Drugs of the Future 2004, 29(5): 479-491

CCC: 0377-8282/2004

Review Article

The therapeutic potential of aryl hydrocarbon receptor (AhR) agonists in anticancer drug development

Andrew D. Westwell

Centre for Biomolecular Sciences, School of Pharmacy, University of Nottingham, University Park, Nottingham, NG7 2RD, U.K.; e-mail: andrew.westwell@nottingham.ac.uk

CONTENTS

Abstract

Agonists of the aryl hydrocarbon receptor (AhR), which in turn induce their own metabolism by cytochrome P-450 isozymes (*e.g.,* CYP1A1) to produce cytotoxic species selectively within cancer cells, represent attractive anticancer drug development candidates. The concept of utilizing the AhR pathway as a vehicle to induce selective cytotoxicity in cancer cells, however, remains untested in the clinic to date. The most promising agents in this class are the 2-(4-aminophenyl)benzothiazole prodrug Phortress (University of Nottingham/National Cancer Institute [NCI] Developmental Therapeutics) and a fluorinated diaminoflavone (Kyowa Hakko/NCI Developmental Therapeutics); both agents are currently in advanced preclinical development. The development of selective antitumor agents targeting the AhR pathway will be reviewed here, and an assessment made of their clinical potential.

Introduction

The aryl hydrocarbon receptor (AhR) pathway was first described in the late 1950s as a metabolic response to environmental pollutants such as polycyclic and polyhalogenated aromatic hydrocarbons. In these early rodent experiments, the administration of polycyclic aromatic hydrocarbons such as benzanthracene, benzo[*a*]-pyrene or 3-methylcholanthrene led to the induction of a number of liver microsomal enzyme activities collectively referred to as aryl hydrocarbon (Ah) hydroxylase (1). The inducibility of Ah hydroxylase activity in both hepatic and extrahepatic tissues appeared to be controlled by a single gene locus encoding a protein known as the Ah receptor (AhR).

The AhR has been found in most, if not all, investigated mammalian and a number of nonmammalian vertebrate species. In humans, it has been found in tissues and cells in culture, including lung, liver, kidney, placenta, tonsils and B-lymphocytes (2), and ovarian (3) and breast (4) cancer cells. Although many of the molecular properties of the AhR are comparable to those of steroid hormone receptors (5), cloning (6) revealed that it belongs to a new family within the helix-loop-helix (HLH) superfamily of proteins, other members of which include the AhR nuclear translocator protein (ARNT), the *Drosophila* proteins SIM and PER [6], as well as the hypoxia-inducible factor HIF-1α (7).

Activation of the AhR pathway was, until relatively recently, associated with the process of chemical carcinogenesis induced by agents such as 3-methylcholanthrene. More recent data from our laboratory and others indicated that certain potent antitumor agents may utilize the AhR pathway in their own activation to cytotoxic species within cancer cells. This renewed interest in the AhR pathway as a therapeutic target will form the focus of the present review. For more general descriptions of AhR biology and ligands, a number of recent review articles are available (8-12).

The aryl hydrocarbon receptor (AhR) signaling pathway

A depiction of the AhR signaling pathway is presented in Figure 1 (13). The Ah receptor exists as an inactive complex with two molecules of heat shock protein Hsp90 and a 43-kDa protein known as AIP, XAP2 or Ara9. The chaperone protein Hsp90 serves a dual role in preventing premature nuclear translocation and/or dimerization with

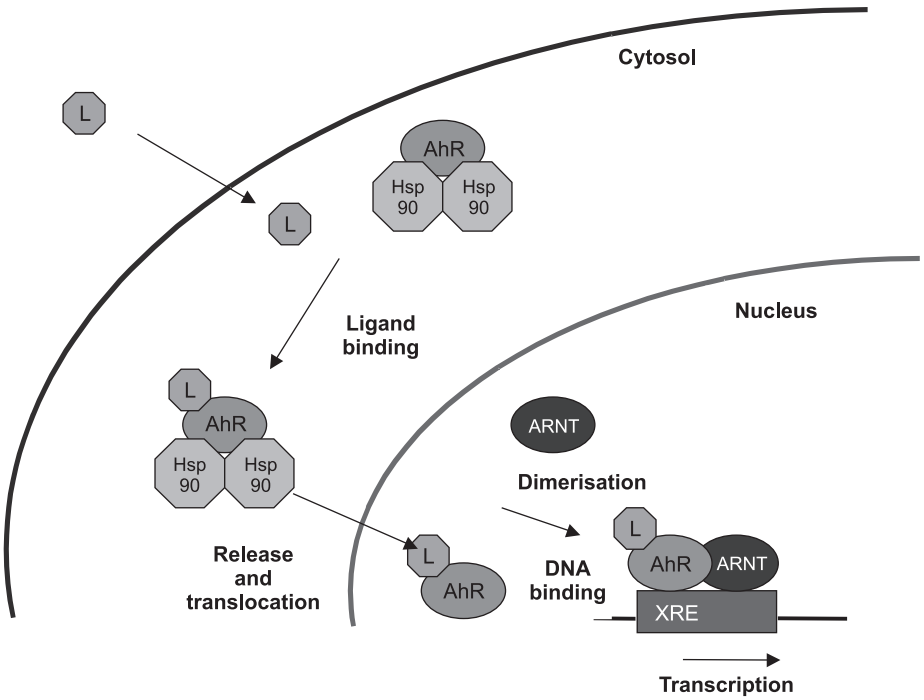


Fig. 1. The aryl hydrocarbon receptor signaling pathway.

DNA-binding partners, and also keeping the AhR in a configuration that favors ligand binding (14). The AIP protein appears to enhance the magnitude and sensitivity of AhR ligand-induced signaling through binding and stabilization of the AhR-Hsp90 complex (15).

Hydrophobic AhR ligands enter the cell by diffusion and bind to the AhR-Hsp90-AIP complex, triggering a conformational change associated with breakdown of the cytosolic complex and rapid translocation of a more DNA-affinic AhR-ligand complex into the nucleus. In the nucleus, the AhR-ligand complex then forms a heterodimer with its partner protein ARNT; this new complex binds with high affinity to a specific DNA sequence (5'-GCGTG-3'), the core binding motif of the dioxin or xenobiotic response element (DRE or XRE, respectively). Binding of AhR-ARNT leads to changes in the chromatin structure, allowing transactivation of AhR-controlled genes (for reviews, see 1, 8, 16).

The AhR battery comprises several genes that encode phase I and phase II drug-metabolizing enzymes, such as the cytochrome P-450 enzymes CYP1A1, CYP1A2 and CYP1B1, NQ01 (NAD[P]H dehydrogenase [quinone], DT-diaphorase) (17), UDP-glucuronosyl-transferase (UGT1A6), glutathione *S*-transferase A1 (GSTA1) and aldehyde dehydrogenase (ALDH3c). Importantly, most of these enzyme systems show a preference for substrates that are themselves AhR agonists; thus the activation of this pathway appears to have evolved as a self-defense system aimed at elimination of hydrophobic xenobiotic substrates (*e.g.*, aromatic hydrocarbons and heteroaromatic amines). This review will focus on attempts to try to turn this xenobiotic defense system to our therapeutic advantage against cancer cells through the selective induction of AhR gene products (mainly CYP1A1) to generate cytotoxic intermediates, leading ultimately to DNA damage and apoptosis.

Studies have suggested a cell cycle-regulatory role for the AhR signaling pathway (18-20), and recent data implicated the AhR in cell cycle arrest (21); however, the role of AhR in the regulation of cell cycle progression is thought not to be critical for survival, since uncontrolled cell proliferation is not one of the results of homozygous deletion of the mouse *AhR* gene (22). The role of AhR signaling in apoptosis has also been studied (23, 24).

Expression of CYP1A1

The role of cytochrome P-450 in tumors in the fate of anticancer drugs has until recently been a rather neglected field, and a number of prodrug antitumor agents have retrospectively been identified as substrates for tumor CYP activation (reviewed in 25). Examples of clinically used anticancer agents where cytochrome P-450 is responsible for metabolic activation (hydroxylation) include the alkylating agents cyclophosphamide (CYP2B6) and ifosphamide (CYP2B6 and CYP3A4) (26).

Activation of the AhR pathway leads to expression of a variety of protein products, of which P-450 family 1 isoforms such as CYP1A1 are amongst the best characterized and understood. Cytochrome P-450 isoforms have been detected at low activity levels in tumor cell lines,

including those comprising the NCI screen (27). Constitutive CYP1A1 levels in particular were found to be low in a range of tumor cell lines, and anticancer drugs have not historically been reported to induce P-450 isoforms. This is not the case for the preclinical drug candidates described in this review, which are found to critically depend on induction of CYP1 isoforms to enable their conversion to cytotoxic species in certain cancer cell lines.

Also relevant to the present discussion are those agents dependent on activation through enzyme systems that comprise part of the AhR gene battery, but where the role of AhR remains to be fully elucidated. The clinically used bioreductive drug mitomycin C is an example, and provides a precedent for the discussion on AhR/CYP1A-activated agents that follows. Mitomycin C, along with other factors such as heat shock and hypoxic stress, induces the activity of the 2-electron bioreductive enzyme NQ01 (DT-diaphorase) (28). The relevance of this example to the present discussion lies in the fact that NQ01 is part of the gene battery associated with AhR (17), and induction of this bioreductive enzyme can be seen with agents that interact with the AhR such as 3-methylcholanthrene. In addition, sensitivity to mitomycin C in the NCI tumor cell line panel was found to correlate with NQ01 enzyme activity (29).

AhR regulation and crosstalk

Multiple signal transduction pathways are implicated in overlap and competition with the AhR pathway, and the AhR signaling system influences diverse cellular processes, including mitogenesis, vascularization and the hypoxia response. Regulation of and crosstalk with other signaling pathways is a complex and only partially understood aspect of AhR biology. It is not the purpose of this review to describe AhR regulation and crosstalk in any detail; the reader should consult a number of recent review articles in this area for further information (8, 9, 11, 12, 30). Crosstalk between the AhR and estrogen receptor (ER) signaling pathways, outlined below, is one area where substantial progress has been made, and where therapeutic possibilities in the cancer field have emerged (11).

AhR-ER crosstalk and anticancer therapy

Inhibitory AhR-ERα crosstalk has been repeatedly observed, for example, in rodent uterus, rodent mammary tumors and breast, ovarian and endometrial cancer cell lines. The high-affinity AhR ligand and environmental toxicant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Fig. 2) (31) elicits distinct antiestrogenic responses in the rodent uterus and human breast cancer cell lines. In addition, TCDD inhibits spontaneous, age-dependent and carcinogen-induced mammary tumor formation and growth in rat models, and the incidence of uterine cancer and breast cancer MCF7 xenograft growth in B6D2F1 immunosuppressed mice (32, 33). In Seveso, Italy, the accidental exposure of humans to TCDD in 1976 led to reduced incidences of mammary and endometrial cancers (34, 35). TCDD has recently been found to down-regulate ERα protein and mRNA levels in human ovarian carcinoma cells (36).

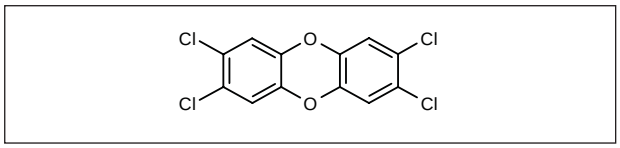


Fig. 2. Structure of the potent AhR agonist TCDD.

AhR ligands such as TCDD are not conventional ER antagonists; however, the use of TCDD and related agents as molecular probes has revealed a great deal regarding AhR-ER signaling crosstalk, and the most pertinent factors relating to this field are outlined below (11):

- AhR agonist-dependent induction of CYP1A1 and CYP1B1 may result in enhanced metabolism of estrogen, and thus depletion of hormone levels.
- Interactions between the nuclear AhR complex and critical regions of estrogen-responsive gene promoters have been identified that repress gene activation.
- Competition has been detected between AhR and ERα signal transduction for the transcription factor nuclear factor 1 (37).

The observation of the antiestrogenic effects of the toxic agent TCDD in a number of tumor models has stimulated a surge of interest led by Safe *et al.* into the development of selective AhR modulators (SAhRMs) for the treatment of estrogen-dependent tumors (11). The major structural class of SAhRM described has been the alternate-substituted (1,3,6,8- and 2,4,6,8-) polychlorinated dibenzofurans (PCDFs) (38-41).

Potent antitumor activity has been associated with alkyl PCDFs in rat mammary tumor models induced by the carcinogen DMBA (7,12-dimethylbenz[*a*]anthracene) (42). Both 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF) and 8-methyl-1,3,6-trichlorodibenzofuran (8-MCDF) (Fig. 3) significantly inhibited mammary tumor growth at doses of 5, 10 or 25 mg/kg/week. Structure-antitumorigenicity relationships correlated with both the relative binding affinities of these compounds for the AhR and structure-antiestrogenicity studies in rat uterus and human breast cancer MCF7 cells. Furthermore, 6-MCDF and tamoxifen were found to act synergistically to inhibit the growth of DMBA-induced mammary carcinomas, and through the reduction of tamoxifen-induced ERα levels, 6-MCDF may confer protection against tamoxifen-induced estrogenic activity in the endometrium (38). Alkyl polychlorinated dibenzofurans may therefore represent a new, relatively nontoxic class of indirectly acting

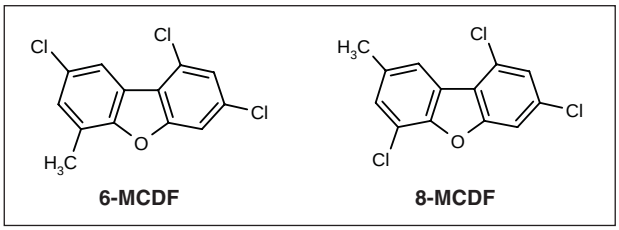


Fig. 3. Structures of alkyl polychlorinated dibenzofurans.

antiestrogens with potential for the clinical treatment of estrogen-driven cancers.

Overview of AhR ligands

A wide range of compounds of diverse structure and lipophilicity, of either synthetic or natural origin, are now known to bind the AhR and induce gene expression. In this sense, the AhR resembles other promiscuous xenobiotic receptors such as the peroxisome proliferator-activated receptor (43) and the pregnane X receptor (44), orphan receptors of the steroid hormone receptor superfamily. As Denison and Nagy point out in their recent review (10), the characteristic of promiscuous ligand binding to the AhR may confer some adaptive advantage to the organism. Since activation of the AhR enhances expression of numerous detoxification enzymes (such as the cytochrome P-450 isozymes), each of which exhibits quite broad substrate specificity, a relatively nonspecific ligand binding site may provide the organism with a wide dynamic range of "chemical detection" and metabolism. Some general observations are made here on the nature of AhR ligands; the discussion throughout this review will focus on those AhR ligands with therapeutic potential in the cancer field.

The first identified and best characterized ligands for the AhR are the polycyclic aromatics that form noncovalent and reversible high-affinity interactions with the receptor. Extensive structure-activity relationship (SAR) studies with polychlorinated aromatic molecules and related analogues have suggested that the ligand binding site preferentially accommodates planar, nonpolar ligands having molecular dimensions approximating a 1.0 x 0.3 nm rectangle (14). Higher affinity binding has also been reported with substantially larger molecules such as substituted indoles and flavonoids, where a binding site of dimensions 0.7 x 1.4 nm must be postulated to accommodate the binding of these ligands. Comparative molecular field analysis using a wider range of ligand types has predicted a single ligand binding pocket of 1.4 x 1.2 x 0.5 nm to accommodate known AhR ligands (45). Alternatively, the AhR may possess more than one ligand binding site.

At the present time, there are no X-ray- or NMR-determined structures of either ligand-bound or unbound AhR, and a lack of information regarding the three-dimensional structure of the AhR ligand binding domain has hindered detailed analysis of the molecular mechanisms involved in AhR-mediated signal transduction. Recently, an AhR ligand binding model for the recognition of polychlorinated dibenzo-*p*-dioxins such as TCDD has been constructed, and preliminary hypotheses on the residues that may be involved in binding have been made (46, 47). A cautionary note should be added here, since the model is only an approximate and probably incomplete picture of the AhR ligand binding domain based on low sequence similarities. The model will, however, serve as a framework for future developments in this area.

Endogenous ligands

A major recent development in the AhR ligand field has been the identification of a number of postulated endogenous ligands for the AhR (reviewed in 9, 10). Proof of the existence of endogenous ligands has, however, been impeded by the fact that the ligands identified have been found to be present in very small quantities and/or have weak AhR-agonist activity. A likely scenario is that distinct endogenous ligands exist with cell-specific distribution, inducing expression of gene products for a desired biological activity in a cell-specific manner. The existence of a number of relatively weak-affinity endogenous AhR ligands, rapidly degraded by the detoxification enzymes that they induce, would then provide the transient induction of gene expression required in the cellular context.

Naturally occurring ligands with therapeutic potential

Naturally occurring compounds can act as agonists of the AhR; perhaps the most well-known example of this is the phytochemical indole-3-carbinol, found in cruciferous vegetables (Fig. 4). Indole-3-carbinol is metabolized within the body to form diindolylmethane and indolo[3,2-*b*]carbazole (Fig. 4), and inhibits the development of hormone-dependent mammary or endometrial cancer in human and animal models. In addition, indole-3-carbinol has been shown to be effective against human papillomavirus-mediated tumors in human patients (48), and is therefore a potential therapeutic agent of considerable interest.

Antitumor 2-(4-aminophenyl)benzothiazoles

Lead discovery

The 2-(4-aminophenyl)benzothiazoles (see Figure 5 for general structure) comprise a potent and highly selective class of novel antitumor agents. The discovery of this class and the subsequent development through to identification of the clinical candidate prodrug Phortress have been reviewed (49, 50); an abbreviated summary

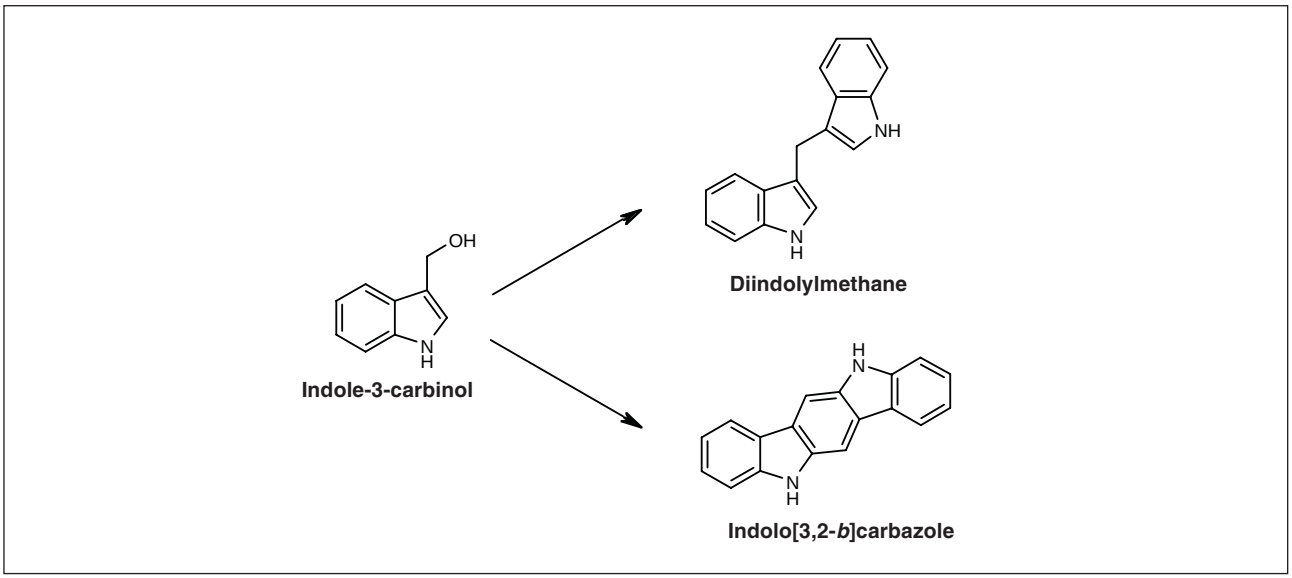


Fig. 4. Metabolism of indole-3-carbinol.

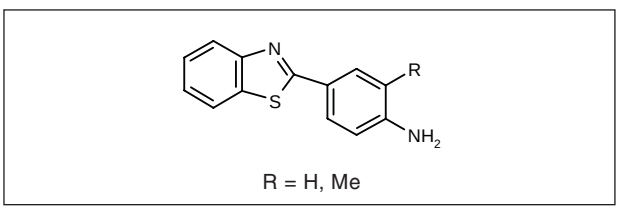


Fig. 5. Structures of early lead antitumor 2-(4-aminophenyl)benzothiazoles.

focusing on mechanistic aspects relating to AhR and downstream targets is given here.

The initial discovery in this area was made when 2-(4-aminophenyl)benzothiazole (R = H) was synthesized as a synthetic intermediate in the early 1990s. This simple lipophilic compound was found to possess remarkable growth-inhibitory activity against human breast cancer cell lines (MCF-7 and MDA-MB-468) in the low nanomolar range, irrespective of cellular estrogen receptor status. Initial SAR studies indicated that the benzothiazole was required for optimal activity (compared to related heterocycles) and that a 3'-substituent (*e.g.,* halogen, methyl, cyano or alkynyl group) was tolerated, and indeed broadened the potent antitumor activity to include other cancer cell lines (*e.g.,* renal and ovarian cancer) (51-54). A particularly notable feature of this new series was the remarkable contrast between sensitive (low nanomolar IC_{50}) and insensitive (IC_{50} > 10 μM) cancer cell lines (NCI 60-cell line panel analysis). *In vivo* antitumor activity was demonstrated for this class of agent; the growth of 5 of 6 breast and 2 of 2 colon tumor xenografts was significantly delayed by 2-(4-amino-3-methylphenyl)benzothiazole (DF-203, NSC-674495; R = Me). On the basis of superior *in vivo* activity in sensitive cell lines, DF-203 was selected as the lead compound for further study.

Target discovery

During initial SAR studies on the active members of the 2-(4-aminophenyl)benzothiazole class, the molecular target was unknown. COMPARE analysis is routinely used by the NCI for analysis of the "fingerprint" of activity in the 60-cell line panel in relation to other agents in the NCI database (55). A high Pearson correlation coefficient (PCC > 0.7) is indicative of a similar pattern of cellular activity and highly suggestive of a conserved mechanism of action. The active compounds in the series possessed a similar profile of antitumor activity across the NCI panel (PCC > 0.7); however, this class of agents was distinct from any known class of antitumor agents used in the clinic (COMPARE-negative, PCC < 0.6).

The role of the AhR signaling pathway

The planar, hydrophobic 2-(4-aminophenyl)benzothiazole analogues, which are readily sequestered by sensitive cell lines only (56), were found to be potent agonists for the AhR (57). Nuclear translocation follows ligand binding to cytosolic AhR, inducing XRE-driven luciferase activity, and the formation of protein-DNA complexes on the XRE sequence of the *CYP1A1* promoter. *In vitro*, *CYP1A1* mRNA activity (58, 59) and CYP1A1 (and to a lesser extent CYP1B1) protein expression (60) were induced exclusively in sensitive cell lines. Induction of *CYP1A1* gene expression is considered one of the most sensitive indicators of exposure to AhR agonists (42). Activation of the AhR pathway was therefore concluded to be an essential feature of the mechanism of action of this novel series.

The working mechanistic model for antitumor 2-(4-aminophenyl)benzothiazoles, following the identification

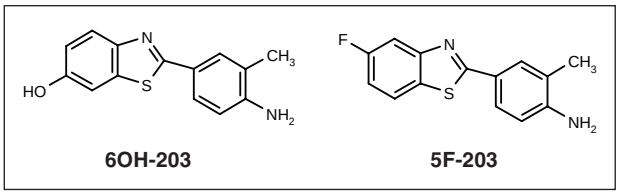


Fig. 6. The DF-203 6-hydroxy metabolite and new lead 5-fluoro isomer.

of the essential role of AhR and CYP1A1, was thought to involve CYP1A1-catalyzed DF-203 bioactivation to a reactive electrophilic species, followed by downstream formation of DNA damage. This hypothesis was supported by the finding that DF-203-derived covalent binding to recombinant CYP1A1 is significantly reduced by glutathione (60), and the detection of DF-203-generated adducts in the DNA of sensitive cells only (61).

Paradoxically, however, CYP1A1 was found to catalyze the formation of a major inactive hydroxylated metabolite from DF-203, 2-(4-amino-3-methylphenyl)-6-hydroxybenzothiazole (6OH-203) (Fig. 6). This 6-hydroxy metabolite, released into the nutrient media of sensitive cells, inhibits the cellular uptake of DF-203 and DF-203-derived covalent binding to CYP1A1, impedes CYP1A1 activity and antagonizes DF-203-induced growth inhibition. The occurrence of a second growth phase of the unusual biphasic dose-response pattern, seen consistently with first-generation benzothiazoles (49), is consistent with the emergence of this hydroxylated metabolite. As a strategy to thwart undesirable deactivating hydroxylation (62, 63), a series of 2-(4-aminophenyl)benzothiazoles fluorinated on the benzothiazole ring was synthesized.

Fluorinated benzothiazoles

The new generation of fluorinated benzothiazole analogues was found to retain the exquisite potency and selectivity profile of the parent nonfluorinated counterparts; crucially, however, in the case of the 5-fluoro and 7-fluoro isomers, eradication of the second growth phase of the biphasic relationship was observed, resulting in a conventional dose-response curve, and negligible amounts of exportable metabolites (64). 2-(4-Amino-3-methylphenyl)-5-fluorobenzothiazole (5F-203; Fig. 6) in particular possessed very similar selectivity and enhanced potency within the NCI cell panel. Unequivocal demonstration of selective antitumor activity *in vivo* has also been demonstrated when sensitive MCF-7 and resistant MDA-MB-435 tumor xenografts were transplanted in opposite flanks of the same mouse (65); only the growth of MCF-7 tumors was significantly delayed upon 5F-203 treatment (4 mg/kg i.p. on 5 consecutive days).

In common with the earlier generation 2-(4-aminophenyl)benzothiazoles, a fully functional AhR signaling pathway was found to be a necessary requisite for the induction of selective cytotoxicity by 5F-203 (66). Following drug treatment of sensitive breast cancer MCF-7 cells, induction of *CYP1A1* and *CYP1B1* transcription, 7-ethoxyresorufin-*O*-deethylase (EROD) activity (indicative of CYP1A1 protein activity) (67), and cell cycle arrest in the G_1 and S phases were observed. These observations were in complete contrast to AhR-deficient AH^{R100} variant MCF-7 cells, indicative of the central role played by the AhR pathway in mediating the cytotoxicity of this class of antitumor agents.

Further work characterizing the involvement of CYP1A1 and CYP1B1 in the unique cytotoxic activity of 5F-203 was recently carried out (68) through measurement of constitutive and 5F-203-induced *CYP1A1* and *CYP1B1* gene expression across the 60 cell lines of the NCI panel. A significant correlation between 5F-203 (and DF-203) sensitivity and induced *CYP1A1* ($r = 0.752$, $p < 0.001$), but not constitutive *CYP1A1* mRNA expression was found. Gene expression changes were found to be concordant with function, since CYP1A1 protein expression mirrored the corresponding gene expression. In addition, induction of *CYP1A1* correlated with sensitivity to 5F-203 following *ex vivo* treatment of fine needle aspirates obtained from a variety of human tumor xenografts. Induction of *CYP1A1* mRNA in response to 5F-203 treatment *ex vivo* may therefore provide a possible surrogate marker for determination of drug-sensitive tumors in patients. Interindividual variability in P-4501A induction (69) will also likely prove to be an important factor in determining the efficacy of the 5F-203 prodrug Phortress in the clinic.

Downstream events in mediating selective cytotoxicity

Antitumor 2-(4-aminophenyl)benzothiazoles, following binding to the AhR and induction of downstream gene products such as CYP1A1, become substrates for their own metabolism. Further evidence for the existence of an electrophilic reactive intermediate has been assimilated through the time- (and concentration-) dependent accumulation of DNA damage (alkaline and neutral Comet assays in breast cancer MCF-7 cells), and through the time- and concentration-dependent generation of 5F-203-derived DNA adducts specifically in sensitive cell lines (*e.g.,* MCF-7 and ovarian cancer IGROV-1 cells) (70).

Questions surrounding the antitumor and metabolic activities of 2-(4-aminophenyl)benzothiazoles and their fluorinated analogues – such as why fluorination in the 5- or 7-position of the benzothiazole ring prevents the formation of the major exportable inactive 6-hydroxy metabolite of DF-203 – cannot easily be explained or predicted by conventional chemical means. Computational quantum mechanical studies on the electronic structures and possible intermediates following CYP1A1 activation have been used to shed some light on this important issue. The counterintuitive patterns of metabolism can only be explained by considering the active intermediate to be a nitrenium ion, since the electronic distribution of

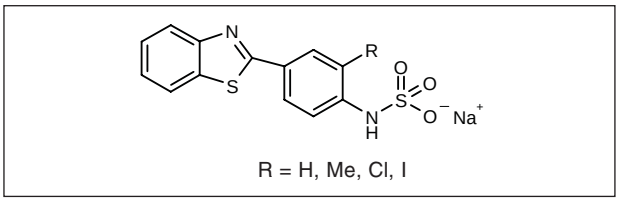


Fig. 7. Sulfamic acid salt prodrugs of 2-(4-aminophenyl)benzothiazoles.

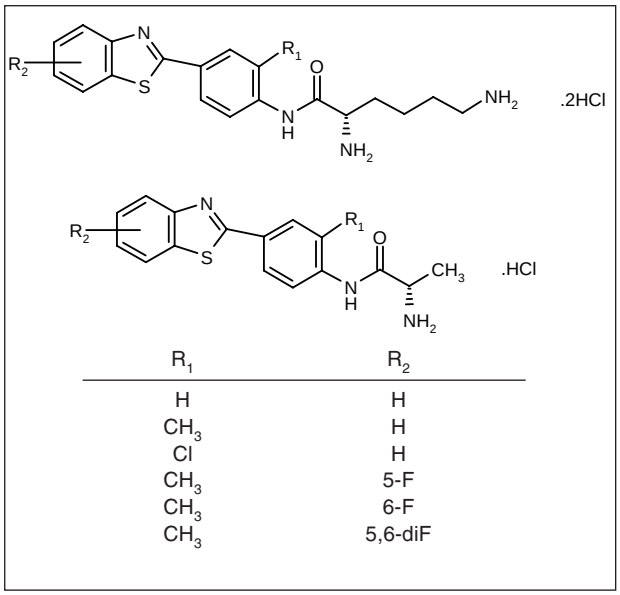
the highest occupied molecular orbital (HOMO) for the nitrenium species derived from each fluorinated analogue correlates perfectly with the production, or otherwise, of an exportable metabolite (71). Further related compounds have been analyzed by this computational method and the predictions of their metabolism have subsequently been verified experimentally.

Prodrug strategy

On the basis of its superior antitumor properties, 5F-203 was chosen as a candidate for preclinical development. 5F-203, as a small-molecule lipophilic agent, possesses negligible water solubility. This property presented something of a dilemma, as ideally an aqueous intravenous (i.v.) formulation would be preferred in order to improve bioavailability and minimize the possibility of first-pass metabolism in the liver (where CYP1A1 is known to be constitutively expressed). Initial aqueous prodrug strategies aimed at overcoming these limitations focused on simple ionic salt derivatives of the parent compounds (72), including a novel prodrug strategy involving the preparation of 2-(4-aminophenyl)benzothiazole sulfamate salts (73). Unfortunately, the sulfamate salts (Fig. 7) were found to be only sparingly soluble under aqueous conditions (pH 4-9), and degradation to the active species (free amine) was found to occur only under strongly acidic conditions.

An ultimately more successful prodrug strategy involved the conjugation of alanyl- and lysyl-amide hydrochloride salts to the exocyclic amine function of 2-(4-aminophenyl)benzothiazoles (Fig. 8) (74), a strategy similar to that used to prepare water-soluble prodrugs of dapsone (75). The synthesized amino acid prodrugs met the criteria of both aqueous solubility and chemical stability. In the presence of sensitive and insensitive cancer cells *in vitro*, rapid (several hours) and quantitative conversion to the parent amine was observed. The amino acid prodrugs retained the stark *in vitro* selectivity profile (NCI 60-cell panel) of the parent drugs (76); total growth inhibition and cytocidal activity were elicited by nanomolar concentrations of prodrug in sensitive cell lines.

The lysyl-amide dihydrochloride salt of 5F-203, known as Phortress (Fig. 9), was chosen as a clinical candidate from this class on the basis of its superior antitumor profile and pharmacokinetic properties amongst the amino acid prodrug candidates. *In vivo*, plasma concentrations



R_1	R_2
H	H
CH_3	H
Cl	H
CH_3	5-F
CH_3	6-F
CH_3	5,6-diF

Fig. 8. Amino acid prodrugs of 2-(4-aminophenyl)benzothiazoles.

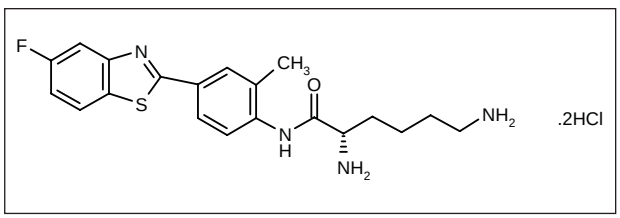


Fig. 9. Structure of the clinical candidate prodrug Phortress.

of 5F-203 regenerated from Phortress, sufficient to elicit cytocidal activity against human mammary carcinoma cell lines, persist for over 6 h.

The NCI cancer cell panel mean graphs for both Phortress and its active component 5F-203 are shown in Figure 10. Comparison of the antitumor activity of Phortress (12, 18 mg/kg i.p.) with that of doxorubicin administered via the clinically optimum route (8 mg/kg i.v. on days 1 and 8) using 9 mammary tumor xenografts revealed that Phortress was equiactive against 6 tumors, slightly (not significantly) less active against 2 tumors, and significantly better than doxorubicin in 1 xenograft model. Phortress significantly delays the growth of a range of breast and ovarian cancer xenografts *in vivo*. The mechanism of action of Phortress is summarized in Figure 11.

Antitumor diaminoflavones

Lead discovery

Flavonoids, of either natural or synthetic orgin, are known to exhibit a wide range of biological activities, including antitumor activity against a range of cancer-relevant targets (77-80). A number of flavonoids exhibit

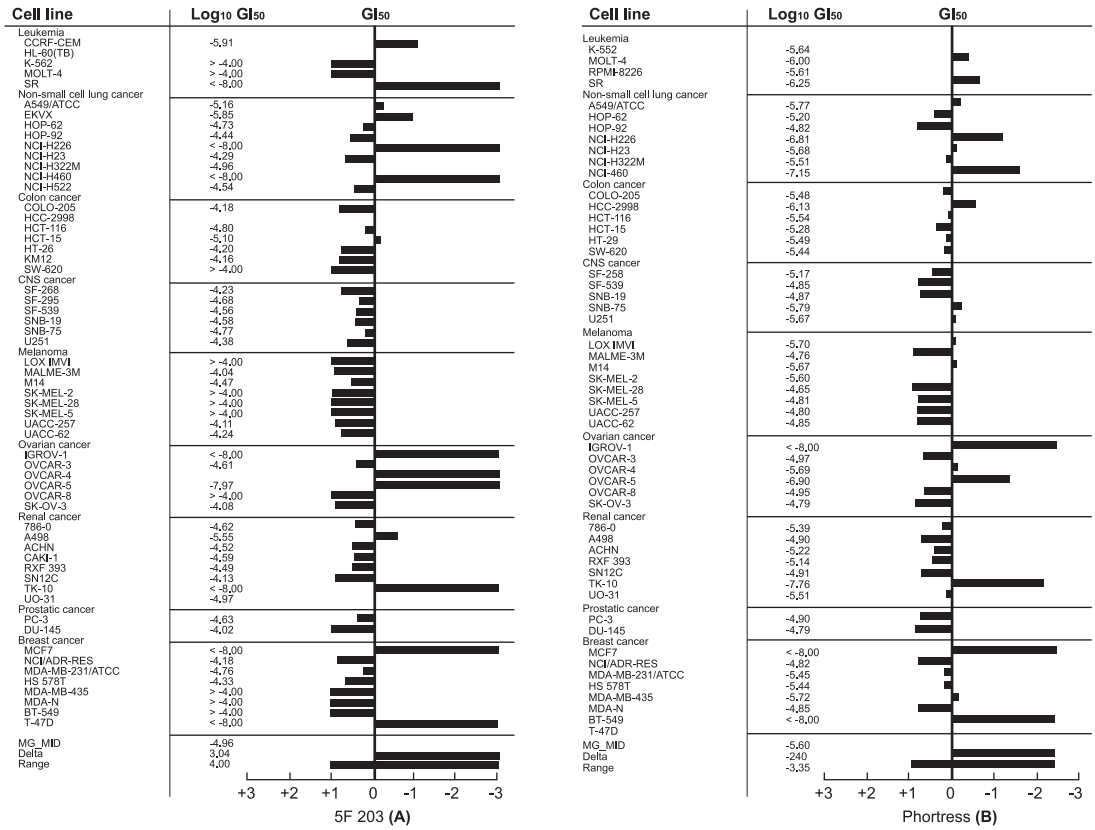


Fig. 10. Comparison of the activities of 5F-203 (**A**) and its lysyl-amide dihydrochloride salt (Phortress, **B**).

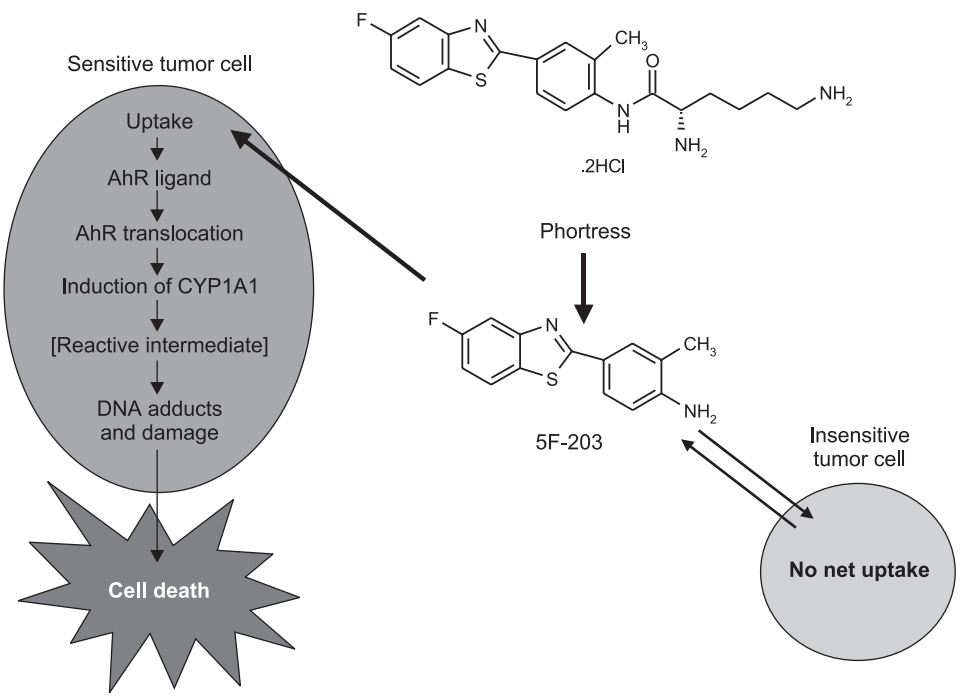
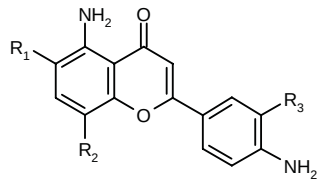


Fig. 11. Summary of Phortress mode of action.



R_1,R_2,R_3 = H; 5,4'-diaminoflavone
R_1,R_2,R_3 = F; 5,4'-diamino-6,8,3'-trifluoroflavone

Fig. 12. Structures of antitumor diaminoflavones.

antiproliferative effects against breast cancer cells or binding affinities for the estrogen receptor; apigenin, for example, possesses antiproliferative activity against the human breast cancer cell line ZR-75-1 (81). As part of a search for novel flavonoids for breast cancer, effective against both estrogen-dependent and -independent growth, Akama *et al.* (Kyowa Hakko) synthesized and tested a range of amino-substituted flavone derivatives for antitumor activity. Among the new flavone derivatives, the 5,4'-diaminoflavone (Fig. 12) was found to possess remarkable antiproliferative activity (low nanomolar IC_{50}) against the ER-positive human breast cancer cell line MCF-7, irrespective of the presence or absence of estrogen (82).

Flavonoids are known to be subject to metabolic oxidation *in vivo* (83), and the two benzene rings of 5,4'-diaminoflavone were suspected to be targets of electrophilic oxidation due to the presence of the two amino electron-donating groups. The Ames test using S-9 mix, the 9000 G supernatant of enzyme-induced rat liver homogenates, is often used to assess metabolic activation, and incubation of 5,4'-diaminoflavone with S-9 mix led to the formation of metabolites with a 16-mass increase (OH addition, LC-MS analysis). Addition of S-9 mix also led to a dramatic decrease in the activity of 5,4'-diaminoflavone against MCF-7 cells, suggestive of potential metabolic deactivation *in vivo*. The scenario of metabolic deactivation *via* aromatic ring hydroxylation has a degree of resonance with the aforementioned deactivating metabolism of 2-(4-amino-3-methylphenyl)benzothiazole (DF-203).

Calculation of the superdelocalizability (Sr) values (84) for 5,4'-diaminoflavone allowed estimates to be made of the likely positions susceptible to metabolism, and, in common with the 2-(4-aminophenyl)benzothiazole series, fluorine atoms were selectively introduced into these positions. Most notably, 5,4'-diamino-6,8,3'-trifluoroflavone (Fig. 12) was found to exhibit potent antitumor activity (nanomolar IC_{50}) against MCF-7 cells, even in the presence of S-9 mix. *In vivo,* this fluorinated diaminoflavone completely suppressed the growth of MCF-7 cells inoculated into nude mice, and exhibited potent antiproliferative activity against a range of human tumor cell lines *in vitro*, including those derived from ER-negative breast, endometrial, ovarian and liver cancers (85). 5,4'-Diamino-6,8,3'-trifluoroflavone was thus selected as a preclinical lead compound from this series.

Further exploration of SAR around the 7-position of 5,4'-diamino-6,8,3'-trifluoroflavone was subsequently carried out, following the observation that the antiproliferative activity of 5,4'-diaminoflavone was modulated by the addition of apigenin (5,7,4'-trihydroxyflavone), which itself exhibits both growth-inhibitory and growth-stimulating activity in certain types of cancer cell lines. A number of these new 7-substituted analogues were found to exhibit comparable or superior antitumor activity against MCF-7 cells compared to 5,4'-diamino-6,8,3'-trifluoroflavone both *in vitro* and *in vivo*. Notably, several of these new potent analogues, *e.g.*, 7-(acyloxy)methyl- and 7-aminomethyl-substituted, were also found to be appreciably soluble in water as compared to the water-insoluble lead 5,4'-diamino-6,8,3'-trifluoroflavone (86).

Antitumor diaminoflavones – mechanism of action

In common with the antitumor 2-(4-aminophenyl)benzothiazoles, the mechanism of action associated with the antitumor diaminoflavones such as 5,4'-diamino-7-methyl-6,8,3'-trifluoroflavone was initially unclear. The antitumor diaminoflavones produced a unique pattern of activity across the NCI cell panel (COMPARE analysis), with no statistically significant correlation to patterns of activity of known classes of antitumor agents, a finding consistent with a novel mechanism of action.

The mechanism of action associated with the lead fluorinated diaminoflavone analogue (5-amino-2,3-fluorophenyl)-6,8-difluoro-7-methyl-4*H*-1-benzopyran-4-one (AF, NSC-686288) has been studied (87-89). A mechanistic scenario has emerged for the fluorinated diaminoflavones that is reminiscent in a number of respects to the mode of action associated with the antitumor 2-(4-aminophenyl)benzothiazoles. AF was found to be an AhR agonist (90). Recombinant human (and rat) CYP1A1, and to a lesser extent CYP1A2, were found to metabolize AF to several products, one of which was identified by mass spectrometry as a potentially reactive hydroxylamine. Following treatment of sensitive human breast cancer MCF-7 cells with AF, increased *CYP1A1* mRNA and CYP1A1/1A2 protein were observed, followed by covalent binding of an AF metabolite to DNA (91). Downstream events included phosphorylation and stabilization of p53, and increased expression of the p53 transcriptional target p21. The induction of CYP1A1 and covalent binding of an AF metabolite were restricted to sensitive cancer cell lines only. Further evidence for the intimate role of the CYP1A family in mediating the antitumor activity of AF came from the observation that pretreatment with the CYP1A inducer 3-methylcholanthrene enhanced covalent binding to macromolecules of the AF metabolite, whereas covalent binding was decreased by coincubation with the CYP1A inhibitor α-naphthoflavone. These observations suggest that AF is able to utilize the AhR signaling pathway to induce CYP1A1/1A2 and its own

metabolic activation to cytotoxic intermediates, causing DNA damage and ultimately cell death in those cancer cells most responsive to CYP1A1/1A2 induction. The development of the antitumor fluorinated diaminoflavones was carried out in parallel in separate laboratories with the antitumor 2-(4-aminophenyl)benzothiazoles; notably, both lead compounds from these two classes are in advanced preclinical development within the Developmental Therapeutics Division of the NCI.

Conclusions and future outlook

This review has mainly focused on two novel classes of AhR agonists, the antitumor 2-(4-aminophenyl)benzothiazoles and fluorinated diaminoflavones, offering the potential of a "first-in-class" agent in clinical development. Clinical trials on the lead benzothiazole prodrug Phortress commenced in April 2004 in the U.K. (under the auspices of Cancer Research U.K.), and results of these initial clinical studies will further define the clinical potential of antitumor agents acting as agonists at the AhR and selectively depending on tumor CYP activation for their cytotoxic activity.

Clinical data will also help to address some of the commonly held concerns regarding this class of agent, namely the fact that a number of known carcinogens (*e.g.*, benzo[*a*]pyrene) utilize the AhR/CYP1A1 pathway to exert their cytotoxic effects, and the known constitutive expression of P-450 isoforms such as CYP1A1 in certain normal tissues (*e.g.*, liver). It is clear, however, that agents such as Phortress differ dramatically from carcinogens such as benzo[*a*]pyrene. For example, inspection of the (indiscriminate) NCI cancer cell line selectivity patterns for such polycyclic aromatic hydrocarbons reveals one striking feature by which the exquisitely selective 2-(4-aminophenyl)benzothiazole series differs dramatically from 1A1-inducing carcinogens. Favorable preclinical toxicology studies (unpublished) allow us to proceed to phase I evaluation with confidence.

It is anticipated that preclinical observations on a number of novel pharmacodynamic endpoints relating to known mechanistic features of the antitumor benzothiazoles (*e.g.*, CYP1A1 induction) may allow selection of patients likely to respond to treatment based on the biochemical phenotype of their tumor.

Acknowledgements

The author would like to acknowledge Cancer Research U.K. for funding as part of a programme grant to the Experimental Cancer Chemotherapy Research group at the University of Nottingham. The Antitumour Benzothiazole project, of which the author has been a part, is a large multidisciplinary research effort involving a number of international academic research laboratories. The support and guidance of Prof. Malcolm F.G. Stevens and Dr. Tracey D. Bradshaw (University of Nottingham, U.K.), the NCI Developmental Therapeutics Program (Director Dr. Edward A. Sausville) and the EORTC Drug Discovery Group (former SPG) is especially acknowledged.

References

1. Schmidt, J.V., Bradfield, C.A. *AH receptor signalling pathways.* Annu Rev Cell Develop Biol 1996, 12: 55-89.

2. Landers, J.P., Bunce, N.J. *The AH receptor and the mechanism of dioxin toxicity.* Biochem J 1991, 276: 273-87.

3. Rowlands, C., Krishnan, V., Wang, X., Santostefano, M., Safe, S., Miller, W.R., Langdon, S. *Characterization of the aryl hydrocarbon receptor and aryl hydrocarbon responsiveness in human ovarian carcinoma cell lines.* Cancer Res 1993, 53: 1802-7.

4. Safe, S., Astroff, B., Harris, M., Zacharweski, T., Dickerson, R., Romkes, M., Biegel, L. *2,3,7,8-Tetrachlorodibenzo-para-dioxin (TCDD) and related compounds as antiestrogens – Characterization and mechanism of action.* Pharmacol Toxicol 1991, 69: 400-9.

5. Poellinger, L., Lund, J., Gillner, M., Gustafson, J.-A. In: Molecular Mechanisms of Steroid Hormone Action. Moudgil, V.K. (Ed.). Walter de Gruyter & Co.: Berlin, 1985.

6. Burbach, K.M., Poland, A., Bradfield, C.A. *Cloning of the AH-receptor cDNA reveals a distinctive ligand-activated transcription factor.* Proc Natl Acad Sci USA 1992, 89: 8185-9.

7. Wang, G.L., Jiang, B.H., Rue, E.A., Semenza, G.L. *Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension*. Proc Natl Acad Sci USA 1995, 92: 5510-4.

8. Bradshaw, T.D., Trapani, V., Vasselin, D.A., Westwell, A.D. *The arylhydrocarbon receptor in anticancer drug discovery: Friend or foe?* Curr Pharm Des 2002, 8: 2475-90.

9. Bradshaw, T.D., Mortimer, C.G., Westwell, A.D. *Update to the arylhydrocarbon receptor in anticancer drug discovery: Friend or foe?* Med Chem Rev 2004, Manuscript submitted.

10. Denison, M.S., Nagy, S.R. *Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals.* Annu Rev Pharmacol Toxicol 2003, 43: 309-34.

11. Safe, S., Wormke, M. *Inhibitory aryl hydrocarbon receptor-estrogen receptor α cross-talk and mechanisms of action.* Chem Res Toxicol 2003, 16: 807-16.

12. Ma, Q. *Induction of CYP1A1. The AhR/DRE paradigm: Transcription, receptor regulation, and expanding biological roles.* Curr Drug Metab 2001, 2: 149-64.

13. Trapani, V. PhD thesis, University of Nottingham, U.K. 2003.

14. Rowlands, J.C., Gustaffson, J.-A. *Aryl hydrocarbon-mediated signal transduction.* Crit Rev Toxicol 1997, 27: 109-34.

15. Bell, D.R., Poland, A. *Binding of aryl hydrocarbon receptor (AhR) to AhR-interacting protein – The role of hsp90.* J Biol Chem 2000, 275: 36407-14.

16. Whitlock, J.P. *Induction of cytochrome P4501A1.* Annu Rev Pharmacol Toxicol 1999, 39: 103-25.

17. Williams, J.B., Wang, R., Lu, A.Y., Pickett, C.B. *Rat liver DT-diaphorase: Regulation of functional mRNA levels by 3-methyl-*

cholanthrene, trans-stilbene oxide, and phenobarbital. Arch Biochem Biophys 1984, 232: 408-13.

18. Ma, Q., Whitlock, J.P. *The aromatic hydrocarbon receptor modulates the Hepa 1c1c7 cell cycle and differentiated state independently of dioxin.* Mol Cell Biol 1996, 16: 2144-50.

19. Vaziri, C., Faller, D.V. *A benzo[a]pyrene-induced cell cycle checkpoint resulting in p53-independent G1 arrest in 3T3 fibroblasts.* J Biol Chem 1997, 272: 2762-9.

20. Weiss, C., Kolluri, S.K., Kiefer, F., Gottlicher, M. *Complementation of Ah receptor deficiency in hepatoma cells: Negative feedback regulation and cell cycle control by the Ah receptor.* Exp Cell Res 1996, 226: 154-63.

21. Puga, A., Xia, Y., Elferink, C. *Role of the aryl hydrocarbon receptor in cell cycle regulation.* Chem-Biol Interact 2002, 141: 117-30.

22. Chan, W.K., Yao, G., Gu, Y.Z., Bradfield, C.A. *Cross-talk between the aryl hydrocarbon receptor and hypoxia inducible factor signaling pathways – Demonstration of competition and compensation.* J Biol Chem 1999, 274: 12115-23.

23. Reiners, J.J., Clift, R.E. *Aryl hydrocarbon receptor regulation of ceramide-induced apoptosis in murine hepatoma 1c1c7 cells – A function independent of aryl hydrocarbon receptor nuclear translocator.* J Biol Chem 1999, 274: 2502-10.

24. Zaher, H., Fernandez-Salguero, P.M., Letterio, J., Sheikh, S.M., Fornace, A.J., Roberts, A.B., Gonzalez, F.J. *The involvement of aryl hydrocarbon receptor in the activation of transforming growth factor-beta and apoptosis.* Mol Pharmacol 1998, 54: 313-21.

25. Patterson, L.H., Murray, G.E. *Tumour cytochrome P450 and drug activation.* Curr Pharm Des 2002, 8: 1335-47.

26. Marinello, A.J., Bansal, S.K., Paul, B., Koser, P.L., Love, J., Struck, R.F., Gurtoo, H.L. *Metabolism and binding of cyclophosphamide and its metabolite acrolein to rat hepatic microsomal cytochrome P-450.* Cancer Res 1984, 44: 4615-21.

27. Yu, L.J., Matias, J., Scudiero, D.A., Hite, K.M., Monks, A., Sausville, E.A., Waxman, D.J. *P450 enzyme expression patterns in the NCI human tumor cell line panel.* Drug Metab Dispos 2001, 29: 304-12.

28. Yao, K.-S., O'Dwyer, P.J. *Involvement of NF-κB in the induction of NAD(P)H:quinone oxidoreductase (DT-diaphorase) by hypoxia, oltipraz and mitomycin C.* Biochem Pharmacol 1995, 49: 275-82.

29. Fitzsimmons, S.A., Workman, P., Grever, M., Paull, K., Camalier, R., Lewis, A.D. *Reductase enzyme expression across the National Cancer Institute tumor cell line panel: Correlation with sensitivity to mitomycin C and E09.* J Natl Cancer Inst 1996, 88: 259-69.

30. Carlson, D.B., Perdew, G.H. *A dynamic role for the Ah receptor in cell signalling? Insights from a diverse group of Ah receptor interacting proteins.* J Biochem Mol Toxicol 2002, 16: 317-25.

31. Fernandez-Salguero, P.M., Hilbert, D.M., Rudikoff, S., Ward, J., Gonzalez, F.J. *Aryl hydrocarbon receptor-deficient mice are resistant to 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced toxicity.* Toxicol Appl Pharmacol 1996, 140: 173-9.

32. Holcomb, M., Safe, S. *Inhibition of 7,12-dimethylbenzanthracene-induced rat mammary tumor growth by 2,3,7,8-tetrachlorobenzo-p-dioxin.* Cancer Lett 1994, 82: 43-7.

33. Gierthy, J.F., Bennett, J.A., Bradley, L.M., Cutler, D.S. *Correlation of in-vitro and in-vivo growth suppression of MCF-7 human breast cancer by 2,3,7,8-tetrachlorodibenzo-p-dioxin.* Cancer Res 1993, 53: 3149-53.

34. Bertazzi, P.A., Pesatori, A.C., Consonni, D., Tironi, A., Landi, M.T., Zocchetti, C. *Cancer incidence in a population accidentally exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin.* Epidemiology 1993, 4: 398-406.

35. Bertazzi, P.A., Bernucci, I., Brambilla, G., Consonni, D., Pesatori, A.C. *The Seveso studies on early and long term effects of dioxin exposure: A review.* Environ Health Perspect 1998, 106(Suppl. 2): 625-33.

36. Rogers, J.M., Denison, M.S. *Analysis of the antiestrogenic activity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in human ovarian carcinoma BG-1 cells.* Mol Pharmacol 2002, 61: 1393-403.

37. Ricci, M.S., Toscano, D.G., Mattingly, C.J., Toscano, W.A. *Estrogen receptor reduces CYP1A1 induction in cultured human endometrial cells.* J Biol Chem 1999, 274: 3430-8.

38. McDougal, A., Wormke, M., Calvin, J., Safe, S. *Tamoxifen-induced antitumorigenic/antiestrogenic action synergized by a selective aryl hydrocarbon receptor modulator.* Cancer Res 2001, 61: 3902-7.

39. Safe, S., Wormke, M., Samudio, I. *Mechanisms of inhibitory aryl hydrocarbon receptor-estrogen receptor crosstalk in human breast cancer cells.* J Mammary Gland Biol 2000, 5: 295-306.

40. McDougal, A., Sethi-Gupta, M., Ramamoorthy, K., Sun, G., Safe, S. *Inhibition of carcinogen-induced rat mammary tumor growth and other estrogen-dependent responses by symmetrical dihalo-substituted analogs of diindolylmethane.* Cancer Lett 2000, 151: 169-79.

41. Koliopanos, A., Kleeff, J., Xiao, Y., Safe, S., Zimmerman, A., Buchler, M.W., Friess, H. *Increased arylhydrocarbon receptor expression offers a potential therapeutic target for pancreatic cancer.* Oncogene, 2002, 21: 6059-70.

42. McDougal, A., Wilson, C., Safe, S. *Inhibition of 7,12-dimethylbenz[a]anthracene-induced rat mammary tumor growth by aryl hydrocarbon receptor agonists.* Cancer Lett 1997, 120: 53-63.

43. Corton, J.C., Anderson, S.P., Stauber, A. *Central role of peroxisome proliferator-activated receptors in the actions of peroxisome proliferators.* Annu Rev Pharmacol Toxicol 2000, 40: 491-518.

44. Watkins, R.E., Noble, S.M., Redinho, M.R. *Structural insights into the promiscuity and function of the human pregnane X receptor.* Curr Opin Drug Discov Develop 2002, 5: 150-8.

45. Waller, C.L., McKinney, J.D. *3-Dimensional quantitative structure-activity relationships of dioxins and dioxin-like compounds – Model validation and AH receptor characterization.* Chem Res Toxicol 1995, 8: 847-58.

46. Denison, M.S., Pandini, A., Nagy, S.R., Baldwin, E.P., Bonalti, L. *Ligand binding and activation of the Ah receptor.* Chem-Biol Interact 2002, 141: 3-24.

47. Petrulis, J.R., Perdew, G.H. *The role of chaperone proteins in the aryl hydrocarbon receptor core complex.* Chem-Biol Interact 2002, 141: 25-40.

48. Bradlow, H.L., Sepkovic, D.W., Telang, N.T., Osborne, M.P. *Multifunctional aspects of the action of indole-3-carbinol as an antitumor agent.* Ann NY Acad Sci 1999, 889: 204-13.

49. Bradshaw, T.D., Stevens, M.F.G., Westwell, A.D. *The discovery of the potent and selective antitumour agent 2-(4-amino-3-methylphenyl)benzothiazole (DF 203) and related compounds*. Curr Med Chem 2001, 8: 203-10.

50. Bradshaw, T.D., Westwell, A.D. *The development of the antitumour benzothiazole prodrug, Phortress, as a clinical candidate.* Curr Med Chem 2004, 11: 1009-21.

51. Shi, D.-F., Bradshaw, T.D., Wrigley, S., McCall, C.J., Lelieveld, P., Fichtner, I., Stevens, M.F.G. *Antitumor benzothiazoles. 3. Synthesis of 2-(4-aminophenyl)benzothiazoles and evaluation of their activities against breast cancer cell lines in vitro and in vivo*. J Med Chem 1996, 39: 3375-84.

52. Bradshaw, T.D., Wrigley, S., Shi, D.-F., Schultz, R.J., Paull, K.D., Stevens, M.F.G. *2-(4-Aminophenyl)benzothiazoles: Novel agents with selective profiles of in vitro antitumour activity.* Brit J Cancer 1998, 77: 745-52.

53. Bradshaw, T.D., Shi, D.-F., Schultz, R.J., Paull, K.D., Kelland, L., Wilson, A., Garner, C., Fiebig, H.H., Wrigley, S., Stevens, M.F.G. *Influence of 2-(4-aminophenyl)benzothiazoles on growth of ovarian carcinoma cells in vitro and in vivo*. Brit J Cancer 1998, 78: 421-9.

54. Hutchinson, I., Bradshaw, T.D., Matthews, C.S., Stevens, M.F.G., Westwell, A.D. *Antitumour benzothiazoles. Part 20: 3'-Cyano and 3'-alkynyl-substituted 2-(4'-aminophenyl)benzothiazoles as new potent and selective analogues.* Bioorg Med Chem Lett 2003, 13: 471-4.

55. Weinstein, J.N., Myers, T.G., O'Connor, P.M., Friend, S.H., Fornace, A.J. Jr., Kohn, K.W., Fojo, T., Bates, S.E., Rubinstein, L.V., Anderson, N.L. et al. *An information-intensive approach to the molecular pharmacology of cancer.* Science 1997, 275: 343-9.

56. Kashiyama, E., Hutchinson, I., Chua, M.-S., Stinson, S.F., Phillips, L.R., Kaur, G., Sausville, E.A., Bradshaw, T.D., Westwell, A.D., Stevens, M.F.G. *Antitumor benzothiazoles. 8. Synthesis, metabolic formation, and biological properties of the C- and N-oxidation products of antitumor 2-(4-aminophenyl)benzothiazoles.* J Med Chem 1999, 42: 4172-84.

57. Loaiza-Pérez, A.I., Trapani, V., Hose, C., Singh, S.S., Trepel, J.B., Stevens, M.F.G., Bradshaw, T.D., Sausville, E.A. *Aryl hydrocarbon receptor mediates sensitivity of MCF-7 breast cancer cells to antitumor agent 2-(4-amino-3-methylphenyl) benzothiazole.* Mol Pharmacol 2002, 61: 13-9.

58. Monks, A., Rivera, M.I., Harris, E., Hose, C., Sausville, E.A. *Gene expression changes induced by a novel antitumor agent, 2-(4-amino-3-methylphenyl)benzothiazole, in MCF-7, a sensitive breast cancer cell line, using cDNA microarrays.* Proc Amer Assoc Cancer Res 2001, 42: Abst 3417.

59. Hose, C., Rivera, M., Sausville, E.A., Monks, A. *Induction of cytochrome P450CYP1A1 and cytochrome P450CYP1B1 by 2-(4-amino-3-methylphenyl)benzothiazole (BZ) in 60 human tumor cell lines: Correlation with BZ toxicity.* Proc Amer Assoc Cancer Res 2001, 42: Abst 2749.

60. Chua, M.-S., Kashiyama, E., Bradshaw, T.D., Stinson, S., Brantley, E., Sausville, E.A., Stevens, M.F.G. *Role of CYP1A1 in modulation of antitumor properties of the novel agent 2-(4-amino-3-methylphenyl)benzothiazole (DF 203, NSC 674495) in human breast cancer cells.* Cancer Res 2000, 60: 5196-203.

61. Stevens, M.F.G., Heydon, R.T., Martin, E.A., Farmer, P.B., Bradshaw, T.D., Hutchinson, I., Westwell, A.D., Browne, H.L., Trapani, V. *Induction of CYP1A1 by 2-(4-aminophenyl)benzothiazoles leads to DNA adducts in sensitive tumor cells.* Proc Amer Assoc Cancer Res 2001, 42: Abst 1754.

62. Liehr, J.G. *2-Fluoroestradiol – Separation of estrogenicity from carcinogenicity.* Mol Pharmacol 1983, 23: 278-81.

63. Morgan, P., Maggs, J.L., Bulman-Page, P.C., Hussain, F., Park, B.K. *The metabolism of 2-fluoro-17-β-estradiol and 4-fluoro-17-β estradiol in the rat and its implications for estrogen carcinogenesis.* Biochem Pharmacol 1992, 43: 985-94.

64. Hutchinson, I., Chua, M.-S., Browne, H.L., Trapani, V., Bradshaw, T.D., Westwell, A.D., Stevens, M.F.G. *Antitumor benzothiazoles. 14. Synthesis and in vitro biological properties of fluorinated 2-(4-aminophenyl)benzothiazoles.* J Med Chem 2001, 44: 1446-55.

65. Bradshaw, T.D., Bibby, M.C., Double, J.A., Fichtner, I., Cooper, P.A., Alley, M.C., Donohue, S., Stinson, S.F., Tomaszewjski, J.E., Sausville, E.A., Stevens, M.F.G. *Preclinical evaluation of amino acid prodrugs of novel antitumor 2-(4-amino-3-methylphenyl)benzothiazoles.* Mol Cancer Ther 2002, 1: 239-46.

66. Trapani, V., Patel, V., Leong, C-O., Ciolino, H.P., Yeh, G.C., Hose, C., Trepel, J.B., Stevens, M.F.G., Sausville, E.A., Loaiza-Pérez, A.I. *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.* Brit J Cancer 2003, 88: 599-605.

67. Kennedy, S.W., Jones, S.P. *Simultaneous measurement of cytochrome P4501A catalytic activity and total protein concentration with a fluorescence plate reader.* Anal Biochem 1994, 222: 217-23.

68. Hose, C., Hollinghead, M., Sausville, E.A., Monks, A. *Induction of CYP1A1 in tumor cells by the antitumor agent 2-[4-amino-3-methylphenyl]-5-fluorobenzothiazole: A potential surrogate marker for patient sensitivity.* Mol Cancer Ther 2003, 2: 1265-72.

69. Ma, Q., Lu, A.Y.H. *Origins of individual variability in P4501A induction.* Chem Res Toxicol 2003, 16: 249-60.

70. Leong, C-O., Gaskell, M., Martin, E.A., Heydon, R.T., Farmer, P.B., Bibby, M.C., Cooper, P.A., Double, J.A., Bradshaw, T.D., Stevens, M.F.G. *Antitumour 2-(4-aminophenyl)benzothiazoles generate DNA adducts in sensitive tumour cells in vitro and in vivo.* Brit J Cancer 2003, 88: 470-7.

71. O'Brien, S.E., Browne, H.L., Bradshaw, T.D., Westwell, A.D., Stevens, M.F.G., Laughton, C.A. *Antitumor benzothiazoles. Part 23 - Frontier molecular orbital analysis predicts bioactivation of 2-(4-aminophenyl)benzothiazoles to reactive intermediates by cytochrome P4501A1.* Org Biomol Chem 2003, 1: 493-7.

72. Wheelhouse, R.T., Shi, D,-F., Wilman, D.E.V., Stevens, M.F.G. *Antitumour benzothiazoles. 4. An NMR study of the sites of protonation of 2-(4-aminophenyl)benzothiazoles.* J Chem Soc Perkin Trans 2, 1996, (7): 1271-4.

73. Shi, D.-F., Bradshaw, T.D., Chua, M.-S., Westwell, A.D., Stevens, M.F.G. *Antitumour benzothiazoles. Part 15: The synthesis and physico-chemical properties of 2-(4-aminophenyl)-benzothiazole sulfamate salt derivatives.* Bioorg Med Chem Lett 2001, 11: 1093-5.

74. Hutchinson, I., Jennings, S.A., Vishnuvajjala, B.R., Westwell, A.D., Stevens, M.F.G. *Antitumor benzothiazoles. 16. Synthesis and pharmaceutical properties of antitumor 2-(4-aminophenyl)benzothiazole amino acid prodrugs.* J Med Chem 2002, 45: 744-7.

75. Pochopin, N.L., Charman, W.N., Stella, V.J. *Amino acid derivatives of dapsone as water-soluble prodrugs.* Int J Pharm 1995, 121: 157-67.

76. Bradshaw, T.D., Chua, M.-S., Browne, H.L., Trapani, V., Sausville, E.A., Stevens, M.F.G. *In vitro evaluation of amino acid prodrugs of novel antitumour 2-(4-amino-3-methylphenyl)benzothiazoles.* Brit J Cancer 2002, 86: 1348-54.

77. Yamashita, Y., Kawada, S., Nakano, H. *Induction of mammalian topoisomerase II delendent DNA cleavage by noninteractive flavonoids, genistein and orobol.* Biochem Pharmacol 1990, 39: 737-44.

78. Ferriola, P., Cody, V., Middleton, E. Jr. *Protein kinase C inhibition by plant flavonoids.* Biochem Pharmacol 1989, 38: 1617-24.

79. Double, J.A., Bibby, M.C., Loadman, P.M. *Pharmacokinetics and antitumor activity of LM 985 in mice bearing transplantable adenocarcinomas of the colon.* Brit J Cancer 1986, 54: 595-600.

80. Beutler, J.A., Cardellina, J.H. II, Lin, C.M., Hamel, E., Cragg, G.M., Boyd, M.R., Centaureidin, A. *A cytotoxic flavone from Polymnia fructicosa inhibits tubulin polymerisation.* Bioorg Med Chem Lett 1993, 3: 581-4.

81. Hirano, T., Oka, K., Akiba, M. *Antiproliferative effects of synthetic and naturally occurring flavonoids on tumor cells of human breast carcinoma cell line, ZR-75-1.* Res Comm Chem Pathol Pharmacol 1989, 64: 69-78.

82. Akama, T., Shida, Y., Sugaya, T., Ishida, H., Gomi, K., Kasai, M. *Novel 5-aminoflavone derivatives as specific antitumor agents in breast cancer.* J Med Chem 1996, 39: 3461-9.

83. Griffiths, L.A. *Mammalian metabolism of flavonoids.* In: Flavanoids: Advanced Research. Harborne, J.B., Mabry, T.J. (Eds.). Chapman and Hall: London 1982, 681-718.

84. Ames, B.M., McCann, J., Yamasaki, E. *Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test.* Mutat Res 1975, 31: 347-64.

85. Akama, T., Ishida, H., Shida, Y., Kimura, U., Gomi, K., Saito, H., Fuse, E., Kobayashi, S., Yoda, N., Kasai, M. *Design and synthesis of potent antitumor 5,4'-diaminoflavone derivatives based on metabolic considerations.* J Med Chem 1997, 40: 1894-900.

86. Akama, T., Ishida, H., Kimura, U., Gomi, K., Saito, H. *Structure-activity relationships of the 7-substituents of 5,4'-diamino-6,8,3'-trifluoroflavone, a potent antitumor agent.* J Med Chem 1998, 41: 2056-67.

87. Kuffel, M.J., Schroeder, J.C., Pobst, L.J., Naylor, S., Reid, J.M., Kaufmann, S.H., Ames, M.M. *Activation of the antitumor agent aminoflavone (NSC 686288) is mediated by induction of tumor cell cytochrome P450 1A1/1A2.* Mol Pharmacol 2002, 62: 143-53.

88. Kuffel, M.J., Bowman, S.L., Browne, J.A., Reid, J.M., Naylor, S., Ames, M.M. *Tumor cell- and tissue-selective cytochromes P450 oxidation of aminoflavone analog NSC 686288 (AF).* Proc Am Assoc Cancer Res 2000, 41: Abst 370.

89. Kuffel, M.J., Squillance, D.P., Reid, J.M., Ames, M.M. *Cytochrome P450-catalysed metabolism of the aminoflavone analog NSC 686288 in human and rat liver microsomes: The role of CYP1A subfamily enzymes.* Proc Am Assoc Cancer Res 1999, 40: Abst 384.

90. Loaiza-Pérez, A.I., Vistica, D., Kennedy, S., Hose, C., Ciolino, H., Yeh, G., Trepel, J., Sausville, E.A. *The aryl hydrocarbon receptor mediates sensitivity of MCF-7 cells to the antitumor agent aminoflavone NSC 686288.* Clin Cancer Res 2001, 7: 413.

91. Loaiza-Pérez, A.I., Kennedy, S., Hose, C., Hollingshead, M., Alley, M., Ciolino, H., Yeh, G., Linehan, M., Vistica, D., Sausville, E.A. *CYP1A1 and CYP1B1 induction and high covalent binding of metabolites are markers to predict sensitivity to aminoflavone in breast and renal cancer cells.* Cancer Epidem Biomar 2002, 11: A224.