1 and 1 μM [^{14}C]**1a** for different incubation periods. Media were removed and plates immediately placed on ice. Cells were rapidly rinsed twice with ice-cold PBS (pH 7.4) and solubilized with 1 N NaOH before measurement of radioactivity and protein content.

Covalent Binding to Cellular Proteins. Cells were grown in 100-mm tissue culture Petri dishes to 50−70% confluency and treated with 0.1 and 1 μM [^{14}C]**1a** for desired incubation periods. Media were removed for HPLC analysis with radioactivity detection of unchanged **1a**, whereas cells were rinsed with cold PBS (pH 7.4) and collected by scraping. After homogenization of cells, proteins were precipitated with cold 50% trichloroacetic acid (TCA; final concentration 10%) and pelleted by centrifugation at 14 000 rpm for 5 min at 4 °C. The protein pellet was exhaustively washed with cold 10% TCA and then with 60% methanol to remove unbound radioactivity. The precipitated protein was solubilized with 1 N NaOH, and radioactivity and protein content were measured.

Other Assays. Protein concentrations were estimated with Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA) by the method of Bradford[16] using bovine serum albumin (Sigma Chemicals) as protein standard. Radioactivity was determined using a Beckman LS 6500 multipurpose scintillation counter (Beckman, Fullerton, CA), after addition of scintillation cocktail (Ecoscint A, National Diagnostics, Atlanta, GA).

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