"*In vitro* antitumor effects of AhR ligands Aminoflavone (AFP 464) and Benzothiazole (5F 203) on human renal carcinoma cells" Luzzani G.A.[1]*, Callero M.A. [1]*, Kuruppu A.I.[2], Trapani V[3]., Flumian C[1]., Todaro L[1]., Bradshaw T.D. [2], and Loaiza Perez A. I[1].

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Short Title: "Antitumor effects of AhR ligands AFP 464 ad 5F 203 on human renal carcinoma cells"

ABSTRACT

We investigated activity and mechanism of action of two AhR ligand antitumor agents, AFP 464 and 5F 203 on human renal cancer cells, specifically examining their effects on cell cycle progression, apoptosis and migration.

TK-10, SN12C, Caki-1 and ACHN human renal cancer cell lines were treated with AFP 464 and 5F 203. We evaluated cytotoxicity by MTS assays, cell cycle arrest and apoptosis by flow cytometry and corroborated a mechanism of action involving AhR signal transduction activation. Changes in migration properties by wound healing assays were investigated: 5F 203-sensitive cells show decreased migration after treatment, therefore we measured c-Met phosphorylation by Western blot in these cells.

Both compounds caused cell cycle arrest and apoptosis in sensitive ~~cell lines~~ TK-10, SN12C and Caki-1 cell lines. 5F 203 induced a decrease in cell viability which was more ~~remarkable~~ marked than AFP 464. This cytotoxicity was reduced after treatment with the AhR inhibitor α-NF for both compounds indicating ~~Ahr~~ AhR signaling activation plays a role in the mechanism of action. 5F 203 is sequestered by TK-10 cells and induces CYP1A1 expression; 5F 203 ~~only~~ potently inhibited migration of TK-10, Caki-1 and SN12C cells, and inhibited c-Met receptor phosphorylation in TK-10 cells.

AhR ligand antitumor agents AFP 464 and 5F 203 represent potential new candidates for the treatment of renal cancer. 5F 203 only inhibited migration of sensitive cells and c-Met receptor phosphorylation in TK-10 cells. c-Met receptor signal transduction is important in migration and metastasis. Therefore we consider that 5F 203 offers potential for the treatment of metastatic renal carcinoma.

INTRODUCTION

The aryl hydrocarbon receptor (AhR) is a cytosolic transcription factor. After binding with its ligand the receptor translocates to the nucleus, binds to xenobiotic response element (XRE) promoter sequences, activating target genes including cytochrome P4501A1 (CYP1A1). Initially, AhR was linked to detoxification functions of cell products and environmental pollutants. Our research group has described a new role for the AhR signaling pathway as a novel molecular target for cancer therapeutics. Currently, there are two antitumor agent AhR ligands, Aminoflavone (AFP 464) and ~~Benzothiazole~~ benzothiazole (5F 203), which have been tested clinically (Fig. 1). It has been ~~proved~~ proven that ~~Benzothiazole~~ 5F 203 and ~~Aminoflavone~~ AFP 464 are AhR-targeted agents (1, 2).The ~~Benzothiazole~~ benzothiazole 5F 203 lysylamide prodrug Phortress has been tested in phase I clinical trials, and disease stabilization was achieved in 28% patients recruited (3). Although neither breast nor ovarian carcinoma patients received Phortress, in~~stabilizing disease in 28% patients recruited(3).In~~ preclinical~~ly models~~, 5F 203 and Phortress evoked potent antiproliferative activity in breast and ovarian tumor models, inducing CYP1A1 expression and generating DNA adducts which are converted to lethal strand breaks in sensitive ~~models~~ cell lines and xenografts only (4,5). We have previously described that 5F 203 induced enhanced AhR nuclear translocation and CYP1A1 expression ~~and AhR~~

~~nuclear translocation~~ in human breast cancer cells (6)~~.~~ ~~and~~ in the human ovarian cell line IGROV-1. ~~and~~In ascites-isolated ovarian cancer cells, ~~that were sensitive~~ sensitivity to 5F 203 correlated with cytosolic AhR translocation to nuclei, upon treatment of ovarian cancer cells *ex vivo*. 5F 203 possessed antiproliferative/pro-apoptotic activity inducing oxidative stress measured as ROS formation, JNK, ERK, and P38MAPK phosphorylation, DNA damage and cell cycle arrest prior to apoptosis. In contrast, 5F 203 failed to induce CYP1A1 expression, AhR translocation or oxidative stress in 5F 203-resistant SKOV-3 cells (7).

~~Also~~Interestingly, preclinical NCI 60 cell line panel data showed that TK10 cells ~~are~~ were consistently sensitive to ~~benzothiazoles 4F~~ 5F 203 and Phortress. Intriguingly, Phortres stabilized disease in the two renal carcinoma patients recruited to trial (in 1 patient stability was maintained for 16 cycles). Therefore, ~~we~~ in this ~~work~~ study we ~~intended to~~sought to examine ~~the~~ AhR pathway activation and CYP1A1 inducibility in TK10 and other renal carcinoma cell lines after treatment with 5F 203. Given the poor prognosis associated with kidney cancer and the paucity of therapeutic options, preclinical investigations of the use of aminophenyl–benzothiazole experimental antitumour agents against these tumo~~u~~rs are warranted.

Figure 1: AFP 464 and 5F 203 chemical structures

Aminoflavone (AF; NSC 686288, AFP 464, NSC710464) is a new anticancer agent undergoing phase II clinical evaluation. It has demonstrated antiproliferative effects in MCF-7 human breast cancer cells mediated by AhR. AF also exhibits noteworthy evidence of antitumor activity *in vitro* and *in vivo* against neoplastic cells of renal origin. AF treatment of sensitive renal cells, in contrast to resistant cells, promoted the induction of CYP1A1, the covalent binding of AF-reactive intermediates and apoptosis (8). AF treatment also induced apoptosis, in human renal cancer sensitive cells e.g. TK-10, Caki-1 and SN12C, which was not observed in ACHN resistant cells. AF induced time-dependent AhR nuclear translocation and AhR transcriptional activity in sensitive renal cancer cell lines. A renal cell strain derived from a human papillary tumor also showed sensitivity to AF, as well as AhR pathway activation and drug-induced apoptosis. AhR translocation was proposed as a marker of sensitivity to AF in sensitive renal tumor cells of different histological origin, for clinical trials (9). Much focus has been emphasized on the vascular endothelial growth factor (VEGFR), platelet derived growth factor, and PI3K pathways, leading to the development, and Food and Drug Administration (FDA)-approval of multiple targeted agents for RCC (10). As these drugs generally slow the progression of disease with only modest objective responses, it is necessary to identify new molecular targets in RCC for the development of more effective therapeutic strategies. Dysregulation of c-Met and its ligand, hepatocyte growth factor (HGF), have been implicated in tumor development, invasion, and angiogenesis in malignancies. Mutations in the c-Met gene have been identified in papillary RCC (11). It has been shown that loss of von Hippel–Lindau (VHL) expression and hypoxia lead to upregulation of c-Met expression in clear cell RCC. Studies performed in clear cell lines demonstrated that c-Met protein was activated in renal cancer cell lines, and cell proliferation was blocked by SU11274 (sunitinib) and ARQ 197(11).

Kidney cancer rapidly acquires resistance to antiangiogenic agents such as ~~Sunitinib~~sunitinib, developing an aggressive migratory phenotype (facilitated by c-Met

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signal transduction). The aim of this study was to investigate the action of AFP464, the ~~Aminoflavone~~ aminoflavone pro-drug currently used in clinical trials, and 5F 203 on renal cancer cells specifically examining their effects on cell cycle progression, apoptosis and cell migration. Both compounds caused cell cycle arrest and apoptosis but only 5F 203 potently inhibited migration of TK-10, Caki-1 and SN12C cells and ~~inhibit a~~the migration ~~involved~~ signal transduction ~~pathway~~cascade, ~~such as~~involving c-Met ~~receptor~~ signaling, in TK-10 cells.

MATERIALS AND METHODS

Cell Lines

The following human renal cancer cell lines were obtained from the National Cancer Institute (NCI) repository: TK-10, SN12C, Caki-1 and ACHN. They were cultured in 25 cm^2 T flasks with 5% CO_2 in RPMI medium (Gibco) supplemented with 10% fetal bovine serum (FBS; PAA).

Antiproliferative activity

Renal cell lines grown in 25 cm^2 flasks were removed by trypsinization and seeded into 96-well culture dishes at a concentration of 750 cells per well. Cells were allowed to grow for 48 h at 37˚C in a humidified atmosphere containing 5% CO_2.

–AFP464 treatment: Cells were treated with AFP464: 10 nM, 100 nM, 500 nM and 1 µM or dextrose water (control) for an additional 120 h.
–5F 203 treatment: Cells were treated with 5F 203; 100 nM, 500 nM, 1 µM, 10 µM and 100 µM or DMSO (0.1%) (control) for an additional 120 h.

In both cases cell viability was determined by the MTS method (Promega). To study AhR pathway involvement, cells were pre-incubated for 1 h with AhR specific antagonist α-naphthoflavone (α-NF, 1 µM).

Cell cycle progression:

Cells were seeded into 6-well culture dishes at a concentration of 3 x 10^5 cells per well. After 24-48 h, cells were treated with test agents.
–AFP 464: Control (dextrose water); α-NF (1 µM); AFP464 (1 µM) for 24 and 48 h; pre-incubation for 1 h with α-NF (1 µM) followed by α-NF (1 µM) + AFP464 (1 µM) for 24 and 48 h.
–5F 203: Control (DMSO 0.1%); α-NF (1 µM); 5F 203 (1 µM) for 24 and 48 h; pre-incubation for 1 h with α-NF (1 µM) followed by α-NF (1 µM) + 5F 203 (1 µM) for 24 and 48 h.
Thereafter, cells were harvested, washed in PBS, and fixed in 70% ethanol. DNA was stained by incubating cells in PBS containing propidium iodide; fluorescence was measured and analyzed using Cyflogic software version 1.2.1. ~~The analysis of the~~ DNA analyses allowed us to determinate the cell distribution in each cell cycle phase.

Detection of 5F 203 in nutrient medium:
TK-10 cells were seeded into 25 mL flasks and allowed 24 h to adhere and begin mitoses. Medium was changed and 5F 203 introduced at a final concentration of 100 nM. Media, collected from flasks at time zero, ~~and~~4 h, 24 h, 48 h and 72 h thereafter, were mixed with 3-fold volumes of HPLC-grade acetonitrile. Protein was precipitated by centrifugation at 14 000 rpm for 10 min. and supernatants analyzed by HPLC. The analytical system consisted of a Hewlett-Packard1050 series module (solvent delivery pump, autosampler, and multiple wavelength detector) and a Hewlett-Packard 1046A fluorescence detector. 5F 203 separation was effected at room temperature on a C18 reversed-phase column (150 x 4.6 mm i.d.) using a mobile phase of 65% methanol and 35% H_2O, delivered at a flow rate of 1 ~~ml~~mL/min. 5F 203 eluted at 338 nm; was identified with UV detection and with fluorescence detection (excitation344 nm; emission 434 nm) and confirmed by chromatographic analysis of authentic 5F 203.

Wound Healing:
To analyze the effect of AFP464 or 5F 203 on migration, cells were seeded in 6-well plates and incubated for 48 h so as to achieve an 80-90% confluent monolayer. Cells were treated for 24 h with 10^{-4} µM to 1µM AFP 464 or 5F 203. After this time had elapsed, a single scratch wound was created in the monolayer with a tip and then immediately photographed at time 0 or T_f(approximately 18 h later, depending on each cell line). Cell migration was assessed by determining the covered area at T_0 and T_f with the program ImageJ and then the percentage migration was calculated using the following equation: $(T_f*100)/T_0$. We considered 20 fields per plate. Migration percentage was analyzed with the program GraphPad Prism5. Wounds were photographed, ~~with a~~imaged under a phase contrast ~~phase~~ microscope.

Western blot:
Cells were seeded in dishes (100×20 mm) at a density of 1-2 x 10^6 per dish, allowed 24 h to attach, before being exposed to ~~benzothiazoles~~5F 203 (1 µM, 5 min, 10 min, 30 min, 1 h, 4 h or 24 h). Following exposure, cell lysates were prepared and protein concentrations evaluated by Bradford assay (12). Proteins (50 µg per simple) were separated by PAGE. CYP-1A1, c-Met, phosphorylated c-Met and GAPDH 1° Abs were purchased from Cell Signaling Technologies. 2° Abs were obtained from Dako. Densitometric analyses of Western blots were performed using Image J.

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RESULTS

AFP464 and 5F 203 induce cytotoxicity in human renal cancer cell lines

In this study, we measured the sensitivity of cells to AFP464 as an *in vitro* regression (shown by a decrease in cell metabolism measured by the MTS assay).
The incubation of TK-10, SN12C and Caki-1 cells with 1 µM AFP464 for 5 days induced a significant decrease in cell viability (compared to control viability, considered to be 100%: TK-10, 21.22±10.9%; SN12C, 50.91±4.9%; Caki-1, 87.24±9.1% cells; Figure 2, ~~with respect to the control which wasconsidered 100%~~). ~~The e~~Effects ~~was~~ were dependent on

drug concentration. In contrast, ACHN cells were not sensitive to AFP464 as significant growth inhibition was not observed at any drug concentration (Fig. 2Ai). In order to investigate whether the sensitivity of renal cancer cells to AFP 464 is mediated by AhR activation, we pre-incubated the cells with the AhR specific inhibitor, α-NF (1 μM), for 1 h prior to treatment with 1 μM AFP464 plus α-NF. The AhR inhibitor significantly reduced the cytotoxic effects of AFP 464 in TK-10 and partially reduced the cytotoxic effects in SN12C with respect to control (Fig.2Aii).

Figure 2: AFP and 5F 203 effect on cellular viability

A) i- Cells were incubated with AFP464 or dextrose water for 5 days. ii- Cells were incubated with AFP 464 (1 μM) for 5 days alone or pre-treated for 1 h with α-NF (1 μM) and then treated with AFP 464 (1 μM) plus α-NF (1 μM) for 5 days. Cellular viability was assessed by MTS assay. The values represent the average of three independent experiments, * $p < 0.05$ with respect to control cells.

B) i- Cells were incubated with 5F 203 or DMSO (0.1%) for 5 days. ii- Cells were incubated with 5F 203 (1 μM) for 5 days alone or pre-treated for 1 h with α-NF (1 μM) and then treated with 5F 203 (1 μM) plus α-NF (1 μM) for 5 days. Cellular viability was assessed by MTS assay. The values represent the average of three independent experiments,* $p < 0.05$ with respect to control cells.

In addition, sensitivity of TK-10, SN12C and Caki-1 cells to 5F 203 was also measured. Interestingly, incubation of these cell lines with 0.1 μM 5F 203 for ~~five~~ 5 days ~~induced a decrease~~ dose-dependently decreased ~~in~~ cell viability, ~~which was~~ more ~~remarkable~~ remarkably than did AFP464 (survival was calculated as TK-10, 45±10%; SN12C, 26±3%; Caki-1, 36±3%, with respect to the control which was considered 100%) ~~, which was maintained with increased concentrations~~ (Fig.2Bi). As with AFP464, the cell line ACHN did not show sensitivity to 5F 203. Involvement of the AhR pathway was ~~also~~ assessed by using the AhR specific inhibitor (α-NF; 1 μM)); results show that the cytotoxic effects ~~obtained with~~of 5F 203 ~~was~~ were significantly ~~reduced~~ diminished in TK-10 and SN12C cells by α-NF-mediated inhibition of AhR ~~with respect to the control~~signal transduction.

AFP464 and 5F 203 cause altered cell cycle distribution and apoptosis in sensitive renal cancer cells

As previous results indicate that AFP464 (NSC710404) induced DNA damage and apoptosis in renal cancer cells (9) and 5F 203 ~~causes~~ evoked DNA damage in breast and ovarian cancer cells, we investigated perturbations in the cell cycle after treatment of renal cells with AFP464 and 5F 203. For this approach, cells were exposed to 1 μM AFP464 ~~,or~~ 1 μM 5F 203 or 0.1% DMSO for 24 and 48 h and subsequently processed for cell cycle analyses. As illustrated (Fig. 3A), AFP464 only caused an increase in phase G0/G1 in TK-10 cells at 24 h (56.7 ± 0.15% control; 61.65 ± 1.65% at 24 h). However, significant accumulation of sub-G0 events was detected in TK-10 cells from 2.5 ± 0.6 % (control) to 8.13 ± 0.2% at 48 h, in SN12C cells 6.2 ± 0.7 % (control) to 17.64 ± 0.9 % at 48 h, and Caki-1 from 2.44 ± 0.2 % (control) to 6.05 ± 1% at 48 h. In contrast, ACHN cell cycle was not perturbed following treatment with AFP464.

Figure 3: AFP464 and 5F 203 altered cell cycle distribution.

A) The effect of 1 µM AFP464 ~~effect~~on cell cycle distribution: Cells were harvested, washed with PBS and fixed in 70% ethanol, then stained with propidium iodide and analyzed by flow cytometry. The figure shows graphs for TK10, SN12-C, Caki-1 and ACHN cells.

B) The effect of 1 µM 5F 203 on cell cycle distribution~~5F 203 effect~~: Cells were harvested, washed with PBS and fixed in 70% ethanol, then stained with propidium iodide and analyzed by flow cytometry. The figure shows graphs for TK10, SN12-C, Caki-1 and ACHN cells.

In both cases, ~~a~~ one representative experiment, ~~out~~ of three, is shown; ~~.~~ 20 000 events were analyzed. The values represent the average of three independent experiments *, $p < 0.05$; **, $p < 0.01$ with respect to control cells.

5F 203 caused an increase in phase G0/G1 in SN12C and Caki-1 cells. While this effect was observed at 24 h in SN12C cells (control: 54.8 ± 0.62 % vs 24 h: 73.48 ± 5.44 %), in Caki-1 cells G0/G1 arrest was seen at 24 and 48 h (control: 52.05 ± 0.47 %, 24 h: 61.9 ± 3.9 %, 48 h: 64.34 ± 2.03 %). Accumulation of Sub-G0 events was observed in TK-10, SN12C and Caki-1 cells at both assayed times. TK-10 cells: from 4.15 ± 2.13 (control) to 6.4 ± 2.4 % and 11.0 ± 4.0 (24 and 48 h, respectively); SN12C cells: from 0.76 ± 0.64 % to 4.6 ±1.4 % and 11.1 ± 0.4 % (24 and 48 h, respectively); Caki-1 cells: from 2.4 ± 0.22 % to 4.2 ± 1.09 % and 4.6 ± 1.6 % (24 and 48 h, respectively). In contrast, ACHN cell cycle was not perturbed following treatment with 1 µM 5F 203 (Fig. 3B).

5F 203 is depleted from medium of TK-10 cells and induces expression of CYP1A1.

It has previously been reported that 5F 203 is stable in medium (13). When added to TK-10 cancer cell nutrient medium(100 nM 5F 203) , 5F 203 was rapidly depleted (Figure 4A). After 24 h ~60% depletion was noted after treatment of cells with 100 nM 5F 203; after 72 h treatment, medium levels of 5F 203 were below detection levels.

Figure 4: 5F 203 depletion from nutrient medium and CYP1A1 induction

A) Time-dependent depletion of 5F 203 from nutrient medium of TK-10 cells exposed to 100 nM 5F 203.

B) Induction of CYP1A1 protein expression following exposure (24 h, 1 µ~~m~~M) of TK-10 cells to fluorinated analogues of 2-(4-amino-3methylphenyl)benzothiazole, including 5F 203*

Potent cytosolic AhR ligands, aminophenylbenzothiazoles (including 5F 203) have also been reported to activate AhR signaling, thereby ~~and induce~~ inducing expression of CYP1A1 (13-15). In lysates of TK-10 cells exposed to fluorinated 2-(4-amino-3-methylphenyl)benzothiazole analogues including 5F 203 (1µM; 24 h) CYP1A1 protein could be detected (Figure 4B).

Impact of AFP464 and 5F 203 treatment on cell migration

We investigated the effect of AFP464 and 5F 203 treatments on migration of renal tumor cells *in vitro*. As shown in Fig. 5, AFP464 treatment decreased migration neither in the sensitive nor in the resistant cell lines (61 ± 8 %, 62 ± 1 %, and 64 ± 7 % of migration percentage for TK-10, SN12C and ACHN respectively as compared to 70 ± 5 %, 67 ± 15 % and 73 ± 8 % ~~of~~ in the controls).

Figure 5: AFP464 and 5F 203 effects on cellular migration.

A) AFP464 wound healing assay: TK10, SN12C and ACHN cells were incubated with AFP464 for 24 h with the dilutions ~~shown~~ described in the methodology. ~~After that, the~~ A wound was made with a yellow tip~~;~~, ~~measuring~~ the initial and final wound areas were measured (Tf= 20 h post wound) and analayzed with the ~~program~~ Image J program. The graph shows the ~~migration~~ percentage migration for each dilution realized. Values that are significantly different from controls ~~were~~ are indicated ~~by~~ *p< 0.05. Representative fields ~~of~~ from one experiment are shown under each graph. Experiments were performed in triplicate. (Andrea confirm)

~~B)~~ 5F 203 wound healing assay: TK10, SN12C, Caki-1 and ACHN cells were incubated with 5F 203 for 24 h with the dilutions ~~shown~~ described in the methodology. A wound was made with a yellow tip; the initial and final wound areas were measured (Tf= 14 h post wound) and analayzed with the Image J program. The graph shows the percentage migration for each dilution realized. Values that are significantly different from controls are indicated *p< 0.05. Representative fields from one experiment are shown under each graph. Experiments were performed in triplicate (Andrea confirm)~~After that, the wound was made with a yellow tip, measuring the initial and final area (Tf= 14 hpost wound) with the program ImageJ. The graph shows the migration percentage for each dilution realized. Values that are significant different were indicated by *p< 0.05. Representative fields of one experiment are shown under each graph.~~ Andrea, I don't know what happened to A), B) – please ignore the cross out, it is the autoformatting!

In contrast, 5F 203 (1 µM) significantly ~~decreased~~suppressed cell migration in the three sensitive cell lines, 42 ± 6 %, 18 ± 7 % and 29 ± 2 % in TK-10, SN12C and Caki-1 cell lines respectively as compared to 69 ± 1 %, 63 ± 3 % and 62 ± 4 % for controls. For the non-sensitive cell line (ACHN), migration percentage was of 46 ± 2 %, as compared to 38 ± 1 % for control. While in TK-10 and Caki-1 cells, treatment ~~decreased migration~~ with 10^{-1} µM and 1 µM 5F 203 decreased migration~~respectively~~, inhibition of SN12C cells` migration ~~inhibition is~~was apparent at all concentrations examined ($\geq 10^{-4}$µM).

Effect of 5F 203 on c-Met phosphorylation in TK-10 cells

As treatment with 5F 203 demonstrated inhibition of TK-10 cells ~~`~~migration, and p-Met is involved in the migration process, cell lysates were subjected to p-Met Western blot analyses. TK-10 cells were exposed to 1 µM 5F 203 for 5 min, 10 min, 30 min, 1 h, 4 h or

24 h. ~~We observed time dependent significant down regulation from 5 min to 1 h where 1 h~~ examined. Particularly significant down-regulation of phosphor C-Met was detected in lysates of TK10 cells exposed to 1 μM 5F 203 for 1 h (P<.001; Fig. 6).

Figure 6: 5F 203 effect on c-Met phosphorylation

A) Western blots illustrating total and phosphorylated c-Met expression in lysates of TK-10 cells treated with 1 μM 5F 203 for 5 min, 10 min, 30 min, 1 h, 4 h or 24 h. Total c-Met 1^0Ab recognizes c-Met (145 KDa) and phospho c-Met 145 KDa. Representative phospho c-Met blots are shown (n=3). Lysates were prepared on 3 separate occasions; ~~1~~ representative total c-Met and GAPDH (loading control) blots are shown.

a) B) Densitometry was performed on all blots to quantify c-Met and phosphorylated c-Met expression. The values represent the average of three independent experiments. * p< 0.05;** p < 0.01 and *** p < 0.001 with respect to control cells.

DISCUSSION

Our previous studies showed that renal cancer cell lines of human origin, such as Caki-1, TK-10 and A498, and human renal cell ~~strains~~ lines derived from patients with renal tumors were very sensitive to AF (2). However, other cell lines and cell strains were classified as resistant. Treatment with AF of sensitive cells resulted in AhR signaling pathway activation which leads to CYP1A1 induction, inducing AF metabolism to reactive intermediates that cause DNA damage and apoptosis, measured by cytokeratin 18 cleavage. In our previous report, we indicated that papillary renal tumors were more sensitive to AF than clear cell tumors (8). The enhanced activity of AF against the papillary variant of renal cell carcinoma is of special value. Except for Temsirolimus and Sunitinib, there are little or no data regarding the safety and efficacy of new targeted drugs in papillary ~~histology~~ renal tumors and there is a need for the development of new effective therapies (16).

We suggested that the AhR may represent a new molecular target for the treatment of these tumors, distinct from proteins currently targeted in the clinic. AhR translocation and activation may be used as a biomarker in tumor biopsies to predict sensitivity to treatment with AF of renal tumors of different histological types. This could be incorporated into phase II clinical trials, together with other markers of sensitivity, such as the induction of CYP1A1, high covalent binding of metabolites and apoptosis, for the selection of patients that potentially could respond to the treatment with this agent (8, 9).

Our studies showed that in sensitive cells AF induced formation of apoptotic bodies~~,~~; incubation with the AhR inhibitor, α-NF, prior to treatment with AF, decreased the number of apoptotic bodies formed (9). Indicating that the AF-induced apoptosis is mediated by AhR activation. Also, we previously studied the molecular pathway involved in AF-induced apoptosis, and we observed the induction of p-P53 and P21 levels after treatment with1 μM AF in these cells between 3 and 6 h, which confirms that this compound caused apoptosis and presumably cell cycle arrest (9). This finding is consistent with the induction of p-P53 and P21 that was previously reported after AF treatment of MCF-7 breast cancer cells (17). In addition, caspase 3 activation and consequent PARP cleavage after treatment with AF in TK-10 cells was found which is consistent with the DNA damage and apoptosis

caused by this antitumor agent. Furthermore, caspase 3-dependent apoptotic body formation was observed in MCF-7 breast cancer cells after treatment (18), which is consistent with our observation in renal cancer cells. Since AFP464 is a prodrug administered to patients which is metabolized to form AF, we assumed that apoptosis *via* p-P53 and P21 and caspase activation also occurred in our system.

In this paper we demonstrated accumulation of TK-10, SN12C and Caki-1 events in Sub-G0 after treatment of cells with AFP464 which indicates apoptosis caused by this clinical used ~~Aminoflavone~~ aminoflavone derivative.

Our current data confirm that AFP464 caused cytotoxicity in sensitive human renal cancer cells which was significantly reduced when we pretreated with the AhR inhibitor α-NF, indicating AhR activation (Fig 2). We confirmed that in sensitive renal cells AFP464 induces translocation of AhR to the nucleus, indicating AhR signaling activation; ~~and~~ in addition, ~~Histone~~ histone H2AX phosphorylation ~~which indicates~~ indicated DNA damage caused by the drug (data not shown; 19).

When we studied cell cycle arrest we observed AFP464 only caused an increase in phase G0/G1 in TK-10 cells at 24 h.

Finally we demonstrated that AFP464 did not change migration properties of renal cancer cells.

~~The~~ Novel data have been generated following treatment of cell lines of renal carcinoma origin ~~obtained~~ with AhR ligand 5F 203 ~~are novel~~. TK-10, SN12C and Caki-1 were sensitive to 5F 203, whereas ACHN represents a resistant cell line.

Interestingly, incubation of these cell lines with 0.1 µM 5F 203 for ~~five~~ 5 days ~~induced~~ decreased ~~a decrease in~~ cell viability which was more remarkable than AFP 464. This cytotoxicity was reduced after treatment of cells with the AhR inhibitor α-NF. Antitumorbenzothiazoles such as 5F 203 are potent AhR ligands (20); activity has been shown to be mediated *via* AhR signal transduction in mammary carcinoma cells, with subsequent induction of CYP1A1 and biotransformation of 5F 203 to cytotoxic nitrenium species (21). Here we demonstrate that this pathway is activated in sensitive renal cell carcinoma lines. Moreover, we demonstrate depletion of 5F 203 from nutrient medium of TK-10 cells and induction of CYP1A1 protein expression (Fig 4).

Apoptosis, detected as accumulation of sub-G0 events was also detected after treatment with 5F 203 in the three sensitive cell lines. 5F 203 caused an increase in phase G0/G1 in SN12C and Caki-1 cells only. Arrest in G1 phase was observed also after treatment of ovarian sensitive cells with 5F 203 (7).

In contrast to AFP464, 5F 203 significantly decreased cell migration in the three sensitive cell lines. A decrease in wound healing ability was observed in sensitive cell lines after 24 h treatment with concentrations of 5F 203, compared to the control. ~~Also the most~~Consistent with loss of migratory potential, ~~a~~significant decrease in c-Met phosphorylation was observed at 1 h in TK-10 cells treated with 1 µM 5F 203. Inhibition of c-Met activity by 5F 203 is consistent with previous observations: 5F 203 (1 µM; 24 h) decreased c-Met phosphorylation by 85% and 69 % in MCF-7 and MDA-MB-435 breast carcinoma cells respectively ~~?????~~ (unpublished data). ~~It was proposed~~ We speculate that 5F 203, a potent AhR ligand triggers activation of a signaling cascade which potentially inhibits HIF signal transduction and hence c-Met transcription and subsequent activation (22). The Met signaling pathway is a key pathway for the treatment of renal cancer (11) and

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is also involved in metastasis progression; therefore we consider that 5F 203 has potential for the treatment of renal metastatic carcinoma.

Andrea, instead of (or as well as) plotting the ration between total and phosphoMet in the bar grapg, do you think you could plot total Met:GAPDH ration and phosphoMet:GAPDH ratio please? It does look as though total Met levels are down regulated – as would make sense according to our speculative hypothesis: 5F 203-triggered AhR signal transduction activation would reduce HIF-1β levels available, therefore inhibit HIF signaling, therefore c-Met transcriotion and expression – but depending on its half-life, lysates may need to be prepared > 24 h.

All that said, it has been shown 5F 203 also inhibits phospho c-Met – as you say a good target in renal carcinoma.

AhR was predominantly expressed in the nuclei of high-grade clear cell RCC (ccRCC) and tumor-infiltrating lymphocytes (TILs), and its expression levels in cancer cells and TILs correlated with the pathological tumor stage and histological grade. A multivariate Cox analysis revealed that the strong expression of AhR in cancer cells was a significant and independent predictor of disease-specific survival. AhR ligands up-regulated the expression of AhR and CYPs and promoted invasion by up-regulating MMPs. Furthermore, siRNA for AhR down-regulated CYPs, and inhibited cancer cell invasion together with the down-regulation of MMPs. These results suggest that AhR regulates the invasion of ccRCC and may be involved in tumor immunity. Therefore, inhibiting the activation of AhR may represent a potentially attractive therapeutic target for ccRCC patients.

Enhanced activation of AhR in renal cancer linked with poor prognosis. It was suggested that *inhibition* of AhR would be therapeutically beneficial. (Activation of aryl hydrocarbon receptor promotes invasion of clear cell renal cell carcinoma and is associated with poor prognosis and cigarette smoke.

Ishida M[1,2], Mikami S[3], Shinojima T[1], Kosaka T[1], Mizuno R[1], Kikuchi E[1], Miyajima A[1], Okada Y[4], Oya M[1].)

YResults section, page 7: 5F 203 is depleted from medium of TK-10 cells and induces expression of CP1A1. That is the relevance of these findings. Are they also tractable in other renal carcinoma lines? **Did you try in other renal cell lines? Yes, I`ll find these data. Attached – for DF 203 3 renal cells line TK10, Caki-1, A498; for 4F 203, just TK10.**

The authors provided data that inhibition of AhR by alpha-NF may impact proliferation inhibition by both compounds (effect of single alpha-NF treatment on proliferation. **We have this control** Figure 2). This statement has to be taken with care as they did not include essential controls on the

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CONCLUSSIONS

AhR ligand antitumor agents, such as AFP–464 and 5F 203, represent potential new candidates for the treatment of renal cancer. Both compounds caused cell cycle arrest and apoptosis. 5F 203 is sequestered by TK-10 cells and induces CYP1A1 expression; 5F 203 only potently inhibited migration of TK-10, Caki-1 and SN12C cells, and inhibited c-Met receptor phosphorylation, in TK-10 cells. C-Met receptor signal transduction promotes migration and metastasis.
Therefore we consider that 5F 203 offers potential for the treatment of metastatic renal carcinoma.

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