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Radiosensitization and DNA Repair Inhibition by the Combined Use of Novel Inhibitors of DNA-dependent Protein Kinase and Poly(ADP-Ribose) Polymerase-1[1]

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ABSTRACT

The DNA repair enzymes, DNA-dependent protein kinase (DNA-PK) and poly(ADP-ribose) polymerase-1 (PARP-1), are key determinants of radio- and chemo-resistance. We have developed and evaluated novel specific inhibitors of DNA-PK (NU7026) and PARP-1 (AG14361) for use in anticancer therapy. PARP-1- and DNA-PK-deficient cell lines were 4-fold more sensitive to ionizing radiation (IR) alone, and showed reduced potentially lethal damage recovery (PLDR) in G_0 cells, compared with their proficient counterparts. NU7026 (10 μM) potentiated IR cytotoxicity [potentiation factor at 90% cell kill (PF_{90}) = 1.51 ± 0.04] in exponentially growing DNA-PK proficient but not deficient cells. Similarly, AG14361 (0.4 μM) potentiated IR in PARP-$1^{+/+}$ (PF_{90} = 1.37 ± 0.03) but not PARP-$1^{-/-}$ cells. When NU7026 and AG14361 were used in combination, their potentiating effects were additive (*e.g.*, PF_{90} = 2.81 ± 0.19 in PARP-$1^{+/+}$ cells). Both inhibitors alone reduced PLDR ~3-fold in the proficient cell lines. Furthermore, the inhibitor combination completely abolished PLDR. IR-induced DNA double strand break (DNA DSB) repair was inhibited by both NU7026 and AG14361, and use of the inhibitor combination prevented 90% of DNA DSB rejoining, even 24-h postirradiation. Thus, there was a correlation between the ability of the inhibitors to prevent IR-induced DNA DSB repair and their ability to potentiate cytotoxicity. Thus, individually, or in combination, the DNA-PK and PARP-1 inhibitors act as potent radiosensitizers and show potential as tools for anticancer therapeutic intervention.

INTRODUCTION

Enzyme-mediated repair of DNA DSBs[3] is a major mechanism of resistance to radiotherapy, and inhibition of DNA DSB repair is thus a strategy for radiopotentiation. This study focuses on the use of novel inhibitors of the DNA-PK and PARP-1, both of which are DNA strand break-activated enzymes and key components of DNA damage recognition, repair, and signaling pathways.

Two DNA DSB repair pathways in eukaryotes are NHEJ and HRR (1). Important components in these repair pathways are the PI 3-K-related protein kinase family of enzymes. These DNA damage-activated serine/threonine protein kinases include DNA-PK, ATM, and ATR (2). The DNA-PK holoenzyme comprises a heterodimer of M_r ~70,000 and ~80,000 polypeptides, known as Ku, which binds to DNA strand breaks, recruiting and activating the M_r 470,000 catalytic subunit, termed DNA-PKcs (3). Numerous studies have shown that cells lacking either functional DNA-PKcs or Ku80 through mutation or gene knockout are hypersensitive to IR and cross-linking agents (3–6). DNA-PK, together with the XRCC4/DNA ligase IV complex and the recently identified cofactor Artemis, is specifically required for NHEJ (7, 8), with ~80% of DNA DSBs repaired by this pathway (9).

The first identified inhibitor of the PI 3-K-related protein kinase enzyme family was the fungal metabolite Wortmannin. Although primarily used as a PI 3-K inhibitor, it was also shown to potentiate IR-induced cytotoxicity and inhibit DNA DSB repair at concentrations that inhibit cellular DNA-PK (10–13). The recently synthesized molecule NU7026, 2-(morpholin-4-yl)-benzo[h]chomen-4-one, a novel and specific inhibitor of DNA-PK, has been evaluated in this study.

PARP-1 is a M_r 116,000 enzyme that is an important component of the BER complex required for DNA SSB repair (14–16). Loss of PARP-1 function, using molecular genetic techniques or inhibition, causes radiosensitization and potentiates the cytotoxicity of monofunctional alkylating agents (17–19). Thus, PARP-1 inhibitors are predicted to improve the efficacy of radio and certain types of chemotherapy. It is generally assumed that the inhibition of BER is the cause of the enhanced cytotoxicity. However, we have recently reported that a PARP-1 inhibitor (NU1025) also inhibits DNA DSB repair in cells exposed to IR, and this may contribute to the cytotoxic mechanism (12).

Convincing evidence for *in vivo* radiopotentiation with a PARP-1 inhibitor was obtained using PD128763 (20). Using a murine tumor model, Leopold and Seebolt-Leopold demonstrated that PD128763+IR caused a 10–15-day increase in growth delay compared with irradiation alone. The clinical potential of this class of agents has led to the development of diverse classes of potent PARP-1 inhibitors (21–23). The tricyclic benzamidizole 1-(4-dimethylaminomethylphenyl)-8,9-dihydro-7H-2,7,9a-benzo[cd]azulen-6-one) AG14361 has been used in this study (23).

Radiosensitization by deficiency or inhibition of either DNA-PK or PARP-1 is not limited to proliferating cells. Nonproliferating cells exposed to IR are known to undergo PLDR leading to substantial increases in survival compared with proliferating cells or cells induced to start a proliferative cycle shortly after irradiation (24, 25). Several early reports, using either cell lines mutationally inactivated in DNA-PK or PARP-1 inhibitors, showed a reduction in PLDR in nonproliferating (quiescent) cells (26–30). Local tumor conditions, such as cell and vascular density, as well as availability of oxygen and nutrients, frequently result in a high fraction of nonproliferating cells. PLDR after irradiation is considered to be an important determinant of radioresistance and has been demonstrated in tumor cell lines and in an experimental tumor model (31–33). Thus, the potential of small molecule inhibitors of DNA-PK and PARP-1 to prevent PLDR is an important consideration in the assessment of their therapeutic application.

Here, we describe the biological effects of both AG14361 and

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[3] The abbreviations used are: DSB, double strand break; ATM, ataxia telangiectasia-mutated kinase; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; LD_{90}, dose of ionizing radiation that causes 90% cell kill; ATR, ataxia telangiectasia and Rad3-related kinase; BER, base excision repair; DNA-PK, DNA-dependent protein kinase; HRR, homologous recombination repair; IR, ionizing radiation; MEF, mouse embryonic fibroblast; NHEJ, nonhomologous end joining; PARP-1, poly(ADP-ribose) polymerase-1; PF_{90}, potentiation factor at 90% cell kill; PI 3-K, phosphatidylinositol 3-kinase; PLDR, potentially lethal damage recovery; SSB, single-strand break; YAC, yeast artificial chomosome.

NU7026 in cell culture models. We have investigated the ability of these inhibitors, used alone or in combination, to radiosensitize both proliferating and quiescent paired cell lines proficient or deficient in either DNA-PK or PARP-1. In addition, their effects on DNA DSB repair were investigated.

MATERIALS AND METHODS

Drugs. NU7026 was synthesized in the Department of Chemistry, University of Newcastle upon Tyne. AG14361 was synthesized by Pfizer GRD, CA (23). NU7026 and AG14361 were dissolved in anhydrous DMSO at stock concentrations of 5 and 10 mM, respectively, and stored at −20°C. Drugs (alone or in combination) were added to cell cultures so that the final DMSO concentrations were kept constant at 1% (v/v).

Cell Lines and Culture. Primary PARP-$1^{+/+}$ and PARP-$1^{-/-}$ MEFs were a gift from Professor Gilbert de Murcia, École Supérieure de Biotechnologie de Strasbourg, France. Spontaneously immortalized cell lines were derived from the primary MEFs to enable clonogenic survival assays to be performed. It was noted that the immortalized PARP-$1^{+/+}$ MEFs expressed very high basal levels of p53 protein compared with the PARP-$1^{-/-}$ MEFs, and therefore, the *p53* gene was sequenced. It was found that the PARP-$1^{-/-}$ cells had a wt p53 sequence, but the PARP-$1^{+/+}$ had an Asp to Glu substitution at codon 278 in p53, within a conserved region of the DNA binding domain. This rendered the p53 unable to act as a transcriptional transactivator.[4] The significance of the difference in the functional status of p53, with respect to the results obtained, is explored in the "Discussion." The Chinese hamster cell lines V3 (mutated in DNA-PKcs) and V3YAC [V3 transfected with a YAC carrying the complementing human DNA-PKcs gene] were kindly provided by Dr. Penny Jeggo, University of Sussex (34).

All cell lines were cultured as monolayers in DMEM medium [supplemented with 10% (v/v) FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin]. Glutamine was added at a final concentration of 2 mM. The V3YAC cell line was maintained under antibiotic selection with genticin (Life Technologies, Inc., Paisley, United Kingdom) at a final concentration of 500 μg/ml to ensure retention of the YAC.

Purified Enzyme Assays. Mammalian DNA-PK (500 ng/μl) was isolated from HeLa cell nuclear extract after chromatography using Q-Sepharose, S-Sepharose, and Heparin agarose. DNA-PK (250 ng) activity was measured at 30°C, in a final volume of 40 μl, in buffer containing 25 mM HEPES (pH 7.4), 12.5 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 10% v/v Glycerol, 0.1% w/v NP-40, and 1 mg of the substrate GST-p53N66 (the NH_2-terminal 66 amino acid residues of human wild-type p53 fused to glutathione *S*-transferase) in polypropylene 96-well plates. To the assay mix, varying concentrations of inhibitor (in DMSO at a final concentration of 1% v/v) were added. After 10 min of incubation, ATP was added to give a final concentration of 50 μM, along with a 30-mer double-stranded DNA oligonucleotide (final concentration of 0.5 ng/ml), to initiate the reaction. After 1 h with shaking, 150 μl of PBS were added to the reaction, and 5 μl were then transferred to a 96-well opaque white plate containing 45 μl of PBS per well, where the GSTp53N66 substrate was allowed to bind to the wells for 1 h. To detect the phosphorylation event on the serine 15 residue of p53 elicited by DNA-PK, a p53 phosphoserine-15 antibody (Cell Signaling Technology, Beverly, MA) was used in a basic ELISA procedure. An antirabbit horseradish peroxidase-conjugated secondary antibody (Pierce) was then used in the ELISA before the addition of chemiluminescence reagent (Renaissance, New England Nuclear) to detect the signal as measured by chemiluminescent counting via a TopCount NXT (Packard).

The protocols used to detect ATM kinase and ATR kinase activities were performed essentially according to the methodologies described previously (35, 36). ATM and ATR were immunoprecipitated using rabbit polyclonal antisera raised to the COOH-terminal 400 amino acids of ATM and antisera raised to amino acids 400–480 of ATR, respectively. The PI 3-kinase assay was performed essentially as described previously (37), using baculoviral derived recombinant p110α and p85α (a kind gift from Prof. Mike Waterfield, Ludwig Institute, London, United Kingdom).

The IC_{50}s for the compounds in all of the enzymes assays were derived from sigmoidal plots using the graphic package Prism, in which the enzyme activity in the varying concentration of compounds was plotted against the concentration of compound.

Cytotoxicity Assays. Clonogenic assays were performed as described previously (12). Briefly, exponentially growing cells (± AG14361 and/or NU7026) were exposed to IR. Drug(s) were added 60 min before irradiation followed by a 16-h postincubation at 37°C before harvesting and reseeding for colony formation in the absence of drugs. Data were normalized to untreated controls (1% v/v DMSO). PF_{90} values were calculated from the ratio of the individual LD_{90} values, *i.e.,* the LD_{90} divided by LD_{90} in the presence of inhibitor(s).

PLDR. The effects of AG14361 and NU7026, alone or in combination, were investigated in growth arrested cells after approximately equitoxic doses of IR. Cells were grown to confluence to induce G_1 arrest (G_0 status) before irradiation and irradiated in conditioned medium obtained from plateau phase cells. Cell cycle distribution to confirm G_1 arrest in plateau phase cells was assessed by flow cytometric analysis (data not shown). Drug(s) were added in conditioned medium 60 min before irradiation. After exposure to IR, cells were trypsinized and reseeded for colony formation in the absence of drugs, either immediately after exposure to IR or after a 24-h postincubation (± AG14361 and/or NU7026) at 37°C.

Western Blot Analysis of Protein Expression. Cell lysates were prepared from exponentially growing cells and loaded onto denaturing polyacrylamide gels using standard protocols. DNA-PKcs was resolved on 3–8% (w/v) Tris acetate gradient gels and all other proteins on 4–20% (w/v) Tris glycine gradient gels (Invitrogen Ltd., Paisley, United Kingdom). After electrophoresis, proteins were electrotransferred onto nitrocellulose (Bio-Rad, Herts, United Kingdom) and probed for PARP-1 (H-250 rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA) and DNA-PKcs (Ab-4 cocktail mouse monoclonal; Stratech Scientific, Cambs, United Kingdom). As a loading control, an antiactin antibody (mouse clone AC-40; Sigma, Dorset, United Kingdom) was used. This was followed by binding of peroxidase-conjugated goat antimouse/rabbit antibody and detection of proteins by enhanced chemiluminescence (DAKO, Ely, United Kingdom).

Cell-based Enzyme Activity Assays. For comparison of enzyme activities in the cell lines, the following techniques were used: DNA-PK was assayed in nuclear extracts. Briefly, DNA was removed from the extracts using high salt elution (fast flow DEAE Sepharose; Amersham Pharmacia, Bucks, United Kingdom). Fifty microgram aliquots were assayed for DNA-PK activity using the SIGNATECT DNA-PK assay system (Promega, Hants, United Kingdom). PARP-1 was assayed by measuring incorporation of radiolabel from [^{32}P] NAD^+ into acid precipitable counts in a permeabilized cell system (38). Cells were permeabilized using digitonin, and a 30-bp blunt-ended oligonucleotide was used in the assay to maximally activate PARP-1.

DNA Strand-Break Assays. DNA DSB levels were measured by neutral filter elution (39). The radiolabelling, drug treatment, postincubation conditions, and sample preparation used in these experiments were exactly as described by Boulton *et al.* (11). In all experiments, cells were exposed to 75 Gy IR. Cell cultures were preincubated ± AG14361 and/or NU7026 for 60 min before exposure to IR, and the drugs remained in the culture medium during the postincubation periods. Regression analysis of each elution profile was performed to calculate the relative retention, *i.e.,* the fraction of sample DNA retained on the filter when 50% of the internal standard has eluted (40). Values for cells treated with IR ± inhibitor(s) were expressed as a percentage of the values of unirradiated controls.

RESULTS

Inhibitor Structures and Evaluation of Enzyme Inhibition. The structure of AG14361 (1-(4-dimethylaminomethyl-phenyl)-8,9-dihydro-7H-2,7,9a-benzo[cd]azulen-6-one) and NU7026 (2-(morpholin-4-yl)-benzo[h]chomen-4-one) is shown in Fig. 1. AG14361 is an extremely potent competitive inhibitor of PARP-1 with a K_i value < 5 nM (16). In the permeabilized cell assay used here, the IC_{50} for AG14361 in the V3YAC cells was 68.4 ± 3.5 nM and 56.3 ± 4.2 nM in the PARP-$1^{+/+}$ cells.

Wortmannin is a noncompetitive, irreversible inhibitor of DNA-PK (10), whereas NU7026 is competitive with ATP. Shown in Table 1 is

[4] P. A. Jowsey, unpublished results.

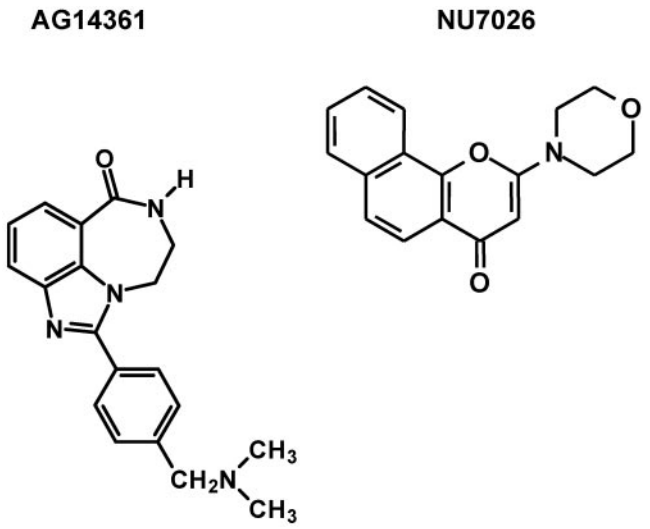

Fig. 1. Chemical structures of AG14361 [1-(4-dimethylaminomethyl-phenyl)-8,9-dihydro-7H-2,7,9a-benzo[cd]azulen-6-one] and NU7026 [2-(morpholin-4-yl)-benzo-[h]chomen-4-one].

Table 1 *Comparison of inhibitor potencies and specificities against the PIKK family enzymes*[a]

Enzyme	NU7026 IC_{50} (μM)	Wortmannin IC_{50} (μM)
DNA-PK	0.23 ± 0.01	0.26 ± 0.02
PI 3-K	13.0 ± 3.00	0.003 ± 0.00
ATM	>100	0.30 ± 0.05
ATR	>100	4.4 ± 0.18

[a] Each IC_{50} value is computed from the mean of more than or equal to three independent experiments ± SE.

a comparison of the relative inhibitory potencies of NU7026 and Wortmannin against PI 3-K, DNA-PK, ATM, and ATR. Although Wortmannin is primarily a PI 3-K inhibitor, being 90-fold more active against PI 3-K than DNA-PK or ATM, NU7026 was more selective for DNA-PK with a 60-fold greater potency against this enzyme than PI 3-K and inactive against both ATM and ATR. Thus, in contrast to Wortmannin, NU7026 demonstrates excellent specificity for DNA-PK.

AG14361 (0.4 μM) completely inhibited purified PARP-1 enzyme activity while having no effect on DNA-PK activity, and similarly, NU7026 (10 μM) completely inhibited purified DNA-PK activity, while having no effect on PARP-1 activity (results not shown). These inhibitor concentrations were used in all subsequent cell culture experiments.

Characterization of Cell Lines. Western blot analyses were carried out to assess PARP-1 and DNA-PK protein levels in the paired cell lines. As expected (Fig. 2*A*), the PARP-$1^{+/+}$ cell line exhibited a band at M_r 116,000 (PARP-1), and this was completely absent in the PARP-$1^{-/-}$ cell line. However, both these cell lines showed bands for DNA-PKcs and Ku80 (results not shown). The V3 cell line lacked the M_r 470,000 DNA-PKcs band, but this was restored in the V3YAC cell line (Fig. 2*A*). Both these cell lines showed the M_r 116,000 PARP-1 band (results not shown). DNA-PK and PARP-1 assays were carried out to confirm that the lack of detectable protein in the V3 and PARP-$1^{-/-}$ cells correlated with an absence of the corresponding enzyme activities, to ensure that DNA-PK activity was present in both the PARP-$1^{+/+}$ and PARP-$1^{-/-}$ cell lines and to ensure that PARP-1 activity was present in both the V3 and V3YAC cell lines (Fig. 2, *B* and *C*). DNA-PK activity was slightly higher in the V3YAC cell line (750 pmol ATP/mg protein) compared with the PARP-$1^{+/+}$ and PARP-$1^{-/-}$ cell lines (500 pmol ATP/mg protein for both cell lines; Fig. 2*B*). This may reflect overexpression of the human DNA-PKcs by the YAC. As expected, DNA-PK activity was undetectable in the V3 cells. PARP-1 activity was similar in all of the cell lines, apart from the PARP-$1^{-/-}$ cells, which retained 3.9 ± 0.5% residual activity compared with the PARP-$1^{+/+}$ cells (Fig. 2*C*). This residual activity is likely to be attributable to PARP-2, which, together with PARP-1, has also been implicated in BER (16).

Radiosensitization by AG14361 and NU7026. The effects of AG14361 and NU7026 alone and on IR-induced cytotoxicity were investigated in exponentially growing cell lines. Both PARP-1- and DNA-PK-deficient cells were ~4-fold more sensitive to IR than their proficient counterparts (note the different IR dose ranges in Fig. 3, *A versus B* and Fig. 3, *C versus D*).

Neither AG14361 (≤40 μM) nor NU7026 (≤50 μM), alone or in combination, reduced clonogenic survival (results not shown). The inhibitors were effective when added immediately before exposure to IR but for convenience were routinely added 1 h before irradiation. Both AG14361 and NU7026 potentiated IR in a concentration-depen-

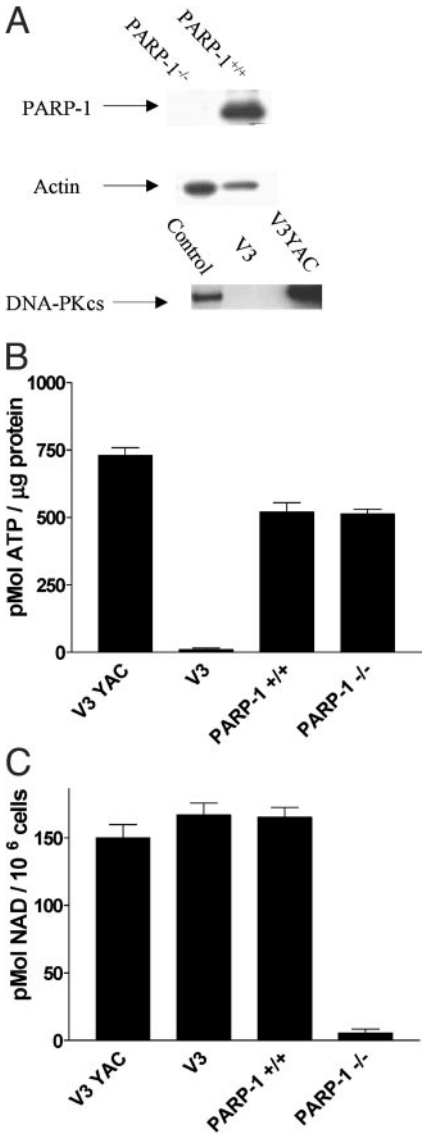

Fig. 2. Characterization of DNA-PK and PARP-1 levels and activities in the cell lines studied. *A*, Western blot analysis of PARP-1 and DNA-PK. Blots were probed using antibodies against PARP-1 and DNA-PKcs; *B*, DNA-PK activity; *C*, PARP-1 activity. Results are the mean of three replicate samples each from three independent experiments ± SE.

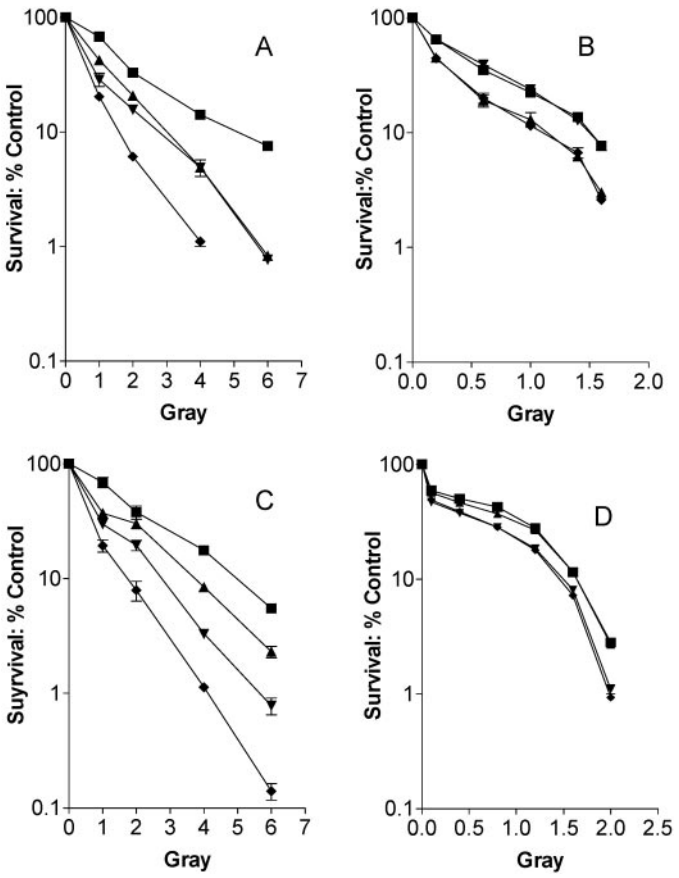

Fig. 3. Effects of increasing doses of IR in the presence or absence of AG14361 and NU7026 on the survival of exponentially growing: *A*, V3YAC cells; *B*, V3 cells; *C*, PARP-$1^{+/+}$ cells; *D*, PARP-$1^{-/-}$ cells; ■, IR alone; ▲, IR + AG14361; ▼, IR+ NU7026; ♦, IR + AG14361 + NU7026. Cells were preincubated with drug(s) for 1 h before exposure to IR, then incubated for 16-h post-treatment before reseeding for colony formation. Data are the mean of at least three independent experiments ± SE.

dent manner, and maximum potentiation was achieved at 10 μM NU7026 and 0.4 μM AG14361 (results not shown). Therefore, these concentrations were used in all subsequent experiments.

The inhibitors used alone, or in combination, potentiated the cytotoxicity of IR in the enzyme proficient exponentially growing V3YAC and PARP-$1^{+/+}$ cells (Fig. 3, *A* and *C*). PF_{90} values were calculated to quantitate the potentiating effects of the inhibitors alone, and in combination, on IR-induced cytotoxicity, and the results are summarized in Table 2. Approximately additive effects were obtained when the inhibitors were used together (Fig. 3, *A* and *C*), *e.g.*, the PF_{90} values for AG14361 and NU7026 in the V3YAC cell line were 1.4 ± 0 and 1.51 ± 0.04, respectively; when used in combination, the PF_{90} value was 2.78 ± 0.04 (Table 2). Significant radiosensitization by AG14361 was observed in all of the cell lines with PARP-1 activity (Fig. 3, *A–C*; PF_{90} values 1.3–1.4) but not in the PARP-$1^{-/-}$ cells (Fig. 3*D*). Similarly, NU7026 caused significant radiosensitization in the DNA-PK proficient cells (Fig. 3, *A*, *C*, and *D*; PF_{90} values 1.1–1.7) but not in the DNA-PK-deficient cell (Fig. 3*B*). These data support the hypothesis that NU7026 and AG14361 mediate potentiation specifically by inhibition of their target enzymes, DNA-PK and PARP-1, respectively.

Prevention of PLDR by AG14361 and NU7026. The effects of the inhibitors on PLDR were investigated. Cells were growth arrested by growing them to confluence. G_0 status was confirmed by flow cytometric analysis (results not shown). Cells were exposed to approximately equitoxic doses of IR (see details in Fig. 4 legend), such that the survival of cells which were immediately replated was ≤5%. After a 24-h delay in replating, which allows quiescent (G_0) cells to repair PLD, the surviving fraction of V3YAC and PARP-$1^{+/+}$ cells was increased 7–8-fold (Fig. 4, *A* and *C*). This recovery was reduced by ~70% by inclusion of AG14361 during the recovery period, whereas NU7026 alone completely abolished PLDR, and the combination of NU7026 and AG14361 reduced survival even further (Fig. 4, *A* and *C*). PLDR was reduced (2–3-fold) in both DNA-PK-deficient (V3) and PARP-deficient (PARP-$1^{-/-}$) cells, compared with their proficient counterparts, and this modest recovery was fully inhibited by AG14361 or NU7026, respectively (Fig. 4, *B* and *D*). As expected, no inhibition of PLDR was obtained by the use of NU7026 in the V3 cell line (Fig. 4*B*). However, when AG14361 was used in the PARP-$1^{-/-}$ cell line (Fig. 4*D*), there was a small, but significant, inhibition of PLDR. A possible explanation is that AG14361 may additionally inhibit PARP-2, which has also been implicated in BER (16).

Inhibition of DNA DSB Repair by AG14361 and NU7026. The kinetics of DNA DSB repair after exposure to IR over a 60-min time period were investigated. In the DNA-PK-proficient (V3YAC) cells, DNA DSBs were rejoined rapidly with ≥80% rejoined by 60 min (Fig. 5*A*). Very similar results were obtained with the PARP-$1^{+/+}$ cells (Fig. 5*B*). By contrast, in the V3 and PARP-$1^{-/-}$ cell lines, higher DNA DSB levels remained 60-min post-IR incubation with only ~50% DNA DSB rejoined (5, *A* and *B*).

To determine whether the inhibitors similarly modulated DNA DSB repair, DNA DSB levels were assessed in cells ± AG14361 and/or NU7026 at 60-min post-IR. (Fig. 6, *A–D*). In the V3YAC and PARP-$1^{+/+}$ cell lines, AG14361 and NU7026 inhibited DNA DSB repair, *e.g.*, in the V3YAC cell line, AG14361 and NU7026 inhibited repair by 40 and 56%, respectively, compared with the drug-free control. When the inhibitors were used in combination in the enzyme-proficient cell lines, repair was reduced by ≥90% (Fig. 6, *A* and *C*). In comparison, NU7026 or AG14361 in the PARP-$1^{-/-}$ or V3 cells, respectively, only reduced repair by 70%, suggesting that inhibition of both enzymes has a more profound effect on repair than inhibition of one enzyme combined with lack of the other enzyme. Finally, as expected, NU7026 and AG14361 exerted no additional inhibitory effect on the reduced DNA DSB rejoining observed in the V3 or PARP-1 cell lines, respectively (Fig. 6, *B* and *D*).

Finally, the longer term effects of the inhibitors on DNA DSB repair were investigated by assessing DNA DSB levels in V3YAC and V3 cells 24-h postirradiation (Fig. 6, *E* and *F*). By this time,

Table 2 *Comparison of the PF_{90} values[a] derived from IR survival curves*

Cell line/treatment	V3YAC	V3	PARP-$1^{+/+}$	PARP-$1^{-/-}$
IR + AG14361	1.4 ± 0.00	1.3 ± 0.01	1.37 ± 0.00	**1.03 ± 0.001**[b]
IR + NU7026	1.51 ± 0.04	**1.0 ± 0.00**[b]	1.69 ± 0.03	1.12 ± 0.01
IR + AG14361 + NU7026	2.78 ± 0.04	1.3 ± 0.03	2.81 ± 0.19	1.13 ± 0.00

[a] All mean PF_{90} values (±SE) were calculated from the ratio of the individual LD_{90} values, *i.e.*, LD_{90} divided by LD_{90} in the presence of inhibitor(s). The mean LD_{90} values for IR + inhibitor(s) were all significantly different from the mean LD_{90} values for IR alone in the same cell line ($P \leq 0.05$, $n = \geq 3$, two-tailed Student *t* test), excluding the LD_{90} values for IR + inhibitor in the cell line lacking its corresponding target enzyme (see below).

[b] The bold PF_{90} values, which are not significantly different from unity, demonstrate the lack of potentiation by the inhibitor in the cell line which lacks its corresponding target enzyme.

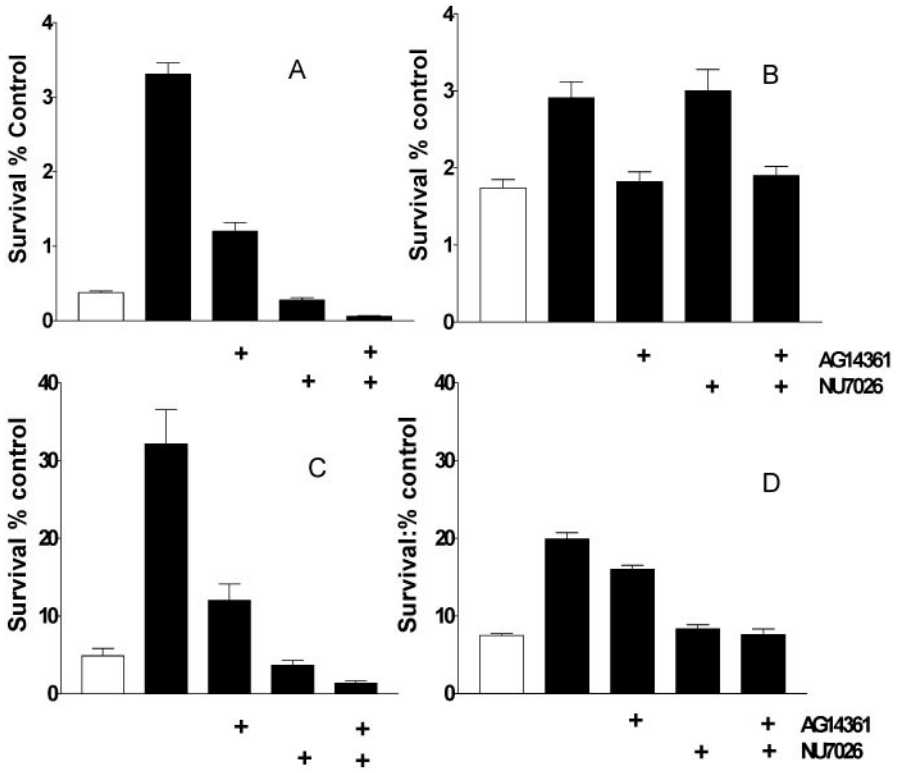

Fig. 4. Effects of AG14361 and NU7026 on recovery from IR-induced potentially lethal damage in growth-arrested cells. Cells were treated with approximately equitoxic doses of IR; 6 Gy for V3YAC, 1.4 Gy for V3, 5 Gy for PARP-$1^{+/+}$, and 1.2 Gy for PARP-$1^{-/-}$. The cells were either replated immediately for colony formation (*white bars*) or after a 24-h postincubation to allow PLDR (*black bars*). *A*, V3YAC; *B*, V3; *C*, PARP-$1^{+/+}$; *D*, PARP-$1^{-/-}$.

96 ± 3% of DNA DSBs were rejoined in the control V3YAC cells, compared with 7 ± 2% and 60 ± 7% when treated with AG14361 and NU7026, respectively (Fig. 6*E*). When AG14361 and NU7026 were used in combination, only 10 ± 2% of the breaks had rejoined. The V3 cell line was only slightly less proficient than the V3YACs at rejoining DNA DSBs by 24 h (77 ± 3%), and this was further reduced by the inclusion of AG14361, whereas NU7026 exerted no additional effect.

DISCUSSION

Here, we describe the effects on cellular responses to IR produced by novel specific inhibitors of the repair enzymes PARP-1 and DNA-PK. We demonstrate that these compounds radiosensitize both proliferating and quiescent cells to IR and inhibit DNA DSB repair. Furthermore, when AG14361 and NU7026 are used in combination, their effects on all these biological endpoints are at least additive. In the survival experiments, cells were incubated for 16 h (approximately one cell cycle) after IR in the presence of the inhibitors before replating for colony formation. Additional experiments are required to determine the minimum exposure time to inhibitors necessary to obtain maximum potentiation. Use of the inhibitors in the paired cell lines, proficient or deficient for DNA-PK and PARP-1, has provided confirmation that the inhibitors mediate their effects on IR-induced cytotoxicity and DNA DSB repair specifically via inhibition of their target enzymes. Additionally, the radiosensitization and reduced DNA DSB repair observed in the deficient cell lines were mimicked by the use of the inhibitors in the proficient cell lines. The effect of enzyme inhibition or deficiency on cell survival closely paralleled the effects on DNA DSB repair, and hence, DNA DSB repair inhibition is a plausible mechanism for the radiosensitization observed.

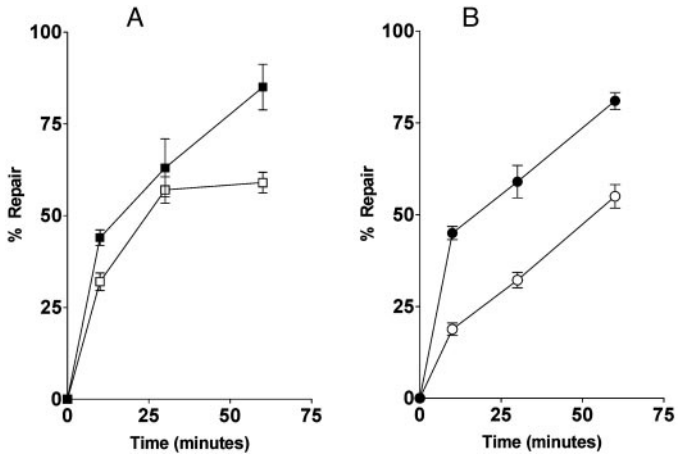

Fig. 5. Kinetics of DNA DSB repair after a fixed dose of IR. *A*, ■ V3YAC cells; □ V3 cells; *B*, ● PARP-$1^{+/+}$ cells; ○ PARP-$1^{-/-}$ cells. Cells were exposed to 75 GY IR and postincubated for increasing amounts of time before being harvested for neutral elution.

Ideally, paired cell lines should be isogenic, differing only in the gene of interest. However, this is rarely the case, and it was established during the course of this work that, in contrast to the PARP-$1^{-/-}$ MEFs, which had retained wild-type p53 after spontaneous immortalization, the PARP-$1^{+/+}$ MEFs had acquired a p53 mutation in the DNA binding domain and did not induce mdm2 in response to IR.[4] In consideration of the potential clinical use of DNA-PK and PARP-1 inhibitors, it is pertinent to point out that both inhibitors were able to cause radiosensitization in this cell line, despite the lack of a functional p53. This ability to radiopotentiate cells, regardless of their p53 status, increases the range of tumors for which the use of the inhibitors may be effective.

The observation that both NU7026 and AG14361 not only radiosensitize proliferating cells but also prevent PLDR in quiescent cells is very important. PLDR is a significant factor in determining tumor responses to radiotherapy (reviewed in Ref. 41). Evidence for PLDR in the nonproliferating compartment of tumors is very sparse, but

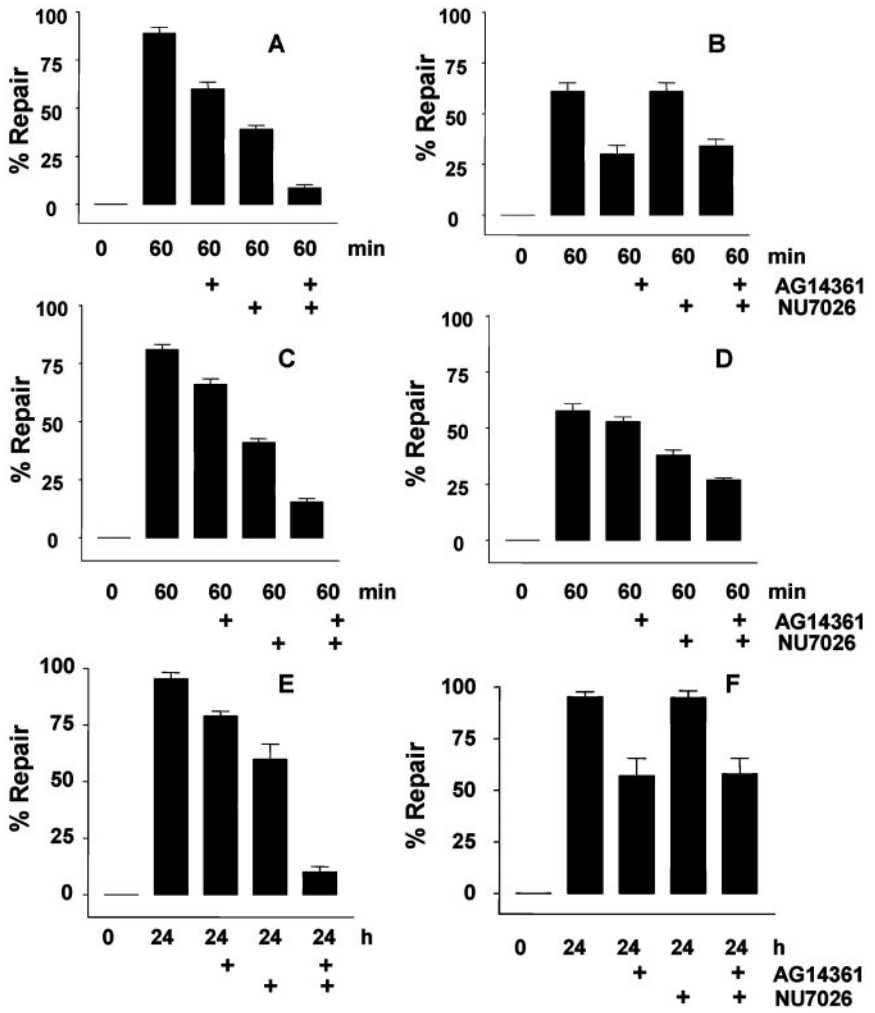

Fig. 6. Effects of AG14361 and NU7026 on DNA DSB repair. *A*, V3YAC cells; *B*, V3 cells; *C*, PARP-$1^{+/+}$ cells; *D*, PARP-$1^{-/-}$ cells. Cells were preincubated with inhibitor(s) for 60 min, exposed to 75 GY IR, and postincubated for 60 min before harvesting for neutral elution (*A–D*) or exposed to 75 GY and postincubated for 24 h. *E*, V3YAC cells; *F*, V3 cells.

experimental and clinical observations suggest that the rapid repopulation after IR-induced killing of the proliferating compartment of tumors results from recruitment of surviving G_0 cells into the proliferative cycle.

Both inhibitors used alone substantially prevented PLDR. More dramatically, when used in combination, AG14361 and NU7026 not only completely abolished PLDR but reduced survival significantly below that obtained in cells exposed to IR alone and replated immediately (*i.e.*, no 24-h recovery period), *e.g.*, when quiescent V3YAC cells were irradiated, survival was reduced to 0.38% if the cells were replated immediately. Survival was increased to 3.3% by a 24-h delay in replating, but the presence of AG14361 and NU7026 during the 24-h delay reduced survival to 0.064%, even lower than the cells which were replated immediately post-IR. This extremely potent radiosensitization (~50-fold) of nonproliferating cells by the combined use of the NU7026 and AG14361 is one of the major aims of radiobiologists and radiotherapists alike. Current interest in clinical rediosensitization has focused on hypoxic radiosensitizers and chemical radiosensitizers, such as the halogenated pyrimidine analogues (42–44), whereas the potential of PLDR inhibitors has largely been ignored. These data point to inhibition of PLDR as a tool for clinical radiosensitization that merits further investigation.

PARP-1 inhibition clearly results in higher levels of IR-induced DNA DSBs, but these data do not prove a direct role for PARP-1 in NHEJ. IR-induced clustered damage involves, *e.g.*, near neighbor base oxidation and hydrolysis of the phosphodiester backbone on opposite DNA strands (45). BER at these sites will generate DNA SSB intermediates. Absence or inhibition of PARP-1 by preventing BER-mediated strand rejoining by ligase IV (46, 47) may result in the longer lived DNA SSBs converting to DNA DSBs, rather than by directly inhibiting NHEJ.

A number of lines of evidence indicate that although DNA-PKcs highly stimulates the NHEJ pathway, in its absence, a slower repair pathway still operates (9, 48). The slower component of repair is probably mediated by DNA-PKcs-independent NHEJ but may also involve HRR, which requires a complex of DNA repair proteins, including RAD51, BRCA1, BRCA2, XRCC2, and ATM (1). Both the slow component of NHEJ and HRR are considered to act independently of DNA-PKcs and PARP-1. It was therefore a striking observation that even 24-h postirradiation, the use of the inhibitors resulted in a reduction in the percentage of DNA DSBs rejoined. In particular, a profound effect on DNA DSB repair was observed in the V3YAC cells with the combined use of the inhibitors at 24 h, where only ~10% of DNA DSBs had rejoined, compared with >95% of the control (Fig. 6*A*). DNA DSBs are considered to be the most cytotoxic lesion that cells encounter, and this almost complete and long-term abrogation of DNA DSB repair by the combined use of the inhibitors would easily explain their potent radiosensitizing effects in both proliferating and growth arrested cells. These data also indicate that the combined inhibition of PARP-1 and DNA-PK has downstream effects on the slow component of DNA DSB repair. One possible mechanism, which is suggested by the data, is that the inhibited

enzymes have more deleterious consequences for DNA DSB repair (and hence survival) than the lack of enzymes. DNA-PK and PARP-1 bind avidly to DNA DSBs, and automodification by phosphorylation and poly(ADP-ribosylation), respectively, is essential for dissociation of these enzymes from the DNA (49, 50). Furthermore, Wortmannin has been demonstrated to block DNA-PK at DNA ends and prevents their processing by either DNA polymerization, degradation, or ligation (51). Similarly, NU7026 and AG14361, by inhibiting the automodification reactions of their target enzymes, are predicted to tether the enzymes irreversibly to the DNA ends. These protein-bound DNA termini could hinder assembly of the enzyme complexes required for the successful execution of NHEJ and HRR.

A number of publications demonstrate interaction and/or cooperation between DNA-PK and PARP-1. Both enzymes have been shown to have high affinities for binding to DNA DSBs (52). Modulation of enzyme activity by poly(ADP-ribosylation) of DNA-PK by PARP-1 and phosphorylation of PARP-1 by DNA-PK has also been demonstrated, suggesting reciprocal regulation of enzyme activity (53, 54). Finally, an intimate association between PARP-1 and Ku has been demonstrated in coimmunoprecipitation studies (55). These observations point to an, as yet, poorly understood functional association of the two enzymes in response to DNA damage.

Increased DNA-PK activity has been widely demonstrated both *in vitro* and *in vivo* and correlates with the resistance of tumor cells to IR and bifunctional alkylating agents (56–59). Conversely, acquired resistance to chemotherapeutic agents has been shown to correlate with increased DNA-PK activity (60–62). Therefore, increased DNA-PK activity has been proposed as a novel cellular and tumor resistance mechanism. Such tumor-specific alterations are likely to be important in terms of therapeutic exploitation. Where DNA-PK is overexpressed, the use of a selective inhibitor is predicted to allow treatment of these tumors which would otherwise be radio and chemoresistant. The effects of NU7026 in model systems with increased DNA-PK activity merits further investigation. Whether there will be a therapeutic gain associated with the use of DNA-PK and PARP-1 inhibitors remains to be established, and an *in vivo* evaluation using human tumor xenografts in nude mice is planned.

In four recent reports, antisense oligonucleotides, small interfering RNAs, and a COOH-terminal peptide which targets Ku80 and prevents DNA-PKcs binding to Ku have been used to selectively deplete or inhibit DNA-PK function in human cell lines (63–66). Loss of DNA-PK activity correlated with radiosensitization, increased mutation, and inhibition of DNA damage repair. Our results, the first to use a selective small molecule inhibitor of DNA-PK, are consistent with these data which highlight the current high interest in this enzyme as a target for radio and chemotherapeutic modulation. A PARP-1 inhibitor is currently entering Phase I clinical trials under the auspices of Cancer Research United Kingdom, and the evidence presented here indicates that the combination of this inhibitor with a DNA-PK inhibitor could prove a powerful chemotherapeutic strategy.

In conclusion, the data presented here suggest that pharmacological inhibition of DNA-PK and PARP-1, both alone and in conjunction, represents a promising strategy for tumor radiosensitization.

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Radiosensitization and DNA Repair Inhibition by the Combined Use of Novel Inhibitors of DNA-dependent Protein Kinase and Poly(ADP-Ribose) Polymerase-1

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