

## The Novel Poly(ADP-Ribose) Polymerase Inhibitor, AG14361, Sensitizes Cells to Topoisomerase I Poisons by Increasing the Persistence of DNA Strand Breaks

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**Abstract** Poly(ADP-ribose) polymerase (PARP) inhibitors enhance DNA topoisomerase I (topo I) poison-induced cytotoxicity and antitumor activity *in vitro* and *in vivo*, but the mechanism has not been defined. We investigated the role of PARP-1 in the response to topo I poisons using PARP-1<sup>-/-</sup> and PARP-1<sup>+/+</sup> mouse embryonic fibroblasts and the potent PARP-1 inhibitor, AG14361 ( $K_i < 5$  nmol/L). PARP-1<sup>-/-</sup> mouse embryonic fibroblasts were 3-fold more sensitive to topotecan than PARP-1<sup>+/+</sup> mouse embryonic fibroblasts (GI<sub>50</sub>, 21 and 65 nmol/L, respectively). AG14361 caused a >3-fold sensitization of PARP-1<sup>+/+</sup> cells to topotecan compared with a <1.4-fold sensitization in PARP-1<sup>-/-</sup> cells. In human leukemia K562 cells, AG14361 caused a 2-fold sensitization to camptothecin-induced cytotoxicity. AG14361 did not affect the cellular activity of topo I as determined by measurement of cleavable complexes and topo I relaxation activity, showing that sensitization was not due to topo I activation. In contrast, repair of DNA following camptothecin removal, normally very rapid, was significantly retarded by AG14361, resulting in a 62% inhibition of repair 10 minutes after camptothecin removal. This led to a 20% increase in the net accumulation of camptothecin-induced DNA strand break levels in cells coexposed to AG14361 for 16 hours. We investigated the DNA repair mechanism involved using a panel of DNA repair-deficient Chinese hamster ovary cells. AG14361 significantly potentiated camptothecin-mediated cytotoxicity in all cells, except the base excision repair-deficient EM9 cells. Therefore, the most likely mechanism for the potentiation of topo I poison-mediated cytotoxicity by AG14361 is via PARP-1-dependent base excision repair.

Topoisomerases catalyze the DNA breakage, unwinding, and religation necessary to relieve torsional strain and are the molecular targets of many anticancer agents. Topoisomerase poisons stabilize the topoisomerase-DNA cleavable complex in the nicked conformation, converting these essential enzymes into cellular poisons. DNA strand breaks, and hence cytotoxicity, produced by topoisomerase poisons correlate directly with topoisomerase activity. Topoisomerase I (topo I) is elevated in some tumors (1), and this has increased interest in the use of topo I poisons in the treatment of cancer. Cytotoxicity is thought to result from the collision between the DNA replication fork and the cleavable complex, which produces a protein-associated single-strand break and a non-protein-associated double-strand break (2). Topo I poisons are

an important class of chemotherapeutic agents; however, clinical resistance is common, with mechanisms including a reduction in topo I levels and/or activity and increased DNA repair. Current strategies to overcome resistance to the topo I poisons are under investigation (3).

One strategy for enhancing topo I poison activity is inhibition of the DNA repair protein poly(ADP-ribose) polymerase-1 (PARP-1). PARP-1 is a 113-kDa nuclear protein that is activated in response to DNA strand breaks and forms polymers of ADP-ribose on itself and other nuclear proteins (4, 5). Although the potential of PARP inhibitors has concentrated on combinations with alkylating agents and ionizing radiation (the most potent activators of PARP-1), PARP inhibitors also enhance topo I poison activity *in vitro* and *in vivo* (6–10). The PARP inhibitor, NU1025, was shown to enhance both camptothecin-induced DNA strand breaks and cytotoxicity 2.5-fold in L1210 cells, suggesting that the two events were related (7). The effect was neither cell line nor p53 dependent because NU1025 and another PARP inhibitor, NU1085, enhanced topotecan-induced cytotoxicity up to 5-fold in 11 of the 12 human cancer cell lines screened (11). These cell-based studies were confirmed *in vivo* using highly potent “third-generation” PARP inhibitors, AG14361 and CEP 6800, which enhanced antitumor activity of irinotecan in three human colon cancer xenograft models (8, 9).

The mechanism underlying sensitization of topo I poisons by PARP inhibitors is not defined. One explanation is that PARP-1 is involved in the repair of topo I poison-mediated

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DNA damage via its role in base excision repair (BER). PARP-1 interacts with the other BER proteins, XRCC1 (12, 13), DNA polymerase  $\beta$  (14), and DNA ligase III (15), and BER is significantly reduced in the absence of PARP-1 (14, 16). Cells deficient in BER (due to lack of XRCC1) are hypersensitive to camptothecin (17, 18), suggesting a role for BER in the response to topo I poisons. Additionally, resistance to camptothecin (via overexpression of XRCC1) can be reversed by the PARP inhibitor, 3-AB (19). Recent studies have shown that poly(ADP-ribosylated) PARP-1 blocks the formation of topo I-DNA covalent complexes, inhibiting topo I-DNA cleavage and accelerating the removal of camptothecin-stabilized topo I-DNA complexes (20). PARP-1 inhibition may therefore prevent topo I poison-mediated DNA damage from being efficiently repaired.

A second hypothesis is that PARP-1 modulates topo I activity. PARP-1 poly(ADP-ribosylates) topo I *in vitro* and in cells causing topo I inhibition (21–23). PARP-1 colocalizes with topo I throughout the cell cycle and this seems to enhance topo I activity (24). Activation of PARP-1 by DNA damage, resulting in poly(ADP-ribosylation), may disrupt this association and hence lead to decreased topo I activity. It follows that PARP-1 inhibition may increase topo I activity, thus sensitizing cells to topo I poisons.

The aim of the current study was to investigate the role of PARP-1 in the cellular response to topo I poisons using the potent PARP inhibitor AG14361 ( $K_i < 5$  nmol/L), PARP-1<sup>+/+</sup> and PARP-1<sup>-/-</sup> cells, and DNA repair-defective and human leukemic cells. PARP inhibitors have entered clinical trials (in combination with temozolomide) for the first time (25) and the data presented here may aid the design of future clinical trials of PARP inhibitors with topo I poisons.

## Materials and Methods

**Reagents.** All reagents were obtained from Sigma (Poole, Dorset, United Kingdom) unless otherwise stated. AG14361 was synthesized and provided by Pfizer GRD (La Jolla, CA; ref. 26). Camptothecin was supplied by Sigma, and topotecan was supplied by SmithKline Beecham Pharmaceuticals (Philadelphia PA). Drugs were dissolved in DMSO and stored as stock solutions at  $-20^{\circ}\text{C}$ . Drugs were added to cells so that the final concentration of DMSO was always 1% (v/v). AG14361 was always used at 0.4  $\mu\text{mol/L}$  unless otherwise stated.

**Cell lines and culture.** Spontaneously immortalized PARP-1<sup>+/+</sup> and PARP-1<sup>-/-</sup> mouse primary embryo fibroblasts were derived by isolation of embryos from PARP-1<sup>+/+</sup> and PARP-1<sup>-/-</sup> mice. These mice were a kind gift from Dr J. Menissier de Murcia (Ecole Supérieure de Biotechnologie, Strasbourg, France; ref. 27). PARP-1<sup>+/+</sup> and PARP-1<sup>-/-</sup> cells were maintained as monolayers in DMEM supplemented with 10% (v/v) FCS, 100 units/mL penicillin, and 100  $\mu\text{g/mL}$  streptomycin. K562 human chronic myelogenous leukemia cells were supplied by the American Type Culture Collection (Manassas, VA) and maintained as a suspension culture in RPMI 1640 supplemented with 10% (v/v) FCS, 100 units/mL penicillin, and 100  $\mu\text{g/mL}$  streptomycin. The Chinese hamster ovary cell lines AA8 (parental) and EM9 (XRCC1 deficient) cells were supplied by the American Type Culture Collection, V3 (DNA-dependent protein kinase catalytic subunit deficient) cells were a kind gift from P. Jeggo (University of Sussex, Sussex, United Kingdom), and irs1SF (XRCC3 deficient) cells were a kind gift from T. Helleday (University of Sheffield, Sheffield, United Kingdom). These cells were maintained as monolayers in RPMI 1640 plus glutamine supplemented with 10% FCS, 100 units/mL penicillin, and 100  $\mu\text{g/mL}$  streptomycin.

**Growth inhibition assay.** Growth inhibition assays were done as described previously (11). Briefly, exponentially growing cells were seeded into 96-well plates (PARP-1<sup>+/+</sup> and PARP-1<sup>-/-</sup> cells) or 6-well plates (K562 cells) and exposed to various concentrations of topo I poison alone or in combination with AG14361 (0.4  $\mu\text{mol/L}$ ) for 5 days. Controls were exposed to 1% DMSO. Cells were fixed, washed, and stained with sulforhodamine B as described previously (28) or fixed and counted in duplicate using a Z1 Coulter counter (Beckman-Coulter, Bucks, United Kingdom). Growth inhibition was expressed as mean absorbance or mean of duplicate cell counts of treated cells expressed as a percentage of the untreated or 0.4  $\mu\text{mol/L}$  AG14361 control. Growth-inhibitory  $\text{IC}_{50}$  values ( $\text{GI}_{50}$ ) were calculated using GraphPad software (San Diego CA). The potentiation factor was calculated as the ratio between the  $\text{GI}_{50}$  of drug alone and the  $\text{GI}_{50}$  drug plus AG14361.

**Clonogenic cell survival assay.** Clonogenic survival of monolayer cells was determined by counting 0.4% (w/v) crystal violet-stained colonies, formed from a known number of cells seeded from cell cultures that had been treated with camptothecin/topotecan for 16 hours as described in the text. Clonogenic survival of suspension cells (by sloppy agar) was done as described previously (7). Briefly, K562 cells were exposed to a range of concentrations of camptothecin in the presence or absence of AG14361 for 16 hours. Drug exposure was terminated by centrifugation and removal of drug and the cells were seeded at known cell densities in 0.125% low melting point agarose (SeaKem, Cambrex, Berks, United Kingdom). Cells were left to grow

**Table 1.** Effect of AG14361 on topotecan-mediated growth inhibition in PARP-1<sup>+/+</sup> and PARP-1<sup>-/-</sup> cells

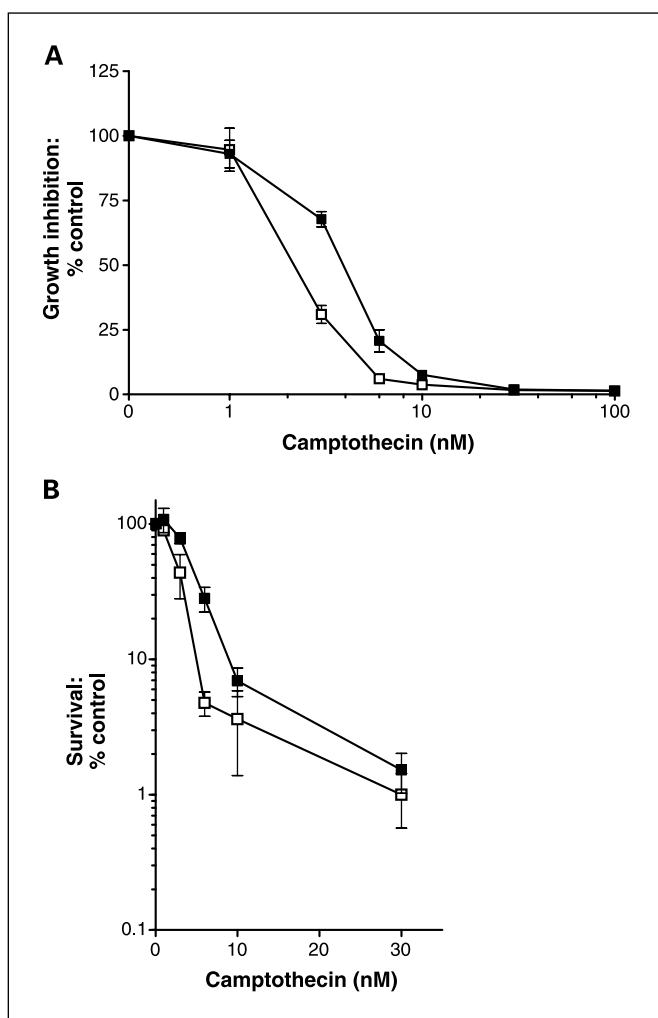
|                       | $\text{GI}_{50}$ (nmol/L) |   | $\text{PF}_{50}$ |
|-----------------------|---------------------------|---|------------------|
|                       | Topotecan                 | Topotecan + 0.4 $\mu\text{mol/L}$ AG14361 |                  |
| PARP-1 <sup>+/+</sup> | 65.0 $\pm$ 7.0            | 19.5 $\pm$ 4.3*                           | 3.4 $\pm$ 0.4    |
| PARP-1 <sup>-/-</sup> | 20.8 $\pm$ 2.0*†          | 15.6 $\pm$ 0.6†                           | 1.4 $\pm$ 0.1    |
| Sensitivity ratio     | 3 $\pm$ 0.2               | 1.3 $\pm$ 0.5                             |                  |

NOTE: Growth of cells treated with topotecan in the presence or absence of AG14361 for 5 days continuously determined by sulforhodamine B assay and expressed as a percentage of the relevant DMSO or 0.4  $\mu\text{mol/L}$  AG14361-treated control. Mean  $\pm$  SE  $\text{GI}_{50}$  of three independent experiments.  $\text{PF}_{50}$  is the potentiation factor, the ratio of  $\text{GI}_{50}$  topotecan alone to  $\text{GI}_{50}$  topotecan plus AG14361 calculated from  $\text{GI}_{50}$  of individual experiments.

\*  $P < 0.001$ , Student's paired  $t$  test compared with PARP-1<sup>+/+</sup> treated with camptothecin alone.

† Not significantly different from PARP-1<sup>+/+</sup> treated with topotecan plus AG14361.

‡  $P < 0.05$ , Student's paired  $t$  test compared with PARP-1<sup>-/-</sup> treated with camptothecin alone.



**Fig. 1.** Effect of AG14361 on topo I poison-induced growth inhibition and cytotoxicity in PARP-1<sup>+/+</sup>, PARP-1<sup>-/-</sup>, and human leukemia cell lines. **A**, camptothecin-induced growth inhibition in exponentially growing K562 cells in the presence (□) or absence (■) of 0.4 μmol/L AG14361. Cells were exposed to drugs for 16 hours followed by 5-day growth in drug-free medium or medium containing 0.4 μmol/L AG14361. Cell growth was measured by cell counting and expressed as a percentage of the relevant DMSO or 0.4 μmol/L AG14361 alone control. Points, mean of three independent experiments; bars, SE. **B**, cytotoxicity in exponentially growing K562 cells exposed to camptothecin in the presence (□) or absence (■) of AG14361 for 16 hours. Cytotoxicity was measured by colony formation in 0.125% agarose and expressed as a percentage of the relevant DMSO or 0.4 μmol/L AG14361 alone control. Points, mean of four independent experiments; bars, SE.

until colonies were visible (~10 days) and then stained with 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to aid counting. Survival was determined by counting the number of colonies, as a fraction of the treated cells seeded, and expressed as a percentage of the number of colonies formed in DMSO or AG14361 alone controls. Samples containing <10 or >300 colonies were not included. Cytotoxic IC<sub>50</sub> (LC<sub>50</sub>) values were calculated using GraphPad Prism software.

**Trapped in agarose DNA immunostaining.** Trapped in agarose DNA immunostaining is an immunofluorescent method for the measurement of drug-stabilized topoisomerase-DNA cleavable complexes in individual cells using isoform-specific antibodies (29) and has been used to detect and quantify stabilized topo I-DNA complexes (30). Exponentially growing K562 cells were treated with camptothecin in the presence or absence of 0.4 μmol/L AG14361 before analysis by trapped in agarose DNA immunostaining. Cells were harvested by

centrifugation and resuspended in 2% (w/v) low melting point agarose (Sea-Prep ultralow melting point agarose, BMA, Rockland, ME) and then smeared onto slides precoated with 0.5% (w/v) low melting point agarose. The slides were placed in lysis buffer [1% (w/v) SDS, 80 mmol/L phosphate buffer (pH 6.8), 10 mmol/L EDTA plus protease inhibitors: 2 μg/mL pepstatin, 2 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L benzamide, and 1 mmol/L DTT] for 30 minutes. The slides were then placed in 1 mol/L NaCl plus protease inhibitors (as above) for 30 minutes. After washing thrice in PBS, slides were exposed to a polyclonal antibody to topo I (2012, Topogen, Port Orange, FL) diluted 1:1,000 in PBS containing 0.1% Tween 20 and 1% (w/v) bovine serum albumin. Slides were then exposed to a FITC-conjugated secondary antibody (FITC-conjugated goat anti-human immunoglobulin G; Sigma). Visualization of the complexes was by fluorescence microscopy using a CCD camera as described previously (29).

**SDS precipitation for detection of cleavable complexes.** This assay was conducted as described by Rowe et al. (31). Briefly, high molecular weight DNA was labeled by exposing the cells to a 24-hour pulse with [<sup>14</sup>C]thymidine followed by 4-hour chase in fresh medium. Cells were treated as described in the figure legend and harvested by centrifugation. Cells were lysed in 2 mL lysis solution [1.25% SDS (w/v), 0.4 mg/mL herring sperm DNA (Promega, Hants, United Kingdom), 5 mmol/L EDTA (pH 8.0) heated to 65°C]. After a 10-minute incubation at 65°C, the KCl concentration was adjusted to 65 mmol/L and the lysates were vortexed for 10 seconds to fragment the DNA, cooled on ice for 10 minutes to allow precipitation of the protein-DNA complexes, and then centrifuged for 10 minutes at 10,000 × g at 4°C. The pellet was washed in wash buffer [10 mmol/L Tris-HCl (pH 8.0), 0.1 mg/mL herring sperm DNA, 100 mmol/L KCl, 1 mmol/L EDTA] heated, and then cooled and the pellet was recovered by centrifugation as before. The wash step was repeated twice and the pellet was resuspended in water preheated to 65°C before measurement of radiolabeled DNA, covalently bound to protein, by scintillation counting (Wallac 1409 DSA β-counter; Perkin-Elmer, Bucks, United Kingdom).

**DNA strand break assay.** The use of alkaline elution to detect topo I poison-induced single-strand breaks has been described previously by Bowman et al. (7). Briefly, exponentially growing cells were labeled with [<sup>14</sup>C]thymidine for 24 hours followed by a further 4-hour growth in fresh medium. Following exposure to camptothecin and/or AG14361 as indicated in figure legends, the cells were coeluted with an internal standard of irradiated (3 Gy) cells labeled with [<sup>3</sup>H]thymidine. Single-strand break frequency was measured by relative elution [i.e., the ratio of the rate of elution of the DNA after treatment compared with untreated control calculated as described by Fornace and Little (32)]. Relative elution = (log control relative retention) – (log sample relative retention); relative retention is the fraction of sample DNA retained when 50% of the internal standard DNA has been eluted.

## Results

**AG14361 enhances the growth-inhibitory and cytotoxic effects of topoisomerase I poisons.** We investigated the role of PARP-1 in the cellular response to topo I poisons by comparing the effect of exposure to topotecan for 5 days on the growth of PARP-1<sup>+/+</sup> and PARP-1<sup>-/-</sup> cells by sulforhodamine B assay. These cells have been characterized previously for PARP-1 protein and activity (33, 34) and we found that there was no difference in their topo I protein levels (data not shown). As shown in Table 1, PARP-1<sup>-/-</sup> cells were three times more sensitive to the growth-inhibitory effects of the topo I poison topotecan than the PARP-1<sup>+/+</sup> cells. AG14361 alone was not growth inhibitory up to a concentration of 10 μmol/L in PARP-1<sup>+/+</sup> and PARP-1<sup>-/-</sup> cells (9). To confirm that the potentiation

**Table 2.** Effect of AG14361 on formation of camptothecin-induced protein-DNA complexes

| Camptothecin ( $\mu\text{mol/L}$ ) | Protein-DNA complexes (% control) |                        |
|------------------------------------|-----------------------------------|------------------------|
|                                    | Camptothecin alone                | Camptothecin + AG14361 |
| 30-min exposure                    |                                   |                        |
| 1                                  | 798 $\pm$ 351                     | 934 $\pm$ 463          |
| 10                                 | 1,202 $\pm$ 620                   | 1,311 $\pm$ 621        |
| 16-h exposure                      |                                   |                        |
| 1                                  | 206 $\pm$ 46                      | 194 $\pm$ 29           |
| 10                                 | 457 $\pm$ 94                      | 516 $\pm$ 62           |

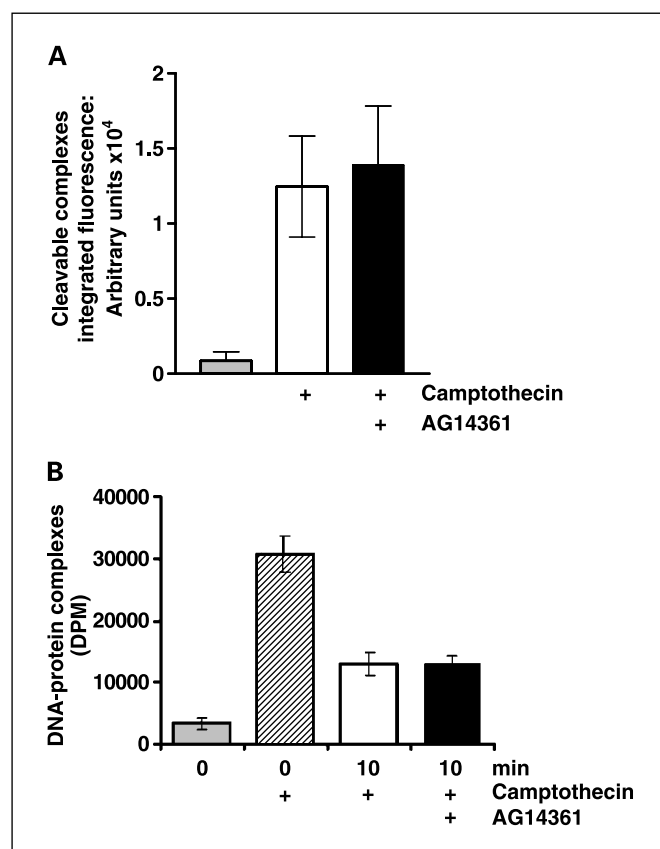
NOTE: K562 cells were exposed to 1 or 10  $\mu\text{mol/L}$  camptothecin in the presence or absence of 0.4  $\mu\text{mol/L}$  AG14361 for 30 minutes or 16 hours. Levels of cleavable complexes were measured by SDS precipitation and expressed as a percent of the DMSO or 0.4  $\mu\text{mol/L}$  AG14361-treated control as appropriate. Mean  $\pm$  SE of three independent experiments.

of topo I poison cytotoxicity by AG14361 seen previously was genuinely a PARP-1-mediated, rather than a nonspecific, effect of the inhibitor, we measured the effect of a 5-day exposure to topotecan in the presence or absence of 0.4  $\mu\text{mol/L}$  AG14361 on the growth of PARP-1<sup>+/+</sup> and PARP-1<sup>-/-</sup> cells. AG14361 caused a >3-fold potentiation of topotecan-induced growth inhibition in PARP-1<sup>+/+</sup> but a <1.4-fold potentiation in PARP-1<sup>-/-</sup> cells.

To investigate the effect of PARP inhibition in a human cell line well characterized for its response to topo I poisons (30), we measured the growth and survival of human chronic myelogenous leukemia K562 cells exposed to camptothecin in the presence or absence of AG14361. Exposure of K562 cells to AG14361 for 16 hours caused significant ( $P < 0.05$ ) ~2-fold potentiation of camptothecin-induced growth inhibition ( $\text{GI}_{50}$ , 16 hours, camptothecin alone  $4.7 \pm 0.4$  nmol/L, camptothecin + AG14361  $2.4 \pm 0.1$  nmol/L) and cytotoxicity ( $\text{LC}_{50}$ , camptothecin alone  $4.86 \pm 0.37$  nmol/L, camptothecin + AG14361  $2.77 \pm 0.55$  nmol/L) as shown in Fig. 1A and B, consistent with the sensitization observed in other cells types (7, 11). However, after only a 30-minute exposure to both drugs, AG14361 did not sensitize K562 cells to camptothecin ( $\text{GI}_{50}$ , 30 minutes, camptothecin alone  $40 \pm 5.3$  nmol/L, camptothecin + AG14361  $38 \pm 6.5$  nmol/L). AG14361 alone was not growth inhibitory up to a concentration of 10  $\mu\text{mol/L}$  in K562 cells (data not shown).

AG14361 has no significant effect on the formation and reversal of cleavable complexes. To determine whether the potentiation of topo I poisons by AG14361 was due to an increase in the number of topo I-DNA cleavable complexes, we measured the formation of cleavable complexes in individual K562 cells by trapped in agarose DNA immunostaining assay and in asynchronous populations of K562 cells by potassium-SDS precipitation. The K562 cell line was used as the formation of cleavable complexes by camptothecin has already been described in this model (30). Cells were exposed to camptothecin rather than topotecan to allow comparison with previously published data. The effect of AG14361 on cleavable complex accumulation was determined after 30-minute or 16-hour camptothecin exposure. We also measured reversal of the complexes following drug removal. AG14361 had no significant effect on either the formation of camptothecin-induced cleavable complexes determined by K-SDS precipitation (Table 2), and confirmed

by trapped in agarose DNA immunostaining immunofluorescence in intact cells (Fig. 2A), or their removal (Fig. 2B). Neither was there any difference between the topo I DNA



**Fig. 2.** Effect of AG14361 on camptothecin-stabilized cleavable complex induction and removal. *A*, K562 cells were treated with 10  $\mu\text{mol/L}$  camptothecin alone (white column) or camptothecin + 0.4  $\mu\text{mol/L}$  AG14361 (black column) for 30 minutes. Gray column, untreated control. Levels of cleavable complexes were measured using the trapped in agarose DNA immunostaining assay. Columns, mean FITC-associated fluorescence values from seven independent experiments; bars, SE. *B*, K562 cells were treated with 10  $\mu\text{mol/L}$  camptothecin in the presence (black column) or absence (white column) of 0.4  $\mu\text{mol/L}$  AG14361 for 30 minutes and then allowed to reverse for 10 minutes in drug-free medium or medium containing 0.4  $\mu\text{mol/L}$  AG14361. Hatched column, a control sample treated with camptothecin and not allowed to repair; gray column, DMSO control. Cleavable complexes were measured by SDS precipitation. Columns, mean  $^{14}\text{C}$ -associated radioactivity (DPM) from three independent experiments; bars, SE.



**Table 3.** Effect of AG14361 on camptothecin-induced DNA strand breaks after 16-hour exposure

| Camptothecin (nmol/L) | DNA single-strand breaks (relative elution) |  | Fold increase in strand breaks |
|-----------------------|---|--|--------------------------------|
|                       | Camptothecin alone                          | Camptothecin + 0.4 $\mu$ mol/L AG14361 |                                |
| 3                     | 0.021 $\pm$ 0.02                            | 0.08 $\pm$ 0.05                        | 2.4 $\pm$ 0.7                  |
| 10                    | 0.12 $\pm$ 0.05                             | 0.15 $\pm$ 0.04                        | 1.6 $\pm$ 0.5                  |
| 15                    | 0.17 $\pm$ 0.06                             | 0.23 $\pm$ 0.02                        | 1.6 $\pm$ 0.4                  |
| 30                    | 0.20 $\pm$ 0.04                             | 0.26 $\pm$ 0.04*                       | 1.3 $\pm$ 0.09                 |
| 40                    | 0.32 $\pm$ 0.06                             | 0.37 $\pm$ 0.02                        | 1.2 $\pm$ 0.17                 |
| 100                   | 0.32 $\pm$ 0.08                             | 0.35 $\pm$ 0.04                        | 1.2 $\pm$ 0.14                 |

NOTE: K562 cells were exposed to camptothecin in the presence or absence of 0.4  $\mu$ mol/L AG14361 for 16 hours before determination of DNA single-strand breaks by alkaline elution. Relative elution was calculated by comparison with DMSO-treated or 0.4  $\mu$ mol/L AG14361 alone control as appropriate. Mean  $\pm$  SE for three independent experiments. Fold increase was calculated from mean  $\pm$  SE relative elution values for individual experiments.

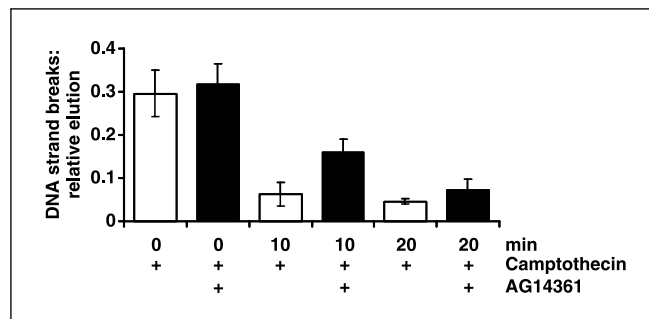
\* $P < 0.005$ , paired  $t$  test compared with camptothecin alone.

relaxation activity of nuclear extracts from untreated K562 cells and those from cells treated with 0.4  $\mu$ mol/L AG14361 for 0.5 or 16 hours (data not shown).

**AG14361 increases the persistence of camptothecin-induced DNA single-strand breaks.** Because AG14361 had no effect on topo I cleavable complex formation, we postulated that PARP-1 plays a role downstream of DNA cleavage, most likely in DNA repair. To investigate this further, we measured the effect of AG14361 on DNA single-strand breaks formed in K562 cells treated with topo I poisons. Coexposure to AG14361 for 16 hours increased camptothecin-induced DNA single-strand breaks 1.2- to 2.4-fold (Table 3), in agreement with previous findings in other cell lines treated with the PARP inhibitor NU1025 (7). Similarly, we also detected higher levels of DNA breaks in PARP-1<sup>-/-</sup> cells compared with PARP-1<sup>+/+</sup> cells following 30-minute exposure to 10  $\mu$ mol/L camptothecin (relative elution PARP-1<sup>+/+</sup> 0.36  $\pm$  0.03, PARP-1<sup>-/-</sup> 0.44  $\pm$  0.04;  $P = 0.002$ , Student's paired  $t$  test). To investigate whether the increased level of DNA strand breaks was due to an increase in formation or a delayed rejoining, we measured the induction and reversal of the strand breaks in K562 cells exposed to 30 nmol/L camptothecin for 30 minutes in the presence or absence of 0.4  $\mu$ mol/L AG14361 followed by removal of camptothecin and incubation for a further 10 or 20 minutes in fresh medium or medium containing AG14361. The data in Fig. 3 show that there was no difference in the level of strand breaks generated in cells exposed for 30 minutes to camptothecin alone or camptothecin plus AG14361, suggesting that the AG14361-induced increase in breaks seen after 16-hour exposure (above) results from the net accumulation of breaks. However, AG14361 did have a significant effect on the reversal of DNA strand breaks following drug removal. After camptothecin withdrawal, the level of breaks decreased rapidly, such that at 10 minutes only 20% of the breaks remained in the absence of AG14361, whereas in the presence of AG14361 50% of the breaks remained. Thus, PARP-1 inhibition hindered the repair of camptothecin-induced strand breaks ~2.5-fold in the 10-minute period following drug removal. This retardation of rejoining persisted such that at 20 minutes after drug removal only 16% of the breaks remained in control cells, whereas 22% remained in the presence of AG14361 (Table 4). The

persistence of DNA breaks was also measured at longer intervals after exposure to a 30-minute pulse of 300 nmol/L camptothecin. AG14361 significantly reduced reversal of these breaks at both 1 and 16 hours (Table 4). AG14361 also significantly retarded DNA break rejoining in PARP-1<sup>+/+</sup> cells (57.3  $\pm$  5.8% breaks remained 60 minutes after withdrawal of 10  $\mu$ mol/L camptothecin in the presence of AG14361 compared with 48.2  $\pm$  4.3% in the absence of AG14361;  $P < 0.05$ ) but not PARP-1<sup>-/-</sup> cells (data not shown), confirming that AG14361 retards DNA break rejoining by inhibition of PARP-1.

**Effect of AG14361 on camptothecin-induced cytotoxicity in DNA repair-deficient cells.** The data above implicate PARP-1 in events downstream rather than upstream of topo I poison-mediated DNA damage (i.e., by modulating DNA repair). PARP-1 plays a role in BER but may also signal to or interact with other DNA repair pathways. To investigate which pathways may be involved in PARP-1-mediated repair of topo I poison-induced DNA damage, we investigated the effect of AG14361 on the cytotoxicity of camptothecin in a variety of DNA repair-deficient cell lines. Chinese hamster ovary cells deficient in the BER scaffold protein XRCC1 (EM9), the nonhomologous end joining enzyme



**Fig. 3.** Repair of camptothecin-induced DNA single-strand breaks in K562 cells in the presence or absence of AG14361. K562 cells were exposed to 30 nmol/L camptothecin for 30 minutes followed by repair in drug-free medium (white columns) or medium containing 0.4  $\mu$ mol/L AG14361 (black columns) for 0, 10, or 20 minutes. DNA strand breaks were measured by alkaline elution. Relative elution was calculated by comparison with DMSO or 0.4  $\mu$ mol/L AG14361 alone control as appropriate. Columns, mean of three independent experiments; bars, SE.

**Table 4.** Effect of AG14361 on repair of camptothecin-induced DNA strand breaks in K562 cells

| Time after camptothecin removal | % Unrepaired DNA |  | Fold increase in strand breaks |
|---------------------------------|------------------|--|--------------------------------|
|                                 | Camptothecin     | Camptothecin + 0.4 $\mu\text{mol/L}$ AG14361 |                                |
| Camptothecin (30 nmol/L)        |                  |  |                                |
| 0                               | 100              | 100  |                                |
| 10 min                          | 20 $\pm$ 5.4     | 50 $\pm$ 1.7*                                | 2.5 $\pm$ 0.2                  |
| 20 min                          | 16 $\pm$ 2.8     | 22 $\pm$ 4.9                                 | 1.4 $\pm$ 0.4                  |
| Camptothecin (300 nmol/L)       |                  |  |                                |
| 0                               | 100              | 100  |                                |
| 1 h                             | 15 $\pm$ 5.5     | 44 $\pm$ 5.1 <sup>†</sup>                    | 1.9 $\pm$ 0.1                  |
| 16 h                            | 10 $\pm$ 9.6     | 39 $\pm$ 6.2 <sup>‡</sup>                    | 2.5 $\pm$ 0.85                 |

NOTE: K562 cells were exposed to 30 or 300 nmol/L camptothecin for 30 minutes followed by repair in drug-free (control) medium or medium containing 0.4  $\mu\text{mol/L}$  AG14361 for the times indicated. DNA strand breaks were measured by alkaline elution. Relative elution was calculated by comparison with DMSO or 0.4  $\mu\text{mol/L}$  AG14361 alone control as appropriate. Mean  $\pm$  SE of three independent experiments.

\* $P < 0.005$ , Student's paired  $t$  test compared with camptothecin alone.

<sup>†</sup> $P = 0.07$ , Student's paired  $t$  test compared with camptothecin alone.

<sup>‡</sup> $P = 0.02$ , Student's paired  $t$  test compared with camptothecin alone.

DNA-dependent protein kinase catalytic subunit (V3), and the homologous recombination repair protein XRCC3 (irs1SF) were compared with their parental (AA8) line. We measured clonogenic survival of these cells after exposure to camptothecin in the presence or absence of 0.4  $\mu\text{mol/L}$  AG14361 for 16 hours (Table 5). The irs1SF and EM9 cells were the most sensitive to camptothecin as has been shown previously (17, 35), confirming that homologous recombination and BER are the most important repair pathways in the survival from topo I poisons. AG14361 potentiated the cytotoxicity of camptothecin 2.25-fold in the AA8 cells and 2.0-fold in the V3 cells but did not significantly potentiate camptothecin in the EM9 cells. The irs1SF cells were found to be hypersensitive to AG14361 and survival of cells treated with 0.4  $\mu\text{mol/L}$  AG14361 was  $<50\%$ . Because of this profound hypersensitivity, we used lower concentrations of AG14361 (15 and 70 nmol/L, corresponding to  $\text{LC}_{50}$  and  $\text{LC}_{90}$ , respectively). Under these conditions, no significant potentiation of camptothecin-mediated cytotoxicity could be detected. However, we cannot exclude the possibility that these concentrations of AG14361 may not have produced sufficient PARP inhibition to enhance camptothecin cytotoxicity.

## Discussion

We have shown that AG14361 potentiates the growth-inhibitory and cytotoxic effects of the topo I poisons camptothecin and topotecan in K562 and PARP-1<sup>+/+</sup> cells at a concentration of AG14361 that alone was neither cytotoxic nor growth inhibitory to the cell lines studied. This is consistent with previous data with AG14361 (9) and other PARP inhibitors in a variety of other cell lines (7, 9, 10). The aim of this study was to elucidate the mechanism that underlies this potentiation of topo I poison-induced cytotoxicity and growth inhibition by PARP inhibitors.

We verified the importance of PARP-1 in the cellular response to topo I poisons in PARP-1<sup>-/-</sup> and PARP-1<sup>+/+</sup> cells.

PARP-1<sup>-/-</sup> cells were 3-fold more sensitive to topotecan than PARP-1<sup>+/+</sup> cells and coadministration of AG14361 caused a 3-fold sensitization of PARP-1<sup>+/+</sup> cells. These data confirm that active PARP-1 plays a role in the protection of cells from topo I poisons and that the effects of AG14361 are indeed due to inhibition of this protective activity. PARP-1<sup>-/-</sup> cells have a residual DNA damage-activated PARP, PARP-2 (5), which due to its structural similarity to PARP-1 is also inhibited by AG14361. AG14361 caused a modest sensitization of PARP-1<sup>-/-</sup> cells, implicating PARP-2 in the cellular response to topo I poisons.

Sensitivity to topo I poisons is related to topo I activity by virtue of the increased number of cleavable complexes stabilized by the poison. PARP-1 and topo I interact with

**Table 5.** Effect of AG14361 on survival of AA8, EM9, V3, and irs1SF cell lines following a 16-hour exposure to camptothecin

| Cell line | $\text{LC}_{50}$ (nmol/L) |   |
|-----------|---------------------------|---|
|           | Camptothecin              | Camptothecin + AG14361                                    |
| AA8       | 25.0 $\pm$ 3.5            | 12.2 $\pm$ 2.4*   |
| EM9       | 5.6 $\pm$ 1.2             | 4.0 $\pm$ 1.1 <sup>†</sup>                                |
| V3        | 16.1 $\pm$ 4.0            | 10.2 $\pm$ 3.6 <sup>‡</sup>                               |
| irs1SF    | 2.7 $\pm$ 0.7             | 1.7 $\pm$ 0.4 <sup>§</sup><br>1.4 $\pm$ 0.4 <sup>  </sup> |

NOTE: Cells were treated with camptothecin  $\pm$  0.4  $\mu\text{mol/L}$  AG14361 and survival was determined using a clonogenic assay. Mean  $\pm$  SE  $\text{LC}_{50}$  of at least four independent experiments. Mean  $\pm$  SE  $\text{PF}_{50}$  values calculated using  $\text{LC}_{50}$  of individual experiments.

\* $P < 0.01$ , compared with camptothecin alone, Student's paired  $t$  test.

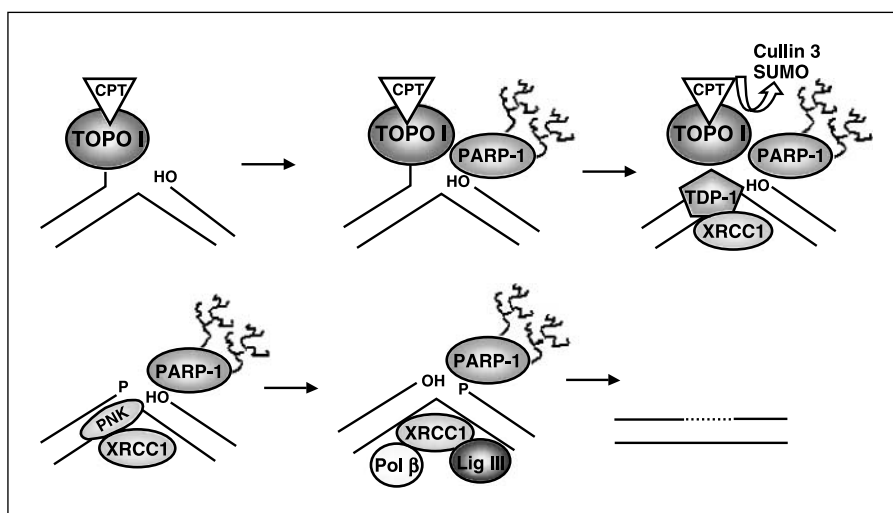
<sup>†</sup> Not significantly different from camptothecin alone.

<sup>‡</sup> $P < 0.05$ , compared with camptothecin alone, Student's paired  $t$  test.

<sup>§</sup> AG14361 (15 nmol/L) was used.

<sup>||</sup> AG14361 (70 nmol/L) was used.

**Fig. 4.** Proposed role of PARP-1 in the repair of topo I poison-mediated DNA damage. Topo I poisons, exemplified by camptothecin (CPT), lead to the formation of a cleavable complex in which topo I is covalently attached to the 3' phosphate. PARP-1 binds the resultant free 5' OH DNA end, stimulating its automodification activity and recruiting XRCC1. XRCC1 in turn recruits tyrosyl DNA phosphodiesterase-1 (*TDP-1*), which removes the topo I from the DNA leaving a 3' phosphate terminus. Before removal, topo I may be partially degraded by Cullin 3 or SUMO-dependent mechanisms, leaving a peptide fragment that is removed by tyrosyl DNA phosphodiesterase-1. XRCC1 also recruits polynucleotide kinase (*PNK*), which converts the DNA ends to 3' OH and 5' phosphate. Finally, XRCC1 acts as a scaffold for DNA polymerase  $\beta$  (*Pol \beta*) and DNA ligase III (*Lig III*) to fill and ligate the gap, completing the repair of the camptothecin-induced DNA damage.



each other, and previously reported data suggest that poly(ADP-ribosylation) may regulate topo I activity (10, 24). By contrast, we found that AG14361 had no significant effect on topo I DNA relaxation activity or on the level or persistence of topo I-DNA cleavable complexes; thus, the sensitization by AG14361 is unlikely to be due to topo I activation. The reason for these differences is currently not understood but may reflect the nature of the experimental system used. By and large, we have investigated the role of PARP-1 activity in intact cells, which we believe gives a more reliable picture of what happens *in vivo*.

Our data indicate that PARP-1 plays a significant part in the repair of topo I poison-induced DNA damage. In K562 cells, AG14361 increased the levels of camptothecin-induced DNA single-strand breaks after a 16-hour exposure but not after 30-minute exposure, suggesting that the AG14361-induced increase in breaks seen after long exposures results from the gradual accumulation of DNA breaks with time. These data are consistent with our observation that AG14361 potentiated camptothecin cytotoxicity after 16-hour coexposure but not after 30-minute coexposure. We propose that PARP-1 facilitates the repair of topo I poison-induced DNA breaks, and as a result, inhibition of PARP-1 and DNA break repair shifts the dynamic equilibrium of break induction and resolution toward net accumulation. We found that AG14361 did indeed significantly delay strand break rejoining at all time points up to 16 hours. These data are largely in agreement with data from Malanga and Althaus (20), showing that poly(ADP-ribosylated) PARP-1 can block the formation of cleavable complexes, inhibit DNA cleavage by topo I, and accelerate the removal of stabilized cleavable complexes.

PARP-1 plays an important role in BER, and BER-deficient EM9 cells are hypersensitive to camptothecin despite normal PARP-1 activity (17, 36). This does not necessarily exclude a role for PARP-1 in other repair pathways [e.g., nonhomologous end joining (37, 38) and homologous recombination (39)]. To test the hypothesis that topo I-induced DNA strand breaks are repaired by PARP-1-dependent BER and to investigate if PARP-1 cooperates with other DNA repair pathways involved in the cellular response to topo I poisons, we used Chinese hamster ovary cells with deficiencies in DNA repair in comparison with

the repair-competent parental AA8 cell line. All of these DNA repair-deficient cell lines were more sensitive to camptothecin than the parental line, with the order of sensitivity being *irs1SF* (homologous recombination) 10-fold, EM9 (BER) 5-fold, and V3 (nonhomologous end joining) 1.5-fold, consistent with previous reports (17, 35). These data implicate all of the DNA repair pathways studied in the response to camptothecin-induced DNA damage, with homologous recombination and BER pathways predominating.

It is unlikely that PARP-1 is involved in the repair of camptothecin-induced DNA breaks by nonhomologous end joining because AG14361 potentiated camptothecin-induced cytotoxicity to a similar extent in both AA8 and V3 cell lines. Additionally, we showed previously that AG14361 increases cellular sensitivity to topotecan regardless of the mismatch repair status (40), indicating that PARP-1 is not acting via this pathway either.

The *irs1SF* cells were hypersensitive to AG14361 alone, confirming recent reports of the hypersensitivity of homologous recombination-deficient cells to PARP-1 inhibitors (41). Studies of AG14361 in combination with camptothecin were therefore conducted at lower concentrations of the inhibitor. There was no significant potentiation of camptothecin-induced cytotoxicity by AG14361 in these cells, which could have been due to insufficient PARP inhibition and/or the cytotoxicity of AG14361 even at these low concentrations.

As expected, AG14361 did not significantly potentiate camptothecin-induced cytotoxicity in the BER-defective EM9 cell line. Based on the results described here, the most likely mechanism for the potentiation of camptothecin-induced cytotoxicity by AG14361 is via inhibition of PARP-1-dependent BER. BER may repair the DNA strand breaks formed after the removal of the cleavable complex. This proposal is supported by the observation that tyrosyl DNA phosphodiesterase-1, the enzyme responsible for the removal of the topo I-cleavable complex, associates with the BER scaffold protein, XRCC1 (42). PARP-1 associates with XRCC1 (12) and PARP-1 is required for the formation of XRCC1 foci in response to DNA damage (43). Camptothecin-induced DNA damage activates PARP-1 (7) and XRCC1 preferentially associates with poly(ADP-ribosylated) PARP-1 (15). Therefore, PARP-1 activation may promote the

recruitment of XRCC1 to the site of the cleavable complex, which in turn could recruit tyrosyl DNA phosphodiesterase-1 and allow removal of topo I from the DNA. Topo I may be partially degraded before removal from the DNA via a Cullin 3 or SUMO-dependent mechanism (44, 45) followed by proteasomal degradation, independently of PARP-1, accounting for the fact that AG14361 did not hinder the apparent removal of topo I (as detected by anti-topo I antibody) from the DNA. PARP-1 and XRCC1 together may then recruit PNK and other BER proteins as illustrated in Fig. 4. Interestingly, a tyrosyl DNA phosphodiesterase-1-dependent repair process for the repair of single-strand breaks caused by abortive topo I activity has been described recently by El-Khamisy et al. (46).

In contrast to the intensively studied and well-understood role of PARP-1 in the repair of monofunctional alkylating agent-induced DNA damage, its role in topo I poison-induced cytotoxicity has only recently begun to be scrutinized.

PARP inhibitors have entered the clinic for cancer treatment for the first time in a phase I trial combining the novel PARP inhibitor AG014699 with the monofunctional alkylating agent, temozolomide (25). An understanding of the role of PARP-1 in topo I poison-mediated cytotoxicity is fundamental in the effective design of any potential further clinical trials using PARP inhibitor-topo I poison combinations. The data we present regarding the importance of PARP-1 inhibition in DNA repair and cellular survival downstream of topo I poisoning are crucial in guiding effective scheduling of these two agents.

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## The Novel Poly(ADP-Ribose) Polymerase Inhibitor, AG14361, Sensitizes Cells to Topoisomerase I Poisons by Increasing the Persistence of DNA Strand Breaks

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