

Synergistic interactions between aminoflavone, paclitaxel and camptothecin in human breast cancer cells

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Abstract

Purpose Aminoflavone is a unique DNA damaging agent currently undergoing phase I evaluation in a prodrug form (AFP464). In anticipation of combination regimens, interactions between aminoflavone and several anticancer drugs were investigated in MCF-7 breast cancer cells to determine whether synergistic cancer cell killing effects were observed.

Methods Colony formation assays were performed to assess the effect of combining aminoflavone with a variety of anticancer drugs. Changes in initial uptake, retention or efflux of aminoflavone and the second agent were compared to the behavior of drugs alone. Key features required for aminoflavone activity in cell culture models were also explored, focusing on the obligatory induction of CYP1A1/1A2 and binding of reactive aminoflavone metabolites to tumor cell total macromolecules and DNA.

Results Aminoflavone was synergistic when co-incubated with paclitaxel, camptothecin or SN38. Uptake of neither aminoflavone nor any of the other three compounds was altered in combination incubations. Paclitaxel did not inhibit DNA binding of aminoflavone metabolites, while camptothecin did. Aminoflavone-induced CYP1A1 induction was observed in the presence of camptothecin or paclitaxel.

Conclusions Aminoflavone is a promising therapeutic agent for breast cancer due to its unique mechanism of action compared to commonly used drugs. Combined treatments utilizing aminoflavone in conjunction with paclitaxel or camptothecin may provide an even greater cytotoxic effect than achieved with aminoflavone alone.

Keywords Aminoflavone · Paclitaxel · Camptothecin · Cytochrome P450 · MCF-7

Abbreviations

AF	Aminoflavone (5-amino-2,3-fluorophenyl)-6,8-difluoro-7-methyl-4H-1-benzopyran-4-one (NSC 686288)
CAMP	Camptothecin
CYP1A1	Cytochrome P450 1A1
CYP1A2	Cytochrome P450 1A2
DMSO	Dimethyl sulfoxide
DNA SSB	DNA single strand break
DPBS	Dulbecco's phosphate-buffered saline
FBS	Fetal bovine serum
G6PD	Glucose-6-phosphodehydrogenase
IC ₅₀	50% Inhibition concentration
3-MC	3-Methylcholanthrene
NCI	National cancer institute
PBS	Phosphate-buffered saline
TAX	Paclitaxel
TBST	Tris-buffered saline-Tween 20
topo I	Topoisomerase I

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Introduction

Aminoflavone (AF or [(5-amino-2,3-fluorophenyl)-6,8-difluoro-7-methyl-4H-1-benzopyran-4-one], NSC 686288) is a

fluorinated diaminoflavone analog with significant antiproliferative activity in a variety of human tumor cell lines [1, 2]. AF is toxic to a number of cell lines in the National Cancer Institute (NCI) 60 cell line assay, particularly breast, ovarian and renal cells. When AF is compared to other known anticancer agents in the NCI screen using the COMPARE algorithm (<http://www.dtp.nci.nih.gov>), the AF profile is unique, suggesting it has a novel mechanism of action [3].

We previously reported that CYP1A1 and 1A2 converted AF into active metabolites that bind to cellular macromolecules both in microsomal preparations and in sensitive, but not resistant, human tumor cell lines [4]. AF induced the expression of CYP1A1/1A2 in these sensitive human tumor cell lines, none of which constitutively express these metabolic enzymes in significant quantities. AF-treated MCF-7 cells showed phosphorylation and stabilization of p53 [4], indicative of DNA damage. In addition, DNA adducts were detected in sensitive, but not resistant, cell lines [4]. Further studies determined that AF causes DNA damage as based on H₂AX phosphorylation and alkaline elution data [5–7]. Based on the activity profile and reported novel mechanism of action, AF was selected by the NCI for Phase I clinical trials, utilizing the prodrug AFP464 (<http://www.clinicaltrials.gov/ct2/show/NCT00369200>, <http://www.clinicaltrials.gov/ct2/show/NCT00348699>).

In anticipation of further development of AF, we sought to determine the effect of combining AF with other drugs used for cancer treatment, each having diverse known mechanisms of action. We screened several agents in combination with AF. Because of our interest in certain breast cancer treatments, we limited additional studies to PaclitaxelTM (TAX), camptothecin (CAMP) and SN38 (the active metabolite of irinotecan, or CamptosarTM).

TAX, a tubulin stabilizer, causes cell cycle arrest and induces apoptosis. It has efficacy in the treatment of head and neck [8, 9], breast [10, 11], lung [12, 13] and ovarian cancers [14–17]. The mechanism of action of TAX involves binding to, and stabilization of, tubulins [18]. Though TAX has been successfully used in combination with anthracyclines, alkylating agents and trastuzumab (Herceptin[®]), tumors often are, or become, resistant [19, 20].

CAMP and SN38 (the active metabolite of irinotecan) interact with topoisomerase I (topo I) and DNA to stabilize cleavable complexes that prevent resealing of topo I-mediated DNA single strand breaks (SSBs) [21, 22]. These SSBs lead to formation of double strand breaks during DNA synthesis and subsequent apoptosis. CAMP was evaluated in phase I [23] and phase II [24] clinical trials for breast cancer but was found to be too toxic for further use. Irinotecan is approved for use in colon cancer and in phase

II clinical trials has shown activity against metastatic breast cancer [25, 26].

The purpose of combining chemotherapeutic agents for cancer treatment is to circumvent the tumor cell's multiple survival mechanisms as well as to avoid drug resistance. By utilizing drugs that have unique mechanisms of action, these goals may be achieved while affording synergistic tumor cell killing. AF has a unique mechanism of action, and it is uncertain what the effect of combining it with other anticancer agents would be.

The present studies were performed to assess the effect of combining AF with other agents. We observed synergistic activity for combinations of AF and TAX, CAMP and SN38 in MCF-7 human breast cancer cells. This synergy was not sequence dependent. Cellular uptake of drugs was not altered by co-incubations. Key aspects of the mechanism of action of AF were not altered by cotreatment conditions as evidenced by AF's ability to bind cellular macromolecules, cause a slight S phase arrest and induce CYP1A1 expression.

Materials and methods

Materials

MCF-7 human tumor cell lines were obtained from the NCI (Bethesda, MD). All cell lines were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) with 5% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) at 37°C with a humidified atmosphere containing 5% (v/v) CO₂. AF was obtained from the Pharmaceutical Resources Branch, NCI (Bethesda, MD). Radiolabeled AF [(5-amino-2,3-fluorophenyl)-6,8-difluoro-7-[¹⁴C]methyl-4H-1-benzopyran-4-one] (23 mCi/mol) was synthesized at Research Triangle Institute (Research Triangle Park, NC) and provided by the National Cancer Institute. TAX (NCI), CAMP (Sigma) and SN38 were purchased from Pfizer (New York, NY). Silicone and mineral oil were purchased from Sigma-Aldrich (St. Louis, MO).

Colony-forming ability assay

MCF-7 cells were plated at 500 cells/60 mm dish in RPMI containing 5% FBS, 100 units/mL penicillin G and 100 µg/mL streptomycin. Each experiment included six untreated plates and three plates/drug concentration. Cells were allowed to adhere overnight and were treated with AF, TAX, CAMP, or SN38 in sequence or in combination for 24 h. For combination experiments, both drugs were added simultaneously at fixed dose ratios corresponding to one-half, five-eighths, three-quarters, seven-eighths, 1, 1.5 and 2.0 times the individual 50% inhibition concentration (IC₅₀)

values. Plates were washed with phosphate-buffered saline (PBS) before fresh medium was added. Colonies were allowed to grow for 8–10 days before the cells were stained with Coomassie blue (methanol:H₂O:acetic acid, 45:45:10, with 2.5 g/L Coomassie blue dye). Colonies were counted by hand. Fractional survival was calculated by dividing number of colonies in drug-treated plates by number of colonies in control plates. Dose–response curves were generated for each agent to estimate IC₅₀ values. Data were analyzed for synergy by the method of Chou and Talalay [27] using CalcuSyn software (BioSoft, Inc., Ferguson, MO). Synergy is indicated by CI values <1, additivity by CI values = 1 and antagonism by CI values >1.

Short-term radiolabeled drug uptake analyses

MCF-7 cells were trypsinized, washed twice with 1× Hanks Balanced Salt Solution and resuspended at a concentration of 1×10^6 cells/0.5 mL in 5 mL total volume of RPMI without FBS. A mix of silicone and mineral oil (0.5 mL, 1.015 density) was added to labeled microcentrifuge tubes warming in a 37°C heat block. One aliquot (0.5 mL) of cell suspension was removed, added to a microcentrifuge tube of oil, and centrifuged (14,000 rpm) to separate the cells from the medium, as an untreated control. Appropriate drug or vehicle was added to the remaining cell suspension and mixed well. Aliquots of this cell suspension were added to the remaining microcentrifuge tubes. At the indicated time points, the tubes were centrifuged to separate the cells from the drug-containing media. After removing the media and oil, each cell pellet was treated with 50 µL 1 N NaOH and incubated for 1 h at 60°C. To neutralize the solution, 50 µL 1 N HCl was added and the samples stored at –20°C. Thawed sample aliquots were analyzed for protein content by DC protein assay (BioRad, Hercules, CA). The remaining sample was incubated with Ultima Gold high flash-point, universal LSC-cocktail scintillation counter fluid (PerkinElmer Life and Analytical Sciences, Boston, MA) for 1 h in darkness, and then analyzed for the amount of radiolabel with a Beckman liquid scintillation counter (Beckman Coulter Inc., Fullerton, CA).

Long-term radiolabeled drug uptake analyses

MCF-7 cells were plated at 1×10^6 cells/well in six-well plates and allowed to adhere overnight. Cells were washed twice with 1× Dulbecco's phosphate-buffered saline (DPBS) containing Ca²⁺ and Mg²⁺. One mL of RPMI without FBS was added to each well. Drug-containing medium (FBS-free) was prepared at 2× concentrations. At the indicated time points, 1 mL of drug-containing medium was added to each well. To harvest the cells,

drug-containing medium was removed, and the cells washed twice with 1 mL of DPBS. To lyse the cells, 1% TritonX-100 was added to each well, and the plate incubated for 30 min at 37°C. The lysed cell solution was collected in microcentrifuge tubes and stored at –20°C. Thawed sample aliquots were analyzed for protein content by DC protein assay (BioRad, Hercules, CA). The remaining sample was subjected to scintillation counting as described earlier.

AF covalent DNA binding in human tumor cell lines

MCF-7 cells were plated at $1\text{--}2 \times 10^6$ cells/10 cm plates and allowed to adhere overnight. Cells were treated with drug-containing media for 24 h and harvested by trypsinization 0, 6, 12 and 24 h post-pulse. DNA was extracted from the cells and purified with the DNeasy Tissue Kit (Qiagen Inc., Valencia, CA). The concentration of DNA was determined by absorbance at 260 nm using a BioRad spectrophotometer (BioRad, Hercules, CA). The remaining sample was subject to scintillation counting as described earlier.

AF covalent macromolecule binding in human tumor cell lines

Measurements were performed as previously described [4]. Briefly, MCF-7 cells were plated at $1\text{--}2 \times 10^6$ cells/10 cm plates, treated with drug-containing media for 24 h and harvested at that time. Following centrifugation, cell pellets were suspended in H₂O, incubated on ice, and sonicated. Acetone was added to precipitate macromolecules on ice. Protein and nucleic acids were pelleted by centrifugation, then resuspended in methanol, and agitated with a probe sonicator. Macromolecules were re-isolated by centrifugation. This methanol wash was repeated six times. The final pellet was dissolved in 1 N NaOH and sample aliquots were analyzed for protein content by DC protein assay (BioRad,

Table 1 Synergy determined by combination index of combining AF with chemotherapeutic drugs. (*N* = 3)

Drug combined with AF	Average combination index (CI ₅₀) ± SD	Average combination index (CI ₉₀) ± SD
Doxorubicin	0.938 ± 0.04	0.671 ± 0.16
Paclitaxel	0.997 ± 0.15	0.685 ± 0.20
Cisplatin	0.898 ± 0.17	0.607 ± 0.02
5-Fluorouracil	0.969 ± 0.26	0.674 ± 0.08
Camptothecin	0.761 ± 0.12	0.670 ± 0.09
SN38	0.896 ± 0.13	0.613 ± 0.10

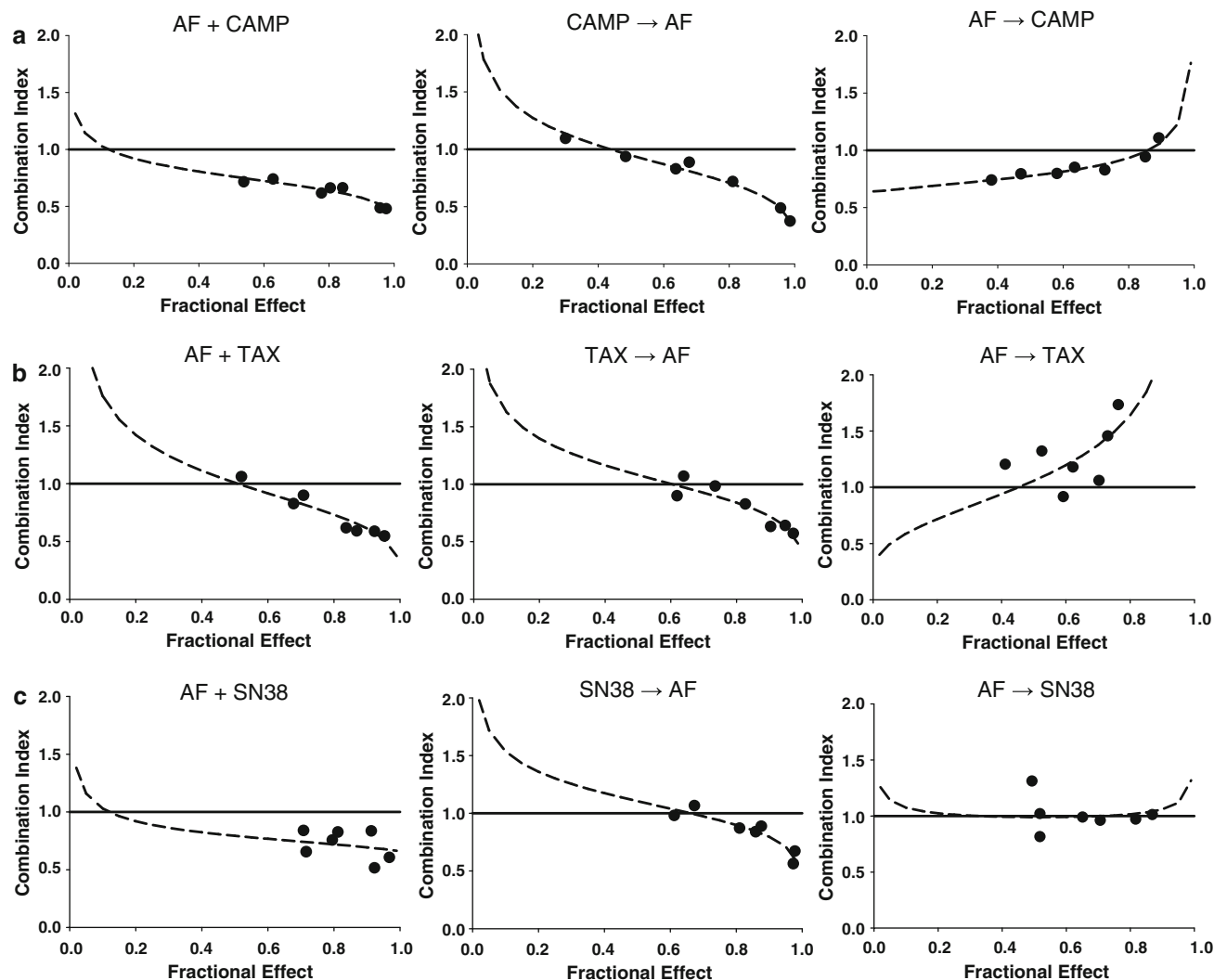


Fig. 1 Effect of AF cotreatment with CAMP, TAX and SN38. Colony-forming ability assays at fixed dose ratios in MCF-7 cells were analyzed with the CalcuSyn program, and combination index plots were generated. (a) Cells treated simultaneously with AF and CAMP (left), cells treated first with CAMP, followed by AF (middle), cells treated first with AF, followed by CAMP (right). (b) Cells treated

simultaneously with AF and TAX (left), cells treated first with TAX, followed by AF (middle), cells treated first with AF, followed by TAX (right). (c) Cells treated simultaneously with AF and SN38 (left), cells treated first with SN38, followed by AF (middle), cells treated first with AF, followed by SN38 (right)

Hercules, CA). The remaining sample was subject to scintillation counting as described earlier.

Cell cycle analysis by flow cytometry

MCF-7 cells were plated $1\text{--}3 \times 10^6$ cells/10 cm plate and allowed to adhere overnight. Cells were treated with drug-containing media for 24 h, and harvested by trypsinization. After centrifugation, cells were washed once with $1 \times$ PBS and resuspended in 0.5 mL PBS. Cells were fixed by adding 0.5 mL of cold 95% ethanol and mixing, then adding an additional 1 mL of cold 95% ethanol, and allowing the cells to incubate for a minimum of 2 h at 4°C . Cells were pelleted by centrifugation and resuspended in 1 mL RNase A

solution (Sigma–Aldrich; $164 \mu\text{g/mL}$ final concentration from a $2,760 \text{ Kunits/mL}$ 30 mg/mL stock diluted into PBS). After incubation at 37°C for 30 min, cells were diluted with 1 mL of PBS and pelleted by centrifugation. The cells were resuspended in 1 mL of propidium iodide (Calbiochem, La Jolla, CA; $50 \mu\text{g/mL}$ in PBS) and incubated at room temperature in darkness for a minimum of 30 min prior to flow cytometer analysis.

Microsome preparations from human tumor cell lines

Following treatment, MCF-7 cells were trypsinized and washed twice with $1 \times$ PBS. Cells were pelleted and prepared by standard differential centrifugation techniques

(adapted from those previously described [28]). Microsomal pellets were suspended in 0.15 M KCl at concentrations of 1–50 mg protein/mL. Sample aliquots were analyzed for protein content by DC protein assay (BioRad, Hercules, CA).

Immunoblotting of CYP1A1

MCF-7 cell microsomes were isolated as described earlier. Immunoblotting was performed as described [4]. Briefly, samples containing 50 µg protein were subjected to electrophoresis for 60 min at 120 V on Criterion XT Precast SDS–polyacrylamide gels containing 12% Bis–Tris (BioRad, Hercules, CA). Separated proteins were electrophoretically transferred to polyvinylidene fluoride membranes for 60 min. Membranes were probed with polyclonal goat α -human CYP1A/1A2 (BD Gentest, Woburn, MA) at 1:5,000, and rabbit α -goat IgG at 1:50,000 (Chemicon International, Temecula, CA). Blots were incubated for 5 min in Pierce Chemical (Rockford, IL) West Femto chemiluminescence substrate detection solution and then exposed to Hyperfilm ECL high performance chemiluminescence film (Amersham Biosciences, Buckinghamshire, UK).

Results

AF synergizes with TAX, CAMP and SN38 in human breast cancer cells

Colony-forming assays with MCF-7 cells were used in initial studies to determine the effect of combining AF with doxorubicin, TAX, cisplatin, 5-fluorouracil, CAMP, or SN38 at fixed dose ratios. Treatments were applied either simultaneously or sequentially. Synergy was observed when MCF-7 cells were cotreated with AF and doxorubicin, TAX, cisplatin, 5-fluorouracil, CAMP or SN38 (Table 1).

Additional studies were conducted with TAX, CAMP and SN38 (Fig. 1) based on our interest in these classes of agents in the treatment of breast cancers. We also addressed the sequential administration of AF and TAX, CAMP or SN38. Little evidence of synergy was observed when AF treatment preceded that of these other agents, and there was some evidence of antagonism (Fig. 1). While synergy was observed when AF treatment preceded that of the other three agents, the extent of synergy was similar to that observed following coadministration. Therefore, additional mechanistic studies utilized coadministration. CAMP was employed in all mechanistic studies as representative of topoisomerase II poisons.

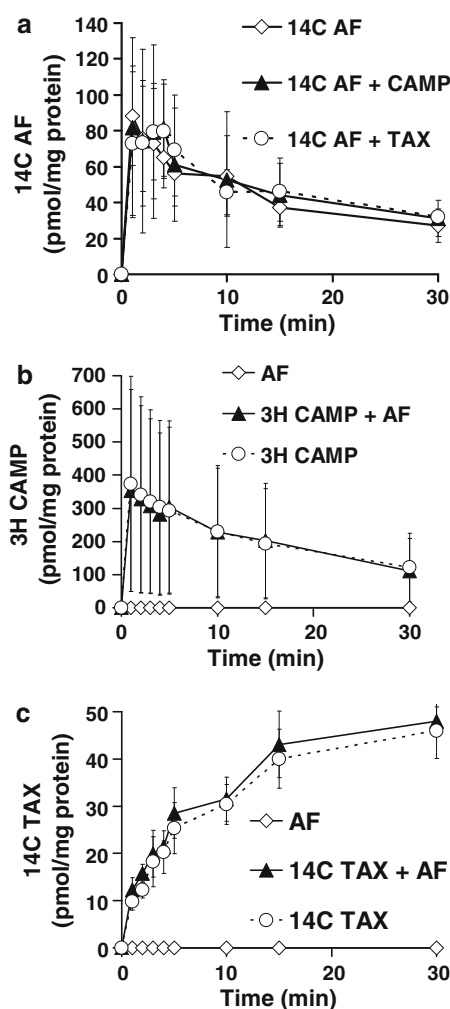


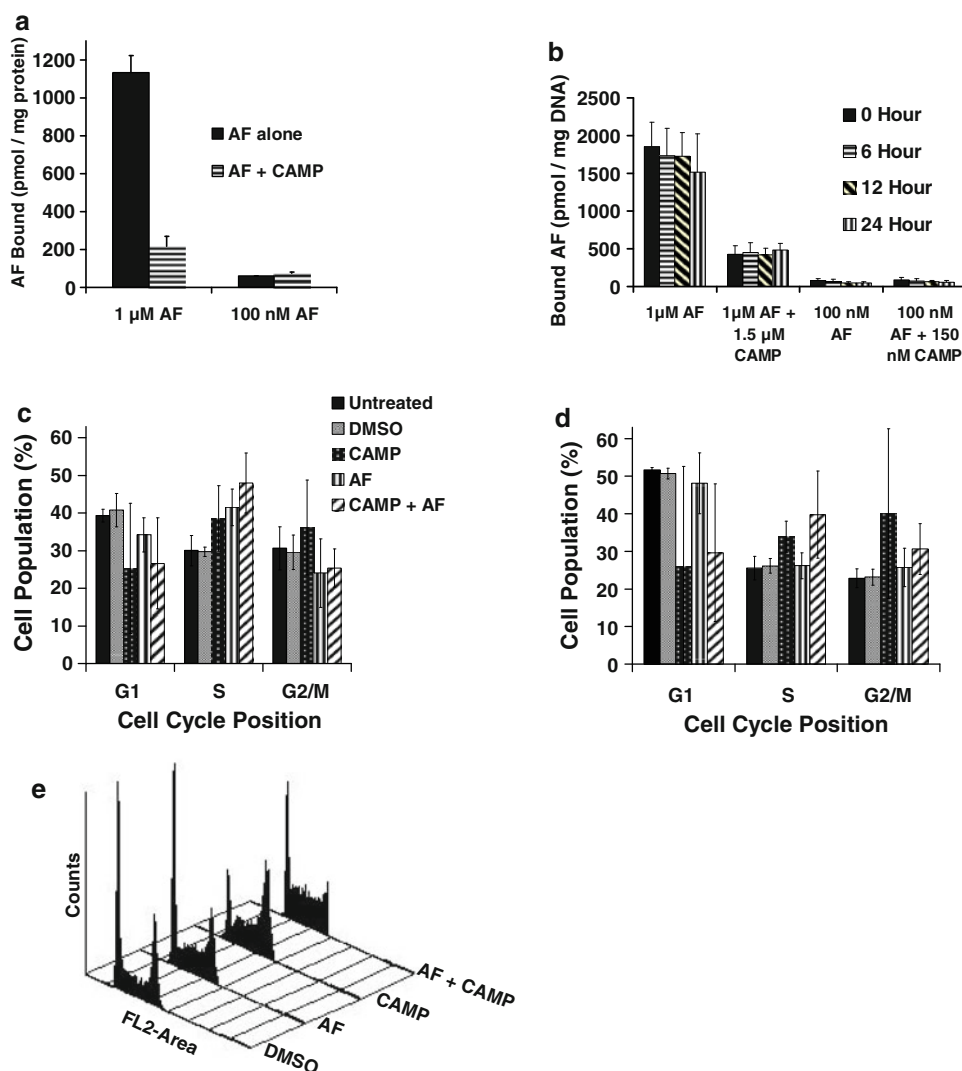
Fig. 2 Effect of cotreatment on drug uptake. MCF-7 cells were treated with (a) 1 µM 14 C-AF $\pm$ 1.5 µM CAMP or 0.4 µM TAX, (b) 1.5 µM 3 H-CAMP $\pm$ 1 µM AF, or (c) 0.4 µM 14 C-TAX $\pm$ 1 µM AF for the time points indicated. Radiolabeled drug uptake was assayed as described in “Materials and methods.” The data are presented as mean $\pm$ standard deviation of three experiments

Cotreatment does not affect drug uptake

Radioactive 14 C-AF, 3 H-CAMP and 14 C-TAX were used to determine whether during cotreatment the uptake, retention or efflux of one or both drugs into MCF-7 cells was altered or inhibited. CAMP and TAX did not alter uptake of AF (Fig. 2a). Similarly, AF did not alter uptake of CAMP (Fig. 2b) or TAX (Fig. 2c).

For additional mechanistic studies, both a “high” (1 µM AF, 1.5 µM CAMP, 0.4 µM TAX) and a “low” (100 nM AF, 150 nM CAMP, 40 nM TAX) set of synergistic dose ratios were utilized to assess effects of the combination in MCF-7 cells.

Fig. 3 Effect of cotreatment with CAMP on AF-DNA, macromolecule binding and cell cycle. MCF-7 cells were treated with (a) ^{14}C -AF $\pm$ CAMP at either a high (1 μM ^{14}C -AF, 1.5 μM CAMP) or a low (100 nM ^{14}C -AF, 150 nM CAMP) fixed dose ratio for 24 h. Cells were harvested at the time points indicated after drug removal and assayed as described in “Materials and methods.” (b) MCF-7 cells were treated with ^{14}C -AF $\pm$ CAMP at a high (1 μM ^{14}C -AF, 1.5 μM CAMP) fixed dose ratio for 24 h. Cells were harvested immediately after the drug treatment and assayed as described in “Materials and methods.” MCF-7 cells were treated with 1 μM AF, 1.5 μM CAMP or both for 24 h and harvested either (c) immediately or (d) 24 h after drug removal. Representative histograms are shown (e). Cell cycle was determined by PI staining and flow cytometry as described in “Materials and methods.” The data are presented as mean $\pm$ standard deviation of three experiments



AF metabolism, macromolecule and DNA binding and cell cycle effects are hindered by high-dose CAMP, but not low-dose CAMP

Radioactive ^{14}C -AF was utilized to determine whether the presence of CAMP altered the metabolism and subsequent total macromolecule binding of AF. MCF-7 cells treated with the high dose ratio of AF and CAMP (1 μM AF + 1.5 μM CAMP) showed a $\sim 80\%$ decrease in macromolecule-bound ^{14}C -AF (Fig. 3a). Cells treated with the low dose ratio (100 nM AF + 150 nM CAMP) showed no change in macromolecule-bound ^{14}C -AF (Fig. 3a). Radioactive ^{14}C -AF was also utilized to determine whether the presence of CAMP altered the metabolism and subsequent DNA binding of AF. MCF-7 cells treated with the high dose ratio of AF and CAMP (1 μM AF + 1.5 μM CAMP) showed a significant $\sim 75\%$ decrease in DNA-bound ^{14}C -AF (Fig. 3b). Cells treated

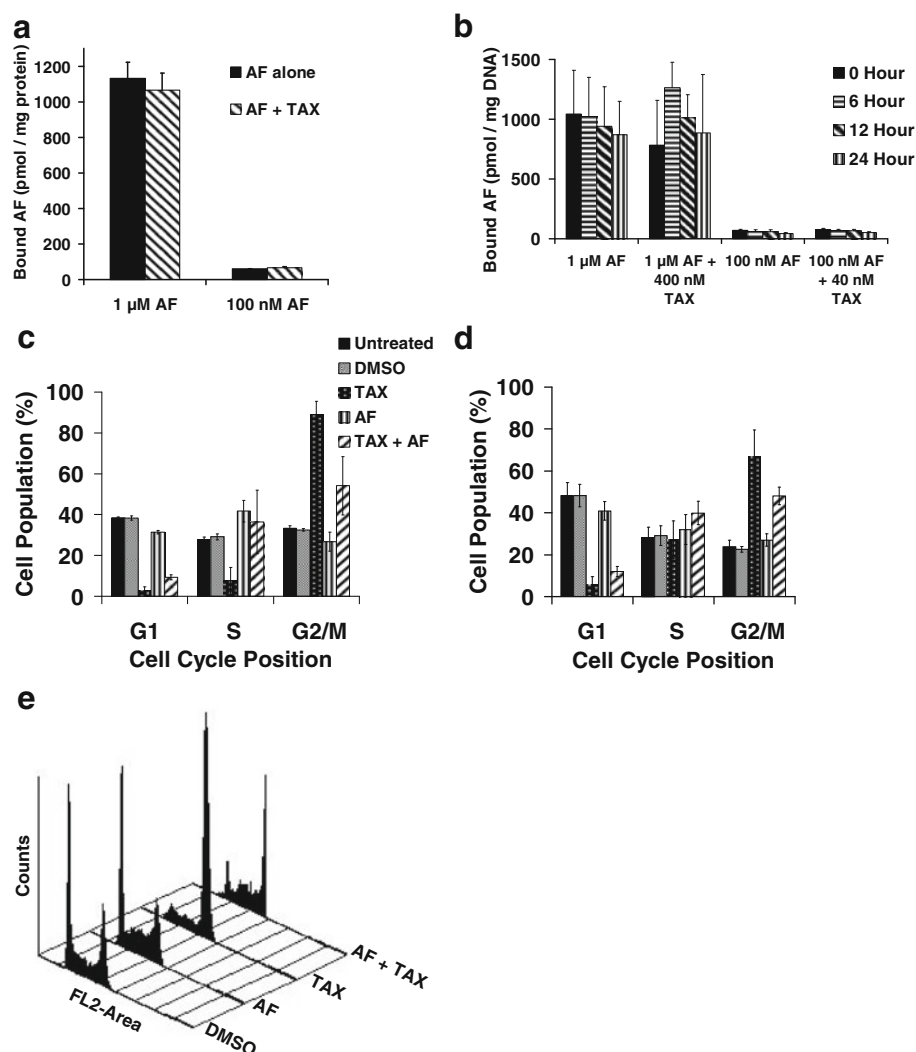
with the low dose ratio (100 nM AF + 150 nM CAMP) showed no change in DNA-bound ^{14}C -AF (Fig. 3b).

AF has been shown to activate the S phase checkpoint [7]. CAMP also causes S phase arrest [29]. Immediately after a 24-h cotreatment of AF and CAMP at the high dose ratio, we observed an increase in S phase arrest (Fig. 3c) compared to cells treated with CAMP alone. A 24 h after drug removal, no change in the population of cells in G2/M was observed (Fig. 3d) in the cotreated sample compared to the sample treated with CAMP alone.

AF metabolism, macromolecule and DNA binding and cell cycle effects are unaltered by TAX

Radioactive ^{14}C -AF was utilized to determine whether the presence of TAX altered the metabolism and subsequent total macromolecule binding of AF. Both high (1 μM AF + 0.4 μM TAX) and low (100 nM AF + 40 nM TAX)

Fig. 4 Effect of cotreatment with TAX on AF-DNA, macro-molecule binding and cell cycle. MCF-7 cells were treated with (a) ^{14}C -AF $\pm$ TAX at either a high (1 μM ^{14}C -AF, 0.4 μM TAX) or a low (100 nM ^{14}C -AF, 40 nM TAX) fixed dose ratio for 24 h. Cells were harvested at the time points indicated after drug removal and assayed as described in “Materials and methods.” (b) MCF-7 cells were treated with ^{14}C -AF $\pm$ TAX at a high (1 μM ^{14}C -AF, 0.4 μM TAX) fixed dose ratio for 24 h. Cells were harvested immediately after the drug treatment and assayed as described in “Materials and methods.” MCF-7 cells were treated with 1 μM AF, 0.4 μM TAX or both for 24 h and harvested either (c) immediately or (d) 24 h after drug removal. Representative histograms are shown (e). Cell cycle was determined by PI staining and flow cytometry as described in “Materials and methods.” The data are presented as mean $\pm$ standard deviation of three experiments



dose ratios of AF and TAX showed no significant difference in macromolecule-bound ^{14}C -AF (Fig. 4a). Radioactive ^{14}C -AF was also utilized to determine whether the presence of TAX altered the metabolism and subsequent DNA binding of AF. MCF-7 cells treated with the high dose ratio of AF and TAX (1 μM AF + 0.4 μM TAX) showed no appreciable difference in DNA-bound ^{14}C -AF (Fig. 4b). Cells treated with the low dose ratio (100 nM AF + 40 nM TAX) also showed no change in DNA-bound ^{14}C -AF (Fig. 4b).

AF has been shown to activate the S phase checkpoint [7], while TAX induces arrest in G2/M phase [30]. Immediately after a 24-h cotreatment of AF and TAX at the high dose ratio a significant increase in S phase arrest and concurrent decrease in G2/M arrest was noted (Fig. 4c) between cells that were cotreated with AF versus those treated with TAX alone. After 24 h of drug removal we observed little change between these cells and those harvested immediately after the 24-h drug treatment (Fig. 4d).

AF-induced CYP1A1 expression is hindered only by high dose CAMP

Microsomal preparation and subsequent immunoblotting were performed to determine whether cotreatment with CAMP or TAX affected the ability of AF to induce CYP1A1 protein expression. MCF-7 cells treated with the high dose ratio of AF and CAMP (1 μM AF + 1.5 μM CAMP) showed no detectable CYP1A1 (Fig. 5, lane 8). Cells treated with the low dose ratio (100 nM AF + 150 nM CAMP) showed a similar level of CYP1A1 induction (Fig. 5, lane 9) to those cells treated with AF alone (Fig. 5, lane 5). Both high (1 μM AF + 0.4 μM TAX) and low (100 nM AF + 40 nM TAX) dose ratios of AF and TAX showed no significant difference CYP1A1 levels (Fig. 5, lanes 12 & 13) compared to cells treated with AF alone (Fig. 5, lanes 4 & 5). Neither CAMP nor TAX alone induced CYP1A1 expression at detectable levels (Fig. 5, lanes 6, 7, 10 & 11).

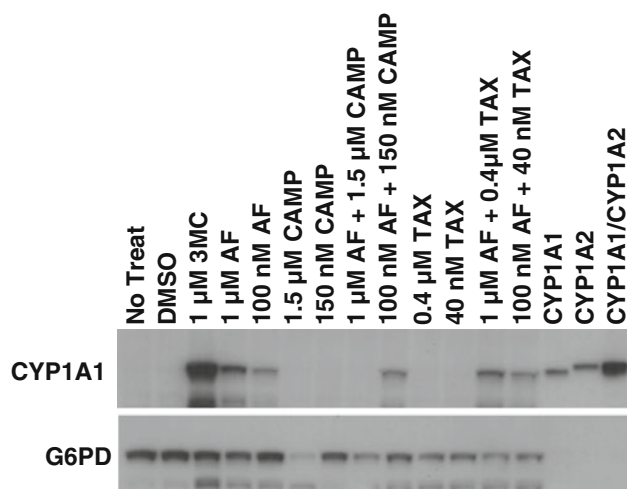


Fig. 5 Effect of cotreatment on AF-induced CYP1A1 expression. MCF-7 cells were treated with AF $\pm$ CAMP at a high (1 μ M AF, 1.5 μ M CAMP) fixed dose ratio or AF $\pm$ TAX at a high (1 μ M AF, 0.4 μ M TAX) fixed dose ratio for 24 h. Cells were harvested immediately after the drug treatment and assayed for CYP1A1 and G6PD expression by immunoblotting as described in “Materials and methods.” 3-methylcholanthrene (3-MC)

Discussion

The activity profile of AF in the NCI 60 human tumor cell line panel was suggestive of a unique mechanism of action [3–5]. Studies performed in our laboratory and others have begun to characterize features of that unique mechanism involving induction of CYP1A1/1A2, metabolic activation of AF to DNA-binding metabolites and subsequent DNA damage as indicated by p53 stabilization and H2AX phosphorylation [4, 5, 7, 31]. The activity profile of AF and the unique features of the mechanism of action have led to ongoing Phase I clinical trials of a prodrug form of AF (AFP-464) at our institution and elsewhere. In anticipation of further development with this agent and with the observed activity against human breast cancer cell lines, we sought to determine the effect of combining AF with selected drugs currently used to treat breast cancer.

Among the drugs relevant for breast cancer therapy assessed in our initial screening studies, synergy was observed with AF in combination with TAX, CAMP or the irinotecan metabolite SN38 during simultaneous incubations (Table 1; Fig. 1). No advantage of sequential administration of agents was observed in our studies (Fig. 1).

In an effort to determine whether the mechanism of action of AF was affected by simultaneous treatment with TAX or CAMP, we first assessed intracellular concentrations of all agents when co-incubated with tumor cells. No changes in initial uptake, retention or efflux of AF were

observed relative to the behavior of the drugs when incubated alone (Fig. 2), consistent with the relative lipophilic nature of these agents and absence of known uptake by active mechanisms. We explored critical cellular affects of AF required for activity in cell culture models, focusing on the obligatory induction of CYP1A1/1A2 (Fig. 5) and binding of reactive AF metabolites to tumor cell DNA and macromolecules (Figs. 3a, b, 4a, b). These experiments were conducted at relatively high and low concentrations producing synergistic activity in combination studies. At high concentrations of CAMP, reduced induction of CYP1A1 was observed relative to AF alone (Fig. 5, lanes 8 & 4). Consistent with those observations, reduced AF binding to DNA and macromolecules was observed under those conditions (Fig. 3a, b). However, at lower concentrations of CAMP, CYP1A1 induction and DNA and macromolecule binding of AF were little changed relative to AF alone (Figs. 3a, b, 5). CAMP is a known inhibitor of RNA and thus protein synthesis at the high concentration of 1.5 μ M [29, 32, 33] and we believe this accounts for the reduced CYP1A1 induction. In the case of TAX, neither CYP1A1 induction (Fig. 5, lanes 12 & 13) nor binding of activated AF to tumor cell DNA or macromolecules were changed relative to AF treatment alone (Fig. 4a, b). Thus, there was no evidence that either of these agents significantly altered key elements associated with AF activity in MCF-7 cells.

AF, TAX and CAMP are known to cause cell cycle arrest [7, 29, 30] at different phases of the cell cycle. AF causes a partial arrest in S phase which enhances CAMP-induced S phase arrest after a 24-h cotreatment (Fig. 3c). AF diminishes TAX-induced G2/M arrest after a 24-h cotreatment (Fig. 4c) and 24 h after drug removal (Fig. 4d). However, we do not believe these combined effects alter the activity of the agents.

Our studies did not reveal alterations in cellular uptake or basic aspects of AF cellular pharmacology that might be responsible for synergy with these agents. We conclude that the three agents' unique mechanisms of action are not hindered by the presence of the other compound. Synergism may well be the result of complimentary downstream death pathways enhancing the effects of each agent.

Considering that AF has a unique mechanism of action, is active in breast cancer cells, and provides provocative results in combination studies, we believe that combined therapies that include AF use may offer a greater therapeutic advantage for breast cancers capable of inducing CYP1A1 for AF metabolism. Animal studies as well as clinical trials are critical components of future studies.

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