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Chapter

Exploring New Molecular Targets in Advanced Ovarian Cancer: The Aryl Hydrocarbon Receptor (AhR) and Antitumor Benzothiazole Ligands as Potential Therapeutic Candidates

Andrea I. Loaiza Perez and Tracey D. Bradshaw

Abstract

Antitumor benzothiazoles, including 2-(4-amino-3-methylphenyl)-5-fluoro-benzothiazole (5F 203; NSC 703786), non-fluorinated parent compound DF 203 (NSC 674495), and Phortress (NSC 710305), the lysyl amide prodrug of 5F 203, are experimental anticancer agents with activity in ovarian and breast cancer models *in vitro* and *in vivo*. These compounds require (and induce their own) metabolism by cytochrome P450 (CYP) enzymes (e.g., CYP1A1) for antitumor action. The aryl hydrocarbon receptor (AhR) is the main transcriptional regulator of CYP1A1, and we have previously demonstrated that DF 203 and 5F 203 are potent AhR ligands and trigger activation of AhR signaling in sensitive breast and ovarian cancer cells, causing nuclear translocation of AhR. We propose that AhR may represent a new molecular target in the treatment of ovarian tumors, and 5F 203 may exemplify a potential novel treatment. Furthermore, putative biomarkers of sensitivity to this agent have been identified.

Keywords: ovarian cancer, AhR, benzothiazoles

1. Introduction

Ovarian cancer is one of the most lethal gynecological cancers. The National Cancer Institute (NCI) estimated ~22,240 new cases with ~14,070 deaths from ovarian cancer in the US in 2018 [1]. Globally, in 2012, 239,000 women were diagnosed with ovarian carcinoma and 152,000 deaths from this disease were recorded [2]. Unfortunately, the majority of cases are only diagnosed at advanced stages (stage III or IV), a consequence of the cancer's asymptomatic nature, and overall survival lies between 5 and 25% [3, 4]. Hence, the inability to detect this disease during early stages has led to poor prognosis. Despite improvements in medicine and patient care, screening for detection of early-stage ovarian cancer is presently lacking. Thus, a better understanding of the molecular events that underlie ovarian cancer development is needed.

The current strategy for treatment of ovarian cancer is surgery followed by radiotherapy and chemotherapy [3, 4]. Although ~70% of ovarian cancer patients respond initially to a combination of platinum and taxane-based chemotherapy, drug-resistance emerges and current treatments are of limited efficacy in preventing tumor recurrence and progression [3, 4]. Thus, despite surgery, radiotherapy, and chemotherapy, most patients present with recurrent disease within 12–18 months, which spreads rapidly within the peritoneal cavity. In platinum-resistant disease, survival rarely exceeds 5 months [1, 5]. Thus, new anti-neoplastic agents are urgently needed to improve the prognoses for ovarian cancer patients. Recently, evidence has emerged revealing the importance of genomic aberrations in the progression of ovarian cancer [6–8]. Through the use of high-throughput technologies (i.e., array comparative genomic hybridization (aCGH), microarray, and SNP arrays), specific genomic regions have been identified to be either amplified or silenced in tumor progression [6, 7].

Molecularly targeted cancer therapies recently introduced into the clinic include drugs designed to interfere with specific proteins ("molecular targets") that are involved in the growth, progression, and spread of cancer. Used in the treatment of ovarian cancer are bevacizumab (a VEGF inhibitor) [9], olaparib [10], and niraparib (PARP inhibitors) [11].

The objective of our work has been for many years to validate the aryl hydrocarbon receptor as a molecular target of antitumor drugs, for the treatment of different cancers including ovarian cancer.

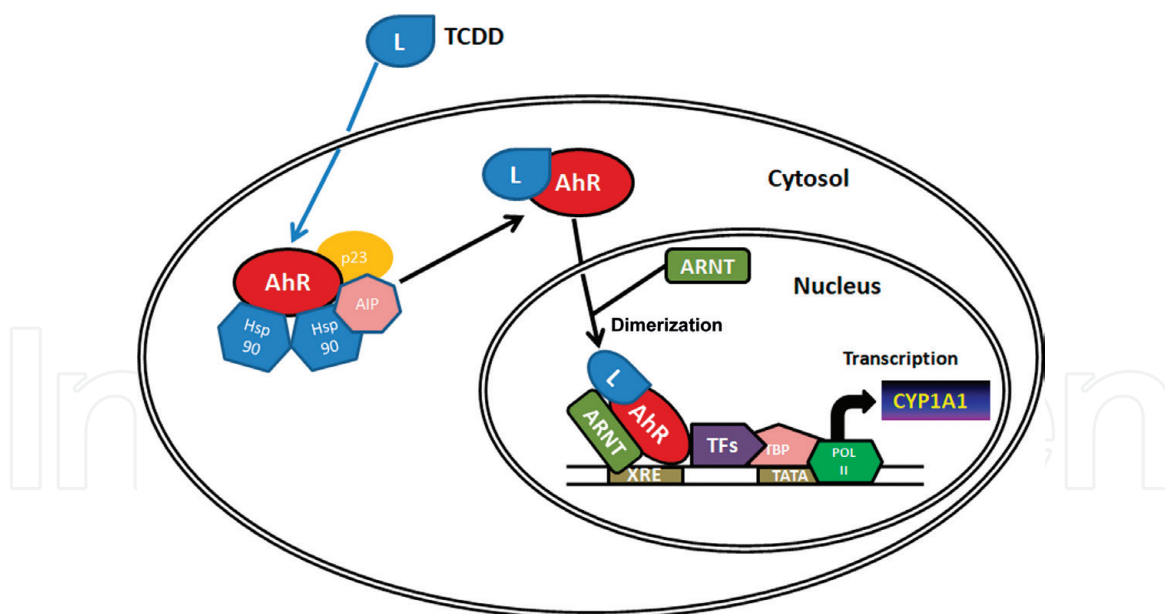## 1.1 The aryl hydrocarbon receptor as a putative molecular target for cancer therapeutics

1.1.1 AhR structure

The aryl hydrocarbon receptor (AhR) was initially defined as a receptor for environmental toxins such as dioxin. It has been described as a "pioneer member" of the basic-helix/loop/helix PER-ARNT-SIM (bHLH/PAS) family of "sensors" of foreign and endogenous signals [12].

As intimated, other members include AhR nuclear translocator (ARNT); *drosophila* proteins, single-minded (SIM) and period (PER); and hypoxia inducible factor 1α (HIF 1α) [13–16]. AhR is a ligand-activated transcription factor. The most commonly known ligands of AhR are polycyclic and polyhalogenated hydrocarbons (benzopyrene, 3-methyl-colanthrene), xenobiotics (phenobarbital), and other pesticides such as tetrachlorodibenzo-p-dioxin (TCDD).

1.1.2 AhR activation and signaling

AhR is localized within the cell cytosol constitutively where it is part of an inactivated complex composed of two heat-shock proteins: heat-shock protein 90 (Hsp90), the aryl hydrocarbon receptor interacting protein (AIP), and a 23-kDa protein (p23) (**Figure 1**). Hsp90 acts as a chaperone maintaining AhR in a favorable ligand-binding configuration while preventing its nuclear translocation. Hydrophobic ligands of AhR enter the cell by diffusion and bind to the receptor associated to Hsp90. Ligand binding to the receptor triggers a conformational change in AhR to a form that exhibits stronger affinity for DNA. This event leads to dissociation of the cytoplasmic complex and AhR nuclear translocation. In the nucleus, AhR interacts with ARNT forming a heterodimer that binds to specific DNA sequences—the xenobiotic response elements (XREs) in the promoter regions of genes within the AhR gene battery. Binding leads to transcriptional activation of

Figure 1.
The aryl hydrocarbon receptor signaling pathway.

genes activated by AhR including those encoding phase I and II metabolic enzymes such as cytochrome P450 (CYP) 1A1, CYP1A2, and CYP1B1. AhR activation was first described as a cellular response to promote elimination of ambient contaminants and xenobiotics [17–19]. In humans, AhR is localized in liver, lungs, kidneys, placenta, lymphocytes, ovary, and breast. AhR/ARNT complex activation is tissue-specific and depends on co-regulators and repressors present in different cell types [18].

1.2 The importance of AhR in ovarian cancer

Functional AhR has been reported in rat and mouse uteri. AhR knockout mice exhibited no histopathological changes in uterine tissues; however, dioxin inhibited estrogen-induced responses including estrogen receptor (ER) binding in rat uteri. In addition, *in utero* and lactational exposure to dioxin results in decreased uterine weights in female offspring during estrus and diestrus. The female reproductive tract expresses mRNA for the transcription factors AhR and ARNT, and changes in their expression at select target sites in specific pathological conditions such as endometriosis and uterine leiomyomas suggest a potential role for these factors in the pathogenesis of these conditions [20].

The role of the AhR and AhR agonists has not been extensively investigated in endometrial and ovarian cancer cell lines; however, there is evidence that AhR-ERα cross talk and growth inhibitory pathways are operative [21–23], requiring further investigation.

Intriguingly, immune suppression in ovarian cancer has been described, with a particular focus on the potential involvement of the c-KIT/PI3K/AKT, wnt/β-catenin, IL-6/STAT3, and AhR signaling pathways in regulation of indoleamine 2,3-dioxygenase expression in tumor-associated macrophages [24].

AhR has important roles in homeostasis and evidence is accumulating to support its contribution to disease pathogenesis—including cancer. AhR expression has been detected in multiple tumor types and its function probed by RNA interference, over-expression, and inhibition studies. AhR knockdown led to decreased cancer cell proliferation, migration, and invasion *in vitro*, and *in vivo*, constitutive over-expression resulted in enhanced stomach and liver cancers, suggesting a pro-oncogenic role. In contrast, loss of AhR in transgenic mice that spontaneously

develop colorectal carcinoma (CRC) and carcinogen-induced tumors led to increased carcinogenesis suggesting a tumor-suppressive role for AhR [25].

Specific to this review, AhR has been found to be widely expressed in many histotypes of ovarian cancer tissue; in the ovarian cancer tissue microarray, the AhR immunoreactivity was present in disgerminoma (DISG), adenocarcinoma (ADEN), teratoma malignant change (TMC), yolk sac tumor (YST), mucinous adenocarcinoma (Mu-ADEN), serous adenocarcinoma (low grade (L-Se-ADEN) and high grade (H-Se-ADEN), but not in normal tissue (NORM). The semi-quantification analysis revealed that the value in NORM was similar to that in DISG and ADEN, but was much lower than that in TMC, YST, Mu-ADEN, and L- and H-Se-ADEN. No difference was detected between the grades, stages, and tumor node metastasis classifications in each histotype of ovarian cancer tissues studied. Moreover, the endogenous AhR ligand 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) inhibited proliferation of OVCAR-3 cells and migration of SKOV-3 cells *in vitro* and suppressed growth of OVCAR-3 cell xenografts in mice [26].

### 1.3 Benzothiazoles and aminoflavone: AhR-targeted anticancer therapies

The benzothiazole (Bz) class of experimental antitumor agent includes 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (5F 203; NSC 703786; **Figure 2**); non-fluorinated parent compound DF 203 (NSC 674495); and Phortress (NSC 710305), the lysyl amide prodrug of 5F 203. These experimental antitumor agents elicit potent and selective antitumor activity *in vitro* against certain cell lines originating from breast (irrespective of ER status) and ovarian carcinomas. Empirical screening in the NCI cell line anticancer drug screen revealed that the Bzs [27–32] and also aminoflavone (AF) [33] were noteworthy for their distinct (selective and potent) patterns of growth inhibitory activity. "Sensitive" cell lines caused total carcinoma cell growth inhibition (TGI) between 0.1 and 1 μM, while "resistant" cell lines are refractory to Bz and AF (TGI concentrations <10 μM). Among the consistently sensitive cell lines to both compound classes were the ER (+) breast cancer cell lines MCF-7 and T47D, certain ovarian (e.g., IGROV-1) and renal (TK10) cancer cell lines [34]. Intriguingly, IGROV-1 cells developed in the laboratory possessing acquired resistance to cisplatin were equi-sensitive to antitumor Bzs as IGROV-1 parental cells. Detailed mechanistic studies for both Bzs and AF identified a mode of action distinct from current clinical chemotherapeutic agents. In "sensitive" cells, Bzs and AF activate AhR signaling, as might be anticipated from their planar structures [35].

AhR signal transduction induces expression of CYP1A1 and (in certain cell lines) CYP1B1. Prior work has demonstrated that CYP1A1 can metabolize (actively biotransform) Bzs and AF to produce DNA-damaging metabolites [30, 33] (**Figure 2**). For example, DNA adducts, single- and double-strand breaks have been detected exclusively in sensitive cells exposed to 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (5F 203), and in Bz-sensitive tumor xenografts extracted from Phortress-treated mice [36–39]. Subsequently, it was irrefutably demonstrated that CYP1A1-bioactivation of 5F 203 resulted in generation of an electrophilic species (nitrenium ion) able to form glutathione conjugates and dGuo adducts [40].

1.4 5F 203 activity in ovarian cancer

Antitumor Bzs, including 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (5F 203; NSC 703786; **Figure 2**), non-fluorinated parent compound DF 203 (NSC 674495), and Phortress (NSC 710305), the lysyl amide prodrug of 5F 203, are experimental anticancer agents with activity in ovarian and breast cancer models *in vitro* and *in vivo* (**Figure 3A** and **B**) [36, 41].
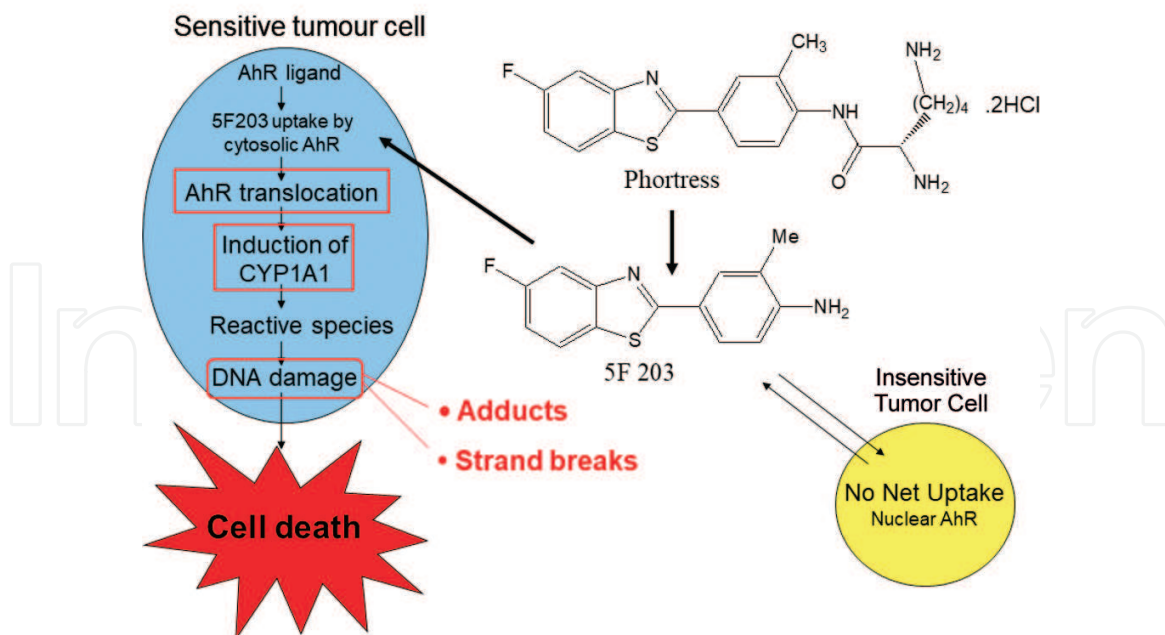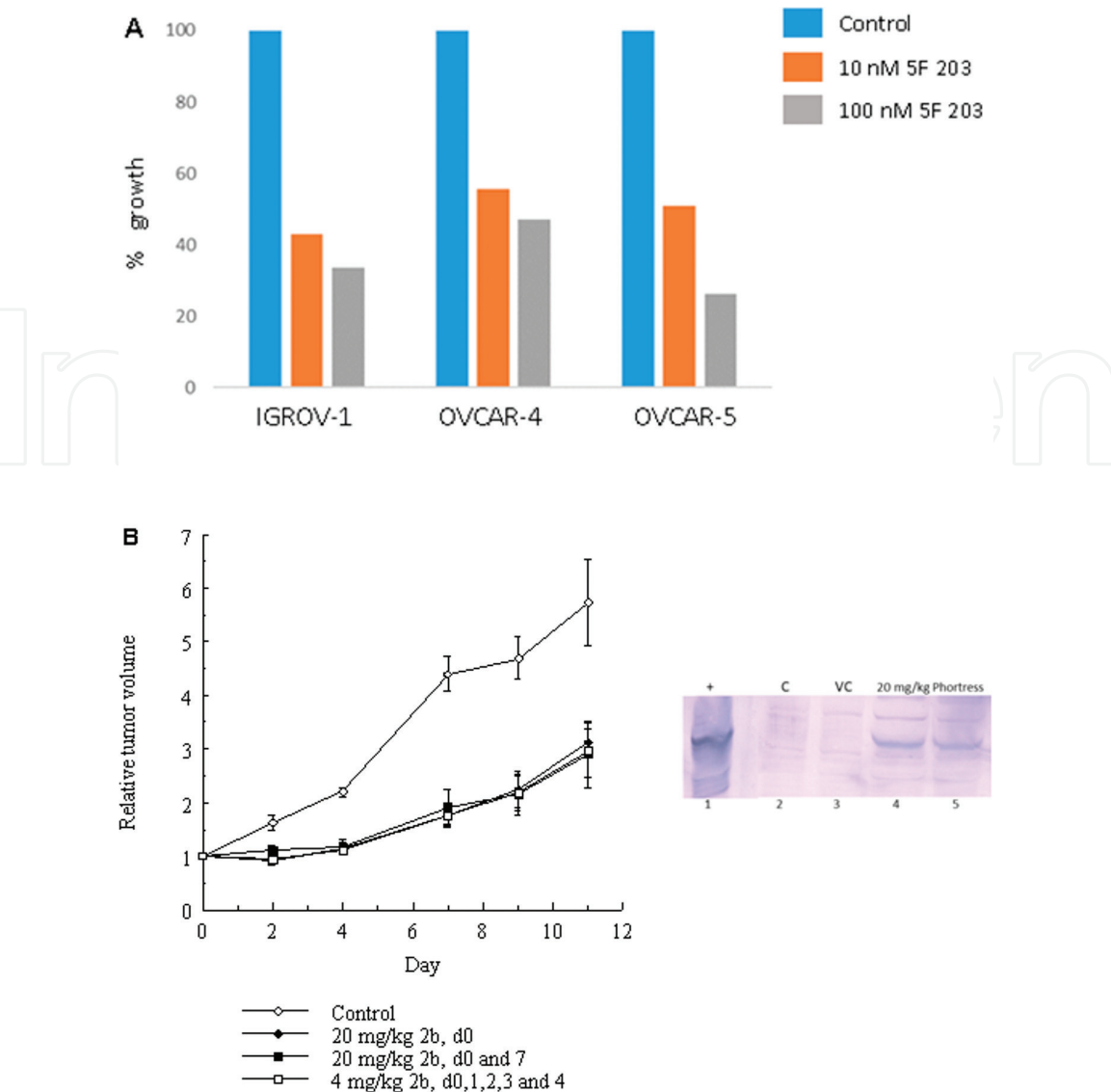
Figure 2.
5F 203, liberated from Phortress binds cytosolic AhR, triggering nuclear translocation and binding to the XRE within the promoter region of AhR-battery genes including CYP 1A1. This enzyme-catalyzed biotransformation of 5F 203 produces nitrenium species that generate lethal DNA adducts at nucleophilic sites precipitating DNA double-strand breaks and apoptosis, exclusively in sensitive cells. In inherently resistant cells, there is no net cellular uptake of lipophilic 5F 203.

As introduced, these compounds require metabolism by cytochrome P450 (CYP) enzymes (e.g., CYP1A1) for antitumor action. The aryl hydrocarbon receptor (AhR) is the main transcriptional regulator of CYP1A1 and we have previously demonstrated that DF 203 and 5F 203 induce activation of AhR signaling in sensitive breast cancer cells, causing nuclear translocation of AhR [27–29]. Also, IGROV-1 human ovarian cancer cells, sensitive to 5F 203 treatment, show induction of CYP1A1 expression by 5F 203 and Phortress (**Figure 3B**), whereas SKOV-3 cells, resistant to these agents, express neither constitutive nor inducible CYP1A1 [42]. Fine needle aspirates obtained from IGROV-1 human xenografts, treated *ex vivo* with 5F 203, resulted in induction of CYP1A1 expression. This was not observed in 5F 203-resistant tumors. It was proposed that induction of *cyp1a1* mRNA in response to 5F 203 treatment *ex vivo* might provide a possible biomarker for determination of drug-sensitive ovarian tumors in patients [42]. Compounds that activate AhR signaling and induce CYP expression frequently generate reactive oxygen species (ROS) in susceptible cells.

Experimental and clinical evidence has emerged linking oxidative stress to pathologies including carcinogenesis [43]. However, oxidative stress is not always detrimental, and may, if induced in a selective manner, be of therapeutic benefit. Many chemotherapeutic agents induce oxidative stress integral to their antitumor mechanism [44]. High-grade ovarian tumors generally present high ROS levels and respond better to treatment with antitumor agents that induce further ROS, such as paclitaxel [45]. In addition, c-JUN amino terminal kinase (JNK), ERK, and P38MAPK sustained activation have key roles in cellular stress-induced apoptosis [46].

1.5 5F 203 induces AhR translocation and activation of CYP1A1-related promoter sequences in 5F 203-sensitive ovarian cancer cells

It has been established that 5F 203 is a potent AhR ligand [35] able to induce nuclear translocation of AhR with subsequent binding to XRE sequences resulting

Figure 3.
A. In vitro growth inhibitory activity of 5F 203 against ovarian carcinoma cell lines. Representative data generated at the NCI are shown. Cells were exposed to test agent for 48 h before growth was assessed by sulforhodamine blue (SRB) assay. B. In vivo Phortress efficacy against IGROV-1 ovarian tumor xenografts. IGROV-1 ovarian xenografts were transplanted s.c. into flanks of NCR-Nu female nude mice. Animals were treated i.v. with Phortress in saline according to the indicated schedules. Control animals received vehicle alone. Tumor volumes were measured using calipers. Western blot showing induction of CYP1A1 protein in IGROV-1 xenograft tumors retrieved from mice treated with Phortress (20 mg/kg; 24 h). Tumor lysates were prepared and proteins separated by PAGE: Lane 1, +ve control, 5 µg CYP1A1 microsomes; lanes 2 and 3, untreated and vehicle control-treated samples; lanes 4 and 5, 24 h 20 mg/kg Phortress [36].

in transcriptional activation of target genes including CYP1A1 and CYP1B1 in breast cancer cells sensitive to this agent [28]. As 5F 203 causes IGROV-1 cytotoxicity and consistent with the hypothesis that 5F 203 is an AhR agonist ligand [47], we wished to determine whether 5F 203 activates AhR signaling in IGROV-1 cells with resulting AhR translocation from cytoplasm to nucleus.

The effect of 5F 203 (1 µM) on subcellular distribution of immunoreactive AhR protein was studied by Western blot. We demonstrated (**Figure 4A**), in IGROV-1 cells treated with DMSO only, the cytoplasmic fraction contained relatively high levels of AhR protein compared with the nuclear fraction. In contrast, after treatment of cells with AhR agonists, 1 µM 5F 203 or 10 nM TCDD (positive control), between 1 and 6 h, immunoreactive AhR protein could be detected in the nuclear fraction, indicating AhR translocation [48].

To identify whether 5F 203 caused AhR nuclear translocation in 5F 203-insensitive ovarian carcinoma cells, the effect of 5F 203 (1 µM) on subcellular distribution

of immunoreactive AhR protein was assessed by Western blot in SKOV-3 cells. In DMSO-treated cells, AhR protein levels were high in cytoplasmic and nuclear fractions. After treatment with 1 µM 5F 203 (0.5–6 h), AhR protein in SKOV-3 cytoplasm remained unchanged. AhR was already present in SKOV-3 nuclei and further translocation was negligible.

After treatment with TCDD, although cytoplasmic AhR was lost, there was no evidence of AhR gain in nuclear fractions (**Figure 4A**). These results were confirmed by immunofluorescence of AhR.

It was then logical to investigate putative activation of CYP1A1-related promoter sequences in 5F 203-sensitive ovarian cancer cells.

CYP1A1 and CYP1B1 promoters are regulated by AhR, which forms a heterodimer with the AhR nuclear transporter (ARNT). Binding of the complete dimer to XRE promoter regions mediates transcription of AhR-responsive genes, including CYP1A1. IGROV-1 and SKOV-3 were transfected with XRE-luciferase reporter construct (pTX.Dir), as a control, the same reporter construct without XRE elements (pT81) was used. Cells were then treated with 0.1% DMSO, 0.1–1 µM 5F 203 or TCDD 10 nM. In IGROV-1 cells transfected with pTX.Dir, 5.5-fold induction of luciferase activity was observed when cells were treated with 1 µM 5F 203 (**Figure 4B**).
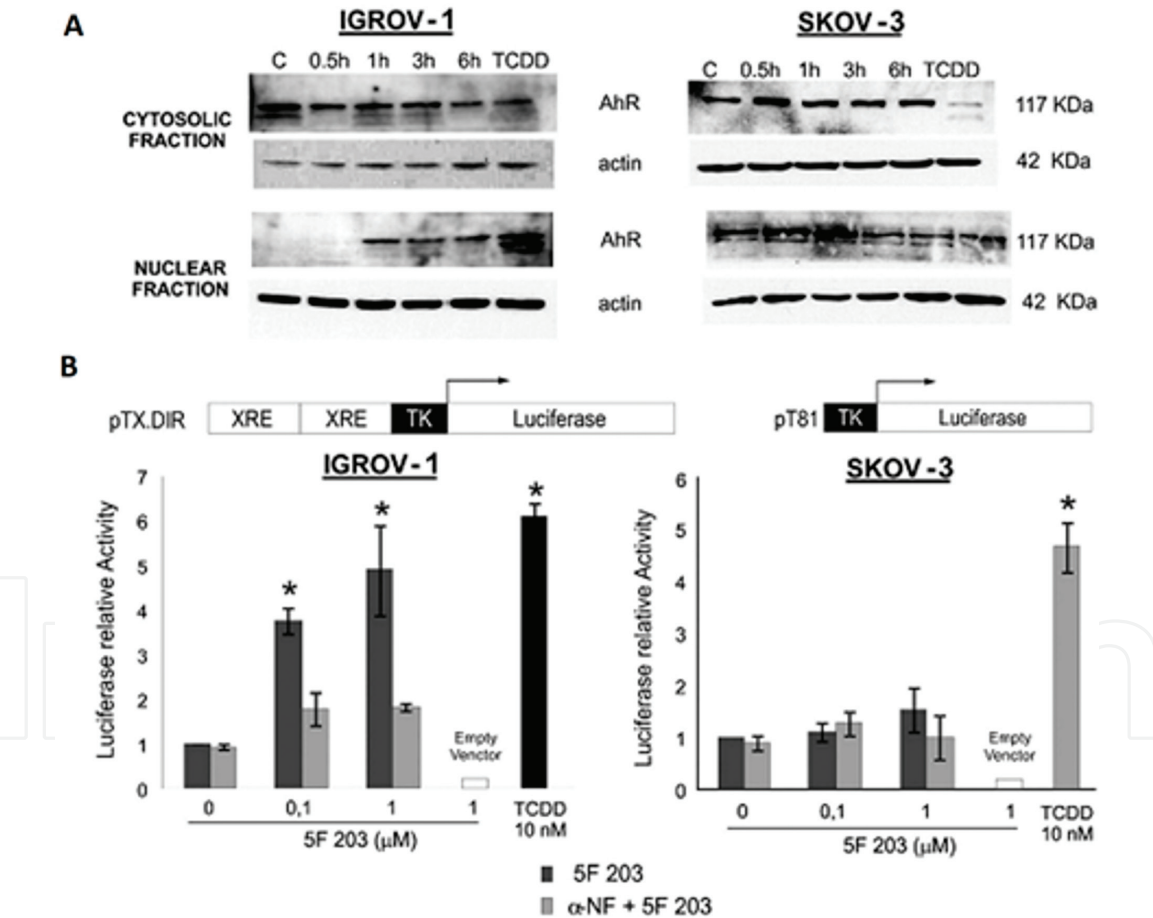
Figure 4.
5F 203 induces AhR nuclear translocation and activation of CYP1A1-related promoter sequences in sensitive ovarian cancer cell lines. A: AhR Subcellular localization. IGROV-1 and SKOV-3 cells were incubated with 5F 203 (1 µM) for indicated times, DMSO (0.1%) for 6 h or TCDD (10 nM) for 1 h as a positive control. Nuclear and cytosolic fractions were isolated using a commercial kit and analyzed for AhR content by Western blot. The figure shows representative Western blots. All Western blots were performed three times for each cell line and revealed the same pattern of AhR subcellular distribution. B: Activation of CYP1A1-related promoter sequences. Cells were transfected with a plasmid containing XRE (AhR consensus sequences) upstream of the luciferase reporter gene and a second plasmid containing R. reniformis luciferase gene as an internal control. After 24 h, cells were incubated with 5F 203 for 18 h or pre-treated for 1 h with 1 µM α-NF followed by 18 h of 5F 203 plus 1 µM α-NF. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega). Values represent the average of three independent experiments.

No induction in luciferase activity was observed when SKOV-3 cells transfected with pTX.Dir were treated with 5F 203 (1 µM). Cells transfected with pT81 and treated with 1 µM 5F 203 showed negligible luciferase activity induction (1.1-fold).

These findings clearly demonstrated that 5F 203 induces activation of promoter sequences known to respond to AhR-mediated signals. This is consistent with interaction of protein complexes induced by treatment with 5F 203 through the XRE CYP1A1 promoter sequence.

In IGROV-1 cells, pre-treatment (1 h) with AhR antagonist α-NF (1 µM) followed by co-treatment (18 h) with 5F 203 plus 1 µM α-NF reduced induction (2.65-fold) of luciferase activity. These results support the requirement of AhR in increased XRE-luciferase activity [48].

1.6 5F 203-induced ROS and γH2AX foci formation in sensitive cells is mediated by AhR

Previous studies demonstrated CYP1A1 induction by 5F 203, and CYP catalyzed 5F 203 biotransformation to DNA reactive species [40]. CYP activity contributed to increased intracellular ROS levels [49]. Oxidative stress is involved in various biological processes including proliferation and apoptosis. Therefore, we compared the effect of 5F 203 on ROS production in Bz-sensitive IGROV-1 cells and Bz-insensitive SKOV-3 cells. To determine whether 5F 203 altered intracellular ROS levels, cells were treated with 5F 203 for 6 h and exposed to 2,7-DCF before ROS levels were evaluated using flow cytometry. 5F 203 increased ROS levels in IGROV-1 cells only. This effect, detectable following 1 h of 1 µM 5F 203 treatment, was partially blocked by pre-incubation of cells with α-NF (**Figure 5A**). Also, pre-treatment with ROS inhibitors N-acetyl cysteine (NAC) and Trolox partially decreased the effect of 5F 203 in IGROV-1 cells (**Figure 5B**). In contrast, 5F 203 strongly inhibited ROS production in SKOV-3 cells, despite detection of neither phenotypic changes nor AhR translocation [48].

It is reported that ROS may cause activation of nuclear factor kappa B (NF-κB) [50]. As activation of this pathway induces NF-κB nuclear translocation, we performed immunostaining of NF-κB using a specific antibody in IGROV-1 and SKOV-3 cells before and after treatment with 5F 203. 5F 203 induced NF-κB translocation in IGROV-1 cells only, and this effect was prevented by pre-treatment of cells with 1 µM α-NF. We then investigated whether 5F 203 caused DNA damage. To determine DNA double-strand breaks (DSB), γH2AX foci were measured in IGROV-1 and SKOV-3 cells following exposure to 5F 203 (1 µM, 2–4 h). DNA DSB formation precipitates serine 139 phosphorylation of histone H2AX, producing γH2AX at DSB sites [48]. γH2AX foci appeared within nuclei of IGROV-1 cells only following treatment with 5F 203 (1 µM; 2–4 h; **Figure 5C**). Foci formation was partially blocked by pre-treatment of cells with α-NF, confirming that activation of AhR is needed for 5F 203-induced DNA damage. In contrast, neither vehicle-treated cells nor SKOV-3 cells challenged with 5F 203 displayed γH2AX foci within their nuclei at any time point examined. These data are consistent with neutral COMET assays performed to examine DNA damage (double-strand breaks) wrought as a consequence of dose-dependent DNA adduct generation which has been detected following treatment of IGROV-1 cells *in vitro* with 5F 203 or Phortress, and in IGROV-1 xenografts retrieved from tumor-bearing mice exposed to Phortress [36].

1.7 5F 203 modulates expression and phosphorylation of stress MAPKs

Mitogen-activated protein kinases (MAPKs) can be modulated by many factors including cell lesions induced by DNA damage [51]. We therefore investigated the effect of 5F 203 on activation of these protein kinases in IGROV-1 cells. As depicted
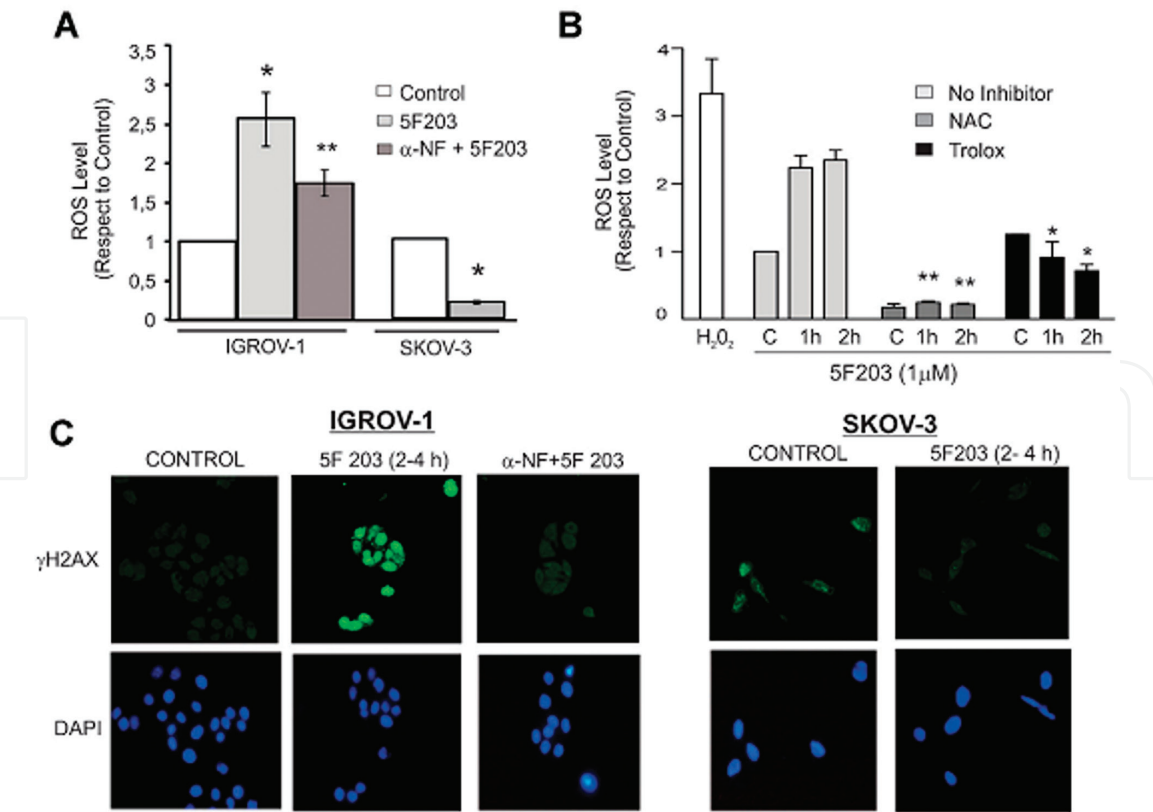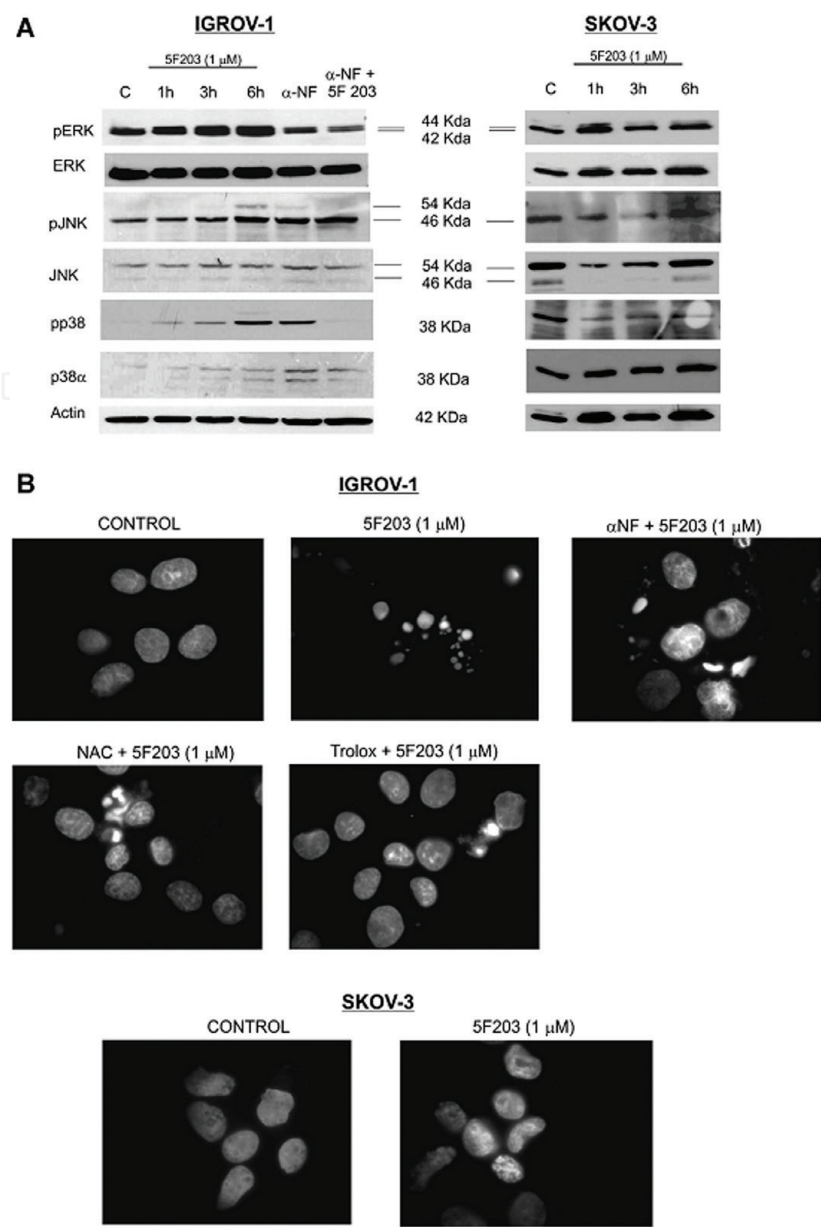
Figure 5.
5F 203 increases ROS levels. Exponentially growing IGROV-1 and SKOV-3 cells were treated with 5F 203 (1 μM) or vehicle control (0.1% DMSO) continuously for 1, 2, 4, or 6 h and ROS levels were measured by flow cytometry after incubation with 2,7-DCF. Data represent the mean of at least two independent experiments where n = 2 per experiment; bars, SEM. *P < 0.05 when compared to untreated cells, **P < 0.05 when compared to cells treated without AhR inhibitor. B: Trolox and NAC inhibition of 5F 203-mediated ROS induction. IGROV-1 cells were exposed to 0.1% DMSO (control), 5F 203 (1 μM) for 1 or 2 h, or pre-treated with Trolox (250 μM) or NAC (100 μM) for 1 h followed by 5F 203 (1 μM) inhibitor for 1 or 2 h. ROS levels were measured by fluorometry after incubation with 2,7-DCF. Data represent the mean of at least two independent experiments where n = 2 per experiment; bars, SEM. *P < 0.05 or **P < 0.01 when compared to cells treated without ROS inhibitor. C: Induces γH2AX foci formation in sensitive IGROV-1 cells. A: Measurement of ROS levels. γH2AX foci following 2–4 h of treatment of cells with 5F 203 (1 μM); IGROV-1 cell nuclei were stained with DAPI. Stained cells were visualized on a fluorescence Nikon C1 confocal microscope using a 60X PlanApo AN 0.95 objective, images were processed and analyzed with Nikon C1-EZ package, version 2.20.

in **Figure 6A**, 5F 203 induced phosphorylation of JNK and P38, the stronger effect was attained after 6 h of treatment in both cases. Also, treatment with 5F 203 increased P38 α levels. Pre-treatment of cells with α-NF decreased phosphorylation of these kinases and P38 α expression, which confirmed that 5F 203 affected expression and activation of these proteins through AhR activation. Exposure to 5F 203 (1 h) also increased phosphorylation of ERK in IGROV-1 cells.

The ability of 5F 203 to induce apoptosis was evaluated. Exposure of IGROV-1 cells to 5F 203 (1 μM; 24 h) induced apoptotic body formation (**Figure 6B**). In contrast, SKOV-3 cells, resistant to 5F 203, did not show such features (**Figure 6B**). Also, pre-treatment of cells with α-NF partially blocked the pro-apoptotic effect of 5F 203 in IGROV-1 cells. These data confirmed that AhR is involved in 5F 203-induced cell death [48].

Trolox (a vitamin E derivative) and NAC are potent ROS scavengers often used as antioxidant agents. We pre-treated IGROV-1 cells with Trolox or NAC in order to investigate the effect of ROS depletion on 5F 203-induced growth inhibition. Cells were exposed to 5F 203 (1 μM, 24 h) after pre-treatment with Trolox (250 μM) or NAC (100 μM) for 1 h. As observed in **Figure 6B,** both inhibitors partially reduced 5F 203-induced apoptotic body formation. These data support the involvement of ROS-generation in 5F 203-induced apoptosis in sensitive IGROV-1 cells [48].
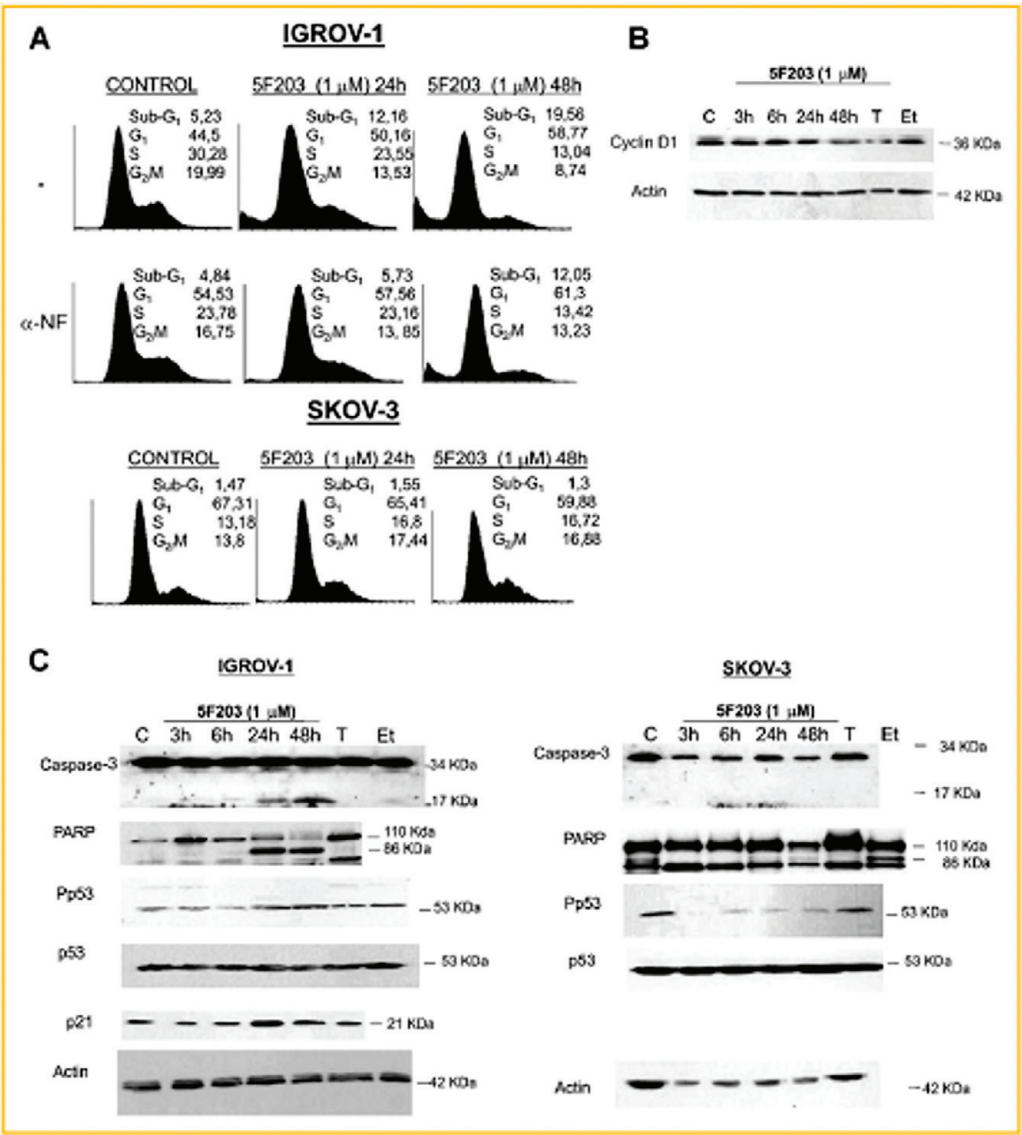
Figure 6.
5F 203 induces MAPK activation and apoptosis in sensitive IGROV-1 cells. A: MAPK expression and activation. IGROV-1 and SKOV-3 cells were incubated with 5F203 (1 µM) for indicated times or DMSO (0.1%) for 6 h. Whole cell extracts were obtained and subjected to SDS-PAGE and Western blotting with pERK, ERK, pJNK, JNK, pp38, and p38 α antibodies. The figure shows representative Western blots. All Western blots were performed three times for each cell line and revealed the same pattern of protein phosphorylation and expression. B: Evaluation of cell apoptosis. Cells were incubated with 1 µM 5F 203 or DMSO (0.1%) for 24 h or pre-treated with α-NF (1 µM) Trolox (250 µM) or NAC (100 µM) for 1 h followed by 5F 203 (1 µM) for 24 h. Then, non-adherent cells were obtained by cytocentrifugation in the culture medium. Once fixed, cells were stained with DAPI and observed under a fluorescence microscope. Condensed and fragmented nuclei were considered apoptotic. Representative fields are shown.

1.8 5F 203 alters cell cycle distribution and evokes apoptosis in sensitive ovarian cancer cells

As results indicated that 5F 203 induced DNA damage (e.g., **Figure 6B**), perturbations in cell cycle were explored. IGROV-1 and SKOV-3 cells were exposed to 1 µM 5F 203 or 0.1% DMSO for 24 and 48 h and prepared for cell cycle analyses. As illustrated (**Figure 7A**), 5F 203 caused an increase in G1 phase IGROV-1 events (44% control; 50% at 24 h and 59% at 48 h), coinciding with decreased G2/M phase (20% control to 13% at 24 h and 9% at 48 h). Accumulation of sub-G1 events was also detected from 5% (control) to 12 and 20% at 24 and 48 h, respectively. When cells were pre-incubated with α-NF, sub-G1 events diminished, indicating that AhR
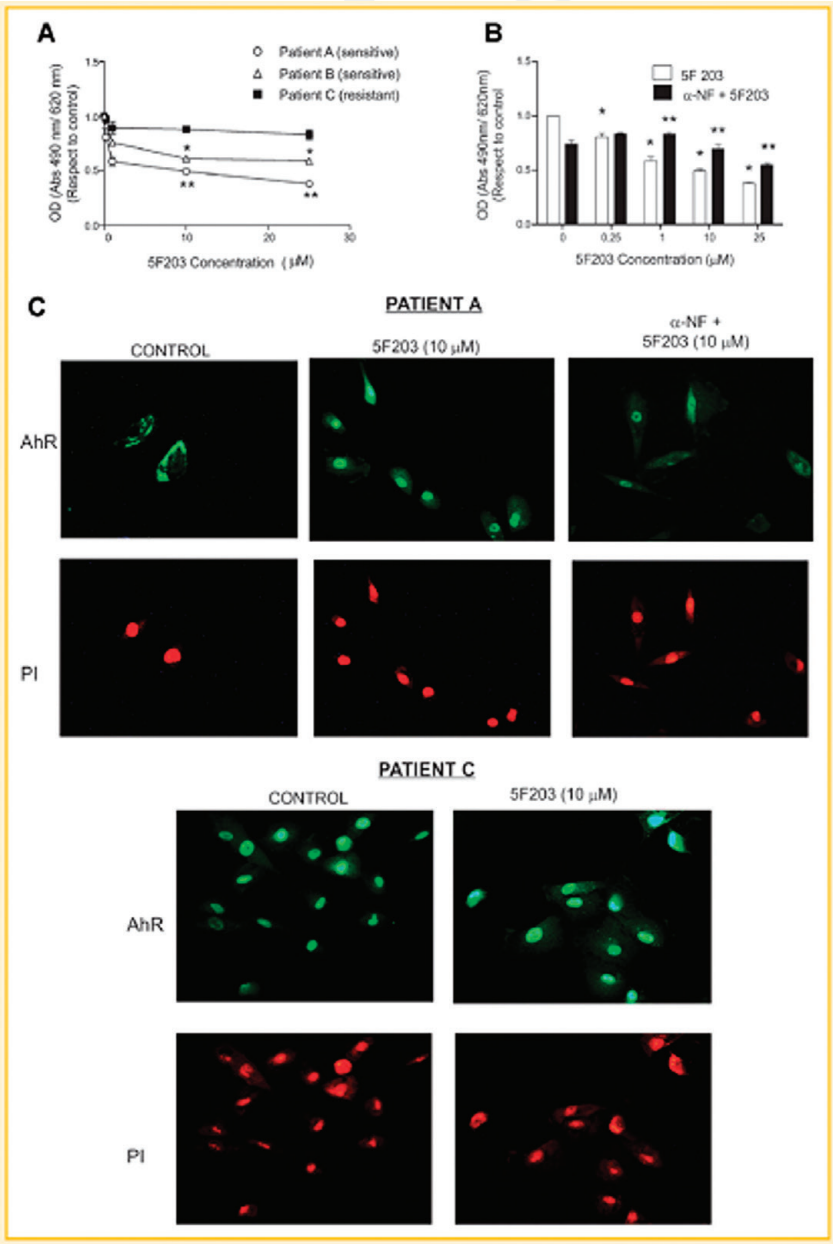
Figure 7.
5F 203 induces AhR-dependent cell cycle arrest in G1 phase, a decrease in cyclin D1 and caspase-3 mediated apoptosis in sensitive ovarian cancer cells. A: Exponentially growing IGROV-1 cells were exposed to either 0.1% DMSO (control) or 5F 203 (1 μM) for 24 and 48 h, (upper panel) or pre-incubated for 1 h with -α NF followed by 24 or 48 h 5F 203 +α -NF (1 μM) (lower panel). Exponentially growing SKOV-3 cells were exposed to either 0.1% DMSO (control) or 5F203 (1 μM) for 24 or 48 h. Then both cell lines were harvested, washed in PBS, and fixed in 70% ethanol. DNA was stained by incubating cells in PBS containing propidium iodide and fluorescence measured and analyzed as described in Materials and Methods section. The experiment was repeated three times (significant difference between treatments with p <0.01). Data of one representative experiment are shown in the figure. B: Effect of 5F 203 on cyclin D1 expression. IGROV-1 cells were exposed to either 0.1% DMSO (control) or 5F 203 (1 μM) for 3, 6, 24, or 48 h. Proteins in total lysates were resolved by SDS-PAGE and Western blot performed with anti-cyclin D1 Ab. Anti-actin Ab was used as a loading control. C: Effect of 5F 203 on p53 signaling pathway, caspase-3 activation, and PARP cleavage in ovarian cancer cells. Cells were incubated with 1 μM 5F 203 during indicated times or TCDD (T) 10 nM for 1 h. Cells treated with 10 μM Etoposide (Et) were used as a positive control for apoptosis. Whole cell extracts were obtained and subjected to SDS-PAGE and Western blotting with pp53 and P21, caspase-3 and PARP antibodies. PP53, phosphorylated form of P53.

activation is necessary for 5F 203-induced apoptosis. In contrast, SKOV-3 cell cycle was not perturbed following treatment with 5F 203.The data demonstrate that 5F 203-induced DNA damage may lead to accumulation of cells in G1 phase concomitant with growth inhibition. As IGROV-1 cells are p53 wild type, their response to 5F 203 is consistent with operation of a G1 checkpoint arrest to cell cycle progression after DNA damage. As G1 phase arrest was observed in 5F 203-sensitive cells, cyclin D1 levels were examined. Exposure of IGROV-1 cells to 5F 203 reduced cyclin D1 protein levels by 50 and 75% after 24 and 48 h, respectively. In contrast, only a 35% decrease in cyclin D1 levels was observed in SKOV-3 cells after 48 h treatment with

5F 203 (**Figure 7B**). In order to study, whether 5F 203 treatment caused caspase 3 activation, PARP cleavage, and p53 phosphorylation in IGROV-1 cells as a result of its pro-apoptotic action, we carried out Western blot experiments upon separated proteins of whole cell lysates following treatment of cells with 1 μM 5F 203 between 3 and 48 h. We observed caspase-3 activation, PARP cleavage, and 2.7- and 4-fold increase in p53 phosphorylation between 24 and 48 h, respectively. A similar pattern of increased p21 protein levels was observed after treatment of IGROV-1 cells

Figure 8.
5F 203 activity in ascites-derived ovarian cancer cell strains isolated from patients. A: 5F 203 cytotoxicity assay. Cells derived from three patients were incubated with 5F 203 for 5 days. Cellular viability was evaluated by MTS assay. Values represent the average of two independent experiments using cells from one patient with n = 4, *P < 0.05 compared with untreated cells. B: Cells derived from patient A (sensitive to 5F 203) were incubated with 5F 203 for 5 days or pre-treated for 1 h with α-NF (1 μM) and then treated with 5F 203 plus α-NF (1 μM) for 5 days. Cellular viability was evaluated by MTS assay. The values represent the average of two independent experiments using cells from one patient with n = 4, **P < 0.01 compared with cells incubated without AhR inhibitor. C: 5F 203 induces translocation of AhR to the nucleus in sensitive ascites-derived ovarian cancer cells. The staining of cells from one representative patient is shown. Cells were grown on coverslips and treated with DMSO for 1 h, 5F 203 for 30 min, or α-NF (1 μM) followed by 1 h of 5F 203, α-NF (1 μM), or 10 nM TCDD for 1 h. After fixation, cells were double-stained for AhR (green) and propidium iodide (red). Cells treated with 10 nM TCDD were incubated only with secondary antibody to determine non-specific background. Stained cells were visualized on a fluorescence microscope using a 40X PlanApo AN 0.95 objective, and images were processed and analyzed with Nikon C1-EZ package, version 2.20.

with 5F 203 (**Figure 7C**). In contrast, caspase activation and PARP cleavage were not detected in SKOV-3 cells treated with 5F 203; these cells showed decreased levels of pp53 after treatment with 5F 203 (**Figure 7C**) [48].

Thus, clear distinction can be seen—in terms of AhR signal transduction activation, CYP1A1 induction, DNA damage, ROS generation and apoptosis—between Bz-sensitive and Bz-insensitive ovarian cancer models *in vitro* and *in vivo*. It is important to evaluate whether such distinction can be translated to the clinic to enable identification of sensitive ovarian cancer phenotypes.

1.9 5F 203-induced cytotoxicity in cells isolated from ovarian cancer ascites is mediated by AhR

It has been proposed that high-grade advanced stage papillary serous ovarian adenocarcinoma ascites fluid is enriched for "metastatic," or "tumor-initiating" cells, and that these cells may represent a therapy-resistant population. Thus, ascites is considered a good model for disease study [52]. Cancer cells derived from ascites fluid produced by ovarian tumors from three patients were authenticated by pathologists. All tumors were high-grade (G3), serous, papillary histological type. Cells were treated *ex vivo* with 5F 203 for 5 days and cytotoxicity measured using MTS assays. Two cell strains were sensitive to 5F 203 and one was resistant (**Figure 8A**). In patient A, 1 and 10 μM 5F 203 decreased cell viability by 40 and 50%, respectively (compared to control considered 100%). This decrease in cell viability diminished to 20 and 30%, respectively, when cells were pre-treated with 1 μM α-NF followed by incubation with 5F 203/α-NF. We observed similar results in cells derived from patient B (data not shown). Results indicate that AhR mediates the effect of 5F 203 in these papillary tumors sensitive to 5F 203 (**Figure 8B**). AhR localization and nuclear translocation were then investigated. As demonstrated in **Figure 4A**, in ovarian cancer cells treated with vehicle (DMSO), high levels of cytosolic AhR protein were detected with some nuclear AhR staining present. However, after treatment for just 1 h with 1 μM 5F 203 or 10 nM TCDD, immunofluorescent AhR protein levels increased in the nucleus and decreased in the cytosol. In contrast, constitutive nuclear AhR localization was detected in cells of patient C, resistant to 5F 203. CYP1A1 mRNA levels were measured by real-time PCR in cells from patients A, B (sensitive to 5F 203), and C (resistant to 5F 203) following exposure to 5F 203 (1 μM; 24 h). In cells from patients A and B, we observed induction of *cyp1a1* expression (**Figure 8A**), which was partially reduced by α-NF. In contrast, reduction of (constitutive) *cyp1a1* expression was observed after treatment of patient C cells with 5F 203. ROS levels were also evaluated after treatment of patient ascites cells with 5F 203 and increased levels were detected only in cells sensitive to 5F 203, ROS were not induced in the 5F 203-insensitive ascites cells of patient C (**Figure 8B**) [48].

These promising data represent only a small clinical sample, but nevertheless support the hypothesis that in a clinical setting, "patient selection" and "precision medicine" are models applicable to antitumor Bzs.

2. Discussion

In this chapter, we propose that AhR represents a novel molecular target for ovarian cancer treatment and that the Bz class signifies AhR-targeted, CYP-activated anticancer agents for the treatment of ovarian cancer.

5F 203 activates AhR signaling in cultured and patient ovarian carcinoma cells sensitive to this agent, demonstrating that 5F 203 cytotoxicity is AhR dependent. In sensitive IGROV-1 cells, 5F 203, a known AhR ligand [35], triggers

AhR translocation from cytosol to nucleus, activating CYP1A1-related pro-moter sequences driving transcription of AhR-responsive genes as reported by XRE-luciferase.

It was recognized several years ago that certain ovarian cancer cell lines were exquisitely sensitive to antitumor Bzs [32]. 5F 203 potency and selectivity against ovarian cell lines within the NCI panel have been demonstrated [32, 53]: IGROV-1, OVCAR4 and OVCAR5 displayed GI_{50} values <100 nM in contrast, whereas GI_{50} values >100 μM were observed in OVCAR8 and SKOV-3 cell lines. Subsequently, induction of CYP1A1 by 5F 203 in sensitive cancer cells only inferred significant correlation between sensitivity and CYP1A1 induction [48]. *In vivo*, significant antitumor efficacy of 5F 203 prodrug Phortress was demonstrated against IGROV-1 ovarian (as well as breast) tumor xenografts. Moreover, CYP1A1 expression was detected in IGROV-1 (and sensitive breast) tumors of mice receiving Phortress treatment. No CYP1A1 protein was detected in insensitive breast tumor tissue following treatment of mice with Phortress [36]. Phortress was well tolerated, it possessed excellent solubility, bioavailability, and pharmacokinetic properties (liberating efficacious, sustained 5F 203 plasma concentrations), and Phase 1 clinical trials were initiated. Clinical evaluation revealed long-term stable disease in lung, colorectal, and kidney cancer patients; however, neither ovarian nor breast cancer patients were recruited to trial and short patent life precluded continuation of development.

AhR ligands such as 5F 203 induce their own CYP-catalyzed bioactivation; therefore, potential drug-drug or indeed drug-xenobiotic interactions were considered prior to commencement of the clinical trial. For example, many oral contraceptives are steroid based and any drug inducing CYP1A1 activity will lead to rapid metabolism and reduced contraceptive efficacy [54]. In the Phortress trial protocol, it was cautioned not to drink grapefruit juice, as this is able to inhibit CYP1A1 potentially reducing Phortress efficacy [55]. Red wine consumption was also discouraged as resveratrol is a competitive antagonist of AhR ligands; it promotes AhR nuclear translocation and binding to DNA, but transactivation of AhR-inducible genes such as *cyp1a1* is inhibited [56].

Differential sensitivity to 5F 203 may be a consequence of differential regulation by AhR of CYP1A1 expression in different cell types. In resistant cells, we observed constitutive nuclear localization of AhR. In resistant cells, AhR may be associated with co-repressors [57]; lack of AhR degradation (by ubiquitination) or recycling may lead to inappropriate AhR function [58]. Also, different AhR nuclear localization sequences [57] or polymorphisms may cause inappropriate receptor function [59]. Additionally, mutations in the CYP1A1 promoter in insensitive cells may lead to decreased CYP1A1 activation [60, 61]. Considering the clinical potential of 5F 203, its mechanism of action was further investigated. 5F 203 induced ROS formation in sensitive cells (**Figure 5**). In IGROV-1 cells, 5F 203 evoked DNA damage detected as H2AX foci (2–4 h), increased pp53 levels and P21 expression, decreased cyclin D1 expression, caused G1 cell cycle arrest and apoptosis. In contrast, SKOV-3 cells showed decreased levels of pp53 after treatment with 5F 203; the reason for this effect is unclear, but it may contribute to cellular resistance to 5F 203 (**Figure 7**). 5F 203-induced growth inhibition and apoptosis in IGROV-1 cells may in part be a consequence of elevated ROS (**Figure 5A**) and caspase-3 activation (**Figure 7C**) resulting in oxidative DNA damage and cell death [48]. Oxidative stress may activate caspases and is implicated in a number of cellular processes including apoptosis. Many chemotherapeutic agents are known to induce cytotoxicity by ROS-mediated mechanisms, for example, doxorubicin [62] and AhR ligand aminoflavone [63]. It was demonstrated that ROS might trigger apoptosis signaling mediated by p53 in IGROV-1 cells [64–66]. IGROV-1 cells possess wild type p53 and show sensitivity

to 5F 203. However, 5F 203 activity is independent of p53 exemplified by (i) potent activity of 5F 203 in MDA-MB-468 p53 mutant cells [32] and (ii) IGROV-1 variant populations demonstrating acquired resistance to cisplatin retaining sensitivity to 5F 203 (Bradshaw et al. unpublished results). Cisplatin resistance is associated with p53 mutations in IGROV-1 cells [64]. In IGROV-1 cells, p53 may be attempting (failed) repair rather than mediating apoptosis. Our results show that antioxidant agents such as NAC and Trolox decrease ROS formation and protect IGROV-1 cells from apoptosis induced by 5F 203 (**Figure 5B**). Previous work has demonstrated that stress signaling pathways are also activated by cisplatin and retinoids in IGROV-1 and cisplatin-resistant IGROV-1 cells; furthermore activation of JNK and P38 by these agents is stronger in cisplatin-resistant IGROV-1 cells. Reflecting the integral to antitumor activity, 5F 203 induces ROS in sensitive IGROV-1 cells and that IGROV-1 cells resistant to cisplatin retain sensitivity to 5F 203, we propose that 5F 203 could be a putative treatment for ovarian tumors. MAPK p38 α acts as an oxidative stress sensor; ROS-induced activation of p38 promotes apoptosis and prevents further oncogenic/carcinogenic ROS formation [44]. Also, micro-RNAs (miRNA) expression can be altered by different stress conditions, and they are well-known stress response regulators. It has been described that two members of the miR-200 family, miR-141 and miR-200a, inhibit p38 α and have an essential role in the redox response. In animal models, accumulation of miRNAs mimics p38 α deficiency and promotes malignancy. Human ovarian adenocarcinomas demonstrating high oxidative stress show high expression of miR-200a and low basal levels of p38 α. Chemotherapy drugs that induce ROS also induce p38 α in these tumors, leading to apoptosis. It was proposed that in ovarian tumors, high levels of miR-200 s and low levels of p38 α could be predictive markers of good clinical response to chemotherapy [45]. Our results are consistent with these observations, IGROV-1 cells have low levels of basal p38 α and treatment with 5F 203 induced p38 α expression and pp38 (**Figure 6A**), which may lead to apoptosis. In contrast, SKOV-3 cells show high basal levels of p38 α and treatment with 5F 203 did not modulate p38 α expression or activation (**Figure 6A**). We hypothesize that this may contribute to the lack of apoptosis induction in SKOV-3 cells. Finally, compatible with results from ovarian cancer cell lines, we identified putative surrogate markers of sensitivity to 5F 203 in a small sample of patient tumors. Clear distinction was demonstrated between ovarian cancer patient tumor cells that were sensitive to 5F 203 and those that were inherently 5F 203-resistant. Only in ascites-isolated patient tumor cells sensitive to 5F 203 were (i) cytosolic AhR translocation to cell nuclei, (ii) CYP1A1 mRNA induction, and (iii) increased ROS levels observed (**Figures 8** and **9**) in response to *ex vivo* treatment. Such pharmacodynamic endpoints are readily obtained from bioassays that could be adopted clinically to detect candidate 5F 203-responsive patients. In this way, unresponsive patients would be spared unnecessary treatment. Sensitivity to 5F 203 and AhR activation should be examined in a larger sample of ovarian carcinoma patient tumors of different histological types in future studies (**Figure 10**). However, the fact that cells isolated from patients with high-grade ovarian tumors were sensitive to 5F 203 shows that this agent may offer alternative treatment for patients with advanced disease. Intraperitoneal (i.p.) chemotherapy is currently used in treatment of ovarian tumors, and both 5F 203 and its prodrug Phortress have demonstrated antitumor efficacy administered either intravenously or i.p. [34].

Nanoformulations of 5F 203 are currently under evaluation to maximize tumor-targeting and sustained, controlled release. Future studies will include investigation of miRNA profiles in 5F 203-sensitive ovarian cancer cells compared with those of insensitive ones. In addition, the activity of 5F 203 against ovarian cancer stem cell-like/initiating populations remains to be evaluated.
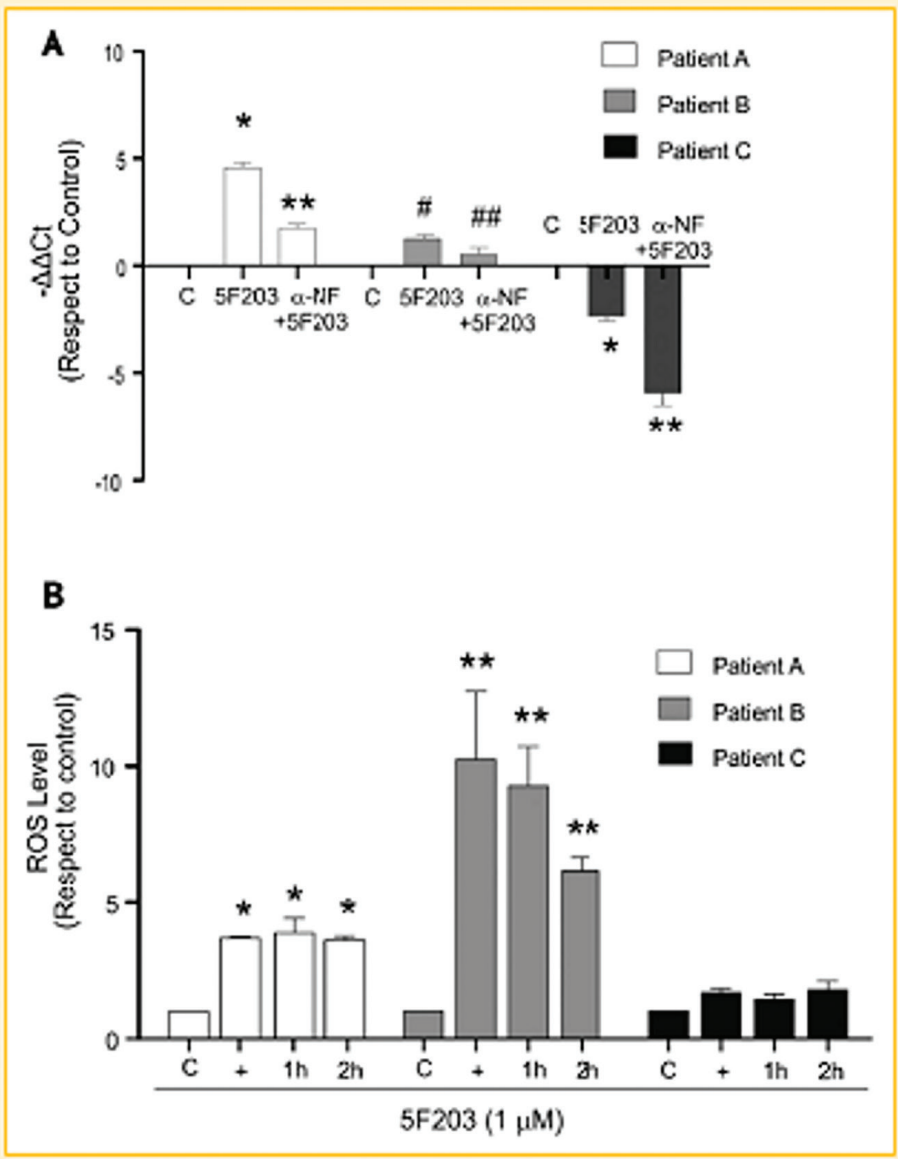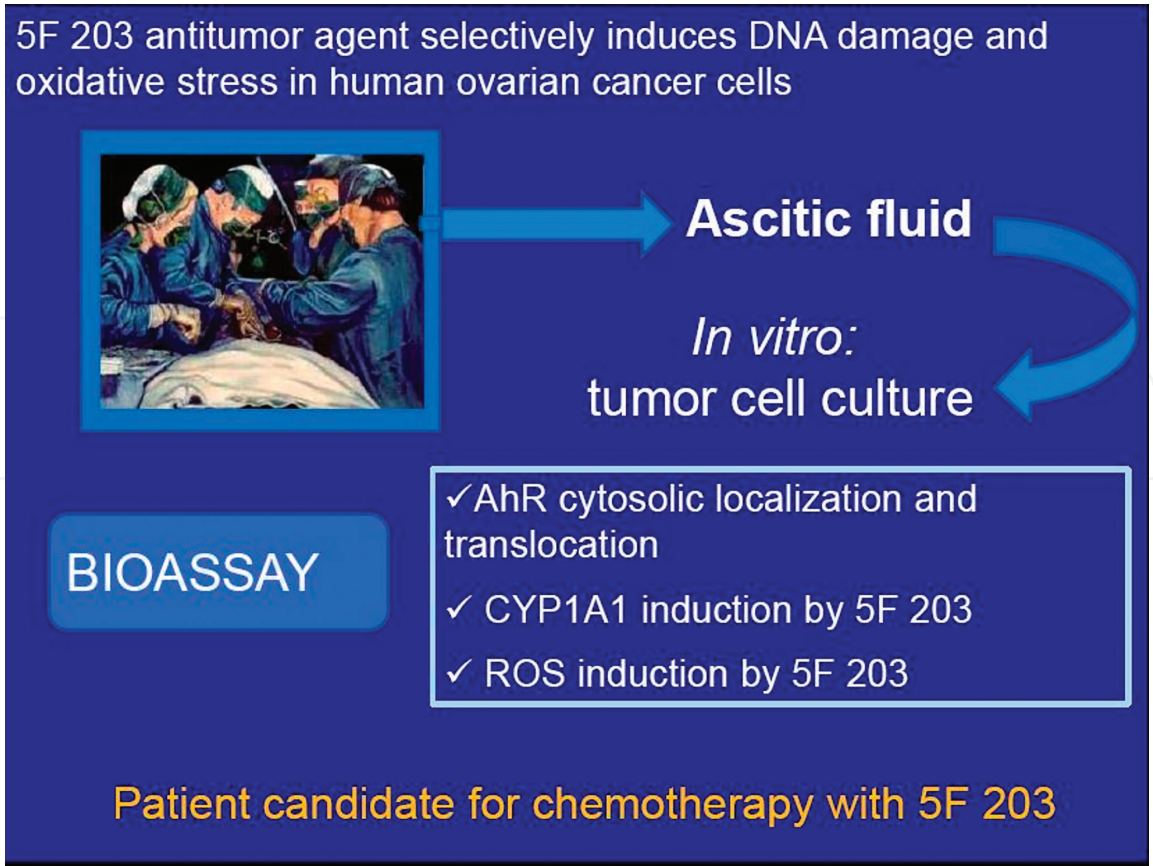
Figure 9.
5F 203 induces CYP1A1 over-expression and increases ROS levels in sensitive ascites-derived ovarian cancer cells isolated from patients. A: Induction of CYP1A1 gene expression. Cells derived from three patients were exposed to 0.1% DMSO (control), 5F 203 (1 μM) for 24 h, or pre-treated with α-NF (1 μM) for 1 h followed by 5F 203 (1 μM) α-NF (1 μM) for 24 h. RNA was isolated and real-time PCR was performed to measure CYP1A1 expression. A: Each bar represents mean ± SD of triplicate measurements in drug treated, compared to untreated cells, using GAPDH expression as an endogenous control. *P < 0.01 or *P < 0.05 when compared to untreated cells **P < 0.01 or **P < 0.05 when compared to cells treated without AhR inhibitor. B: Measurement of ROS levels. Cells derived from three patients were treated with 5F 203 (1 μM) or vehicle control (0.1% DMSO) continuously for 1 or 2 h and ROS levels were measured by fluorometry after incubation with 2,7-DCF. Incubation with H2O2 (100 μM) was used as positive control. Data represent the mean of at least two independent experiments where n = 4 per experiment; bars, SEM. *P < 0.05, **P < 0.01 when compared to untreated cells.

In summary, we have demonstrated AhR-dependent cytotoxicity of 5F 203 in ovarian carcinoma cells, we conclude that AhR may represent a new molecular target in the treatment of ovarian cancer and that 5F 203 may offer a potential novel treatment for newly diagnosed and cisplatin-resistant disease.

Tumor cells will be isolated from ascites fluid and cultured *ex vivo*. Following exposure of carcinoma cells *ex vivo* to escalating Bz concentrations (i) Bz sensitivity; (ii) AhR localization and nuclear translocation; (iii) CYP 1A1 expression and inducibility (by Bz); and (iv) ROS generation will be determined. If tumor cells are identified that show dose-dependent growth inhibition, AhR translocation,

Figure 10.
Proposed procedure for identification of patients whose tumors may be responsive to Bz treatment.

CYP 1A1 induction, and ROS generation following exposure to Bz, the patient from whom cells were isolated may be identified as a suitable candidate to receive Bz therapy.

Acknowledgements

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