Preclinical Development

The Clinically Active PARP Inhibitor AG014699 Ameliorates Cardiotoxicity but Does Not Enhance the Efficacy of Doxorubicin, despite Improving Tumor Perfusion and Radiation Response in Mice

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Abstract

AG014699 was the first inhibitor of the DNA repair enzyme PARP-1 to enter clinical trial in cancer patients. In addition to enhancing the cytotoxic effect of DNA-damaging chemotherapies, we have previously shown that AG014699 is vasoactive, thereby having the potential to improve drug biodistribution. The effectiveness of the clinical agent doxorubicin is confounded both by poor tumor penetration and cardiotoxicity elicited via PARP hyperactivation. In this study, we analyzed the impact of AG014699 on doxorubicin tolerance and response in breast (MDA-MB-231) and colorectal (SW620, LoVo) tumor models in vitro and in vivo. As anticipated, AG014699 did not potentiate the response to doxorubicin in vitro. In vivo, AG014699 did not influence the pharmacokinetics of doxorubicin; however, it did ameliorate cardiotoxicity. Both toxicity and extent of amelioration were more pronounced in male than in female mice. AG014699 improved vessel perfusion in both MDA-MB-231 and SW620 tumors; however, this neither led to improved tumor-accumulation of doxorubicin nor enhanced therapeutic response. In contrast, when combined with radiotherapy, AG014699 significantly enhanced response both in vitro and in vivo. Real-time assessment of tumor vessel function and companion histologic studies indicate that doxorubicin causes a profound antivascular effect that counters the positive effect of AG014699 on perfusion. These data indicate that although AG014699 can enhance response to some chemotherapeutic drugs via improved delivery, this does not apply to doxorubicin. PARP inhibitors may still be of use to counter doxorubicin toxicity, and if the gender effect translates from rodents to humans, this would have greater effect in males. Mol Cancer Ther; 10(12); 2320–9. ©2011 AACR.

Introduction

The abundant nuclear enzyme PARP-1 is activated by DNA breaks and facilitates their repair by loosening chromatin and recruiting repair proteins (1). PARP-1 and its structural homologues, PARP-2 and PARP-3, are the only members of a superfamily of PARP enzymes that actively participate in DNA repair (1, 2). Initial studies with early PARP inhibitors, nicotinamide and 3-amino-benzamide, indicated that they would be useful as anticancer chemosensitizers (3). Subsequent studies showed that PARP inhibitors increase the persistence of DNA damage and cytotoxicity induced by DNA-methylating agents, topoisomerase-I poisons, and ionizing radiation (4). These observations have been verified using genetic depletion of PARP-1. Potent PARP inhibitors have been developed for testing in clinical trial in cancer patients, the first being AG014699 in 2003 (5). Other companies rapidly followed suit and 9 have PARPi in clinical development (ref. 6; www.clinicaltrials.gov). BSI-201 impressively enhanced gemcitabine þ carboplatin activity in triple-negative breast cancer patients without increasing toxicity (7). There is scant and/or contradictory evidence of the activity of PARPi in combination with these agents in cell-based studies (8–12), but there is evidence of anticancer activity in xenografts (9–12). Similarly, we observed that the PARPi, AG14361, enhanced the anticancer activity of temozolomide against SW620

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xenografts but did not increase its cytotoxicity against SW620 cells (13). Taken together with the observation that 3-aminobenzamide and nicotinamide increased cisplatin antitumor activity in mice through hemodynamic effects (14), these data indicated that PARPi may improve drug delivery to tumors by altering blood flow. Further investigations revealed that both AG14361 and AG014699 caused arterial relaxation ex vivo and vasodilatation in tumor xenografts (15). Thus, the exciting clinical data with BSI-201 could be attributable to improved drug delivery rather than chemosensitization. Fluctuations in tumor blood flow have been observed in patients (16) and affect microregional oxygenation and drug delivery (17). Furthermore, tumor blood flow can correlate with surgery, chemotherapy, and radiotherapy outcomes (18). PARPi may, therefore, improve the activity of all chemotherapeutic drugs limited by poor drug delivery.

Doxorubicin is widely used in the treatment of breast and other cancers (http://www.cancerhelp.cancerresearchuk.org/about-cancer/treatment/cancer-drugs/doxorubicin), but it exhibits poor tissue penetration, resulting in pronounced gradients of doxorubicin autofluorescence with distance from blood microvessels in breast cancer biopsies (19). There is some evidence of enhancement of doxorubicin activity by PARP inhibition in vivo (20, 21) but not in vitro (22, 23), indicating that the mechanism could be improved drug delivery. Doxorubicin treatment is limited by dose-limiting cardiotoxicity, in which oxidative damage-induced hyperactivation of PARP has been implicated and PARPi have a protective effect (24–26). The aim of this study was to determine whether the clinically active PARPi AG014699 improved the therapeutic index of doxorubicin by both increasing tumor drug delivery and reducing cardiotoxicity. Using a human breast cancer xenograft model (MDA-MB-231), relevant to doxorubicin therapy, and a colon cancer model (SW620), in which we had previously observed AG014699-induced hemodynamic effects, we determined the following: (i) the effect of AG014699 alone and in combination with doxorubicin on tumor blood flow; (ii) the resultant effect on doxorubicin antitumor activity; and (iii) doxorubicin-induced cardiotoxicity in male and female mice.

Materials and Methods

Reagents

Chemicals and reagents were from Sigma unless otherwise stated. For in vivo evaluation, we prepared all agents immediately before administration. Doxorubicin was dissolved in sterile water and AG014699 (Fig. 1A) in sterile saline.

Cell lines and culture

MDA-MB-231, SW620, and LoVo cells (American Type Culture Collection; authenticated by LGC Standards) were maintained in RPMI1640 containing 10% (v/v) fetal calf serum and 2 mmol/L glutamine. Cells were verified as being Mycoplasma-free (MycoAlert; Cambrex Bioscience).

In vitro chemo and radiosensitization

Chemosensitization. MDA-MB-231 cells were seeded into replicate wells in 6-well plates. Doxorubicin was added at a range of concentrations in the presence or absence of 0.4 μmol/L AG014699 for 3 hours. Cells
were seeded in drug-free medium for colony formation.

**Radiosestization.** Exponentially growing MDA-MB-231 cells were cultured in medium containing 0.4 \( \mu \text{mol/L} \) AG014699 or control medium for 1 hour before irradiation (IR; 2.9 Gy/min; Gulmay Medical Ltd.) and for a further 24 hours before seeding for colony formation in drug-free medium. Potentially lethal damage repair (PLDR) was measured in confluent G1-arrested MDA-MB-231 cells, exposed to 6 Gy \( \gamma \)-irradiation and seeded for colony formation immediately or after a 24-hour recovery period. Where indicated, AG014699 (0.4 \( \mu \text{mol/L} \)) was added 30 minutes before irradiation and was present in the recovery incubation.

**Analysis.** Colonies were stained with crystal violet after 10 to 14 days and counted with an automated colony counter (ColCount; Oxford Optronics Ltd.). Percentage cell survival was calculated by comparison with untreated cells incubated in the presence or absence of AG014699, as appropriate.

**\( \gamma \)H2AX assay**

Cells were seeded on to sterile coverslips in 6-well plates and exposed to doxorubicin (1 \( \mu \text{mol/L} \) for 3 hours) or 6 Gy radiation with or without AG014699 (0.4 \( \mu \text{mol/L} \)) for 3 hours. Cells were washed twice, replenished with fresh medium, and duplicate samples harvested at various times thereafter. Cells were formalin-fixed, washed 3 times with PBS, and blocked using 1% bovine serum albumin (BSA) in PBS for 30 minutes at room temperature. Cells were permeabilized by adding 7% Triton X-100 in PBS for 7 minutes, and \( \gamma \)H2AX foci were revealed using mouse anti-phospho-Histone H2AX (Ser-139; 1:500 in 1% BSA/PBS) followed by anti-mouse Alexa Fluor-488 (1:1,000 in 1% BSA/PBS). 4',6'-Diamidino-2-phenylindole (1:2,500 in PBS) was used to reveal nuclei. Samples were mounted (DAKO) and stored in the dark at 4°C until analysis.

**In vivo studies**

All in vivo studies were approved by the Home Office Inspectorate, local ethics committees, and conducted under PPL40/3212 (Manchester) and PPL60/3554 (Newcastle) according to UK–CCCR Guidelines (27) and in compliance of The Scientific Procedures Act 1986. Animals were bred in-house and maintained using the highest possible standard of care, and priority was given to their welfare.

**In vivo pharmacokinetics and toxicity**

**Pharmacokinetic studies.** Doxorubicin pharmacokinetics were determined following i.p. injection of doxorubicin-HCl, alone or in combination with AG014699 (10 mg/kg administered i.p. 30 minutes before doxorubicin) in 3 studies. Initially, plasma pharmacokinetics of 10 mg/kg doxorubicin was determined in BALB/c mice over a 3-hour time-course. Plasma and tumor pharmacokinetics were assessed using a limited sampling schedule over a 3-hour time-course in CD-1 nude mice using 2 models: LoVo (colorectal cancer) and MDA-MB-231. Mice bearing the LoVo xenografts were given 10 mg/kg doxorubicin but, because tumor levels were found to be low, mice bearing the MDA-MB-231 xenografts received 20 mg/kg. In this latter study, plasma and tumor concentrations of AG014699 were also determined.

In all studies, mice were bled by cardiac puncture under terminal anesthesia at various times posttreatment (3 mice/time point). Where appropriate, tumors were removed, snap-frozen in liquid nitrogen, and homogenized in 3 volumes of PBS. Plasma and tumor concentrations of doxorubicin and, where stated, AG014699 were determined by reference to standards prepared in mouse plasma using high-performance liquid chromatography (HPLC; Waters) with fluorescence detection (\( \lambda_{ex} \) 475/\( \lambda_{em} \) 555 nm). Briefly, samples and standards were spiked with 5 \( \mu \text{L} \) daunomycin internal standard, acetonitrile precipitated, and 50 \( \mu \text{L} \) supernatant was resolved on a Supelcosil LC-CN 5 \( \mu \text{m} \) column (Sigma–Aldrich) with a mobile phase of 0.02 mol/L phosphate buffer at pH 3.5 in acetonitrile (5:3 w/v). Pharmacokinetic parameters were calculated using a noncompartamental model with terminal elimination rate estimated by log-linear regression.

**Toxicity studies.** Chronic toxicity of doxorubicin. Male and female CD-1 mice were administered doxorubicin (5 mg/kg \( \pm \) AG014699 once weekly for 3 weeks (\( n = 5 \) per group). Body weights and physical condition were monitored. Toxicity was expressed as average body weights as a percentage of the starting body weights.

**Acute toxicity of high-dose doxorubicin.** The acute toxicity of high-dose doxorubicin was assessed in male CD-1 mice treated with 20 mg/kg doxorubicin i.p. Four days after administration, there was unexpectedly high toxicity (~15% loss of body weight, failure to groom, hypokinesia) and the study was terminated on grounds of animal welfare. Mice were bled from the tail vein and the level of creatine kinase was assessed as a measure of muscle damage.

**Troponin I and creatine kinase assays**

Troponin I (Mouse Cardiac Tn-I 96-well ELISA; Life Diagnostics, Inc.) and creatine kinase (QuantChrom Creatine Kinase Assay Kit; Universal Biologicals) were assessed using commercially available ELISA kits. Blood was collected from the tail vein of anesthetized mice (to avoid mechanical damage to the heart) and the plasma was immediately removed by centrifugation and stored frozen at –80°C. Assays were conducted according to the manufacturer’s instructions.

**In vivo efficacy studies**

MDA-MB-231 (3 \( \times 10^7 \)) or SW620 (1 \( \times 10^7 \)) cells were implanted into adult athymic nude mice maintained and handled in isolators under specific pathogen-free conditions. Mice bearing tumors that were 200 to 250 mm\(^2\) in volume were randomized into treatment groups (\( n = 5 \) per.
group). Doxorubicin was administered once weekly (5 mg/kg, i.p.) for 3 weeks or daily for 5 days (2 mg/kg, i.p.) with or without additional AG014699 (10 mg/kg, i.p.), once daily for 5 days. Tumor-localized radiotherapy (X-ray) was administered as 5 daily 2-Gy fractions as described previously (28). AG014699 (10 mg/kg, i.p.) was administered 30 minutes before each fraction/doxorubicin dose. Tumor volumes and mouse weights were monitored at least 3 times weekly. The experiments were terminated before the tumor volume exceeded 1,000 mm³.

**Dorsal window chamber studies**

Dorsal window chamber surgery has been described previously (28). Dorsal window chambers were implanted with MDA-MB-231 or SW620 cells (50 μL of a 1 × 10⁶/mL stock). Once tumors established, mice were anesthetized and vascular parameters were assessed by intravitral microscopy. Images were taken using bright-field and fluorescence microscopy (Nikon Eclipse E800) both before and after administration of doxorubicin (10 mg/kg, i.v.) or AG014699 (1–10 mg/kg, i.p.). Real-time evaluation of the vascular effects of AG014699 has been described previously (15). Briefly, BSA labeled with Alexa-Fluoro-647 (BSA-647; lex 647 nm, 1 mg/mL in sterile saline; Molecular Probes; Invitrogen) was administered at a dose of 0.1 mL per mouse i.v. before AG014699 administration, and changes in fluorescence (ex 668 nm) were monitored (Metamorph analysis system).

**Histologic assessment of vasculature and hypoxia**

Mice bearing MDA-MB-231 and SW620 tumors (300–400 mm³) were randomly assigned to receive saline (0.1 mL/10 g), doxorubicin (10 mg/kg, i.v.), AG014699 (10 mg/kg, i.v.), or both. Thirty minutes later, mice received the hypoxic marker pimonidazole (60 mg/kg, i.p.; Chemicon International Inc.) and, after a further 30 minutes, were administrated the perfusion marker Hoechst 33342 (0.1 mL of a 6 mg/kg stock, i.v.). One minute later, tumors were excised and rapidly snap-frozen in liquid nitrogen. Then, 8-μm cryostat sections were scanned using a NIKON Eclipse E800 fluorescent microscope to determine the number of Hoechst-stained (perfused) vessels. Sections were then contained to reveal endothelial structures (rat anti-CD31; BD Biosciences Pharmingen) and regions of hypoxia (pimonidazole binding; Hypoxyprobe antibody; Chemicon International) as described previously (29). Vessel density was analyzed per unit area of the tumor section in addition to hypoxia as the fraction of tumor exhibiting positive staining on observation using a NIKON Eclipse E800 fluorescent microscope (28).

**Results**

**AG014699 does not influence the sensitivity of MDA-MB-231 cells to doxorubicin in vitro but causes sensitization to ionizing radiation in vitro and in vivo**

Initial studies were undertaken to confirm that PARP inhibition via AG014699 had no direct impact on the response of MDA-MB-231 cells to doxorubicin. Cells were treated with increasing concentrations of doxorubicin alone or in combination with AG014699 (0.4 μmol/L). Cytotoxicity was assessed by clonogenic assay. Untreated cells had a clonogenic potential of 0.69 ± 0.05. AG014699 alone had no significant impact on cell survival and had no effect on the dose–response of MDA-MB-231 cells to doxorubicin (Fig. 1B). Consistently, the level of DNA damage, determined by the number of γH2AX foci at various time points after a 24-hour exposure to doxorubicin (1 μmol/L) did not significantly differ between cells treated with doxorubicin alone or with AG014699 (Fig. 1C). In contrast, when MDA-MB-231 cells were treated with AG014699 in combination with radiation, cytotoxicity was potentiated (Fig. 2). In exponentially growing MDA-MB-231 cells, AG014699 significantly (P = 0.0268) enhanced the radiation response, reducing the LD₅₀ of 4.24 ± 0.23 Gy for IR alone to 3.53 ± 0.05 for IR + AG014699 (Fig. 2A). γH2AX foci were higher in cells cotreated with AG014699 and radiation than those treated with radiation alone (Fig. 2B). To evaluate whether AG014699 also inhibited PLDR, confluent MDA-MB-231 cells were exposed to 6 Gy radiation, with or without AG014699, and seeded for colony formation immediately or after a 24-hour recovery period. AG014699 significantly (P = 0.0033) reduced survival compared with radiation treatment alone, implying a strong inhibitory effect against PLDR (Fig. 2B).

**AG014699 had no effect on the pharmacokinetics of doxorubicin in vivo**

We investigated whether AG014699 affected the plasma pharmacokinetics of doxorubicin in female BALB/c mice by administering doxorubicin (10 mg/kg, i.p.) alone or 30 minutes after AG014699 (10 mg/kg, i.p.). Plasma levels of doxorubicin were determined by HPLC. Pharmacokinetic parameters were determined using noncompartamental analyses. The plasma elimination of doxorubicin was rapid and well described by a mono-exponential decay curve, both when administered alone and when given in combination with AG014699. AG014699 did not markedly affect any measured parameters (Fig. 3A and B; Supplementary Table S1). A second limited sampling study was undertaken in CD-1 nude mice. As in the BALB/c model, AG014699 did not influence the plasma pharmacokinetics of doxorubicin. In contrast, doxorubicin did appear to increase the concentration of AG014699 in the plasma, largely as a result of decreased clearance of AG014699 (Fig. 3C; Supplementary Tables S1 and S2).

**Male mice exhibit worse toxicity than females in response to doxorubicin which is ameliorated by AG014699**

Previous studies have indicated that doxorubicin toxicity is worse in male than in female rodents (30, 31). Furthermore, PARP hyperactivation in response to doxorubicin-induced oxidative stress has been implicated as a key mediator of normal tissue damage (24). To assess chronic toxicity, male and female mice were
administered doxorubicin once weekly (5 mg/kg), either alone or in combination with AG014699 (10 mg/kg) for 3 weeks, and toxicity was monitored by assessing body weight. All drug-treated mice lost weight. The greatest loss was in male mice treated with doxorubicin alone (20%). Cotreatment with AG014699 resulted in only 10% weight loss, indicating reduced toxicity. In contrast in female mice, doxorubicin only caused a 10% reduction in body weight, which was unaffected by coadministration of AG014699. The weight of control mice receiving saline or mice treated with AG014699 alone was stable or increased over the same time frame (data not shown). Troponin I levels were monitored in the plasma of the male mice as a direct marker of cardiac damage. Doxorubicin treatment alone increased plasma troponin I (Fig. 4B). However, the addition of AG014699 reduced plasma troponin I levels toward that observed in controls or animals treated with AG014699 alone (Fig. 4B). Acute toxicity was also monitored following high-dose doxorubicin (20 mg/kg) treatment. Body weight was monitored for 4 days posttreatment. Doxorubicin alone resulted in a 13% weight loss. When combined with single (day 1) or multiple (daily x 4) dosing with AG014699 (10 mg/kg), weight loss was 8%, indicating that AG014699 partially ameliorated toxicity (data not shown). Troponin I could not be detected in plasma upon sacrifice at day 4. However, creatine kinase was assessed as a potential marker of (cardiac) muscle damage. Again, consistent with the weight change data, AG014699 modulated the increase in creatine kinase levels compared with that observed with doxorubicin treatment alone (Fig. 4C).

Figure 2. AG014699 enhances the response of MDA-MB-231 cells to radiation treatment in vitro. Clonogenic survival (A) of cells treated with radiation (closed symbols) + AG014699 (open symbols) while in exponential growth phase. The γH2AX foci were analyzed to track DNA damage (B) following radiation alone (6 Gy, gray bars) versus that observed following treatment with radiation + AG014699 (closed bars) or AG014699 alone (open bars). *, P < 0.03 versus radiation. Clonogenic survival of cells irradiated (6 Gy) while growth arrested at confluence and seeded immediately (0 hour) or 24 hours later for colony formation (C).

Figure 3. AG014699 does not affect the plasma pharmacokinetics of doxorubicin. A, doxorubicin, detected by HPLC using fluorescence detection, in plasma from BALB/c mice treated with a single i.p. dose of doxorubicin (10 mg/kg) either alone (solid symbols) or 30 minutes after a single i.p. dose of AG014699 (10 mg/kg; open symbols). B, limited sampling study in CD-1 nude mice in which AG014699 (white bars indicate 20 mg/kg doxorubicin + 10 mg/kg AG014699) had no effect on doxorubicin (black bars) plasma pharmacokinetics. C, plasma AG014699 after administration alone (10 mg/kg; black bars) or with doxorubicin (20 mg/kg; white bars).
AG014699 increases the IR response but not the response of MDA-MB-231 or SW620 xenografts to doxorubicin treatment in vivo, despite improving tumor perfusion

To confirm that MDA-MB-231 in vivo radiosensitization translated to in vitro radiopotentiation, MDA-MB-231 tumors were established in nude mice and treated with 5 daily fractions of 2 Gy alone or combined with AG014699 (10 mg/kg; 30 minutes before each IR fraction). Control tumors reached 3 times the treatment volume in 13 ± 1 days. AG014699 alone had no effect on tumor regrowth (14 ± 1 days; Fig. 5A). Radiation significantly delayed tumor growth to 33 ± 4 days, which was further delayed by the addition of AG014699 (43 ± 2; P = 0.05 compared with radiotherapy alone; Fig. 5A). Similarly, dephosphorylated-AG014699 significantly enhanced radiation-induced tumor growth delay in LoVo xenografts (tumor growth delay with radiation alone, 32 ± 1 day; radiation + AG14447, 41 ± 2 days; P = 0.002; Supplementary Fig. S1).

We have previously shown that AG014699 is vasoactive in colorectal xenografts (15). We hypothesized that an AG014699-mediated improvement in vascular perfusion would enhance the efficacy of doxorubicin in vivo. Female cba nude mice bearing MDA-MB-231 tumor xenografts were treated with doxorubicin (5 mg/kg) once weekly alone or with AG014699 (10 mg/kg daily, ×5). Tumor growth delay (4× treatment volume) was 17 ± 4 days in doxorubicin-treated mice compared with 14 ± 3 days in controls. AG014699 neither had any effect on tumor growth alone, nor did it enhance the growth delay observed when combined with doxorubicin (Fig. 5B). The weight loss observed over the duration of the experiment was approximately 5% in doxorubicin- and doxorubicin/AG014699-treated mice. The inability of AG014699 to enhance the effect of doxorubicin was not “mouse-strain specific” as we undertook a similar study with female CD-1 nude mice bearing MDA-MB-231 tumors treated weekly with doxorubicin (5 mg/kg) ± AG014699 (10 mg/kg). Doxorubicin caused a modest delay in tumor growth (20 ± 6 days) compared with controls (14 ± 3 days). AG014699 had no significant effect either alone (15 ± 3 days) or in combination with doxorubicin (22 ± 4 days). To ensure that the previously reported vasoactivity of AG014699 was not restricted to colorectal xenografts, MDA-MB-231 tumors were established in dorsal window chambers to allow real-time assessments of vessel function following AG014699 administration. Mice were administered Alexa-Fluoro–conjugated BSA-647 and fluorescence uptake into the tumors was assessed as an indicator of tumor perfusion. AG014699 treatment (10 mg/kg) led to a marked enhancement in tumor fluorescence (Fig. 5C) in the MDA-MB-231 model, as previously observed in colorectal tumors. In contrast, when mice received saline, fluorescence did not rise above the baseline achieved before administration (Fig. 5C). There was a dose-dependency to the fold enhancement in fluorescence observed over the basal plateau that increased from 1.5 with 1 mg/kg AG014699 to 1.7 at 3 mg/kg and 2.2 at 10 mg/kg (data not shown). The lack of correlation between vascular effects and doxorubicin response could still have been model dependent. We, therefore, evaluated doxorubicin efficacy with and without AG014699 treatment in male and female mice bearing SW620 xenografts. In accordance with the MDA-MB-231 data, AG014699 did not potenti ate doxorubicin response either on the weekly or daily ×5 schedule in either gender (Fig. 5D; data not shown).

Doxorubicin causes acute vascular changes that counteract the positive effects of AG014699 on vessel perfusion

Failure of AG014699 to enhance doxorubicin could be based upon several mechanisms. We decided to evaluate
whether doxorubicin itself caused changes in vascular function and whether this could impair the influence of AG014699 on perfusion. MDA-MB-231 xenografts were established in dorsal window chambers. Doxorubicin (10 mg/kg) was administered and vessels were monitored at various time points thereafter under transillumination. Doxorubicin caused an acute vessel shutdown, consistent with a pronounced antivascular effect (Fig. 6A). These acute vascular changes were also apparent in SW620 tumors (Fig. 6A). To complement these real-time observations, a histologic study was undertaken. SW620 tumors were treated with either saline, AG014699 (1 mg/kg), doxorubicin (10 mg/kg), or doxorubicin + AG014699 and excised 60 minutes later. The hypoxia marker pimonidazole and the perfusion marker Hoechst 33342 were administered 30 and 1 minute(s) before tumor excision, respectively. The extent of tumor hypoxia did not vary significantly from that observed in control tumors (13 ± 3% tumor area). The perfused vessel density increased on AG014699 treatment (Fig. 6B; 26 ± 0.4 mm −2, compared with 16 ± 7 in controls). Doxorubicin alone caused a slight reduction in vascular perfusion and, in combination, ablated the enhanced perfusion associated with AG014699 treatment (Fig. 6B). Similarly in MDA-MB-231 tumors, AG014699 increased perfusion from 6 ± 5 mm −2 to 13 ± 7 mm −2, which was impaired on combination with doxorubicin (9 ± 4 mm −2). We determined the effect of AG014699 on the distribution of doxorubicin to tumor tissue in female CD-1 nude mice bearing MDA-MB-231 tumors using a limited sampling schedule (Fig. 6D). AG014699 did not affect the levels of doxorubicin detected within the MDA-MB-231 tumors. However, doxorubicin did increase AG014699 within tumors, consistent with our previous observations that doxorubicin decreases AG014699 clearance resulting in elevated levels within tissue and plasma (Supplementary Table S2). Similar observations were made in mice bearing LoVo colorectal tumors (data not shown).

**Discussion**

Therapeutic inhibition of the DNA repair enzyme PARP-1 has emerged as an exciting field in anticancer therapeutics. We have previously shown that PARPi can...
improve tumor perfusion, and potentially drug delivery, which would explain why PARPi can enhance the in vivo therapeutic tumor response in models where little sensitization is observed in vitro. In this study, we aimed to evaluate the benefit of combining the clinical PARPi, AG014699, with doxorubicin, a chemotherapeutic agent whose anticancer activity is known to be limited by poor tumor distributions and whose dose-limiting toxicity appears dependent on hyperactivation of PARP (24).

We anticipated that PARP inhibition would have little direct effect on doxorubicin cytotoxicity in vitro. Accordingly, neither doxorubicin-induced cytotoxicity nor DNA damage was potentiated by AG014699 in MDA-MB-231 breast cancer cells. These data are consistent with previously published observations using earlier, less potent PARPi (22). In contrast, AG014699 significantly enhanced the in vitro response to radiotherapy, illustrating that a potentiating effect can be observed in the MDA-MB-231 model when an appropriate DNA-damaging agent is used.

We hypothesized that in vivo, combining AG014699 with doxorubicin would be beneficial in 2 ways; first, by ameliorating cardiotoxicity and, second, by allowing greater tumor accumulation of doxorubicin and, therefore, greater efficacy. We first established that AG014699 had no effect on the pharmacokinetics of doxorubicin that could have influenced the outcomes of both studies. Interestingly, although AG014699 did not alter the plasma pharmacokinetics of doxorubicin, doxorubicin altered the clearance of AG014699 resulting in higher plasma concentrations in mice treated with the combination than those treated with AG014699 alone. Potentially, doxorubicin-induced nephrotoxicity (32, 33) impairs renal clearance of AG014699. Cardiotoxicity studies were undertaken using both male and female mice as previous reports in the literature have shown that the detrimental effects of doxorubicin are more prevalent in males (30, 31). In keeping with these findings, chronic dosing with doxorubicin caused greater weight loss in male than in female mice. This was associated with increased plasma concentrations of troponin I, a marker of cardiac muscle damage. Both weight loss and troponin I levels were ameliorated by coadministration of AG014699. Because doxorubicin-induced cardiotoxicity is proposed to be due to PARP hyperactivation, a recent demonstration that PARP activity is higher in male mice and humans than in females (34) is highly pertinent to our observations. Similar findings were observed on acute dosing of doxorubicin using creatine kinase, which appeared to be a more sensitive read-out for toxicity than troponin I over the 4-day time frame used. Previous data have shown that nephrotoxicity occurs before cardiotoxicity in rodents (33) and, similar to cardiotoxicity, involves peroxidation (32, 35) and, presumably therefore, PARP hyperactivation.

Figure 6. Doxorubicin causes acute vascular effects that counteract the improved perfusion observed with AG014699 alone. A, MDA-MB-231 and SW620 tumors were established in a dorsal window chamber, and vessels were observed immediately before or 30 minutes after administration of doxorubicin (10 mg/kg). B, histologic assessment of tumor perfusion after AG014699 and doxorubicin (Dox) treatment determined by assessing the number of Hoechst-stained (perfused) vessels 1 hour after drug treatment in SW620 tumors. Values shown are average ± SD (n = 5 per group). C, representative images of Hoechst distribution. D, AG014699 does not enhance tumor uptake of doxorubicin. MDA-MB-231 tumor-bearing mice were treated with doxorubicin alone (20 mg/kg; closed bars) or doxorubicin + AG014699 (10 mg/kg; open bars). Doxorubicin levels were determined by HPLC with fluorescence detection (n = 3 per time point).
Creatine kinase possibly reflects this nephrotoxicity or more generalized toxicity (32). Importantly, the observation that AG014699 prevents an increase in plasma creatine kinase indicates a protective effect of PARPi against various normal toxicities beyond cardiotoxicity.

Doxorubicin is clinically used in breast cancer; therefore, we analyzed the effect of doxorubicin with and without combined treatment with AG014699 in MDA-MB-231 tumors. Surprisingly, we saw no enhancement of response despite AG014699 alone remaining in markedly improved perfusion in the MDA-MB-231 model observed using real-time intravital microscopy. In our previous studies, such marked vascular responses were sufficient to enhance the therapeutic response of SW620 colorectal xenografts to temozolomide, yielding prolonged tumor regression and cure in 40% of treated animals despite having no sensitizing effect in vitro (36). We analyzed whether the ability of AG014699 to potentiate the response to doxorubicin was model and/or gender dependent by repeating the efficacy studies in both male and female mice bearing SW620 tumors using multiple treatment protocols. Although previous data have indicated that SW620 tumors are unresponsive to doxorubicin (37), in our studies we observed a significant response at the dose used (5 mg/kg weekly). However, as with the MDA-MB-231 studies, no improvement was observed in the SW620 model when doxorubicin was combined with AG014699. This led us to speculate that there was a common mechanism that ablated the potential impact of AG014699 vascular effects on doxorubicin biodistribution in both models.

We concentrated on vascular effects and found that doxorubicin produced a profound, acute impact on tumor vascularization. Real-time imaging suggested acute vessel shutdown that appeared focused on smaller, potentially less mature vessels. Histologic assessment revealed that AG014699 alone increased the number of perfused vessels, but when combined with doxorubicin, the positive effect of AG014699 was completely ablated in SW620 tumors and significantly reduced in MDA-MB-231 xenografts. Consequently, tumor levels of doxorubicin were not improved by AG014699 cotreatment as would have been anticipated with a classic enhancement in delivery. These studies are consistent with previous preclinical studies showing that doxorubicin decreases tumor blood flow (38) and blunts the response of isolated aortas to the endothelium-dependent vasodilator, acetylcholine (39). In contrast, AG014699 did potentiate radiotherapy response that could be attributed both to improved oxygenation and increased cytotoxicity in the presence of a PARP inhibitor.

Taken together, these data indicate that, although AG014699 can enhance perfusion and improve the therapeutic response of some chemotherapeutic regimens through improved tumor delivery, this cannot be extrapolated to all agents. Doxorubicin appears to have a direct effect on tumor vessels that confounds the ability of AG014699 to enhance perfusion. We have previously shown that AG014699 can inhibit the function of myosin light-chain kinase, potentially preventing vasoconstriction (15). Whether the doxorubicin effect is mediated through this same pathway is as yet unknown, but it is certainly rapid and observed within 30 minutes. In the context of doxorubicin, PARP-inhibitors would still be useful in ameliorating dose-limiting toxicity. Recent studies have shown that women have lower PARP activity than men, analogous to the gender differences in mice (30, 31, 34). If the gender influences on cardiotoxicity translate from rodent to humans, PARP inhibitors would have greater usefulness in males.

Disclosure of Potential Conflicts of Interest

N. J. Curtin receives grant support from Pfizer, and both N. J. Curtin and K. J. Williams are named inventors on patents relating to AG014699. No potential conflicts of interest were disclosed by the other authors.

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The Clinically Active PARP Inhibitor AG014699 Ameliorates Cardiotoxicity but Does Not Enhance the Efficacy of Doxorubicin, despite Improving Tumor Perfusion and Radiation Response in Mice


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