FLUORINATED 2-(4-AMINO-3-METHYLPHENYL)BENZOTHIAZOLES INDUCE CYP 1A1 EXPRESSION, BECOME METABOLIZED AND BIND TO MACROMOLECULES IN SENSITIVE HUMAN CANCER CELLS

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List of nonstandard abbreviations: 5F 203, 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (NSC 703786); 6F 203, 2-(4-amino-3-methylphenyl)-6-fluorobenzothiazole (NSC 702156); 5,6 di-F 203, 2-(4-amino-3-methylphenyl)-5,6-difluorobenzothiazole (711671); 4F 203, 2-(4-amino-3-methylphenyl)-4-fluorobenzothiazole (NSC 706705); 7F 203 2-(4-amino-3-methylphenyl)-7-fluorobenzothiazole (NSC 711670); DF 203, 2-(4-amino-3-methylphenyl)benzothiazole (NSC 674495); 6-OH 203, 2-(4-amino-3-methylphenyl)-6-hydroxybenzothiazole (NSC 703785); Phortress, lysylamide prodrug of 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole; HPLC, high-performance liquid chromatography; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide; AhR, aryl hydrocarbon receptor; QRT-PCR, quantitative real-time reverse transcription-polymerase chain reaction; PBS, phosphate buffered saline; XRE, xenobiotic response element; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; PVDF, polyvinylidene fluoride; TBS, tween buffered solution; GI₅₀, concentration of compound needed to cause 50% growth inhibition; TGI, total growth inhibition; LC₅₀, concentration of compound needed to cause 50% cell death (cytocidal activity).
Abstract

Fluorinated 2-(4-amino-3-methylphenyl)benzothiazoles possess potent anti-proliferative activity against certain cancer cells similar to the unfluorinated 2-(4-amino-3-methylphenyl)benzothiazole (DF 203, NSC 674495). In “sensitive” cancer cells, DF 203 is metabolized by, can induce expression of, and bind covalently to CYP1A1. Metabolism appears to be essential for its anti-proliferative activity through DNA adduct formation. However, a biphasic dose-response relationship compromises its straightforward development as a chemotherapeutic agent. We investigated whether fluorinated benzothiazoles inhibit cancer cell growth without the biphasic dose-response, and whether the fluorinated benzothiazoles are also metabolized into reactive species, with binding to macromolecules in sensitive cancer cells. One fluorinated benzothiazole, 2-(4-amino-methylphenyl)-5-fluorobenzothiazole (5F 203, NSC 703786) did exhibit potent, anti-proliferative activity without a biphasic dose-response. The fluorinated benzothiazoles were also metabolized only in cells, which subsequently showed evidence of cell death. We used microsomes from genetically engineered human B-lymphoblastoid cells expressing cytochromes P450 (CYP1A1, CYP1A2 or CYP1B1) to clarify the basis for fluorinated benzothiazole metabolism. 5F 203 induced CYP1A1 and CYP1B1 mRNA expression in sensitive breast and renal cancer cells, while 5F 203 induced CYP1A1 mRNA but not CYP1B1 mRNA expression in sensitive ovarian cancer cells. 5F 203 did not induce CYP1A1 or CYP1B1 mRNA expression in any “resistant” cancer cells. The fluorinated benzothiazoles induced CYP1A1 protein
expression exclusively in sensitive cells. $[^{14}C]$.-F 203 bound substantially to subcellular fractions in sensitive cells but only minimally in resistant cells. These data are concordant with the anti-proliferative activity of fluorinated benzothiazoles deriving from their ability to become metabolized and bind to macromolecules within sensitive cells.
Benzothiazoles contain a benzene ring fused to a thiazole ring (Shi et al., 1996). These small molecules display anti-tumor properties that are modulated by substitutions at specific positions on the benzothiazole pharmacophore (Chua et al., 1999; Kashiyama et al., 1999b; Wells et al., 2000; Hutchinson et al., 2001; Hutchinson et al., 2002). 2-(4-Amino-3-methylphenyl)benzothiazole (DF 203) represents the lead compound for a recently explored series, and possesses potent anti-tumor properties in select breast, ovarian and renal cancer cell lines of the 60-cell line, disease-oriented NCI Anticancer Drug Screen (Hose et al., 2003). DF 203 displays a unique biphasic dose-response relationship, with more potent activity at concentrations below 1 µM but reduced anti-proliferative activity at concentrations ranging from 1-100 µM. The precise mechanism by which DF 203 and similar benzothiazoles display their anti-proliferative effects has not been fully elucidated. Recent studies, however, suggest that their ability to become metabolized and covalently bind to subcellular targets contributes to their anti-cancer effects (Kashiyama et al., 1999b; Chua et al., 2000; Hutchinson et al., 2001). Metabolism and covalent binding to subcellular macromolecules have been shown to play a pivotal role in the anti-cancer activity of other small molecules (Rivera et al., 1999; Efferth et al., 2001; Kuffel et al., 2002). A prior study demonstrated that co-incubation of DF 203 with alpha-naphthoflavone, a CYP1A1 inhibitor, in sensitive cells can inhibit anticancer activity, which reveals the critical involvement of CYP1A1 in the anticancer activity of the benzothiazoles (Chua et al., 2000).
The fluorinated benzothiazoles (Figure 1) represent a novel class of anti-cancer drug candidates that, similar to DF 203, potently and selectively inhibit the growth of renal, breast and ovarian cancer cell lines and thus retain a pattern of differentiated growth inhibition (Chua et al., 2000). 5F 203 represents one of a series of fluorinated 2-(4-amino-3-methylphenyl)benzothiazoles synthesized to circumvent the biphasic dose-response relationship thought to thwart the anti-proliferative activity of the benzothiazoles (Hutchinson et al., 2001).

As prior reports have shown, DF 203 covalently binds to DNA forming adducts exclusively in sensitive, growth-inhibited cells (Kashiyama et al., 1999b; Trapani et al., 2003). Other recent studies have shown that sensitive MCF-7 breast and IGROV1 ovarian cancer cells treated with 5F 203 formed DNA adducts, including a dominant adduct distinct from that formed following their treatment with DF 203 (Leong et al., 2003; Trapani et al., 2003). Therefore, we sought to clarify whether 5F 203 binds to subcellular macromolecules in addition to DNA in growth-inhibited sensitive cancer cells.

In this study, we determine the ability of the fluorinated benzothiazoles to induce CYP1A1 mRNA and protein expression in sensitive cells and whether CYP1A1 might play a pivotal role in the metabolism and anti-proliferative activity of these drug candidates corroborating previous investigations (Hose et al., 2003). Radiolabeled 5F 203 was used to reveal the extent that 5F 203 binds to subcellular fractions in several cancer cell lines. Metabolism of the fluorinated benzothiazoles and their binding to subcellular macromolecules may constitute a basis for the mechanism of growth inhibition for these and related compounds.
Materials and Methods

Chemicals and Reagents. The fluorinated benzothiazoles were synthesized following published methods (Hutchinson et al., 2001). Stock solutions (10 mM) of the fluorinated benzothiazoles were prepared in DMSO or acetonitrile and stored protected from light at -20°C. [14C]5F 203 (14C at the C-2 position of the thiazole ring, 40.5 mCi/mmol) was synthesized at the Research Triangle Institute (Research Triangle Park, NC). The product was 98% pure as determined by radiochemical detector (TLC) and HPLC. For all experiments, [14C]5F 203 was dissolved in DMSO and stored as a 10 mM stock solution at -70°C. HPLC-grade acetonitrile was purchased from Baker (Philipsburg, NJ). All other chemicals were purchased from Sigma-Aldrich (Milwaukee, WI) and were of the highest commercial grade available. NADPH was purchased from Calbiochem (La Jolla, CA). Cell culture medium and supplements were purchased from Quality Biological, Inc. (Gaithersburg, MD) except for heat-inactivated fetal bovine serum, which was purchased from Hyclone Laboratory, Inc. (Logan, UT). Leupeptin, AEBSF and Aprotinin were purchased from Sigma (St. Louis, MO). Antibodies specific for CYP1A1 (polyclonal antiserum for human CYP1A1/1A2), and microsomes from genetically engineered human B-lymphoblastoid cells expressing cytochromes P450 (CYP1A1, CYP1A2 or CYP1B1) were obtained from Gentest Corp. (Woburn, MA). ECL kits were purchased from Amersham (Piscataway, NJ). Gentest Corp. (Woburn, MA).

In vitro cell culture. The human breast (MCF-7 and MDA-MB-435), ovarian (IGROV1 and SKOV3) and renal (TK-10 and CAKI-1) cancer cell lines were
obtained from the NCI-Frederick repository. The cell types, sources, stocks, morphological characteristics and methods of propagation of human cancer cell lines have been previously described (Alley et al., 1988; Stinson et al., 1992). Monolayers of cells were cultured at 37°C in an atmosphere of 5% CO₂/95% air in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100-µg/ml streptomycin. Cells were maintained continuously in the logarithmic (sub-confluent) growth phase with routine sub-culturing.

**Sulforhodamine B dye assay.** The methods used for the 60-cell line panel have been described elsewhere (Monks et al., 1997). Briefly, compounds were solubilized in dimethylsulfoxide at 200X. The compounds were diluted into RPMI-1640 containing 10% FBS and serial 1-log dilutions were prepared for a total of 5 concentrations. Generally, the working range for initial testing of each benzothiazole was 10⁻⁴ through 10⁻⁸ molar. The compounds were added to cultures of each of the 60 cell lines used in the panel. Following 48 h of exposure to the benzothiazoles, media was removed; the cells were fixed and stained with sulforhodamine B and the total stain quantitated by optical density determinations. Using time 0 as control, cell growth was determined for each cell line and the concentrations needed to inhibit growth 50% (GI₅₀), to cause total growth inhibition (TGI) and to produce 50% cytocidal activity (LC₅₀) were calculated.

**In vitro time course MTT assay.** The cell lines and concentration ranges of benzothiazoles evaluated in the concentration x time (c x t) assays were chosen on the basis of 60-cell line screening data. As described elsewhere
(Hollingshead et al., 2004), cells were exposed to the fluorinated benzothiazoles for increasing periods of time before anti-cancer activity was determined using each of several exposure durations ranging from \(< 1\) h to 144 h. A “stable endpoint” MTT formazan colorimetric endpoint permitted comparative quantitation of anti-cancer activity in replicate cell culture plates. From the plot of the composite \(c \times t\) data, the minimum exposure conditions (both concentration and time) required to achieve anti-proliferative (GI\(_{50}\)) cytostatic (TGI) and/or cytotoxic (LC\(_{50}\)) activity in a given cell line were readily determined. Use of \(c \times t\) indices of GI\(_{50}\), TGI, and LC\(_{50}\), readily permit numerical characterization and comparison of the sensitivities of multiple cell lines as well as the relative activity of the fluorinated benzothiazoles toward one or more cell lines. In addition, such indices provide a means to determine the concentration ranges of a given agent required to confer growth inhibition, cytostasis and/or cytotoxicity.

**Microsomal metabolism of 5F 203, 6F 203 and 5,6 di-F 203.** The ability of microsomes from human B-lymphoblastoid cells expressing human CYP1A1, CYP1A2 and CYP1B1 to metabolize the fluorinated benzothiazoles was determined by previously described methods (Kashiyama et al., 1999b; Chua et al., 2000). A typical reaction contained 100 mM K\(_2\)PO\(_4\), 5 mM MgCl\(_2\), the appropriate fluorinated benzothiazole (10 \(\mu\)M), 1 mg/mL microsomal protein and 10 mM NADPH. Control reactions contained microsomes from lymphoblastoid cells containing empty vector. Mixtures were incubated at 37°C with gentle agitation for 2 h. Aliquots were mixed with a 3-fold volume of cold acetonitrile to
terminate the reaction. The mixture was centrifuged and supernatants analyzed by HPLC with concurrent UV and fluorescence detection.

**Cellular uptake and metabolism of 5F 203, 6F 203 and 5,6 di-F 203.** MCF-7, MDA-MB-435, TK-10, CAKI-1, IGROV1 and SKOV3 cells in the logarithmic growth phase were passaged into 25 cm² flasks and allowed to adhere for 24-48 h until they reached 50-60% confluency. Cell medium was then replaced with medium containing 1.0 µM of either 5F 203, 6F 203 or 5,6-diF 203. Control flasks contained either the respective benzothiazoles without cells or cells treated with vehicle alone (0.1% DMSO). Aliquots of cell medium were removed at 24-h intervals for at least 3 days of exposure. In 5F 203-treated MCF-7 cells, aliquots of cell medium were also removed following 12, 14 and 16 h of incubation. All aliquots were analyzed by HPLC as described previously (Chua et al., 2000; Hutchinson et al., 2001). The percentage of each fluorinated benzothiazole remaining was determined by comparing chromatographic peak areas of the fluorinated benzothiazoles at time zero with those detected during specific periods of incubation.

**5F 203 cell treatment and RNA extraction.** MCF-7, TK-10, IGROV1, MDA-MB-435, CAKI-1 and SKOV3 cells grown to 60-80% confluency were treated with 0.01, 0.1 or 1.0 µM 5F 203 for 12 or 24 h. Control flasks received vehicle alone (0.1% DMSO). Cells were harvested with Trypsin and total cellular RNA extracted from 1.5-5.0 x 10⁵ cells according to the RNeasy method (Qiagen Inc., Chatsworth, CA). The extracted RNA was stored at -20°C until further evaluation. The concentration and purity of the RNA were determined by
measurement of the optical densities at 260 and 280 nm. A ratio of > 1.7 for $A_{260}/A_{280}$ was required for use in these studies.

**Real-time quantitative RT-PCR analysis.** Real-time quantitative RT-PCR analysis was used to evaluate CYP1A1 and CYP1B1 gene expression in 5F 203-treated cells. The methodology including the primers and probes used has been previously described in detail (Loaiza-Perez et al., 2002). PCR efficiencies were validated by means of a standard curve.

**Western Immunoblot Analysis of CYP1A1.** CYP1A1 expression was determined in MCF-7, TK-10 and MDA-MB-435 cells treated with the fluorinated benzothiazoles (24 h, 1 µM) using a method previously described in detail (Chua et al., 2000). In brief, whole cell lysates were prepared and protein content estimated by Coomassie Blue assay (Pierce Biotechnology, Rockford, IL) using bovine serum albumin as standard. Solubilized proteins (50 µg per well) were loaded on 10% Tris-Glycine pre-cast gels (Novex) and transferred in a submerged transfer unit (NOVEX Xcell II) to polyvinylidene fluoride (PVDF) membranes, in Tris glycine transfer buffer (Novex) containing 5% methanol for 90 min at 125 V and 4°C. Membranes were then blocked with blocking buffer (PBS-0.1% Tween 20 (T) and 10% non fat dried milk) for 1 h at room temperature and rinsed (3 x 5 min with TBS-0.1%T and 2 X 5 min with TBS). Blots were then incubated for 2 h at room temperature with polyclonal goat anti-human primary antibody (1:500 dilution in TBS-0.02% T, 1% milk) and rinsed (3 x 5 min with TBS-0.1%T and 2 X 5 min with TBS). Membranes were incubated for 1 h at room temperature with the horseradish peroxidase-conjugated rabbit anti-
goat secondary antibody (Gentest Corp, Woburn, MA, 1:80,000 dilution in TBS-
0.02% T, 1% dried non fat milk) and rinsed (3 x 5 min with TBS-0.1% T and 2 X 5
min with TBS). Enhanced chemiluminescence (ECL) Western Blotting Detection
Kit (Amersham) was used as recommended by the manufacturer to detect
CYP1A1 expression.

Subcellular binding analysis. MCF-7, MDA-MB-435, TK-10, CAKI-1, IGROV1
and SKOV3 cells were grown to 60-80% confluency in 560 cm² tissue culture
plates (PGC Scientifics, Frederick, Maryland). Cells were treated with 0.1 µM
[^14C]5F 203 (40.5 mCi/mmol) for 16 h at 37°C before being harvested. After
cells were centrifuged and homogenized, subcellular fractions were isolated by
differential centrifugation in a similar manner as described previously (Jones et
al., 1995). Protein concentrations were determined by the Coomassie Blue
assay (Pierce Biotechnology, Rockford, IL) using bovine serum albumin as
standard. The resultant fractions were stored at –70°C until further analysis.
Proteins in samples were precipitated with trichloroacetic acid (TCA) and
covalent binding of[^14C]5F 203 to subcellular fractions was determined using a
previously described method (Rivera et al., 1999).

Statistics. All data were analyzed with a one-way ANOVA with Tukey’s post test
performed using GraphPad InStat version 3.00 for Windows 95, GraphPad
Software, San Diego California USA, www.graphpad.com. Data are reported as
means ± SD.
Results

5F 203, 6F 203 and 5,6 di-F 203 inhibit the growth of selected cancer cell lines. Sulforhodamine B dye and MTT assays were used to determine the anti-proliferative activity of select fluorinated benzothiazoles. The fluorinated benzothiazoles 5F 203, 6F 203 and 5,6 di-F 203 had potencies similar to DF 203 in suppressing the growth of specific breast, renal and ovarian cancer cell lines in the NCI primary anti-cancer drug screen (Figure 2). MCF-7 and TK-10 cells displayed exceptional sensitivity to 5F 203, 6F 203 and 5,6 di-F 203 (GI_{50} < 15 nM, Table 1). The IGROV1 cell line was more sensitive to 5F 203 and 5,6 di-F 203 (GI_{50} < 10 nM) than to 6F 203 which had a GI_{50} value of 1.28 µM. CAKI-1, SKOV3 and MDA-MB-435 cells were resistant to the fluorinated benzothiazoles (GI_{50} > 20 µM). For this reason, those resistant cell lines were chosen as negative controls for subsequent studies.

The fluorinated benzothiazoles were synthesized in an attempt to overcome the biphasic dose-response curve in MCF-7 and TK-10 cells treated with DF 203; yet a distinct biphasic dose-response curve resulted when these cells were treated with 6F 203. This biphasic response was present, albeit less pronounced, when MCF-7 and TK-10 cells were treated with 5,6 di-F 203 (Figure 2A, B). In contrast, 5F 203 potently inhibited the growth of MCF-7, IGROV1 and TK-10 cells without this characteristic biphasic dose-response curve (Figure 2A-C). Interestingly, we did not observe a biphasic dose-response curve in IGROV1 cells treated with either of the fluorinated benzothiazoles or with DF 203 (Figure 2C).
In addition to anti-proliferative activity (which is defined as the ability of these drug candidates to inhibit cancer cell growth), the fluorinated benzothiazoles at sub-micromolar concentrations conferred cytostasis (which is defined as that concentration needed to cause total growth inhibition) following brief exposures in multiple cell lines. In the *in vitro* assays, as summarized in Table 2, each of the fluorinated benzothiazoles inhibited the growth of MCF-7 cells with GI$_{50}$ values < 0.03 µM and TGI values < 0.5 µM. However, none of these derivatives displayed substantial cytotoxicity in MCF-7 cells even with 48- or 144-hour exposures to 100 µM (tables 1 and 2). LC$_{50}$ activity was achieved in TK-10 following < 1 hr x 6 µM, whereas IGROV1 required >24 hours x 0.74 µM. By contrast, 5F 203, 6F 203 and 5,6 di-F 203 failed to confer LC$_{50}$ activity in MCF-7 cells even following 144 h x 100 µM (Table 2, Figure 3C).

**Sensitive ovarian, breast and renal cancer cells take-up and metabolize the fluorinated derivatives.** Previous studies demonstrated that DF 203 uptake and biotransformation occurs exclusively in growth-inhibited, sensitive cells (Chua et al., 1999; Kashiyama et al., 1999b; Chua et al., 2000). To determine whether sensitive cells also take-up and biotransform the fluorinated benzothiazoles, aliquots of cell culture medium from both sensitive cells and resistant cells treated with 1 µM 5F 203, 6F 203 or 5,6 di-F 203 for at least 3 days were analyzed using HPLC. The concentrations of fluorinated benzothiazoles in nutrient medium from MCF-7, IGROV1 and TK-10 sensitive cell lines decreased with increasing exposure times (Figure 4A-C). In MCF-7 and IGROV1 cells, the decline in peak areas corresponding with 5F 203, 6F 203 and 5,6 di-F 203 was
accompanied by the concurrent emergence of chromatographic peaks of yet uncharacterized metabolites (data not shown). When incubated with TK-10 cells, 5F 203 and 5,6 di-F 203 completely disappeared from culture medium within 2 to 3 days, but 40% of 6F 203 remained following the 3-day incubation (Figure 4C). Complete disappearance of 6F 203 from the culture medium of TK-10 cells required more than 6 days of incubation (data not shown). Following a 12-h incubation, 5F 203 peak areas were reduced by 40% and 50% (10 µM and 5 µM respectively) in MCF-7 cells resulting in the emergence of three uncharacterized, minor metabolites (Figure 5A). The concentration levels of these metabolites in the culture medium increased with incubation time (Figure 5B, C).

In contrast, the concentrations of all three fluorinated benzothiazoles remained largely unchanged even after 3 days of incubation in resistant MDA-MB-435, CAKI-1, and SKOV3 cells (Figure 4D-F). Only 70% of the original amount of 6F 203 remained in medium after 2 days of incubation in CAKI-1 cells, however we detected no additional 6F 203 disappearance even following 6 days of incubation (data not shown). No metabolite peaks were observed in aliquots of media from any of the insensitive cells incubated with the fluorinated benzothiazoles.

**CYP1A1, CYP1A2 and CYP1B1 metabolize 5F 203, 6F 203 and 5,6 di-F 203.** DF 203 has been previously shown to mediate its anticancer activity by activating the aryl hydrocarbon receptor (AhR) signaling pathway (Loaiza-Perez et al., 2002). The AhR signaling pathway modulates the transcription of genes
encoding CYP1A1, CYP1B1 and CYP1A2. Since these microsomal proteins are able to metabolize DF 203, we suspected that these CYPs might also play a role in fluorinated benzothiazole metabolism. The metabolism of 5F 203, 6F 203 and 5,6 di-F 203 was investigated using microsomes from human B-lymphoblastoid cells expressing human recombinant CYP1A1, CYP1A2 or CYP1B1. Following a 2-h incubation, peak areas of the fluorinated benzothiazoles in the chromatograms declined (Figure 6) while peak areas corresponding to uncharacterized metabolites emerged. These metabolites were initially detected after 30 min of incubation (data not shown). All three CYP1 isoforms extensively metabolized fluorinated benzothiazoles following the 2-h incubation, but CYP1A1 exhibited the greatest capacity to metabolize the benzothiazoles (10 \( \mu \)M, Figure 6A). Isoteric replacement of hydrogen with fluorine at the 5’ position successfully blocked the formation of 2 metabolites, which likely included the 6-OH 203 metabolite. CYP1A1 microsomes produced metabolites with greater peak areas compared with those produced from CYP1A2 and CYP1B1 microsomes. No peaks corresponding to metabolites resulted from reaction mixtures containing microsomes derived from cells with empty vector.

**5F 203 induces CYP1A1 and CYP1B1 mRNA expression in sensitive cancer cell lines.** Previous studies have demonstrated that benzothiazoles inhibit the growth of sensitive cancer cells concurrent with induction of CYP1A1 and, to a lesser extent, CYP1B1 expression (Chua et al., 2000; Loaiza-Perez et al., 2002; Trapani et al., 2003). We used quantitative real-time RT-PCR (QRT-PCR) to determine whether 5F 203, which lacks a multi-phasic quality to the dose-
response curve for growth inhibition, induced CYP1A1 and CYP1B1 mRNA expression in sensitive (MCF-7, TK-10 and IGROV1) cells or resistant (MDA-MB-435, CAKI-1 and SKOV3) cells. In general induction of CYP1A1 expression was greater than that seen for CYP1B1 expression relative to controls. CYP1A1 expression increased 2.5-fold when the concentration increased from 0.01 µM to 0.1 µM and 8-fold when the concentration increased from 0.1 to 1.0 µM following 12 h 5F 203 treatment of MCF-7 cells. We detected a 4-fold and 2.5-fold increase in CYP1A1 expression corresponding to increases in concentration from 0.01 µM to 0.1 µM and from 0.1 µM to 1.0 µM respectively following 24 h 5F 203 treatment of these cells (Figure 7A). CYP1A1 expression increased in a time- and dose-dependent manner (1.5-2-fold) in TK-10 cells treated with 5F 203 compared to untreated controls (Figure 7B). A 10-fold increase in CYP1A1 expression occurred when IGROV1 cells were treated with 5F 203 corresponding to an increase in concentration from 0.1 µM to 1.0 µM (24 h, Figure 7C). However, lower concentrations and shorter exposure times resulted in no increases in CYP1A1 expression relative to untreated controls. CYP1B1 expression increased 8-fold in MCF-7 cells treated with 1.0 µM compared with 0.1 µM 5F 203 (12 h; Figure 8A). CYP1B1 expression increased approximately 5-fold in MCF-7 cells treated with 0.1 µM compared with 0.01 µM 5F 203 (24 h; Figure 8A). Similarly to induction of CYP 1A1 expression, CYP1B1 expression increased in a time- and dose-dependent manner (1.5-2-fold) in TK-10 cells treated with 5F 203 (Figure 8B). On the other hand, none of the resistant cells demonstrated increases in either CYP1A1 or CYP1B1 mRNA expression in
comparison to untreated controls (Figures 7-8). CYP1B1 mRNA induction observed under all exposure conditions in IGROV1 cells resembled untreated controls (Figure 8C).

**Fluorinated benzothiazoles induce CYP1A1 expression in sensitive MCF-7 and TK-10 cells.** Previous studies indicated that DF 203 induced CYP1A1 expression in MCF-7 cells but not in MDA-MB-435 cells (Chua et al., 2000). To determine whether the fluorinated benzothiazoles induced CYP1A1 protein expression differentially in sensitive cells compared with resistant cells, we used immunoblot analysis to detect CYP1A1 protein expression in MCF-7, TK-10 and MDA-MB-435 cells that were treated with several fluorinated benzothiazoles including the lysylamide prodrug of 5F 203 (Phortress). Western blot analysis revealed that 4F 203, 5F 203, 6F 203, 5,6 di-F 203 and Phortress induced CYP1A1 expression in sensitive MCF-7 cells while 7F 203 caused only minimal CYP1A1 induction (1.0 µM, 24 h, Figure 9A). CYP1A1 induction was less pronounced in sensitive TK-10 cells, similarly to what has been previously shown for DF 203 (Chua et al., 2000), even though TK-10 and MCF-7 cells showed comparable sensitivities to the fluorinated benzothiazoles (1.0 µM, 24 h, Figures 2, 3, and 9B). CYP1A1 induction was prominent after TK-10 cells were exposed to 5F 203, 6F 203, 5,6 di-F 203 and Phortress and to a much lesser extent when these cells were treated with 4F 203 and 7F 203. Figure 9C shows that, similar to DF 203, the fluorinated benzothiazoles were unable to induce CYP1A1 protein expression in resistant MDA-MB-435 cells (Chua et al., 2000; Bradshaw et al., 2002a; Loaiza-Perez et al., 2002).
5F 203 binds to subcellular macromolecules in sensitive cancer cells. DF 203 covalently binds to DNA exclusively in sensitive cancer cells (Kashiyama et al., 1999b). We measured subcellular localization of \([^{14}\text{C}]5\text{F 203}\) within sensitive and resistant cancer cells to determine if a correlation exists between the ability of 5F 203 to bind to subcellular macromolecules within cells and the sensitivity of cancer cells to 5F 203. We found that \([^{14}\text{C}]5\text{F 203},\) or one or more metabolite(s), bound to macromolecules in nuclear, mitochondrial, microsomal and cytosolic fractions of the sensitive cell lines (Figure 10). In MCF-7 cells, the majority of bound radioactivity was localized in the nuclear and microsomal fractions along with a considerable amount in the mitochondrial fraction (Figure 10A). A similar pattern was observed when ZR-75-1 cells (another estrogen receptor-positive breast cancer cell line) were treated with \([^{14}\text{C}]5\text{F 203}\) (data not shown). The subcellular fractions of IGROV1 cells showed relatively indistinct differences in their capacities to bind to 5F 203 (Figure 10B). The extent of binding to subcellular fractions in this cell line was less pronounced than in MCF-7 and TK-10 cells, possibly because CYP1A1 expression is not induced to as great extent in IGROV1 cells as MCF-7 and TK-10 cells following 5F 203 treatment. Substantial \([^{14}\text{C}]5\text{F 203}\) binding occurred in the microsomal fraction of TK-10 cells while the mitochondrial and nuclear fractions also showed considerable binding (Figure 10C). A minimal amount of bound radioactivity in subcellular fractions of resistant cell lines MDA-MB-435 and SKOV3 was detected (Figure 10A,B). Nuclear binding of \([^{14}\text{C}]5\text{F 203}\) was minimal yet more apparent in
nuclear fractions of CAKI-1 cells (Figure 10C). This was anticipated since CAKI-1 cells were less resistant to 5F 203 than either MDA-MB-435 or SKOV3 cells.
Discussion

In this investigation, we provide additional insight into the mechanism governing the anti-proliferative effects of the fluorinated 2-(4-amino-3-methylphenyl)benzothiazoles in certain cancer cell types. Similar to DF 203, and consistent with previous studies, the fluorinated benzothiazoles induced CYP 1A1 mRNA and protein expression exclusively in “sensitive” breast, renal and ovarian cancer cells (Kashiyama et al., 1999a; Chua et al., 2000; Hose et al., 2003; Monks et al., 2003). Our data clearly demonstrate that sensitive cells and cytochromes P450 (1A1, 1A2 and 1B1) readily metabolized the fluorinated benzothiazoles. In addition, we show that [14C]5F 203 or its metabolite(s) bound to macromolecules in cells proportionate to the cells’ sensitivity to the growth inhibitory effects of 5F 203. Interestingly, we demonstrate that these metabolites bound not only to the nuclear fraction, as would be expected from a prior demonstration of DNA adduct formation (Leong et al., 2003; Cain, 2003), but also to mitochondrial, microsomal and cytosolic targets.

The fluorinated 2-(4-amino-3-methylphenyl)benzothiazoles were originally synthesized to circumvent the formation of the inactive metabolite 6-OH 203, which likely contributes to the biphasic dose-response relationship of DF 203 (Bradshaw et al., 1998b; Bradshaw et al., 2001; Hutchinson et al., 2001). In this study, fluorination of the benzothiazole in the 5-position resulted in a monophasic dose-response curve as opposed to a biphasic dose-response curve for growth inhibition. This finding therefore obviates the concern that dose escalation in
clinical trials would need to account for the possibility that higher doses may actually compromise potential efficacy.

Fluorination of small molecules frequently blocks metabolic hydroxylation, which can modulate their anti-cancer activity (Akama et al., 1997). 6F 203-treated MCF-7 and TK-10 cells displayed a biphasic dose-response curve, corroborating findings from a previous investigation (Hutchinson et al., 2001). The biphasic dose-response detected, albeit in a less pronounced fashion, in 5,6-diF 203-treated MCF-7 and TK-10 cells was unanticipated since a previous investigation predicted that the difluorinated benzothiazoles, including 5F 203, would produce neither exportable metabolites nor a biphasic dose-response curve (O'Brien et al., 2003). Using an HPLC with an exceptionally low limit of detection, we were able to detect minor metabolites resulting from both 5,6 di-F 203-treated cells and 5F 203-treated cells to a comparable extent (data not shown). It is possible that “low level” metabolites were produced which antagonized the anti-proliferative activity of 5,6 di-F 203. A biphasic dose-response curve was previously observed when IGROV1 cells were treated with DF 203 containing 3’ halogen substitution and a pronounced biphasic dose-response curve was detected in OVCAR-5 ovarian cells treated with nonhalogenated DF 203 (Bradshaw et al., 1998a). Thus, the tendency of a benzothiazole to produce a biphasic dose-response curve may depend on the cancer cell type, the presence or absence of functional groups on the benzothiazole pharmacophore, or the extent the benzothiazole metabolizes into products that might antagonize its anti-proliferative activity.
This study demonstrated that the fluorinated benzothiazoles were capable of producing anti-proliferative, cytostatic and in some instances cytotoxic effects exclusively in sensitive cancer cell lines. It was unanticipated that the fluorinated benzothiazoles would not produce cytotoxic activity even following extensive exposures (> 6d) in MCF-7 cells since previous studies indicate that the most potent fluorinated benzothiazole 5F 203 produces apoptosis and DNA damage in MCF-7 cells which is characteristic of cytotoxic activity (Loaiza-Perez et al., 2002; Trapani et al., 2003). While the endpoints of protein mass measured in SRB assays and the viable cell mass measured in MTT assays used in this study were unable to reveal substantial cytotoxicity (LC$_{50}$ activity) in MCF-7 cells with 6-day exposure to high drug concentration, such activity was readily detectible in IGROV-1 and TK-10 cells under the same in vitro assays conditions.

Cytochromes P450 (CYP1A1, CYP1A2 and CYP1B1) have been shown to metabolize anti-cancer drugs (Kashiyama et al., 1999b; Rochat et al., 2001) and pro-drugs (Sladek, 1994). CYP1A1 preferentially metabolizes DF 203 to an inactive hydroxyl metabolite as well as a reactive electrophilic species, which frontier molecular orbital studies predict as being a nitrenium ion (O’Brien et al., 2003). In this study, CYP1A1 preferentially metabolized 5F 203, 6F 203 and 5,6 di-F 203. This is consistent with observations that a few anti-cancer agents induce the expression of cytochromes P450 in human cancer cells, subsequently resulting in their metabolism to display their cytotoxic effects (Chase et al., 1998; Jounaidi et al., 1998). 5F 203 treatment caused a stronger induction of CYP1A1 mRNA and protein expression in sensitive MCF-7 cells than in sensitive TK-10
cells, supporting results from previous investigations (Bradshaw et al., 2002a; Hose et al., 2003; Monks et al., 2003).

The AhR signaling pathway activates the transcription of CYP1A1, CYP1B1 and CYP1A2 genes (Whitlock, 1999) and mediates the sensitivity of MCF-7 cells to the anti-cancer effects of DF 203 and 5F 203 (Loaiza-Perez et al., 2002; Trapani et al., 2003). These candidate anticancer agents activate the AhR signaling pathway in sensitive but not in resistant cells. Once this pathway is activated, AhR heterodimerizes with the AhR nuclear translocator (ARNT) protein (Jain et al., 1994; Safe and Krishnan, 1995). This heterodimer complex translocates from the cytosol into the nucleus where it binds to the xenobiotic response element (XRE) located within the 5'-flanking region of responsive genes to initiate transcription, leading to the synthesis of their protein products (Denison et al., 1998; Lampen et al., 1998).

Others and we show that cell sensitivity to a particular anti-cancer agent can depend upon a cell’s ability to take-up and metabolize this agent (Kashiyama et al., 1999b; Hutchinson et al., 2001; Bradshaw et al., 2002a; Bradshaw et al., 2002b). Previous reports indicate that a few drug candidates, including DF 203, become metabolically activated subsequent to inducing CYP1A expression in sensitive cancer cells (Chua et al., 2000; Kuffel et al., 2002; Pichard-Garcia et al., 2004). In this study, metabolism occurred exclusively in sensitive cancer cells, suggesting that the fluorinated benzothiazoles are metabolically activated.

The fluorinated benzothiazoles retain potent and selective anticancer activity and the capacity to become metabolized in vivo (Chua et al., 1999;
Bradshaw et al., 2002a; Bradshaw et al., 2002b). Observed concentrations of 5F 203 and 6F 203 in mouse plasma during a previous study exceed the “c x t” parameters of cell exposure required to achieve cytostasis in MCF-7 and IGROV-1 cells, and cytocidal activity in TK-10 cells observed in this current study (Alley et al., 2000). Pharmacological evaluations of L-lysylamide pro-drugs of 5F 203 and 6F 203 following single i.v. bolus administrations to mice at well-tolerated dosages demonstrate efficient in vivo conversion of each pro-drug to 5F 203 and 6F 203 (Alley et al., 2000).

We demonstrate that fluorinated benzothiazoles disappeared from nutrient media correlating with their capacity to be metabolized and bind covalently to macromolecules within “sensitive” cells but not “resistant” cancer cells. Prior studies have demonstrated the ability of benzothiazoles or their metabolites to bind covalently to DNA in vitro to mediate their anti-cancer effects (Leong et al., 2003; Trapani et al., 2003). A 5F 203 derivative has previously been shown to covalently bind to DNA in IGROV-1 and MCF-7 cells in vivo in proportion to its ability to inhibit tumor growth (Bradshaw et al., 2002a). Although previous studies demonstrated the benzothiazoles’ ability to bind to nuclear and microsomal macromolecules (Chua et al., 2000; Loaiza-Perez et al., 2002), ours is the first to demonstrate the affinity of 5F 203 for mitochondrial and cytosolic macromolecules in sensitive cells. Since 5F 203 has been previously shown to induce apoptosis (Trapani et al., 2003), its affinity for the mitochondria raises the possibility that this drug candidate facilitates cytochrome C release to initiate the caspase cascade essential for executing apoptosis (Cain, 2003). Further
experiments will seek to explore the consequences of 5F 203 interaction with the functional properties of mitochondria.

This work demonstrates that the fluorinated benzothiazoles, and in particular 5F 203, exhibit promising anti-cancer activity that warrants their continued development. Efforts are underway to characterize any 5F 203 metabolites that may possess anti-cancer activity, thereby yielding additional clues regarding the mechanism of anti-proliferative activity for 5F 203. In the United Kingdom, Phortress (L-lysylamide pro-drug of 5F 203) will enter clinical trials under the auspices of the Cancer Research Campaign.
Acknowledgments

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References


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Trapani V, Patel V, Leong CO, Ciolino HP, Yeh GC, Hose C, Trepel JB, Stevens MFG, Sausville EA and Loaiza-Perez Al (2003) DNA damage and cell cycle arrest induced by 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (5F 203, NSC 703786) is attenuated in aryl hydrocarbon receptor deficient MCF-7 cells. *Br J Cancer* **88**:599-605.


Footnotes

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This is part 25 of the series “Antitumor 2-(4-aminophenyl)benzothiazoles.”
Figure Legends

**Figure 1.** (A) Chemical structures of the unfluorinated benzothiazole and its primary metabolite and the fluorinated 2-(4-amino-3-methylphenyl)benzothiazoles with numbering scheme. The (*) represents the position of the $^{14}$C label within the 5F 203 molecule. (B) Chemical structure of the lysylamide prodrug of 5F 203.

**Figure 2.** Growth inhibitory effects of 5F 203, 6F 203, 5,6 di-F 203 and DF 203 in select sensitive human cancer cells. Growth inhibition was measured by the Sulforhodamine B (SRB) protein assay after (A) MCF-7 breast, (B) TK-10 renal and (C) IGROV1 ovarian cancer cell lines were treated with the benzothiazoles for 72 h in accordance with *Materials and Methods*. Dose-response curves were constructed showing percentage growth relative to untreated control cells. Data represent the mean ± SD of at least three independent experiments.

**Figure 3.** 5F 203 concentrations required to produce 50% growth inhibition ($\text{GI}_{50}$, A), total growth inhibition ($\text{TGI}$, B) or 50% net cell kill ($\text{LC}_{50}$, C) in MCF-7, TK-10 and IGROV1 cells relative to untreated controls. Cells in 96-well plates were treated with the fluorinated benzothiazoles at selected exposure durations as described in *Materials and Methods*. Growth of cells following 5F 203 treatment was determined using the MTT assay (Alley et al., 1995). Data represent means ± SD (N = 8). The difference between values ($\text{GI}_{50}$, TGI or $\text{LC}_{50}$) for a given cell line was determined as significantly different from *MCF-7 cells or ¶ IGROV1 cells at a given time point (P < 0.05). Bars shown to reach 100 $\mu$M actually exceed this value but by no more than 10%.
Figure 4. Metabolism of 5F 203, 6F 203 and 5,6 di-F 203 in (A) MCF-7, (B) IGROV1, (C) TK-10, (D) MDA-MB-435, (E) SKOV3 and (F) CAKI-1 cells. Cells were treated with 1.0 µM fluorinated benzothiazoles for up to 3 days. Control flasks were treated with either vehicle (0.1% DMSO) or contained the respective fluorinated benzothiazoles without cells. At specified time points, aliquots of media were precipitated and supernatants were analyzed by HPLC as described in Materials and Methods. Data represent the mean of 3 experiments (bars, SD). Values significantly different from a5F 203 (P < 0.05) and b6F 203 (P < 0.05). All fluorinated benzothiazoles were stable in culture medium for the entire duration of the experiment.

Figure 5. Time-dependent depletion of 5F 203 from nutrient RPMI 1640 medium (A) and emergence of metabolites following 5.0 µM (B) and 10.0 µM (C) 5F 203 treatment of MCF-7 cells. MCF-7 cells were incubated with 5.0 µM or 10.0 µM 5F 203 for 0, 12, 14 or 16 h. Control flasks were treated with 0.1% DMSO. At selected time points, aliquots of media were analyzed by HPLC as described in Materials and Methods. Data represent the mean of two independent experiments.

Figure 6. Metabolism of 5F 203, 6F 203 and 5,6 di-F 203 (10 µM) by microsomes of human B-lymphoblastoid cells expressing cytochrome P450s (A) CYP1A1, (B) CYP1B1 and (C) CYP1A2. Metabolites were detected once fluorinated benzothiazoles were incubated with microsomes, and aliquots of reaction mixtures were analyzed by HPLC as described under Materials and Methods. Unknown metabolites were detected using the fluorescence excitation
and emission wavelengths of the parent fluorinated benzothiazole calibration standards. Each bar corresponds with an uncharacterized metabolite detected in the chromatogram following HPLC analysis. Elution times for the parent fluorinated benzothiazoles 5F 203, 6F 203 and 5,6 di-F 203 were 30, 33 and 31 respectively. Elution times for metabolites are indicated in numerical values above each bar. Data represent mean ± SD of three independent experiments. CYP1A1 was significantly (P < 0.05) more effective at metabolizing the fluorinated benzothiazoles than CYP1B1 or CYP1A2 as indicated by the activity levels of resultant metabolites.

**Figure 7.** CYP1A1 gene expression in sensitive and resistant cancer cell lines. RNA was extracted from (A) breast, (B) renal and (C) ovarian cells treated with varying concentrations of 5F 203 or 0.1% DMSO (control) for 12 or 24 h. Gene expression was assessed with QRT-PCR as described in detail elsewhere (Loaiza-Perez et al., 2002). Data represent the mean ± SD of at least three independent experiments.

**Figure 8.** CYP1B1 gene expression in sensitive and resistant cancer cell lines. RNA was extracted from (A) breast, (B) renal and (C) ovarian cells treated with varying concentrations of 5F 203 or 0.1% DMSO (control) for 12 or 24 h. Gene expression was assessed with QRT-PCR as described in detail elsewhere (Loaiza-Perez et al., 2002). Data represent the mean ± SD of at least three independent experiments.

**Figure 9.** CYP1A1 induction in cells treated with fluorinated benzothiazoles. (A) MCF-7, (B) TK-10 and (C) MDA-MB-435 cells were treated with 1.0 µM of the
respective fluorinated benzothiazoles for 24 h and cellular proteins from these cells were immunoblotted as described in Materials and Methods. Lane 1 contains recombinant CYP1A1 (2.5 µg) which served as a positive control, lane 2 is a blank well, lane 3 contains untreated control cells, lanes 4-9 contain cells treated with 4F 203, 5F 203, 6F 203, 7F 203, 5,6 di-F 203 and lysylamide prodrug of 5F 203 respectively.

Figure 10. Subcellular binding of [14C] 5F 203 in sensitive and resistant cancer cell lines. (A) Breast (B) ovarian and (C) renal cancer cells were exposed to 0.1 [14C] 5F 203 for 16 h. Cells were homogenized and isolated into the desired subcellular fractions (i.e., nuclear, mitochondrial, microsomal, cytosolic) using differential centrifugation as described in detail elsewhere (Jones et al., 1995). Subcellular binding was determined by measuring radioactivity in each fraction by scintillation counting and protein content was estimated using the Coomasie Blue Protein Assay as described in Materials and Methods. Each data point represents the mean ± SD of at least four experiments. The difference between the extent of subcellular binding of [14C] 5F 203 to a particular fraction of a given sensitive cell line was significantly different from the corresponding resistant cell line (P < 0.05; i.e. MCF-7 vs. MDA-MB-435) as indicated by *.
TABLE 1 In vitro activity of fluorinated benzothiazoles in select breast, ovarian and renal cancer cell lines\textsuperscript{a}

Cancer cell lines were treated with the fluorinated benzothiazoles (10\textsuperscript{-4}-10\textsuperscript{-8}M) and analyzed for in vitro anti-cancer activity using the SRB assay in accordance with \textit{Materials and Methods}.

<table>
<thead>
<tr>
<th>Benzothiazole (NSC)</th>
<th>MCF-7</th>
<th>MDA-MB-435</th>
<th>TK-10</th>
<th>CAKI-1</th>
<th>IGROV</th>
<th>SKOV3</th>
</tr>
</thead>
<tbody>
<tr>
<td>5F 203 (703786)</td>
<td>\textit{G_I}_{50}</td>
<td>&lt;0.01</td>
<td>&gt;100</td>
<td>&lt;0.01</td>
<td>&gt;20</td>
<td>&lt;0.01</td>
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<tr>
<td></td>
<td>TGI</td>
<td>44.7</td>
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<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<td>\textit{L_C}_{50}</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>6F 203 (702156)</td>
<td>\textit{G_I}_{50}</td>
<td>0.01</td>
<td>&gt;100</td>
<td>&lt;0.01</td>
<td>&gt;100</td>
<td>1.28</td>
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<tr>
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<td>TGI</td>
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<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>5,6 di-F 203 (711671)</td>
<td>\textit{G_I}_{50}</td>
<td>0.012</td>
<td>&gt;100</td>
<td>&lt;0.01\textsuperscript{b}</td>
<td>&gt;100</td>
<td>&lt;0.01\textsuperscript{b}</td>
</tr>
<tr>
<td></td>
<td>TGI</td>
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<td>\textit{L_C}_{50}</td>
<td>&gt;100</td>
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<td>&gt;100</td>
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<td>&gt;100</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Values (GI\textsubscript{50}, TGI and LC\textsubscript{50}) are expressed in µM determined from plotted dose-response curves.

\textsuperscript{b}Indicates value is an approximation since higher concentrations resulted in values exceeding 50%.

Values represent means of at least two independent experiments. NT, not tested.
TABLE 2  In vitro activity following brief exposure of MCF-7 breast cancer cells\textsuperscript{a} to the fluorinated benzothiazoles

MCF-7 cells were treated with 5F 203, 6F 203 and 5,6 di-F 203 at varying concentrations (10\textsuperscript{-4}-10\textsuperscript{-8}M) for 45 min followed by a 144-h incubation in drug-free culture medium. Samples were analyzed for in vitro anti-cancer activity using the MTT assay in accordance with Materials and Methods.

\begin{tabular}{lccc}
\hline
 & 5F 203 (703786) & 6F 203 (702156) & 5,6 di-F 203 (711671) \\
\hline
\textit{GI}_{50} & 0.018 ± 0.002 & 0.020 ± 0.009 & 0.022 ± 0.004 \\
TGI & 0.20± 0.15 & 0.19± 0.11 & 0.38 ± 0.19 \\
\textit{LC}_{50} & >100 & >100 & >100 \\
\hline
\end{tabular}

\textsuperscript{a}All values (\textit{GI}\textsubscript{50}, TGI, \textit{LC}\textsubscript{50}) are expressed in \textmu M and were determined from plotted dose-response curves.

Values represent means ± SD of three independent experiments.
A.

![Chemical structure](image)

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<th>$R_3$</th>
<th>$R_4$</th>
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<td>H</td>
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<td>H</td>
<td>F</td>
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</table>

B.

![Chemical structure](image)

L-lysyl-amide prodrg of 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (Phortress)
Figure 2

A. Percentage Growth vs. Concentration (Molar)

B. Percentage Growth vs. Concentration (Molar)

C. Percentage Growth vs. Concentration (Molar)
Figure 3

A. 

G_{50} \text{ (µM)}

<table>
<thead>
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<th>TK-10</th>
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<td>144.00</td>
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</table>

B. 

TGI (µM)

<table>
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C. 

L_{50} \text{ (µM)}

<table>
<thead>
<tr>
<th>Time (h)</th>
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<td>144.00</td>
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</table>

MCF-7, IGROVI, and TK-10
Figure 8

(A) Expression Relative to Control

- MCF-7 12 h
- MCF-7 24 h
- MDA-MB-435 12 h
- MDA-MB-435 24 h

Concentration (μM)

(B) Expression Relative to Control

- TK-10 12 h
- TK-10 24 h
- CAKI-1 12 h
- CAKI-1 24 h

Concentration (μM)

(C) Expression Relative to Control

- IGROVI 12 h
- IGROVI 24 h
- SKOV3 12 h
- SKOV3 24 h

Concentration (μM)