Synthesis and Biological Properties of Benzothiazole, Benzoxazole, and Chromen-4-one Analogues of the Potent Antitumor Agent 2-(3,4-Dimethoxyphenyl)-5-fluorobenzothiazole (PMX 610, NSC 721648)[1]

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New fluorinated 2-aryl-benzothiazoles, -benzoxazoles, and -chromen-4-ones have been synthesized and their activity against MCF-7 and MDA 468 breast cancer cell lines compared with the potent antitumor benzothiazole **5**. Analogues such as **9a**,**b** and **12a**,**d** yielded submicromolar GI_{50} values in both cell lines; however, none of the new compounds approached **5** in terms of antitumor potency. For **5**, binding to the aryl hydrocarbon receptor appeared to be necessary but not sufficient for growth inhibition.

Introduction

The versatile and synthetically accessible 2-arylbenzothiazole scaffold has provided the inspiration for the discovery of a number of new antitumor agents with unusual mechanisms of action in recent years.[2] The 2-(4-aminophenyl)benzothiazoles (Figure 1) provide a case in point and illustrate the wider benefits of a "chemistry-led" approach to drug discovery.[3] The original (non-fluorinated) lead compound in this class[4] (**1**, DF 203) was found to activate the arylhydrocarbon receptor (AhR[a]),[5,6] inducing selective expression of the cytochrome P450 CYP1A1 in sensitive cancer cell lines (e.g., breast and ovarian) following translocation to the nucleus.[7,8] The major exportable 6-hydroxylated metabolite of **1** from drug−CYP1A1 interaction was found to be inactive and antagonistic to the antitumor activation process,[9] leading to the development of fluorinated benzothiazoles to thwart the deactivation process.[10,11] Among the fluorinated analogues, 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (**2**, 5F 203) emerged as the lead compound and, based on a favorable comparison with doxorubicin against a panel of breast cancer xenografts,[12] is now in phase 1 clinical trial in the U.K. in the form of its water soluble L-lysyl amide prodrug (Phortress).[13] It is noteworthy that the ^{11}C-methylated derivative of one of the inactive hydroxylated metabolites has recently re-emerged as an amyloid-affinic agent (**3**, [^{11}C]PIB) in clinical trials for early diagnosis of Alzheimer's disease using positron emission tomography (PET).[14,15] Once again, metabolic stability (phase II metabolism) may limit the clinical application of **3**, and the potential use of metabolically stabilized (fluorinated) benzothiazoles such as **4** in the Alzheimer's diagnosis setting has been reported.[16]

More pertinent to the present work, a related simple benzothiazole 2-(3,4-dimethoxyphenyl)-5-fluorobenzothiazole (**5**, PMX 610, formerly GW 610; NSC 721648) has been shown to exhibit exquisitely potent (GI_{50} < 0.1 nM) and selective in vitro antitumor properties in human cancer cell lines (e.g., colon, non-small-cell lung and breast subpanels) of the National Cancer Institute (NCI) 60 human cancer cell line screen.[17] Lead compound **5** was developed from the related antitumor 2-(4-hydroxyphenyl)benzothiazole.[18] Surprisingly, SAR studies indicated that minor modifications of the dimethoxyphenyl group, removal of the fluoro group, or its replacement with other halogens had a profoundly dyschemotherapeutic effect with respect to in vitro growth-inhibitory activity. Despite a selectivity profile reminiscent of the fluorinated aminophenylbenzothiazoles **1** and **2**, preliminary pharmacological investigations revealed that **5** showed distinctive features in cell line selectivity and mechanism of action.[17] The synthesis of an ^{11}C-labeled version of **5** as a potential PET probe has been reported.[19]

In our earlier work we were intrigued by the fact that benzothiazole (**5**) appeared to represent a "pinnacle" of antitumor activity surrounded by a desert of inactive benzothiazole analogues.[17] Structurally related 2-phenylbenzimidazole derivatives such as **6a**,**b** have also been shown to be devoid of the potent antitumor effects of **5**.[20] We have now reinvestigated this idiosyncratic effect and report the synthesis of further phenolic 2-arylbenzothiazoles not reported in our earlier work, plus isosteric related 2-arylbenzoxazole and 4*H*-chromen-4-one heterocycles, all bearing additional substituents in the heterocycle moiety (generally fluoro) and one or more oxygen substituents in the 2-aryl fragment. Thus, we anticipated that the present work would reveal novel agents at least equiactive to **5** in in vitro screens but also with reduced lipophilicity consequent on replacing the benzothiazole bicycle with its benzoxazole and 4*H*-chromen-4-one counterparts and related

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[a] Abbreviations: AhR, arylhydrocarbon receptor; CYP, cytochrome P450; PET, positron emission tomography; NCI, National Cancer Institute; SAR, structure−activity relationship; DMAP, 4-dimethylaminopyridine; MTT, 1-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; α-NF, α-naphthoflavone.

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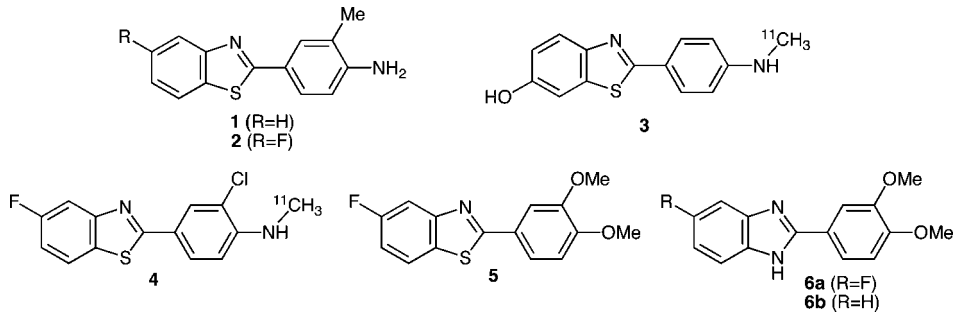
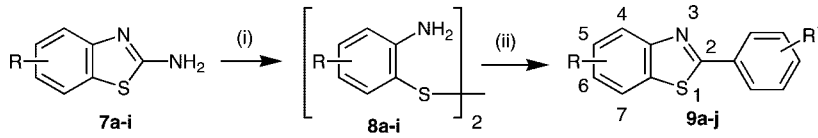


Figure 1. Potential therapeutic/diagnostic 2-arylbenzazoles.

Scheme 1[a]



	R		R
7/8a:	5-F	**7/8f:**	6-Et
7/8b:	5-CF_3	**7/8g:**	6-OMe
7/8c:	6-F	**7/8h:**	6-OEt
7/8d:	6-Cl	**7/8i:**	6-SO_2Me
7/8e:	6-Br		

	R	R^1		R	R^1
9a:	5-F	3-OH	**9f:**	6-Br	4-OH
9b:	5-F	4-OH	**9g:**	6-Et	4-OH
9c:	5-CF_3	4-OH	**9h:**	6-OMe	4-OH
9d:	6-F	4-OH	**9i:**	6-OEt	4-OH
9e:	6-Cl	4-OH	**9j:**	6-SO_2Me	4-OH

[a] Reagents: (i) KOH(aq), reflux; (ii) substituted benzaldehyde, PPh_3, *p*-TsOH, toluene, reflux.

structures (**5** is highly lipophilic with a calculated log *P* of 4.2 [ChemDraw Ultra, version 9.0]).

Chemistry

Synthesis of 2-Arylbenzothiazoles (9a−j). Substituted 2-aminobenzothiazoles **7a−i** were oxidatively ring-opened to the corresponding bis(2-aminophenyl) disulfides **8a−i** in aqueous KOH according to our previous work.[10] Cyclization to benzothiazoles **9a−j** was achieved by condensation with hydroxybenzaldehydes in refluxing toluene containing triphenylphosphine and catalytic *p*-toluenesulfonic acid (general method A, Scheme 1; see Experimental Section). ^{1}H NMR data for intermediate disulfides **8a−i** and spectroscopic data of **9a−j** are recorded in Supporting Information.

Synthesis of 2-Phenylbenzoxazoles (12a−r). 2-Phenylbenzoxazoles were obtained in good overall yields via a simple two-step procedure based on the methodology reported by Evindar and Batey (general methods B and C, Scheme 2; see Experimental Section).[21] DMAP-promoted Schotten−Baumann reaction of commercially available 5- or 4-fluoro-2-bromoaniline (**10a**,**b**) with benzoyl chlorides bearing oxygen substituents under basic conditions gave the intermediate *o*-bromobenzanilides **11a−r**, which were cyclized without further purification by treatment with a catalyst system comprising copper (I) iodide and 1,10-phenanthroline ligand, plus cesium carbonate as base, to give the desired substituted 2-phenylbenzoxazole products **12a−r**. The regiochemistry of cyclization was governed by the position of the bromide leaving group, using a strategy similar to that employed for the regiospecific synthesis of antitumor fluorinated benzothiazoles.[22]

An alternative synthesis of benzoxazole (**12d**) was achieved starting from 4-fluoro-2-nitrophenol (**13**), which was reacted with 3,4-dimethoxybenzoyl chloride in pyridine to yield the ester (**14**), which was reduced by catalytic hydrogenation over 5% Pd/C to yield amine (**15**). Cyclization of **15** was achieved in boiling toluene containing catalytic *p*-toluenesulphonic acid to give 2-(3,4-dimethoxyphenyl)-5-fluorobenzoxazole **12d** (Scheme 2; see Supporting Information for details). Analytical and spectroscopic data of intermediate benzanilides **11a−r** and benzoxazoles **12a−r** are recorded in Supporting Information.

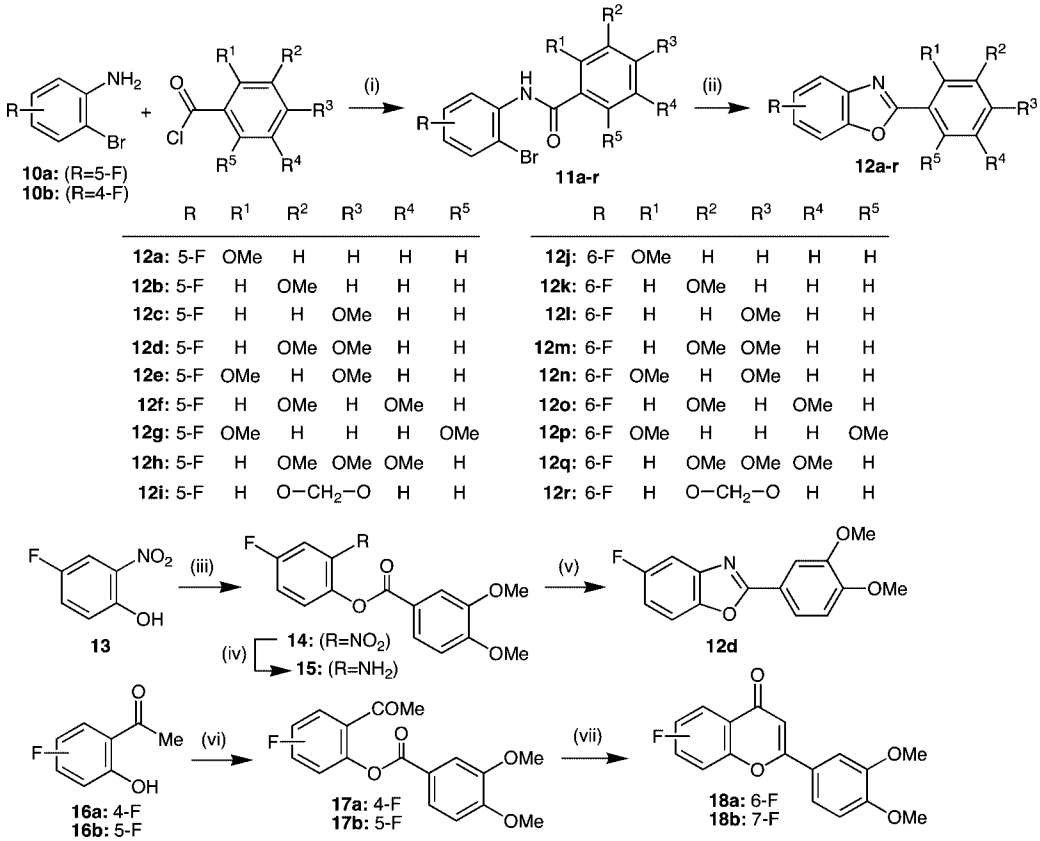
Synthesis of 2-Phenyl-4*H*-chromen-4-ones (18a,b). Related syntheses from 4-fluoro- (**16a**) and 5-fluoro-2-acetylphenol (**16b**) with 3,4-dimethoxybenzoyl chloride in dry pyridine afforded intermediate esters (**17a**,**b**), which were used without further purification and cyclized under basic conditions to 2-arylbenzopyranones (**18a**,**b**) in 42% and 34% yield, respectively (Scheme 2, experimental details in Supporting Information).

Biological Results and Discussion

In Vitro Growth-Inhibitory Assays. In earlier work we showed that the human breast cancer cell lines MDA 468 (estrogen receptor negative, ER−) and MCF-7 (estrogen receptor positive, ER+) employed in MTT assays following 3-day drug exposure were suitable for study of SAR in the 2-arylbenzothiazole class of compound.[17] Table 1 shows the antiproliferative data for newly synthesized benzothiazoles **9a−j**, benzoxazoles **12a−r**, and chromenones **18a**,**b**. The previously reported potently active 2-(3,4-dimethoxyphenyl)-5-fluorobenzothiazole **5**[17] and inactive 2-(3,4-dimethoxyphenyl)-5-fluorobenzimidazole **6a**[20] were used here as reference compounds.

It was previously noted that demethylation of one or other of the methoxyl groups of lead **5** resulted in less active compounds; however, we were intrigued to explore the activity of monohydroxylated 2-phenylbenzothiazoles bearing fluorine or related substituents on the benzothiazole ring. Since demethylation could potentially be an initial antitumor mechanistic step, it was desirable to synthesize and evaluate biologically representative 2-hydroxyphenyl derivatives in the benzothiazole series. Accordingly compounds **9a−j** were tested for growth inhibitory activity in both MCF-7 and MDA 468 human cancer cell lines (see Table 1). In general the new benzothiazoles were more active against the ER −ve breast cancer cell line MDA 468, giving GI_{50} values in the submicromolar range for compounds bearing halogen (F, Cl, Br) or trifluoromethyl substituents on the benzothiazole ring (**9a−f**).

Scheme 2[a]



	R	R^1	R^2	R^3	R^4	R^5
12a:	5-F	OMe	H	H	H	H
12b:	5-F	H	OMe	H	H	H
12c:	5-F	H	H	OMe	H	H
12d:	5-F	H	OMe	OMe	H	H
12e:	5-F	OMe	H	OMe	H	H
12f:	5-F	H	OMe	H	OMe	H
12g:	5-F	OMe	H	H	H	OMe
12h:	5-F	H	OMe	OMe	OMe	H
12i:	5-F	H	O−CH_2−O		H	H

	R	R^1	R^2	R^3	R^4	R^5
12j:	6-F	OMe	H	H	H	H
12k:	6-F	H	OMe	H	H	H
12l:	6-F	H	H	OMe	H	H
12m:	6-F	H	OMe	OMe	H	H
12n:	6-F	OMe	H	OMe	H	H
12o:	6-F	H	OMe	H	OMe	H
12p:	6-F	OMe	H	H	H	OMe
12q:	6-F	H	OMe	OMe	OMe	H
12r:	6-F	H	O−CH_2−O		H	H

[a] Reagents: (i) NEt_3, DMAP, CH_2Cl_2; (ii) CuI, 1,10-phenanthroline, Cs_2CO_3, DME, reflux; (iii) 3,4-dimethoxybenzoyl chloride, pyridine, room temp; (iv) H_2 (40 psi), 5% Pd/C, EtOH; (v) *p*-TsOH, toluene, reflux; (vi) 3,4-dimethoxybenzoyl chloride, pyridine, room temp; (vii) KOH, pyridine, 50 °C, then AcOH, H_2SO_4, reflux.

In the MCF-7 (ER +ve) cell line the most active compounds were found to be the fluorinated (3-hydroxyphenyl)- and (4-hydroxyphenyl)benzothiazoles derivatives **9a** and **9b** (GI_{50} of 0.57 and 0.40 μM, respectively). Compounds bearing ethyl, alkoxy, or methylsulfonyl substitutents on the benzothiazole ring (**9g**−**j**) were found to be much less active.

2-(3,4-Dimethoxyphenyl)-5-fluorobenzoxazole (**12d**), the direct benzoxazole analogue of **5**, exhibited submicromolar potency against both cell lines (Table 1). Overall, compounds in the benzoxazole series (**12**) were more active against the MDA 468 cell line with IC_{50} varying over a >5000-fold range (0.017−98.6 μM). Against the generally less sensitive MCF-7 cell line, benzoxazoles displayed a >250-fold range of activities. It was difficult to discern what influence the position of the fluoro group, or position and multiplicity of the methoxyl groups, was having on growth-inhibitory activity; thus, 5-fluoro-2-(2-methoxyphenyl)benzoxazole (**12a**) and its 6-fluoro analogue (**12j**) had submicromolar potency against MCF-7, whereas benzoxazole (**12p**) with a 6-fluoro group and 2,6-dimethoxy substitution in the 2-aryl moiety was the least potent agent in this class. 6-Fluorobenzoxazole compounds **12m** and **12q** (bearing 3,4-dimethoxy and 3,4,5-trimethoxy substituents on the phenyl ring) were found to be potently active on the MDA 468 cell lines, giving GI_{50} values of 17 and 37 nM, respectively. The two 4*H*-chromen-4-one derivatives **18a**,**b** gave rather modest growth inhibitory activity in these two cell lines.

For the 5-fluoro heterocycles (6-fluoro in the benzopyranones) with a 3,4-dimethoxy disposition in the 2-aryl group, activity across the heterocyclic series was in the order benzothiazole > benzoxazole ≫ benzimidazole[20] and chromen-4-one, with a 10^6-fold difference between the most potent agent (**5**) and the inert benzimidazole **6**. We conclude, and remarkably so, that none of the new compounds remotely approach benzothiazole (**5**) in potency against these two breast cancer cell lines. This new finding, that replacement of the (fluorinated) bicyclic benzothiazole moiety of **5** with benzoxazole and chromen-4-one congeners impairs activity, builds on our earlier work that confirmed similar dyschemotherapeutic effects consequent on modifying the oxygen substituents on the 2-aryl group of **5**.[17]

Cell Cycle Perturbations. Flow cytometric analysis of cellular DNA content was used to determine cell cycle phase perturbations during treatment of MDA 468 cells with 2-(3,4-dimethoxyphenyl)-5-fluorobenzothiazole, benzoxazole, and benzimidazole congeners **5**, **12d**, and **6a**. Figure 2 charts the percentage of cells in the different stages of cell cycle (pre-G_1, G_1, S, and G_2/M), data taken directly from the flow cytometric DNA histograms (not shown). Consistent with growth inhibitory potency, profound G_2/M cell cycle arrest followed treatment of MDA 468 cells with **5** (0.1 μM) over 24 h (Figure 2b, $p < 0.03$), compared with control (nontreated) MDA 468 cells (Figure 2a). The G_2/M block observed after 24 h in MDA 468 cells was typically maintained for 48 and 72 h (Figure 2b), although treatment with higher concentrations of **5** (1 μM) revealed decreasing numbers of cells arrested within G_2/M as events accrue in a pre-G_1 apoptotic peak, consistent with DNA damage induction (data not shown). At 0.1 μM, the less potent analogues **12d** and **6a** failed to perturb MDA 468 cell cycle profiles (24 h). However, following treatment of cells with 1 μM **12d** (24 h), G_2/M events were enhanced in MDA 468 cell populations (Figure 2c). Noticeably in MDA 468 cells treated with 1 μM **12d** (24 h), events accumulated in S phase and an apoptotic pre-G_1 population emerged (Figure 2c). In line with

Table 1. Activity of New Substituted 2-Phenyl-benzazoles and -Chromen-4-ones against Human Breast Cancer Cell Lines[a]

	GI_{50} (μM)[b] in cell lines[c]	
compd	MCF-7	MDA 468
5	<0.0001[d]	<0.0001[d]
6a	85.7[e]	94.4[e]
9a	0.57	0.20
9b	0.40	0.21
9c	13.8	0.10
9d	54.0	0.50
9e	26.5	0.28
9f	4.16	0.28
9g	>100	>100
9h	45.8	9.67
9i	>100	>100
9j	62.3	42.2
12a	0.36	0.27
12b	39.5	0.87
12c	22.7	0.76
12d	0.83	0.33
12e	2.16	0.54
12f	45.4	0.79
12g	58.1	22.0
12h	16.0	2.58
12i	61.4	14.5
12j	0.82	1.19
12k	35.1	2.02
12l	41.0	9.55
12m	3.53	0.017
12n	69.9	3.29
12o	3.72	0.46
12p	90.7	98.6
12q	1.09	0.037
12r	19.2	6.77
18a	83.4	20.6
18b	27.9	0.45

[a] Determined by MTT assay (72 h drug exposure); see biological experimental section for details. [b] Compounds tested in triplicate, data expressed as mean values of three independent experiments. [c] Cancer cell line origin: MCF-7 (breast, ER +ve), MDA 468 (breast, ER −ve). [d] Data previously obtained; see ref 17. [e] Data previously obtained; see ref 20.

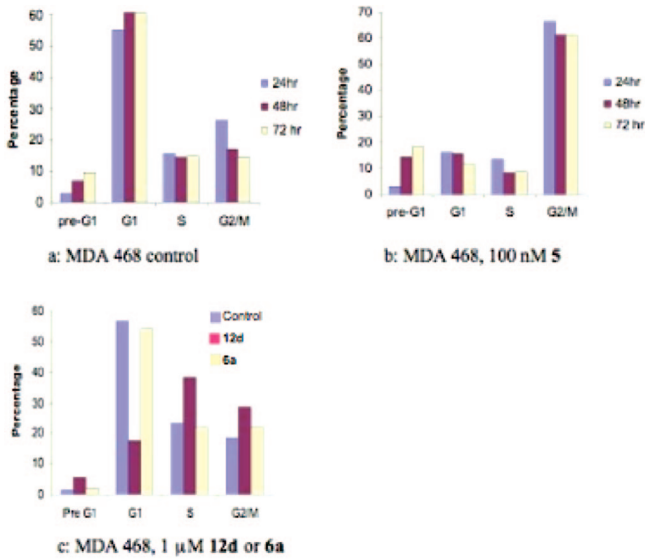


Figure 2. Cell cycle analyses following exposure of mammary carcinoma cells to **5**, **12d** or **6a**. Representative bar charts illustrate perturbations in cell cycle phases following treatment of MDA 468 cells with 100 nM **5** for 24−72 h (b), compared to nontreated control (a), and 1 μM **12d** or **6a** for 24 h (c). Independent analyses were performed ≥3 times, and 10 000 events per sample were analyzed.

inactivity against MDA 468 cells, benzimidazole **6a** (1 μM, 24 h) effected neither perturbation of MDA 468 cell cycle nor an apoptotic population.

Binding to the Aryl Hydrocarbon Receptor. (AhR). As part of wider efforts to uncover mechanistic targets underpinning the antitumor activity of the *O*-substituted 2-phenylbenzazole series, we have examined the potential role of the aryl hydrocarbon receptor (AhR) in mediating the potent antitumor activity of lead benzothiazole **5**. By use of a previously described CRL:WI rat liver cytosol [^{3}H]tetrachlorodibenzo-*p*-dioxin (TCDD) displacement AhR assay[23] (see Supporting Information for details), an IC_{50} of 25 nM was obtained for benzothiazole **5** (K_i = 6.8 nM). Lead compound 2-(3,4-dimethoxyphenyl)-5-fluorobenzothiazole **5** is therefore a high affinity ligand for the aryl hydrocarbon receptor (AhR).

Depletion of 5 from Nutrient Media. Depletion of benzothiazole **5** from nutrient media was monitored by HPLC across a range of sensitive and insensitive cell lines (see Supporting Information for details). Rapid depletion of **5** from nutrient media supporting sensitive MCF-7 cells was observed; 50% depletion occurred within 6 h. Insensitive breast cancer cell lines, MDA 231 and MDA 435, were significantly less able to sequester **5**, and only 30% was depleted from the nutrient media after 72 h. Sensitive colon cell lines KM12 and HCC 2998 consumed **5** from nutrient media less avidly than MCF-7 cells, with approximately 50% depletion after 24 h (Supporting Information).

Intriguingly, the AhR antagonist α-naphthoflavone (α-NF)[24] drastically reduced sequestration of benzothiazole **5** by sensitive MCF-7 cells, with <45% depletion of **5** from medium after 72 h. Correspondingly, α-NF (10 μM) diminished the activity of **5** in MCF-7 and MDA 468 breast cancer cell lines, whereas alone, **5** inhibited the growth of MCF-7 and MDA 468 cell lines with GI_{50} < 1 nM (Table 1). In the presence of 10 μM α-NF, **5** elicited significantly weakened (>2000-fold) growth inhibitory activity against MCF-7 and MDA 468 cells, where GI_{50} values of 0.514 and 0.27 μM were achieved, respectively (Supporting Information).

Conclusions

The synthesis of a range of 2-phenyl-benzothiazoles, -benzoxazoles, and -chromen-4-ones related to the potent antitumor lead compound 2-(3,4-dimethoxyphenyl)-5-fluorobenzothiazole **5** has been accomplished. Evaluation against the MCF-7 and MDA 468 breast cancer cell lines revealed compounds within the new series with potent (submicromolar GI_{50}) activity in both cell lines (e.g., **9a**,**b** and **12a**,**d**). Although none of the new series was able to recapitulate the potent antitumor properties of **5**, the new compounds were significantly more active than the structurally related benzimidazoles.[20] Antitumor potency appeared to correlate with the emergence of a G_2/M cell cycle block. For lead compound **5**, binding to the aryl hydrocarbon receptor appeared to play an important role in growth inhibition. Work continues to identify further potent antitumor compounds from this intriguing class and to further delineate the antitumor mode of action.

Experimental Section

Chemistry. Melting points were measured on a Griffin apparatus and are uncorrected. Mass spectra were recorded on a Bruker MicroTOF LC instrument. NMR spectra were recorded on a Bruker AVANCE 500 MHz instrument; coupling constants (*J*) are in Hz. Merck silica gel 60 (40−60 μM) was used for column chromatography. All commercially available starting materials were used without further purification.

General Method A for the Synthesis of 2-Arylbenzothiazoles (9a−j). Substituted disulfides (**8a**−**i**, 0.71 mmol), prepared in 35−75% yields by hydrolysis of 2-aminobenzothiazoles (**7a**−**i**) in

refluxing aqueous KOH followed by air oxidation as previously described,[10] were heated under reflux in toluene (20 mL) containing PPh_3 (0.185 g, 0.71 mmol) and *p*-TsOH (2 mg) for 3 days. After the mixture was cooled, solvent was removed in vacuo and the benzothiazoles were purified by column chromatography (EtOAc/hexane, 1:4). ^{1}H NMR data for disulfides (**8a**−**i**) are given in Supporting Information. Microanalytical data and spectroscopic properties for benzothiazoles **9a**−**j** in given in Supporting Information.

General Method B for the Synthesis of *o*-Bromobenzanilides (11a−**r).** Substituted benzoyl chloride (10 mmol) was added to a mixture of 4- or 5-fluoro-2-bromoaniline (**10a**,**b**, 10 mmol), triethylamine (11 mmol), and 4-dimethylaminopyridine (2 mmol) in CH_2Cl_2 (20 mL), and the mixture was stirred at room temperature for 18 h. The mixture was diluted with further dichloromethane (30 mL), then washed with 1 M HCl (50 mL), and the aqueous fraction was extracted with dichloromethane (50 mL). The combined organic layers were dried ($MgSO_4$), filtered, and concentrated in vacuo. The crude product benzanilides were checked for purity (^{1}H NMR) and then used directly in the next step.

General Method C for the Synthesis of 2-Phenylbenzoxazoles 12a−**r.** To a mixture of *o*-bromobenzanilide **11a**−**r** (5 mmol), copper(I) iodide (47.5 mg, 0.25 mmol), 1,10-phenanthroline (94 mg, 0.5 mmol), and cesium carbonate (2.45 g, 7.5 mmol) was added DME (50 mL) at room temperature, under a nitrogen atmosphere. The mixture was heated under reflux for 18 h and then allowed to cool to room temperature. The mixture was diluted with water (100 mL) and then CH_2Cl_2 (200 mL). The organic layer was extracted, then dried ($MgSO_4$), filtered, and concentrated in vacuo. The crude product was purified by column chromatography (diethyl ether/petroleum ether) to give the required substituted 2-arylbenzoxazole in 61−91% yields.

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Supporting Information Available: Spectroscopic and analytical data for new compounds and intermediate structures; microanalytical data (CHN) for new compounds; details on cell culture, growth inhibitory assay, cell cycle analyses, aryl hydrocarbon receptor binding assay, uptake/stability studies; two figures showing depletion of **5** from nutrient media and effect of α-naphthoflavone on growth and viability of MCF-7 and MDA 468 cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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