Cancer Chemother Pharmacol (2006) 57: 569–576
DOI 10.1007/s00280-005-0075-7

ORIGINAL ARTICLE

Lori J. Pobst · Matthew M. Ames

CYP1A1 activation of aminoflavone leads to DNA damage in human tumor cell lines

Received: 26 April 2005 / Accepted: 22 June 2005 / Published online: 11 August 2005

Abstract *Purpose*: Aminoflavone (5-amino-2,3-fluorophenyl)-6,8-difluoro-7-methyl-4H-1-benzopyran-4-one; AF; NSC 686288) is a novel anticancer agent with a unique pattern of growth inhibitory activity in the National Cancer Institute (NCI) 60 tumor cell line screen. Phase I clinical trials with AF will begin soon. We previously demonstrated extensive metabolism of AF by cytochrome P450 (CYP) 1A1 and CYP1A2, metabolic activation, formation of irreversible protein and DNA adducts and p53 stabilization in sensitive, but not resistant, human tumor cell lines treated with AF [9]. The present studies focus on the effects of AF on cellular DNA and cellular responses to DNA damage. *Methods*: Phosphorylation of H2AX in MCF7 cells treated with AF was determined with immunofluorescence. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4- sulfophenyl)-2H-tetrazolium) assays were used to determine the effect of cotreatment with caffeine or wortmannin, inhibitors of ataxia-telangiectasia-mutated protein (ATM) and ATR (ATM- and *rad3*-related protein), on the AF IC_{50} values for MCF7 cells. DNA damage in MCF7 cells treated with AF was determined by alkaline elution. DNA-topoisomerase complex stabilization was ascertained by the ICE (in vitro complex of enzyme) assay. *Results*: Treatment of sensitive cells with AF resulted in phosphorylation of H2AX, a histone 2A variant that is phosphorylated in response to DNA damage. AF IC_{50} values for MCF7 cells were lowered by cotreatment with caffeine or wortmannin, further implicating DNA damage in AF cytotoxicity. There was no evidence of DNA–DNA cross-linking in sensitive cells, but protein-associated single-strand breaks were observed after AF treatment. Although this pattern of DNA damage is commonly associated with topoisomerase poisons, there was no evidence for AF-induced stabilization of either topoisomerase I- or II-DNA complexes. *Conclusions*: These studies further implicate DNA damage in the cytotoxicity of AF and identify biochemical features of that damage including formation of protein-associated single-strand breaks not involving topoisomerase I or II.

Keywords Aminoflavone · Cytochrome P450 · H2AX · Alkaline elution · MCF7 · Topoisomerase

L. J. Pobst · M. M. Ames (✉)
Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic College of Medicine,
Guggenheim 13, 200 First Street S.W.,
Rochester, MN 55905, USA
E-mail: ames.matthew@mayo.edu
Tel.: +1-507-2842424
Fax: +1-507-2843906

Introduction

Aminoflavone (5-amino-2,3-fluorophenyl)-6,8-difluoro-7-methyl-4H-1-benzopyran-4-one; AF; NSC 686288) is a fluorinated diaminoflavone analog with substantial antiproliferative activity in a variety of cultured human tumor cell lines, including MCF7 breast cancer cells [2] as well as in mice with implanted human tumors [3]. AF was active in a number of cell lines in the NCI 60 cell line assay. When the pattern of activity was compared to the profiles of known anticancer agents using the COMPARE program (http://www.dtp.nci.nih.gov), the AF profile was unique, which is highly suggestive of a novel mechanism of action [25].

Flavone analogs have many biological activities including topoisomerase inhibition [26], protein kinase inhibition [5, 6], and the inhibition of tubulin polymerization [19]. Many flavones are metabolized by CYP isoforms, especially CYP1A isoforms [13], and some flavones inhibit certain CYP isoforms [27]. We have shown that AF is metabolized predominantly by CYP1A1 and, to a lesser extent, CYP1A2 [9]. HPLC/MS/MS analysis of metabolites produced by incubation of AF in a liver microsomal preparation with the cofactor NADPH showed a number of oxidative metabolites, including alcohols, phenols, and oxidative

deflourination products. Of particular interest was the identification of an aromatic hydroxylamine metabolite [9] because the aromatic hydroxylamine moiety is electrophilic and, therefore, chemically reactive with nucleophilic compounds such as cellular macromolecules (i.e., proteins and DNA).

We previously reported that AF is metabolically activated by CYP1A1 and 1A2 to reactive metabolites capable of binding to cellular proteins in both microsomal preparations and in human tumor cell lines [9]. AF induced the expression of CYP1A1/1A2 in human tumor cells, which do not constitutively express these metabolic enzymes in significant quantity. Treatment of sensitive MCF7 cells resulted in phosphorylation and stabilization of p53 [9], suggestive of DNA damage. Further, DNA adducts were detected in sensitive, but not resistant, cell lines [9]. Based on the in vitro and in vivo activity profile and novel elements of the mechanism of action, AF has been selected by NCI for Phase I clinical trials.

The present studies were performed to better characterize the interactions between AF and cellular DNA and the nature of the DNA damage. We report evidence of DNA damage as measured by H2AX phosphorylation. Caffeine and wortmannin co-treatment with AF resulted in decreased IC_{50} values for MCF7 cells indicating that DNA damage observed is at least partially responsible for the cellular toxicity of AF. Alkaline elution studies demonstrated evidence of protein-associated DNA single-strand break formation. However, there was no evidence of DNA-topoisomerase complex formation in cells treated with AF.

Methods and materials

Materials

MCF7 and M14-MEL cells were obtained from the National Cancer Institute (NCI, Bethesda, MD, USA). The cell lines were grown in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) with 5% fetal calf serum (FCS, Invitrogen). AF was obtained from the Pharmaceutical Resources Branch, NCI. Rabbit polyclonal anti-γ-H2AX was provided by Junjie Chen (Mayo Clinic). Rhodamine-tagged goat antirabbit IgG was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Topoisomerase I and II antibodies were purchased from Topogen (Columbus, OH, USA). ^{14}C-labeled thymidine (specific activity 52 mCi/mmole, label on C2) and ^{3}H-labeled thymidine (specific activity 20 Ci/mmole, label on methyl group off of C5) were purchased from New England Nuclear (Boston, MA, USA). Polycarbonate filters were purchased from Osmonics, Inc. (Minnetonka, MN, USA). Etoposide (VP16), camptothecin, caffeine, wortmannin, α-naphthoflavone, and proteinase K were purchased from Sigma-Aldrich (St. Louis, MO, USA). MTS reagent was purchased from Promega (Madison, WI, USA) and PMS (phenazine methosulfate) was purchased from Serva Electrophoresis (Heidelberg, Germany).

H2AX phosphorylation

Cells were seeded (1×10^6 cells/well) into 6-well plates containing cover slips and incubated for 24 h before treatment. Cells were treated with AF (50 nM or 1 μM) or 0.1% (v/v) DMSO for 6 h. The cells were fixed for 12 min at room temperature with a 3% paraformaldehyde solution [in phosphate-buffered saline (PBS) with 20 mg/ml sucrose] and then treated with a 0.5% Triton X-100 solution (in 20 mM Hepes, 50 mM NaCl, 3 mM $MgCl_2$, 300 mM sucrose, pH 7.4) for 5 min at room temperature and washed (twice with PBS) before incubation with rabbit polyclonal anti-γ-H2AX antibody (1:100 dilution in H_2O with 5% goat serum, for 20 min at 37°C). The cells were washed (twice with PBS) and incubated with rhodamine-labeled goat antirabbit IgG (1:200 dilution in H_2O with 5% goat serum, for 20 min at 37°C). Following secondary antibody incubation, the cover slips were stained with Hoechst 33258 for 30 s, mounted and viewed with a Nikon ECLIPSE E800 fluorescence microscope. Images were processed using Adobe Photoshop (Adobe Systems Inc., San Jose, CA, USA) and Canvas software (ACD Systems International Inc., Saanichton, BC, Canada).

Alkaline elution

Single-strand DNA breaks and DNA–DNA cross-links were measured using the alkaline elution technique [8]. MCF7 cells were seeded (1×10^5 cells/plate) in 5 ml RPMI 1640 containing ^{14}C-labeled thymidine (0.1 μCi/plate) and incubated for 24 h. The medium was removed and fresh medium was added for another 24 h incubation prior to treatment. The cells were treated with AF (100 nM or 1 μM) for 12 h, VP16 (0.5 μM) for 1 h, or nitrogen mustard (NM, 0.6 μM) for 30 m. Drug-treated MCF7 cells were placed on a polycarbonate filter (2 μ pore size) along with approximately 5×10^5 [^{3}H]thymidine-labeled L1210 cells that had been irradiated (3 Gy) to provide an internal standard. For the cross-linking experiments, the MCF7 cells were irradiated with 6 Gy, in addition to drug treatment. Cells were lysed on the filter with 2% SDS and 0.02 M Na_2EDTA, pH 10. Certain samples (as indicated) were then treated with proteinase K (0.5 mg/ml in the SDS lysis buffer) for 1 min. Elution buffer (tetrapropylammonium hydroxide-0.02 M EDTA, pH 12.1) was run though the filter at a rate of 0.057 ml/min and samples were collected in 3 h fractions for a total of 15 h. The samples were then analyzed for ^{14}C and ^{3}H radioactivity using a Beckman liquid scintillation counter. The apparent DNA single-strand break frequency (rad equivalents) was determined using calculations previously described [20].

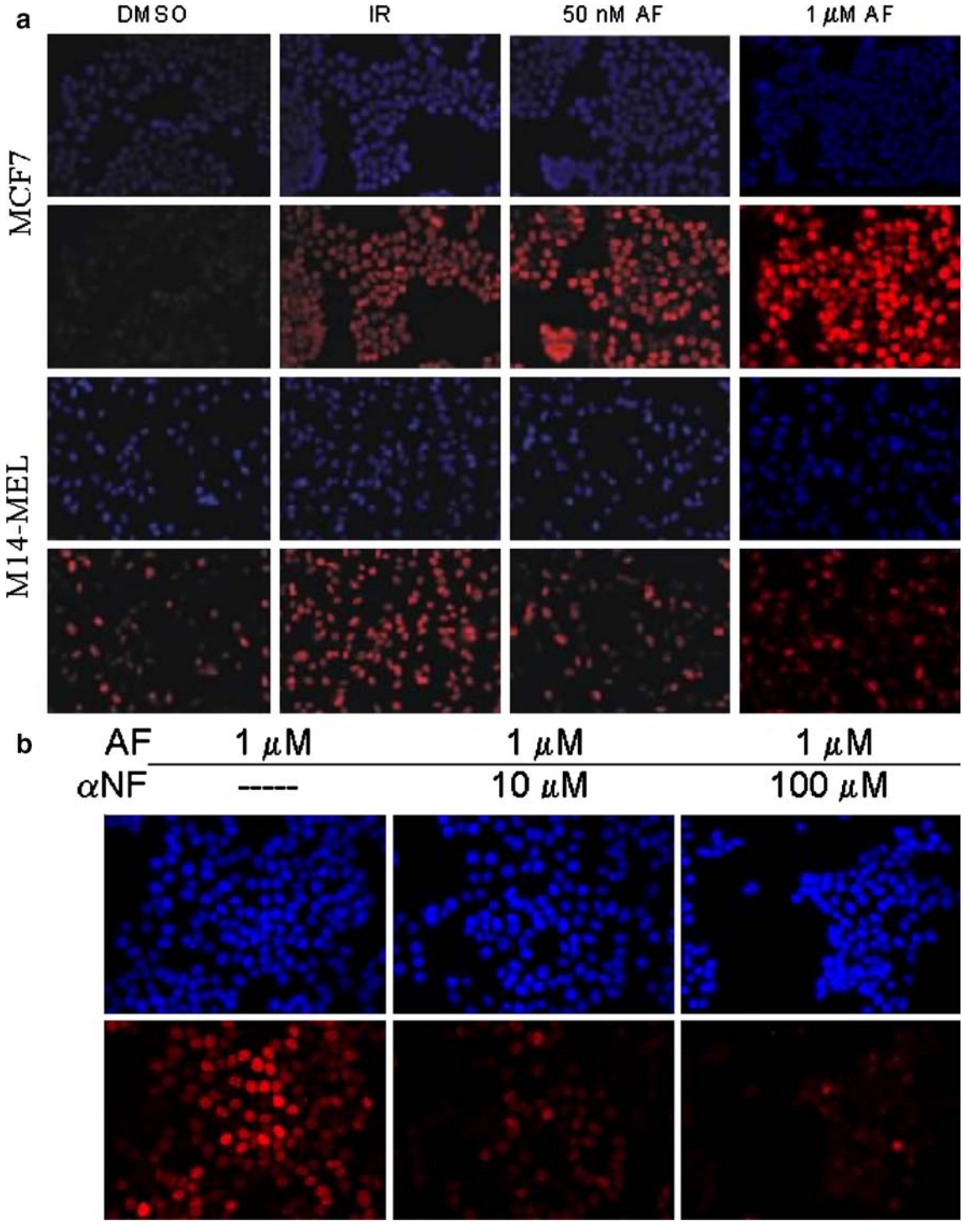

Fig. 1 Effect of AF on H2AX phosphorylation. **a** H2AX phosphorylation in MCF7 and M14-MEL cell lines. Cells were treated with AF (50 nM or 1 μM) or vehicle (DMSO, 0.1% v/v) for 6 h. Ionizing radiation (IR, 10 Gy) was used as the positive control for DNA damage. The cells were incubated with an anti-γH2AX antibody followed by incubation with a rhodamine-conjugated secondary antibody (*red*) and Hoechst dye (*blue*) to identify the cell nucleus. The cells were then examined with a fluorescence microscope. Data are representative of three independent experiments. **b** MCF7 cells cotreated with AF (1 μM) and αNF (10 or 100 μM). Data are representative of two independent experiments.

In vivo complex of enzyme (ICE) bioassay

DNA-topoisomerase complex formation was measured using the ICE assay [11]. Briefly, MCF7 cells were plated at 3×10^6 cells/100-mm plate and allowed to grow for 48 h. The cells were then exposed to AF (50 nM, 100 nM, and 1 μM) for 6 h. The negative control cells were treated with 0.1% (v/v) DMSO for 6 h. The posi-

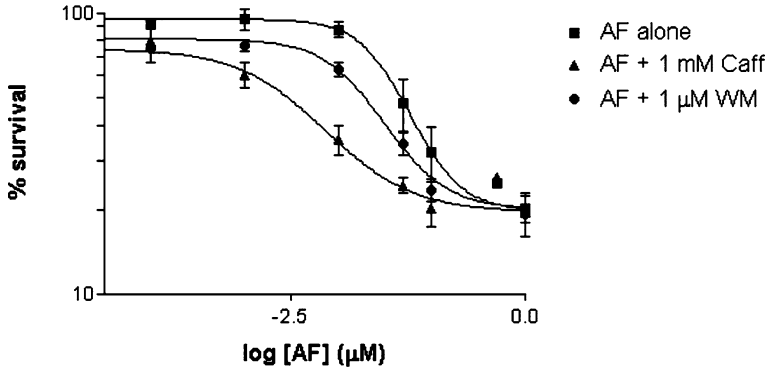

Fig. 2 Cotreatment with caffeine or wortmannin decreases the IC_{50} in MCF7 cells. Cells were plated in 96-well plates and treated with AF (concentration range 0.001–25 μM) in the absence or presence of caffeine (1 mM) or wortmannin (1 μM). Growth inhibition was measured with the MTS assay. Survival is determined by comparison with untreated controls. Data represent the mean ± -SEM for three independent experiments (two experiments for wortmannin data). The curves shown are best fit based on a nonlinear regression sigmoidal dose-response equation performed by GraphPad Prism software

tive control cells were treated with etoposide (100 μM) or camptothecin (10 μM) for 30 min in serum-free medium. After drug treatments, the cells were lysed with a 1% sarkosyl solution in TE buffer (1 M Tris–HCl, 0.1 M EDTA, pH 8.0). The lysates were applied to the top of a CsCl "cushion" (1.50 g/cc). The gradients were centrifuged at 90,000 rpm for 5 h at 25°C. The supernatant was discarded and the DNA pellet was washed with 100% EtOH and resuspended in TE buffer. An aliquot of each fraction was diluted and analyzed spectrophotometrically to determine DNA content. Another aliquot of each fraction was diluted and applied to a nitrocellulose membrane using a slot blot device. The membrane was probed with both Topo I and Topo II antibodies as described previously [9]. Signals were detected using a chemiluminescent system (Pierce West Pico).

MTS assay

Cells were plated at 2,000 cells/well in 96-well plates in RPMI medium with 5% FCS (100 μl/well). After a 24-h incubation, the cells were treated with AF in medium (0.001–25 μM final concentration, 50 μl/well) and either caffeine (1 mM final concentration, 50 μl/well), wortmannin (1 μM final concentration, 50 μl/well) or DMSO (0.25% (v/v), 50 μl/well). Due to the instability of wortmannin in aqueous solutions, the wortmannin solution was not prepared until immediately prior to treatment. After 72 h, MTS solution (2 mg/ml) with PMS (50 μg/ml) was added to each well (40 μl per well). Following a 2-h incubation, the plates were read in a microtiter plate reader at 490 nm. Data were analyzed, including curve fits and IC_{50} determinations, with GraphPad Prism software (San Diego, CA, USA).

Results

Treatment with AF causes H2AX phosphorylation

H2AX is an H2A variant that is phosphorylated in response to DNA double-strand breaks and other replication stresses [16, 24]. H2AX phosphorylation was assessed in resistant and sensitive cell lines treated with AF. H2AX phosphorylation was observed in sensitive MCF7 breast cancer cells (Fig. 1a) after AF treatment at a pharmacologically relevant concentration (50 nM). H2AX phosphorylation was also seen in the OVCAR5 ovarian cancer and the Caki-1 renal carcinoma cell lines (data not shown). There was no evidence of H2AX phosphorylation in the resistant M14 melanoma cell line (IC_{50} > 37 μM) in response to AF treatment (Fig. 1a) even at concentrations as high as 1 μM. There was no evidence of H2AX phosphorylation in two additional resistant cell lines, MDA-MB-435 breast and HT29 colon cell lines (data not shown). To verify that the H2AX phosphorylation was caused by a reactive metabolite of AF, MCF7 cells were treated with AF and the CYP1A inhibitor, α-naphthoflavone (α-NF). H2AX phosphorylation was decreased in the presence of α-NF (Fig. 1b).

Caffeine and wortmannin sensitize MCF7 cells

H2AX is phosphorylated as a part of the DNA damage response. To further examine the role these pathways play, cells were treated with AF in the absence or presence of caffeine and wortmannin, which are inhibitors of the PIKK kinases ATM and ATR [17, 18]. These proteins are involved in DNA damage responses by activation of downstream proteins in the cell cycle checkpoint signaling pathways [1]. Co-treatment of MCF7 cells with AF and 1-mM caffeine lowered the AF IC_{50} from 36.8 to 3.6 nM (Fig 2). Co-treatment with 1 μM wortmannin decreased the IC_{50} value to 18.9 nM. The concentrations used for caffeine and wortmannin were at or near the reported IC_{50} values for ATM and ATR inhibition [17, 18], but not high enough to kill a significant number of cells alone (25% for caffeine and 10% for wortmannin, data not shown).

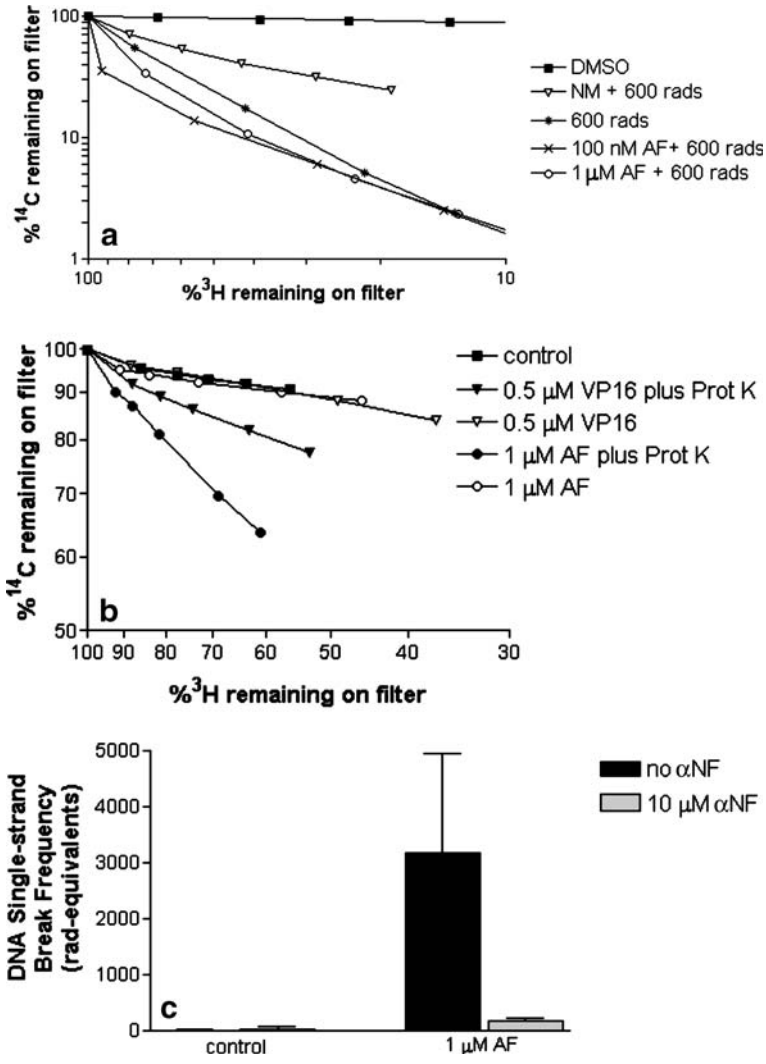

Fig. 3 Alkaline elution in MCF7 cells. **a** ^{14}C-thymidine-labeled MCF7 cells were incubated with AF (100 nM or 1 μM) for 12 h. The cells were then irradiated on ice with 6 Gy. Control cells were treated with 0.1% (v/v) DMSO only. Cells treated with nitrogen mustard (NM, 0.6 μM, 30 min exposure) followed by 6 Gy IR were used as a positive control. The cells were placed on polycarbonate filters (0.2 micron pore size) and lysed in 2% SDS. All cell lysates were treated with 0.5 mg/ml proteinase K before elution. ^{3}H-thymidine-labeled L1210 cells (irradiated on ice with 3 Gy) were used as internal standards for each elution column. Data are representative of two independent experiments. **b** Alkaline elution results of ^{14}C-thymidine-labeled MCF7 cells treated for 12 h with 1 μM AF. Some cell lysates were treated with proteinase K before elution as indicated. Etoposide (VP16) was used as a positive control for protein-associated single-strand breaks. Control cells were treated with 0.1% (v/v) DMSO only. ^{3}H-thymidine-labeled L1210 cells were used as internal standards for each elution column. These cells were irradiated on ice with 4 Gy just before applying the cells to the filters. Data are representative of three independent experiments. **c**. Alkaline elution results of ^{14}C-thymidine-labeled MCF7 cells treated for 12 h with 1 μM AF and α-naphthoflavone (10 μM). All cell lysates were treated with proteinase K before elution. Rad equivalents are calculated based on comparison of internal standard results and experimental results. Data represent the mean ± SEM for two independent experiments

AF causes protein-associated DNA single-strand breaks, but not DNA–DNA cross-links

Alkaline elution was performed to assess the biochemical features of DNA damage in cells treated with AF, including DNA–DNA cross-links and single-strand breaks. There was no evidence for DNA–DNA cross-links in MCF7 cells even after treatment with high concentrations of AF (Fig. 3a). In contrast, there was evidence of single-strand DNA breaks in MCF7 cells treated with AF, but only when cell lysates were treated with proteinase K prior to alkaline elution (Fig. 3b). Co-treatment with the CYP1A inhibitor α-NF decreased the observed DNA damage (more than 90%, Fig. 3c), consistent with the hypothesis that DNA damage is caused by a CYP1A-generated reactive metabolite of AF.

AF does not stabilize topoisomerase-DNA covalent complexes

Protein-associated DNA single-strand breaks, like those detected in Fig. 3b, c, are most commonly induced by

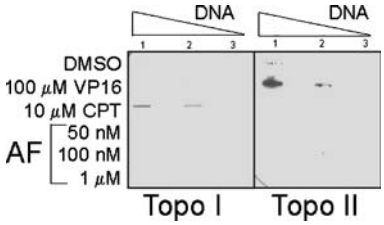

Fig. 4 In vivo complex of enzyme assay. MCF7 cells were treated with AF (50 nM, 100 nM or 1 μM) for 6 h. Negative control cells were treated with 0.1% (v/v) DMSO for 6 h, while positive control cells were treated with VP16 (100 μM) or CPT (10 μM) for 30 min in serum-free media. After drug treatments, cells were lysed and the cellular DNA was isolated using a CsCl "cushion". The DNA pellet was washed, resuspended, and 10 μg (column 1), 5 μg (column 2) or 1 μg (column 3) of DNA was applied to a nitrocellulose membrane using a slot blot device. The membrane was probed for Topo I and II. Signals were detected using the Pierce West Pico chemiluminescence system. Data are representative of two independent experiments.

topoisomerase poisons [15]. Topoisomerase poisons inhibit topoisomerase enzymes after cleavage of the phosphodiester bond, prior to religation, while the enzyme is covalently bound to DNA [10], thus leading to stabilization of DNA-topoisomerase complexes and protein-associated DNA single-strand breaks [12, 23]. These complexes "mask" the single-strand breaks as measured by alkaline elution unless samples are exposed to proteinase K to lyse the protein.

Formation of DNA-topoisomerase complexes was measured with the ICE assay [11]. While there was evidence of DNA-topoisomerase complex formation in cells treated with classic topoisomerase poisons (camptothecin for topoisomerase I and etoposide for topoisomerase II), we could not detect topoisomerase proteins bound to the DNA from AF-treated cells (Fig. 4).

Discussion

Aminoflavone has a unique cytotoxicity pattern in the NCI 60 cell line screen, consistent with a unique mechanism of action. Previous work in our laboratory [9] suggests that the unique mechanism involves metabolic activation by CYP1A1 to form reactive species capable of binding to cellular DNA in sensitive, but not resistant cells. Stabilization of p53, induction of p21 expression and DNA adduct formation [9] provided previous evidence of DNA damage in sensitive cell lines.

In the present studies, DNA damage was further documented by the observation of AF-induced H2AX phosphorylation in sensitive cells. H2AX is phosphorylated in response to double-strand DNA damage and other replication stresses [16, 24]. While this manuscript was in preparation, Meng et al. reported in abstract form (Proc Amer Assoc Cancer Res 2005, 46:599) that H2AX is phosphorylated in AF-treated cells, confirming the results shown here.

Further support for the role of DNA damage in the cytotoxicity of AF comes from the observation that AF cytotoxicity was increased when AF was combined with the ATM and ATR inhibitors, caffeine or wortmannin. ATM and ATR are activated in response to DNA damage and, subsequently, activate downstream proteins, including Chk1, Chk2, and p53 [1]. ATR plays a role in the response to DNA-damaging agents such as UV irradiation and hydroxyurea, while ATM is activated in response to ionizing radiation and other agents that induce DNA double-strand breaks [1]. Inhibition of the ATM and ATR kinases by either caffeine or wortmannin has been shown to sensitize tumor cells to ionizing radiation [17, 18].

The nature of the AF-induced DNA damage was assessed by alkaline elution assays. Results of these studies did not provide evidence of DNA–DNA cross-links, but rather protein-associated DNA single-strand breaks. Protein-associated DNA single-strand breaks are commonly associated with topoisomerase poisons [15]. However, the sensitivity profile of the NCI cell line panel to AF does not correlated with the profile of topoisomerase inhibitors. This lack of correlation could be due to the fact that, based on our data, sensitivity to AF requires induction of CYP1A1 and metabolism of AF by this enzyme in tumor cell lines. AF metabolites could be topoisomerase poisons. It has been shown, for example, that polycyclic aromatic hydrocarbons are capable of trapping topoisomerase complexes on DNA following their metabolic activation [7, 14]. However, there was no evidence for AF-induced trapping of DNA-topoisomerase complexes, even at high concentrations of AF. Further, when we compared the sensitivity pattern of the 16 cell lines sensitive to AF (Fig. 5) to those of the topoisomerase inhibitors etoposide (VP16) and camptothecin (CPT), there was no correlation, indicating that it is not likely that AF works via topoisomerase inhibition by an active metabolite.

Ecteinascidin 743 (Et743), a novel chemotherapeutic agent, has also been shown to cause protein-associated DNA single-strand breaks by alkaline elution [21]. Et743 has a unique activity pattern on the NCI 60 cell line screen, although there was some correlation with other DNA binders, such as actinomycin D. Et743 binds to DNA in the minor groove and bends the DNA toward the major groove. While Et743 inhibited topoisomerase I at high concentrations, this interaction does not appear to play a role in the DNA damage and cytotoxicity of Et743 [21]. The activity of Et743 has been linked to the transcription-coupled nucleotide-excision repair system (TC-NER) [22]. It was shown that cells lacking proteins in the TC-NER pathway were more resistant to Et743. It has been proposed that Et743 poisons the TC-NER machinery, trapping one or more of the NER proteins in DNA-protein complexes and not allowing religation of damage [4]. There has been no direct evidence showing which or how many of the NER proteins are involved in the DNA-protein complexes. Studies to determine the identity of the protein involved in the DNA-protein

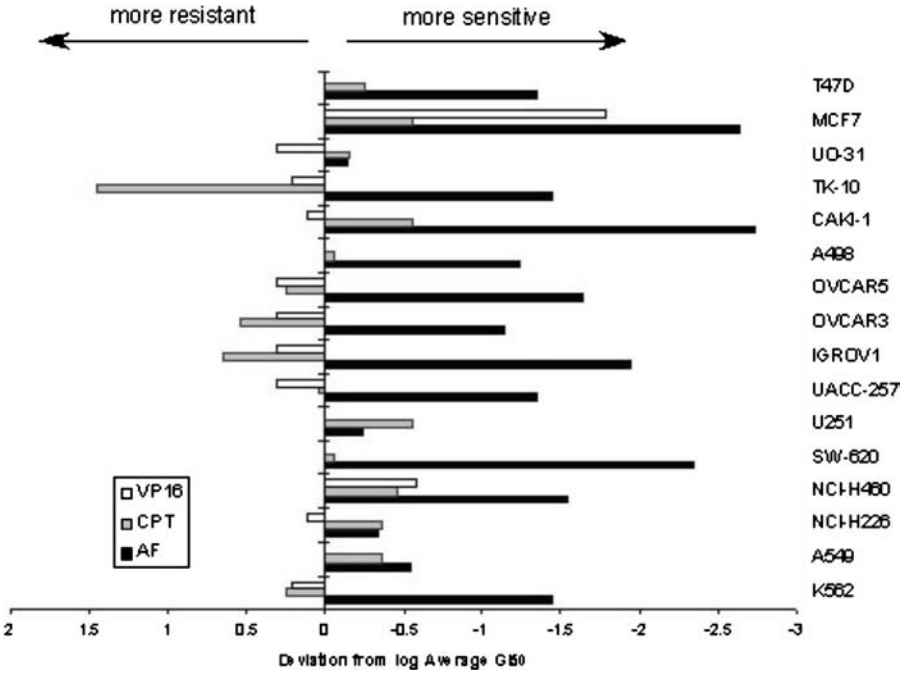

Fig. 5 Subset of NCI screen data comparing the pattern of cytotoxity for etoposide (*VP16*) and camptothecin (*CPT*) in the 16 cell lines that are sensitive to AF. These data are available at http://www.dtp.nci.nih.gov

complexes (including those in the TC-NER pathway) in AF-treated cells are underway.

Acknowledgements The authors wish to thank Dr. Junjie Chen for generously providing the γ-H2AX antibody. This work was funded by a Mary Kay Ash Foundation Grant (MMA) and the Mayo Comprehensive Cancer Center (CA15083).

References

1. Abraham RT (2001) Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev 15(17):2177–2196
2. Akama T, Shida Y, Sugaya T, Ishida H, Gomi K, Kasai M (1996) Novel 5-aminoflavone derivatives as specific antitumor agents in breast cancer. J Med Chem 39:3461–3469
3. Alley MC, Stinson SF, Pacula-Cox CM, Camalier RF, Phillips LR, Daw TW, Sausville EA (1999) Pharmacologic evaluations of a novel amino-substituted flavone (NSC 686288) exhibited unique in vitro and in vivo anticancer activities. Proc Am Assoc Cancer Res 40:119
4. Aune GJ, Furuta T, Pommier Y (2002) Ecteinascidin 743: a novel anticancer drug with a unique mechanism of action. Anticancer Drugs 13(6):545–55
5. Cushman M, Zhu H, Geahlen RL, Kraker AJ (1994) Synthesis and biochemical evaluation of a series of aminoflavones as potential inhibitors of protein-tyrosine kinases p561cd, EGF4, and p60v-src. J Med Chem 37:3353–3362
6. Ferriola PC, Cody V, Middleton E Jr (1989) Protein kinase C inhibition by plant flavonoids: kinetic mechanisms and structure-activity relationships. Biochem Pharmacol 38:1617–1624
7. Khan QA, Kohlhagen G, Marshall R, Austin CA, Kalena GP, Kroth H, Sayer JM, Jerina DM, Pommier Y (2003) Position-specific trapping of topoisomerase II by benzo[a]pyrene diol epoxide adducts: implications for interactions with intercalating anticancer agents. Proc Natl Acad Sci USA 100(21):12498–503
8. Kohn KW, Ewig RA, Erickson L, Zweeling L (1981) Measurement of strand breaks and crosslinks in DNA by alkaline elution. In: Friedberg EC, Hanawalt PC (eds) DNA Repair: a laboratory manual of research techniques. 379 p
9. Kuffel MJ, Schroeder JC, Pobst LJ, Naylor S, Reid JM, Kaufmann SH, Ames MM (2002) Activation of the antitumor agent aminoflavone (NSC 686288) is mediated by induction of tumor cell cytochrome P450 1A1/1A2. Mol Pharmacol 62(1):143–153
10. Liu LF, Rowe TC, Yang L, Tewey KM, Chen GL (1983) Cleavage of DNA by mammalian DNA topoisomerase II. J Biol Chem 258(24):15365–15370
11. Mao Y, Okada S, Chang LS, Muller MT (2000) p53 dependence of topoisomerase I recruitment in vivo. Cancer Res 60(16):4538–4543
12. Nelson EM, Tewey KM, Liu LF (1984) Mechanism of antitumor drug action: poisoning of mammalian DNA topoisomerase II on DNA by 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide. Proc Natl Acad Sci USA 81(5):1361–1365
13. Nielsen SE, Breinholt V, Justensen U, Cornett C, Dragsted LO (1998) In vitro biotransformation of flavonoids by rat liver microsomes. Xenobiotica 28(4):389–401
14. Pommier Y, Kohlhagen G, Laco GS, Kroth H, Sayer JM, Jerina DM (2002) Different effects on human topoisomerase I by minor groove and intercalated deoxyguanosine adducts derived from two polycyclic aromatic hydrocarbon diol epoxides at or near a normal cleavage site. J Biol Chem 277(16):13666–13672
15. Pommier Y, Schwartz RE, Zwelling LA, Kerrigan D, Mattern MR, Charcosset JY, Jacquemin-Sablon A, Kohn KW (1986) Reduced formation of protein-associated DNA strand breaks in Chinese hamster cells resistant to topoisomerase II inhibitors. Cancer Res 46(2):611–616
16. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM (1998) DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J Biol Chem 273(10):5858–5868
17. Sarkaria JN, Busby EC, Tibbetts RS, Roos P, Taya Y, Karnitz LM, Abraham RT (1999) Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. Cancer Res 59(17):4375–4382
18. Sarkaria JN, Tibbetts RS, Busby EC, Kennedy AP, Hill DE, Abraham RT (1998) Inhibition of phosphoinositide 3-kinase related kinases by the radiosensitizing agent wortmannin. Cancer Res 58(19):4375–4382
19. Shi Q, Chem K, Morris-Natschke SL, Lee KH (1998) Recent progress in the development of tubulin inhibitors as antimitotic antitumor agents. Curr Pharm Des 4:219–248

20. Szmigiero L, Erickson LC, Ewig RA, Kohn KW (1984) DNA strand scission and cross-linking by diaziridinylbenzoquinone (diaziquone) in human cells and relation to cell killing. Cancer Res 44(10):4447–4452
21. Takebayashi Y, Goldwasser F, Urasaki Y, Kohlhagen G, Pommier Y (2001) Ecteinascidin 743 induces protein-linked DNA breaks in human colon carcinoma HCT116 cells and is cytotoxic independently of topoisomerase I expression. Clin Cancer Res 7(1):185–191
22. Takebayashi Y, Pourquier P, Zimonjic DB, Nakayama K, Emmert S, Ueda T, Urasaki Y, Kanzaki A, Akiyama SI, Popescu N, Kraemer KH, Pommier Y (2001) Antiproliferative activity of ecteinascidin 743 is dependent upon transcription-coupled nucleotide-excision repair. Nat Med 7(8):961–966
23. Trask DK, Muller MT (1988) Stabilization of type I topoisomerase-DNA covalent complexes by actinomycin D. Proc Natl Acad Sci USA 85(5):1417–1421
24. Ward IM, Chen J (2001) Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. J Biol Chem 276(51):47759–47762
25. Weinstein JN, Myers TG, O'Connor PM, Friend SH, Fornace AJ Jr, Kohn KW, Fojo T, Bates SE, Rubinstein LV, Anderson NL, Buolamwini JK, van Osdol WW, Monks AP, Scudiero DA, Sausville EA, Zaharevitz DW, Bunow B, Viswanadhan VN, Johnson GS, Wittes RE, Paull KD (1997) An information-intensive approach to the molecular pharmacology of cancer. Science 275(5298):343–349
26. Yamashita Y, Kawada S, Nakano H (1990) Induction of mammalian topoisomerase II dependent DNA cleavage by nonintercalative flavonoids, genistein and orobol. Biochem Pharmacol 39:737–744
27. Zhai S, Dai R, Friedman FK, Vestal RE (1998) Comparative inhibition of human cytochromes P450 1A1 and 1A2 by flavonoids. Drug Metab Dispos 26(10):989–992