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

The fluorinated benzothiazole analogue 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (5F 203, NSC 703786) is a novel agent with potent and selective antitumour properties and, in the form of its i-lysylamide prodrug Phortress (NSC 710305), is a current candidate for early phase clinical studies. Previous findings have indicated that cytochrome P450 1A1 (CYP1A1) may play a role in the antitumour activity of molecules in the benzothiazole series including the nonfluorinated parent compound 2-(4-amino-3-methylphenyl)benzothiazole (DF 203, NSC 674495) (Kashiyama et al, 1999; Chua et al, 2000; Loaiza-Pérez et al, 2002). In this study, we assessed and verified that a fully functional aryl hydrocarbon receptor (AhR) signalling pathway is a necessary requisite for the induction of efficient cytotoxicity by 5F 203 in MCF-7 wild-type sensitive cells. Drug exposure caused MCF-7 sensitive cells to arrest in G1 and S phase, and induced DNA adduct formation, in contrast to AhR-deficient AhR100 variant MCF-7 cells. In sensitive MCF-7 cells, induction of CYP1A1 and CYP1B1 transcription (measured by luciferase reporter assay and real-time reverse transcriptase-polymerase chain reaction (RT–PCR)), and 7-ethoxyresorufin-O-deethylase (EROD) activity was demonstrated, following treatment with 5F 203. In contrast, in resistant AhR100 cells, drug treatment did not affect CYP1A1 and CYP1B1 transcription and EROD activity. Furthermore, AhR100 cells failed to produce either protein/DNA complexes on the xenobiotic responsive element (XRE) sequence of CYP1A1 promoter (measured by electrophoretic mobility shift assay) or DNA adducts. The data confirm that activation of the AhR signalling pathway is an important feature of the antitumour activity of 5F 203.

Keywords: 2-(4-amino-phenyl)benzothiazoles; aryl hydrocarbon receptor; CYP1A1; DNA damage; S-phase arrest; MCF-7

2-(4-Amino-3-methylphenyl)benzothiazoles are novel compounds with potent and unique antitumour properties (Shi et al, 1996; Bradshaw et al, 1998a,b; Kashiyama et al, 1999). It was demonstrated that selective metabolism in vitro of the parent agent DF 203 (NSC 674495) correlated very highly with its antiproliferative activity, with uptake and biotransformation observed only in those cell lines that are sensitive to the compound, such as MCF-7 and T47D breast carcinoma cells (Kashiyama et al, 1999). CYP1A1, whose expression and activity are induced only in sensitive cells, appears to be responsible for the metabolic hydroxylation of DF 203 in position 6, which produces an inactive and antagonistic molecule (Chua et al, 2000). CYP1A1 is also postulated to have a crucial role in mediating the antitumour activity of DF 203, possibly generating an electrophilic intermediate responsible for the formation of DNA adducts in sensitive cells (Stevens et al, 2001).

Fluorinated derivatives of the parent drug DF 203 (see Figure 1 for structures) were synthesised in order to prevent deactivation resulting from metabolic ring hydroxylation (Hutchinson et al, 2001). Fluoro-analogues retain potency and selectivity, and successfully reduce or abolish the production of inactive and antagonistic metabolites and the consequent biphasic dose–response phenomenon. Drug-induced induction of CYP1A1, a crucial event in determining the antitumour specificity of this series of benzothiazoles (Chua et al, 2000), was not compromised by fluorination (Hutchinson et al, 2001).

2-(4-Amino-3-methylphenyl)-5-fluorobenzothiazole (5F 203, NSC 703786) has emerged as the most potent of the new generation of antitumour benzothiazoles both in vitro and in vivo and is currently the focus of pharmaceutical and preclinical development, as it can be converted to a readily soluble prodrug with appropriate pharmaceutical properties (Bradshaw et al, 2002).

As for the parent drug DF 203, depletion of 5F 203 from culture media and subsequent induction of CYP1A1 correlate with cell...
responsiveness. Only in sensitive cells does drug uptake occur and CYP1A1 enzyme expression increase to detectable levels; naturally occurring drug-resistant cells do not show significant changes in either drug levels in medium or CYP1A1 expression (Brantley et al., 2001).

We have previously reported that the parent compound DF 203 induces activation of the AhR in sensitive cells such as human breast epithelial cancer MCF-7 cells. In contrast, nonresponsive cells, for example, breast carcinoma MDA-MB-435 or prostate carcinoma PC-3 cells, showed no activation of the AhR and no induction of CYP1A1 after drug treatment (Loaiza-Pérez et al., 2002). We also have reported a suitable model to investigate the role of the AhR in mediating drug cytotoxicity (Loaiza-Pérez et al., 2001), consisting of AhR-deficient AHR100 cells, derived from MCF-7 human breast epithelial cancer cells by continuous exposure to escalating concentrations of benzo[a]pyrene (Yeh et al., 2001). AHR100 cells display relative resistance to the cytotoxic effects of several polycyclic aromatic hydrocarbons (PAHs), such as benzo[a]pyrene and dimethylbenz[a]anthracene (DMBA), due to impairment of the AhR signal transduction pathway (Yeh et al., 2001; Ciolino et al., 2002).

We therefore have used wild-type MCF-7 and AHR100 cells as a model, to investigate the molecular determinants for sensitivity to the clinical candidate 5F 203, including toxicity, cell cycle distribution, DNA damage, CYP1A1 activity and AhR signalling pathway activation. The results presented in this paper confirm that activation of the AhR plays an essential role in 5F 203 antitumour activity, mediating responsiveness and efficacy.

**MATERIALS AND METHODS**

**Drug and cell culture**

5F 203 was synthesised by the Cancer Research Laboratories at the University of Nottingham, UK and the Drug Synthesis and Chemistry Branch, NCI, following published methods (Hutchinson et al., 2001). The compound was dissolved in DMSO to make a 100 mM stock concentration and further diluted to the working concentration (1 nM – 100 μM) for experimental procedures. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was prepared as 10 mCi of [3H]-TCDD (New England Nuclear, Boston, MA, USA) and was used as a positive control for AhR activation. Cells were exposed to DMSO (0.1%) or 5F 203 (1 μM) for 24 h. Floating cells contained in the supernatant were collected by centrifugation, and stained with 0.4% DAPI. Experiments were repeated at least three times. Stained cells were then visualised on a Zeiss Axiophot microscope using ×63 objective and images were captured with an Optronics CCD camera (Loaiza-Pérez et al., 2002).

**Assessment of cytotoxicity**

Cells were inoculated in 96-well plates and maintained for 24 h before treatment with 5F 203, which consisted of five serial dilutions (1:10) ranging from 10 nM to 100 μM. Treated cells were subsequently maintained for an additional 48 h, and cellular protein was measured by the sulphorhodamine-B (SRB) assay as described previously (Monks et al., 1991). Briefly, cells were fixed with trichloroacetic acid and stained with SRB. Protein-bound SRB was solubilised and measured spectrophotometrically to determine relative cell viability in treated and untreated cells.

**4,6-Diamidino-2-phenylindole (DAPI) staining**

Approximately 2 × 10⁵ cells were grown on coverslips overnight. Cells were exposed to DMSO (0.1%) or 5F 203 (1 μM) for 24 h. Floating cells contained in the supernatant were collected by cytocentrifugation, and stained with 0.4% DAPI. Experiments were repeated at least three times. Stained cells were then visualised on a Zeiss Axiovert microscope using ×63 objective and images were captured with an Optronics CCD camera (Loaiza-Pérez et al., 2002).

**³²P-postlabelling assay**

Pellets of control and treated (0.1% DMSO or 1 μM 5F 203 for 24 h) MCF-7 and AHR100 cells were used for DNA extraction using Qiagen DNA extraction columns according to the manufacturer’s protocol. Of each extracted DNA 5 μg was digested to deoxyribonucleoside 3’-monophosphates by incubating with micrococcal nuclease (175 mU) and calf-spleen phosphodiesterase (6 mU) at 37°C overnight. DNA adduct enrichment was carried out by butanol extraction (Gupta., 1985). Adducts were then radiolabelled by 5’-phosphorylation using 62.5 μCi of [γ-³²P]ATP and 5 U of T4 polynucleotide kinase.

**HPLC detection and quantification of DNA adducts**

³²P-labelled products were separated on a Hypersil BDS C18 analytical column (250 × 4.6 mm, 5 μm; Shandon). The mobile phase consisted of 88% 2 M ammonium formate (pH 4.0) and 12% acetonitrile for 50 min, followed by a linear gradient of 20 – 45% acetonitrile for 15 min. Radioactivity was monitored by a radiochemical detector (Lab Logic, β-RAM) lined to a Varian Star 9012 pump. Data analysis was done by Laura, an MS Windows package (Lab Logic Inc.). The relative adduct levels (RAL) were calculated by the method of Reddy and Randerath (1986), based on the specific activity of [γ-³²P]ATP and the amount of DNA used. RAL values were then translated into fmol adducts per μg DNA (Gupta, 1985).

**Cell cycle analysis**

Cell cycle analysis on MCF-7 and AHR100 cells was performed as described previously (Sherwood and Schimke, 1995). Briefly,
exponentially growing cells were exposed to either DMSO (0.1%) or 5F 203 (1 µM) for 24 h, then harvested, washed briefly, in cold PBS and fixed in 70% ethanol. DNA was stained by incubating the cells in PBS, containing propidium iodide (50 µg ml⁻¹) and RNAase A (1 mg ml⁻¹) for 30 min at 37°C and fluorescence was measured and analysed using FACSCaliber (Becton Dickinson Immunocytometry Systems, San José, CA, USA) and ModFit (Verity Software, Topsham, ME, USA).

CYP1A1 activity in intact MCF-7 and AHRI100 cells

MCF-7 and AHRI100 cells were grown in 24-well plates to 60–70% confluence prior to treatment with 0.1% DMSO (control) or 5F 203 (1 nM–1 µM). At the end of the incubation period, the medium was aspirated and the cells were washed with PBS. CYP1A1 enzyme activity was subsequently determined by 7-ethoxyresorufin-O-deethylase (EROD) activity in intact cells as described by Kennedy and Jones (1994). Briefly, the fluorescence of resorufin generated from the conversion of ethoxyresorufin by CYP1A1 was measured spectrophotometrically and resorufin content was derived from a standard curve.

Real-time RT–PCR

Evaluation of CYP1A1 and CYP1B1 gene expression in MCF-7 and AHRI100 cells was performed by real-time RT–PCR, using 15 cycles of PCR, primers and probes described in a previous report (Loaiza-Pérez et al., 2002). RNA was isolated using Qiagen kits (Qiagen, Valencia, CA, USA). PCR efficiencies were validated by means of a standard curve.

Transfections

Cells were transfected using LipofectAMINE (Invitrogen), with 0.5 µg Renilla reniformis luciferase (pRL-TK) (Promega, Madison, WI, USA), and 1.5 µg of pTX.Dir or pT81 as described previously (Loaiza-Pérez et al., 2002). Luciferase activity was measured by the Dual-Luciferase Assay System (Promega, Madison, WI, USA) following the manufacturer’s instructions, and transfection efficiency was monitored by the activity of the R. reniformis plasmid.

Electrophoretic mobility shift assay (EMSA)

Synthetic oligonucleotides containing the AhR-binding site of the human CYP1A1 promoter (5’-CTC CGG TCC TTC TCA CGC AAC GCC TGG GCA-3’) (Invitrogen) were used as probes. Electrophoretic mobility shift assays were performed as described previously (Loaiza-Pérez et al., 2002). Briefly, nuclear extracts (20 µg) from control (0.1% DMSO) and treated (1 µM 5F 203 for 1 h, or 10 nM TCDD for 1 h) MCF-7 and AHRI100 cells were incubated in binding buffer with the [32P]DNA probe and the resulting DNA/protein DNA complexes were separated on a 6% polyacrylamide gel (Novex) under nondenaturing conditions and high ionic strength. Gels were dried and imaged by autoradiography.

RESULTS

5F 203-induced cytotoxicity correlates with AhR activation in sensitive MCF-7 cells

Previous observations have shown that cytotoxicity of benzothiazoles is mediated via activation of the AhR signalling pathway (Kashiyama et al., 1999; Chua et al., 2000; Loaiza-Pérez et al., 2002). In addition, cytotoxicity across a large panel of human tumour cell lines correlates with CYP1A1 inducibility (Hose et al., 2001). We then sought to investigate responsiveness of MCF-7 wild-type cells to the clinical candidate 5F 203 as compared to that of MCF-7-derived AhR-deficient cells (AHRI100). Specifically, we sought to establish whether AhR-mediated signalling could account for all the cytotoxic potential of this agent. Figure 2A indicates that MCF-7 cells are sensitive to this compound (GI₅₀ = 121 nM, 48 h exposure). In contrast, AHRI100 cells show a decreased growth inhibition after treatment with high drug concentration (GI₅₀ > 100 µM) and can therefore be considered resistant.

Figure 2 (A) Selective cytotoxicity of 5F 203 to MCF-7 cells. Growth inhibition induced by 5F 203 was assessed by sulphforhodamine B assay as detailed in Materials and Methods. Values are means ± s.d. (n = 10). (B) 5F 203 induces changes in nuclear morphology of 5F 203-sensitive MCF-7 breast cells but not AHRI100 cells. Cells prepared as described in Materials and Methods were exposed for 24 h to 0.1% DMSO (control) or 1 µM 5F 203, and stained with 4,6-diamidino-2-phenylindole (DAPI).
As a complementary approach, MCF-7 and AH R100 cells treated with 0.1% DMSO (control) or 5F 203 (1 μM) for 1 h were subsequently stained by DAPI (Figure 2B). Only MCF-7 cells treated with the drug showed altered nuclear morphology, which included chromatin condensation and marginalisation to the nuclear membrane. AH R100 cells failed to show any significant morphological changes. Taken together, Figure 2A and B suggests that activation of the AhR signalling pathway participates in the antiproliferative activity of 5F 203, which results in growth inhibition and modified nuclear features.

5F 203 induces DNA adduct formation in MCF-7 cells

We next questioned whether DNA adducts could be produced in MCF-7 and AhR-deficient AH R100 cells. DMSO-treated MCF-7 and AH R100 cells showed a similar profile of DNA adducts, with a few adducts eluting within the early retention time (≤20 min) (Figure 3). After treatment with 1 μM 5F 203, no significant changes in these peaks were found in either cell line, while several other adducts with longer retention time were detected. The major adduct formed in AH R100 cells (adduct 17 in Figure 3Bi) is chromatographically equivalent to an adduct detected in MCF-7 cells (adduct 7 in Figure 3Ai). Although DNA adduct profiles appear similar in the two cell lines, the number of total adducts/10⁸ nucleotides induced by drug treatment was greatly decreased in AH R100 cells compared to wild-type MCF-7 cells. These findings suggest that impairment of the AhR signalling pathway may result in reduced activation of 5F 203 into reactive species able to damage DNA.

5F 203 causes altered cell cycle distribution

As our results in Figures 2 and 3 indicated that 5F 203 was inducing the formation of DNA adducts, we investigated whether this could result in an altered cell cycle profile. For this approach, cells sensitive (MCF-7) and insensitive (AH R100) to 5F 203 were exposed to 1 μM of the drug or 0.1% DMSO for 24 h and subsequently processed for cell cycle analysis. As illustrated in Figure 4, 5F 203 treatment of MCF-7 cells caused a notable increase in G₁ (37 to 52%) and S (20 to 27%) phase cells, which coincided with a decrease in G₂/M (42 to 16%) phase cells. Accumulation of cells in sub-G₁ was minimal when compared to control. In contrast, 5F 203-insensitive cells (AH R100) failed to demonstrate any notable effect on the cell cycle, in particular S, G₂/M and sub-G₁ phases. The data demonstrate that 5F 203-induced DNA damage may lead to cell accumulation in G₁ and S phase concomitant with growth inhibition. As MCF-7 cells are p53 wild type, their response to 5F 203 treatment is consistent with the operation of a G₁- and possibly S-phase checkpoint to cell cycle progression after DNA adduct formation.

CYP1A1 and CYP1B1 induction

5F 203 induces DNA damage and cell cycle arrest most obviously in MCF-7 cells with a fully functional AhR signalling pathway. The hypothesis that deficient activation of CYP1A1 might occur in AhR-deficient AH R100 cells was then addressed. The enzymatic activity of CYP1A1 in intact MCF-7 and AH R100 cells treated with 5F 203 was assayed by measuring EROD activity. Although both CYP1A1 and CYP1B1 can catalyse the dealkylation of ethoxyresorufin, the specific activity of CYP1A1 is approximately 40-fold higher than that of CYP1B1 (Doostdar et al, 2000). Incubation with 5F 203 for 24 h resulted in a concentration-dependent increase in EROD activity over a range of 50 nM to 1 μM in MCF-7 cells, whereas the drug had no effect on EROD activity in AH R100 cells (Figure 5A).

In order to compare the effect of 5F 203 on CYP1A1 and CYP1B1 gene expression, MCF-7 and AH R100 cells were treated with the compound (1 μM) for 24 h and mRNA levels for these two genes were measured by real-time RT-PCR. 5F 203 caused an increase in the mRNA levels of both cytochromes in MCF-7 cells, where relative levels of CYP1A1 mRNA were approximately 100-fold higher when compared to control; only a minimal increase of CYP1B1 mRNA (10-fold) was observed (Figure 5B). In contrast, in AH R100 cells, levels of CYP1A1 and CYP1B1 mRNA after treatment remained similar to control (Figure 5B). These findings are in agreement with previous studies which described that the parent compound DF 203 causes induction of CYP1A1 protein and increase in mRNA levels in MCF-7 cells but not in DF 203 inherently resistant cells (Chua et al, 2000; Loaiza-Pérez et al, 2002).

Figure 3 DNA adduct profiles in (A) MCF-7 and (B) AH R100 cells. Cells were incubated with (i) 0.1% DMSO or (ii) 1 μM 5F 203 for 24 h. ³²P-labelled adducts were analysed using 2 μg digested DNA on a Hypersil C18 column with a gradient of acetonitrile in 2 M ammonium formate as detailed in Materials and Methods. The early peak is due to the presence of free [³²P]ATP.
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pretreated with an antagonist of AhR, and AH R100 cells, electrophoretic mobility shift assays were performed. Previous reports from our laboratory (Loaiza-Pérez et al., 2002) described that, in MCF-7 cells, three protein/DNA complexes were found in nuclear extracts of control cells, although the intensity of the uppermost complex was lower than the others

CYP1A1 and CYP1B1 promoters are regulated by the AhR, which forms a heterodimer with ARNT. Binding of the complete dimer to the XREs in the promoter region mediates transcription of AhR-responsive genes, including CYP1A1 and CYP1B1 (Whitelock, 1999). MCF-7 and AH R100 cells were transfected with an XRE-luciferase reporter construct (pTX.Dir); as control, the same luciferase reporter construct but without the XRE (pT81) was used. Cells were then treated with DMSO (0.1%), TCDD (10 nM) or 5F 203 (1 μM). TCDD was used as a prototypic compound activator of CYP1A1 transcription and included as a positive control. As shown in Figure 6A, in MCF-7 cells transfected with pTX.Dir, a 15-fold induction of luciferase activity was observed with TCDD, and 5F 203 treatment similarly resulted in a 15-fold induction. However, when AH R100 cells were transfected with XRE and treated with 5F 203 (1 μM), XRE-luciferase activity was demonstrated to be only approximately 1.7-fold higher than control. No induction in luciferase activity was observed when cells transfected with pT81 were treated with 5F 203 (1 μM) or TCDD (10 nM). Similar results were obtained when cells were transfected with a fragment of mouse native CYP1A1 promoter (inclusive of four dioxin responsive elements (DREs)) (pGudLuc1.1) (data not shown) (Garrison et al., 1996). When pTX.Dir-transfected cells were pretreated with an antagonist of AhR, α-naphthoflavone, before treatment with 5F 203, luciferase activity was reduced approximately 25%, suggesting competition between the two ligands for binding to the AhR (results not shown).

In order to compare protein/DNA complex formation on the XRE of CYP1A1 in MCF-7 but not AH R100 cells

We report here that treatment with 5F 203 causes accumulation in the G1 and S phase, along with growth inhibition in wild-type MCF-7 cells, but it does not affect cell cycle progression of AH R100 cells. We also confirm the production of DNA damage in these sensitive cells (Stevens et al., 2001), whereas the formation of DNA adducts is significantly reduced in the resistant subclone (Figure 3). These results corroborate the view that antitumour activity of 5F 203 is highly dependent on functional AhR signalling, whose impairment results in reduced efficacy of the agent.

Activation of the AhR signalling pathway may be an important factor determining sensitivity to 5F 203 because it triggers metabolic transformation of the drug through CYP1A1 into reactive species damaging DNA. Our data show that exposure of AH R100 cells to 5F 203 produces markedly fewer DNA adducts, as compared to wild-type MCF-7 cells. The chemical structure of these adducts has not yet been characterised. Computational studies using frontier molecular orbital calculations point to the putative reactive species being a nitrenium ion (Dr SE O’Brien, personal communication), but further studies are required to confirm this hypothesis and identify the adducts. A more detailed description of the formation of DNA adducts induced by benzoazinones in sensitive cells will be published separately.

5F 203 increases protein/DNA complex formation on the XRE sequence of CYP1A1 in MCF-7 but not AH R100 cells

5F 203 is highly dependent on functional AhR signalling, whose inhibitory properties of the parent compound, DF 203, were demonstrated in MCF-7 cells. The chemical structure of 5F 203-induced cytotoxicity

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species that cause DNA damage (Szeliga and Dipple, 1998; Burczynski and Penning, 2000) resulting in G1 cell cycle arrest (Vaziri and Faller, 1997). In contrast, the environmental pollutant TCDD, which is the most potent AhR agonist, induces CYP1A1 and modulates CYP1B1 expression in MCF-7 cells (Dohr et al, 1995), but is not directly genotoxic. It has been hypothesised that TCDD-induced alterations in estrogen metabolism may lead to increased generation of reactive oxygen species and consequent induction of DNA adducts (Wyde et al, 2001).

In addition to selective metabolism, differential modulation of AhR functions could underlie specific cytotoxicity of the agent. Activation of the AhR signalling pathway consists of several steps, including translocation, pairing with nuclear factors, transactivation of gene transcription and degradation (Rowlands and Gustaffson, 1997), each of which might be differentially regulated in different cells. Other proteins, which integrate the function of the AhR, like heat-shock protein 90 or ARNT, may also be related to differential sensitivity to 5F 203. For example, Qin et al (2001) have demonstrated in patients that there is an association of ARNT splice variants with ER-negative breast cancer, poor induction of vascular endothelial growth factor under hypoxia, and poor prognosis. Further studies are necessary to determine, in greater detail, aspects of the pathway that may be altered in 5F 203-sensitive cells.
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