Radiosensitization by the poly(ADP-ribose) polymerase inhibitor 4-amino-1,8-naphthalimide is specific of the S phase of the cell cycle and involves arrest of DNA synthesis

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Abstract
Radiosensitization caused by the poly(ADP-ribose) polymerase (PARP) inhibitor 4-amino-1,8-naphthalimide (ANI) was investigated in 10 asynchronously growing rodent (V79, CHO-Xrs6, CHO-K1, PARP-1+/+ 3T3, and PARP-1−/− 3T3) or human (HeLa, MRC5VI, IMR90, M059J, and M059K) cell lines, either repair proficient or defective in DNA-PK (CHO-Xrs6 and M059J) or PARP-1 (PARP-1−/− 3T3). Pulse exposure to ANI (1-hour contact) potentiated radiation response in rodent cells except in PARP-1−/− 3T3 fibroblasts. In contrast, ANI did not significantly enhance radiation susceptibility in asynchronously dividing human cells; yet, single-strand break rejoining was lengthened by ca. 7-fold in all but mouse PARP-1+/+ 3T3s. Circumstantial evidence suggested that radiosensitization by ANI occurs in rapidly dividing cells only. Experiments using synchronized HeLa cells consistently showed that ANI-induced radiosensitization is specific of the S phase of the cell cycle and involves stalled replication forks. Under these conditions, prolonged contact with ANI ended in the cell cycle and involves stalled replication forks. Under these conditions, prolonged contact with ANI ended in arrest of DNA synthesis. De novo DNA double-strand breaks hours after irradiation, evoking collision with uncontrolled replication forks of DNA lesions whose repair was impaired by inhibition of the PARP catalytic activity. The data suggest that increased response to radiotherapy by PARP inhibitors may be achieved only in rapidly growing tumors with a high S-phase content. [Mol Cancer Ther 2006; 5(3):564–74]

Introduction
Poly(ADP-ribose) polymerases (PARP) are present in higher eukaryotes only (for a review, see ref. 1). PARP-1, the founding family member, has been the most extensively studied. PARP-1 is a nuclear 113-kDa enzyme that detects and binds DNA single-strand breaks (SSB) through a zinc-finger domain located at its NH2-terminal end (2). PARP-1 is involved in many important cellular pathways, including early DNA damage recognition (3), base excision repair (BER; ref. 4), replication (5, 6), transcription (7), and cell death (8, 9). Accordingly, PARP-1 null mice and cells display increased genomic instability and are extremely sensitive to ionizing radiation (5, 10, 11).

PARP-1 activation is initiated by binding to SSB (12, 13) and triggers synthesis and transfer of ADP-ribose polymers on various nuclear proteins associated with chromatin, including PARP-1 (2). A role for PARP-1 in the repair of DNA double-strand breaks (DSB) has also been suggested (14, 15). However, PARP-1−/− cells display normal DSB repair through nonhomologous end joining as well as homologous recombination (16), and it has been shown that PARP-1 is not involved in recovery from DSB induced by γ-rays (17), etoposide (18), or neocarzinostatin (19); yet, post-transcriptional ADP-riboseylation of some repair proteins affects their DNA-binding ability and catalytic activity (6).

Numerous PARP inhibitors have been identified. Most of them are derived from the benzamide structure and act through competitive inhibition of the catalytic domain of the enzyme (20, 21). PARP inhibitors are currently developed to reduce inflammatory disease or reperfusion injury, or to potentiate the cytotoxic effect of ionizing radiation or antitumor drugs, such as temozolomide and camptothecin derivatives (22). PARP inhibitors have been reported to act as radiosensitizers in rodent cells, but their effect against human cells was marginal only (23–33). To elucidate the origin of such differential radiosensitization, repair-deficient cells and their repair-proficient parents in the PARP-1 (PARP-1−/− versus PARP-1+/+ mouse 3T3 fibroblasts), Ku86 (CHO-Xrs6 versus CHO-K1, Chinese hamster), and DNA-PKcs (M059J versus M059K, human) pathways were assayed for radiation survival in the absence and presence of 4-amino-1,8-naphthalimide (ANI), a well-documented, potent PARP inhibitor (21, 34, 35).
Nontransformed (IMR-90) and SV40-transformed (MRC5VI) human fibroblasts, Chinese hamster V79 fibroblasts, and asynchronous or synchronized human HeLa cells were also used. The results suggest that radiosensitization by ANI depends primarily on the percentage of cells engaged in DNA replication. Indeed, in synchronized HeLa cells, radiosensitization by ANI was found to occur specifically in the S phase of the cell cycle, in agreement with the known role of PARP-1 in the control of replication forks.

**Materials and Methods**

**Reagents**

Products and their suppliers were as follows: [2-14C]thymidine and [methyl-3H]thymidine (Amersham Biosciences, Orsay, France); agarose and molecular weight markers for pulsed-field gel electrophoresis (Bio-Rad, Hercules, CA); 4,6-diamidino-2-phenylindole, tetrapropylammonium hydroxide and proteinase K (Sigma-Aldrich Chemicals, Saint Quentin Fallavier, France); ANI (ACROS Organics, Geel, Belgium); other chemicals and solvents (Merck, Darmstadt, Germany); polycarbonate filters (Nuclepore, 2.0 μm pore size; Whatman, Banbury, Oxon, United Kingdom); rabbit polyclonal anti-poly(ADP-ribose) antibody (Alexis Biochemicals, San Diego, CA); mouse monoclonal anti-human PARP-1 antibody (clone 4C10-5; Becton Dickinson, Le Pont de Claix, France); TRITC-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, Soham, Cambridgeshire, United Kingdom); products for cell culture and Alexa 488–conjugated goat anti-mouse IgG (Invitrogen, Cergy Pontoise, France).

**Cells and Cell Cultures**

Spontaneously, immortalized mouse embryo fibroblasts (3T3) from PARP-1+/− and knockout PARP-1−/− mice (11) were provided by Dr. G. de Murcia (UPR 9003 Centre National de la Recherche Scientifique, ESBS, Illkirch, France). Human cervix carcinoma HeLa cells were a gift from Dr. J. Coppey (Institut Jacques-Monod, Paris, France). MRC5VI, a SV40-transformed cell line derived from normal human fibroblasts, and the Chinese hamster ovary (CHO) Xrs6 mutant were provided by Dr. P.A. Jeggo (Genome Damage and Stability Centre, University of Sussex, Brighton, United Kingdom). Xrs6 cells carry the Ku86 gene deletion on one allele and the Ku70 gene deletion on the other (36, 37). The parental Chinese hamster CHO-K1 cell line (ATCC CCL-61) and normal human embryonic IMR-90 fibroblasts (ATCC CCL-186) were purchased from American Type Culture Collection (Rockville, MD). Chinese hamster embryonic lung V79 fibroblasts (clone 79-1/379A) came from Flow Laboratories (Irvine, Scotland). Human glioblastoma M059J and M059K cells were provided by Dr. J-P. de Villartay (INSERM U 429, Hôpital Necker, Paris) on behalf of Dr. J. Turner (Cross Cancer Institute, University of Alberta, Edmonton, Canada). M059J cells do not express the catalytic subunit of the DNA-dependent protein kinase, DNA-PKcs (38). None of the cell lines used bears a mutation on BRCA1 or BRCA2.

All cell lines were maintained as exponentially growing monolayers, either in α-MEM supplemented with nonessential amino acids (CHO-K1), or in DMEM (all other cell lines), with 10% or 15% (IMR-90) FCS and 5% (3T3) or 7% CO2 (all other cell lines) in air.

Synchronization of HeLa cells at the G1-S junction was achieved using a double thymidine block (39). Cell cycle progression was monitored by dual-variable flow cytometry using a FACStarPLUS cytofluorometer (Becton Dickinson) with pulse bromodeoxyuridine labeling (10 μmol/L, 15 minutes) of S-phase cells as described (40).

**Clonogenic and Growth Assays**

For colony formation assays, enough cells from mid-log growing subcultures were plated in triplicate at constant density in 25-cm² vented flasks to obtain ca. 500 colonies in untreated samples and incubated at 37°C for 4 hours to allow cell adhesion before treatment. Following radiation and/or drug exposure, the flasks were rinsed with HBSS and returned to fresh medium for 7 to 11 days. Colonies were fixed with methanol, stained with Coomassie blue R-250, and scored visually under lens magnification. Colonies of ≤50 cells were disregarded. The intra-assay variability was ≤10%.

For growth assays (IMR-90, PARP-1+/+ 3T3, and PARP-1−/− 3T3), 10⁵ cells were plated in duplicate or triplicate and incubated overnight. After treatment, cells were rinsed twice with HBSS and returned to fresh medium for five doubling times. At that time, cells were harvested by 0.05% trypsin/0.02% EDTA, pelleted, resuspended in medium, and scored under microscope in a Malassez cuvette.

In experiments using synchronized HeLa cells, the cellular multiplicity [i.e., the number of cells (n) per potential colony-forming unit] was measured by digital microscope examination of the culture flasks at the time of treatment. The single-cell surviving fraction (SCSF) was subsequently calculated using the discrete distribution equation (41):

\[ S_{exp} = \sum_{i=1}^{n} \frac{a_i}{n} \left[ 1 - (1 - SCSF)^i \right] \]  

where \( S_{exp} \) is the experimental cell survival determined from bulk colony scoring and \( a_i \) is the fraction of colony-forming units containing \( i \) cells.

**Radiation and Drug Treatment**

γ-Ray irradiation of cells was done at room temperature in culture medium, using an IBL-637 (137Cs) irradiator (CIS Biointernational, Saclay, France) at 1.0 or 56.6 Gy/min. Aliquots of ANI (30 μmol/L, final concentration) were introduced at the time required from a 3 mmol/L stock solution in pure DMSO. The DMSO concentration in medium was 1%. Controls were made with DMSO alone at the same and twice the same concentration.

Radiation survival (S) determined from clonogenicity or growth assays was fitted to either one of the following equations:
where $D$ is the radiation dose. For each experiment, the mean lethal dose $D_{37}$ (i.e., the dose of radiation leaving $1/e = 0.37$ survival) was calculated from the curves drawn for best fit to the experimental data.

**Determination of PARP-1 Activity by Immunofluorescence**

For each cell line, immunodetection of poly(ADP-ribose) was carried out to determine the amount of ANI necessary to reach inactivation of PARP-1. Cells were grown for 48 hours on coverslips and exposed to H$_2$O$_2$ (1 mmol/L, 10 minutes, 37°C) with or without prior introduction of ANI. After fixation and Triton X-100 permeation, cells were incubated with an anti-poly(ADP-ribose) primary antibody (1:200 dilution), rinsed, incubated with an Alexa 488- or tetramethylrhodamine isothiocyanate–conjugated secondary antibody (1:500 dilution), and finally counterstained with 4,6-diamidino-2-phenylindole. Immunofluorescence was visualized using an Axiophot microscope (Carl Zeiss, Jena, Germany) equipped with a Micromax chilled camera (Princeton Applied Research, Oak Ridge, TN).

**Analysis of SSB Repair by Alkaline Filter Elution**

Radiation-induced SSBs were measured by alkaline filter elution over polycarbonate filters according to Filipinski and Kohn (42). This method allows titration of direct SSB, alkali-labile, and abasic sites altogether. Cells were grown in the presence of [2,14C]thymidine (0.02–0.06 Ci/ml) for two doubling times. Radioactive thymidine was removed 3 hours before treatment to allow ligation of Okasaki fragments. The culture flasks were given 5 Gy and returned to the incubator for varying times (up to 60 minutes) without or with ANI. When present, ANI (30 μmol/L) was introduced 1 hour before radiation and was present for the whole duration of post-irradiation incubation. The medium was removed at the end of the incubation period; the flasks were rapidly rinsed once with ice-cold PBS and maintained on ice for no more than 10 minutes before collecting cells. Cells were harvested with the aid of a cell scrapper, and ca. 6 × 10⁷ cells were deposited onto polycarbonate filters and lysed, and DNA fragments were eluted as described (43). [Methyl-³H]thymidine-labeled cells (2 × 10⁸) that had received 3 Gy of γ-rays in ice were used as internal standards. DNA retention was calculated relative to 50% retention of the internal standard (42, 43).

**Determination of DSB by Pulsed-Field Gel Electrophoresis**

Clamped homogeneous electrical field electrophoresis allows the separation of DNA molecules up to 10 Mbp. The fraction of DNA fragments of this size is correlated with the number of DSB induced at random in chromosomes by radiation. Clamped homogeneous electrical field is therefore suitable for the determination of DSB in mammalian cell DNA. However, the sensitivity of the method is low, such that large doses of radiation must be applied for quantitative analysis of DSB formation and rejoining. The results are usually expressed as the fraction of activity released [i.e., the fraction of cell DNA (≤10 Mbp) migrating out of the plugs].

Cells for pulsed-field gel electrophoresis were prepared according to Stenèrlov et al. (44). In this method, embedded cells are lysed in the cold to prevent the conversion of abasic sites BD and SSB into DSB. Briefly, cells grown with [2,14C]thymidine as above were rinsed twice with HBSS, fed with fresh medium, and synchronized at the G$_1$-S junction with a double-thymidine block (see above). ANI (30 μmol/L) was introduced 1.5 hours after thymidine block release. After 30 minutes, cells were given 20 or 80 Gy or mock-irradiated and returned to the incubator for varying times in the presence of ANI. The flasks were finally washed with ice-cold PBS, and cells were harvested by gentle scraping in ice-cold 2 mmol/L EDTA (pH 8). DNA was resuspended in 160 μL of PBS buffer at 37°C and mixed under mild vortexing with an equal volume of 1.6% w/v low melting point agarose (Bio-Rad) maintained in PBS buffer at 37°C. The suspension was immediately pipetted into prechilled 4 × 10-mm-wide moulds and allowed to form plugs on ice for 30 minutes. Embedded cells were subsequently lysed in buffer containing 0.5 mol/L EDTA, 2% N-lauroylsarcosine, and 1 mg/mL protease K (pH 8) for 24 hours at 4°C. The plugs were rinsed twice with 1 mmol/L EDTA, 10 mmol/L Tris-HCl (pH 8, 30 minutes, 4°C) and incubated (48 hours, 4°C) in buffer (pH 7.5) containing 1.85 mol/L NaCl, 0.15 mol/L KCl, 5 mmol/L MgCl₂, 2 mmol/L EDTA, 4 mmol/L Tris, and 0.5% Triton X-100. The plugs were finally washed with PBS and stored in 40 mmol/L EDTA buffer at 4°C for no more than 10 days before electrophoresis.

The plugs were inserted into the wells of an 0.8% w/v agarose gel made in TAE buffer [40 mmol/L Tris-acetate, 2 mmol/L EDTA (pH 8)], and the gels were submitted to pulsed-field gel electrophoresis at 14°C in a CHEF-DR III apparatus (Bio-Rad) with buffer recirculation. Migration (2 V/cm) was done using three blocks, 24 hours each, with switch times (angles) of 20 minutes (96 degrees), 25 minutes (100 degrees), and 30 minutes (106 degrees), respectively. The molecular weight markers used (Bio-Rad) were Schizosaccharomyces pombe (3.5–5.7 Mbp), Hansenula wingei (1.0–3.1 Mbp), and Saccharomyces cerevisiae DNA (0.2–2.2 Mbp).

After electrophoresis, the gels were stained with 1 μg/mL ethidium bromide under mild agitation (30 minutes) followed by destaining for 1 hour in buffer. DNA fluorescence was visualized over an UV transilluminator with camera recording. The gels were subsequently dried in vacuo over a piece of Whatman paper, and the radioactivity profiles were determined using a Phosphorimager apparatus and ImageQuant densitometry software (Amersham Biosciences) to determine the fraction of activity released.
Measurement of DNA Synthesis in Synchronized HeLa Cells

HeLa cells were synchronized at the G1-S junction using a double-thymidine block (see above). Cells were initially plated at a suitable density to obtain as close to 2 × 10^6 cells as possible at the time of the second thymidine block release. After release from the thymidine block, cells were allowed to progress in S phase for 1.5 hours; 1% DMSO or 30 μmol/L ANI was then introduced and cells irradiated or sham-irradiated 30 minutes later. DMSO or ANI was present for up to the end of the experiment (up to 8 hours after thymidine block release). At the time desired, [methyl-^3H]thymidine (0.2 μCi/mL) was introduced without medium change, and cells were returned to the incubator for 15 minutes. The flasks were carefully washed with warm HBSS, fed with fresh medium, returned to the incubator for 15 minutes, washed again twice with PBS buffer, and finally lysed in 1 mL lysis buffer [50 mmol/L Tris, 25 mmol/L EDTA, 0.1 mol/L glycine, 2% lauryl sulfate (pH 9.7)]. The lysates were transferred to scintillation vials and counted in a WinSpectral 1414 counter (Perkin-Elmer Wallac, Orsay, France).

Results

Assay of Experimental Conditions

Immunofluorescence detection of H2O2-induced elongation of poly(ADP-ribose) was used for assays of PARP-1 inhibition. As expected (21, 34), 30 μmol/L ANI was sufficient to abolish PARP-1 activation (Fig. 1) and was consistently used throughout.

Radiosensitization of Asynchronous Cells by ANI Occurs in Rodent Cells Only

To determine whether inhibition of the catalytic activity of PARP-1 in the various cell lines of interest resulted or not in enhanced cell kill by radiation, the response of asynchronous cells was investigated through a range of radiation doses in the presence of 30 μmol/L ANI and compared with that of drug-free cultures incubated with an equal amount of DMSO for the same length of time. When present, ANI was introduced 30 minutes before irradiation and removed 30 minutes later. The effect of DMSO on radiation response was carefully investigated, because DMSO may induce radioprotection (45, 46) or counteract the response to PARP inhibitors (47). Altered radiation, susceptibility by 1% DMSO was similar in all cell lines and resulted in an increase of the D37 value (see Materials and Methods) by a factor of 1.33 ± 0.15 (data not shown).

Survival curves without or with ANI (Fig. 2) were generated for the 10 cell lines used in this study and adjusted to the most appropriate mathematical model, depending on whether the radiation response curve fitted an exponential (Eq. B) or a linear-quadratic dose dependence (Eq. C; see Materials and Methods). With the exception of PARP-1−/− 3T3 fibroblasts, ANI acted as a radiosensitizer in rodent cells as determined by the increase of the initial slope (α) of the survival curve, paralleling a decrease of the mean lethal dose (D37). In contrast, no significant increase of radiosensitivity was found in human cells (Fig. 3).

ANI Lengthens SSB Rejoining

The rejoining of radio-induced SSB without and with ANI was investigated by alkaline elution in 5 of the 10 cell lines studied above. The results are shown in Fig. 4. The curves generated from the experimental data determined two groups: PARP-1−/− 3T3 fibroblasts on one hand and MRC5VI, HeLa, V79, and PARP-1+/+ 3T3s on the other hand. The latter four were nearly indistinguishable in their capacity to rejoin SSB in the absence of ANI, and ANI considerably retarded SSB rejoining in those cells. PARP-1−/− 3T3s unexpectedly developed an intermediate capacity of SSB rejoining (Fig. 4).

Radiosensitization by ANI Occurs Specifically in the S Phase

Rapidly dividing cells only responded to ANI (Fig. 5). This prompted us to determine whether a particular compartment of the cell cycle was involved in the radiosensitizing
Radiosensitization by PARP Inhibitors

ANI Elicits Differential Radiosensitization in Asynchronous Rodent and Human Cells

The results show that in asynchronous cultures, ANI delays SSB repair in all but 3T3 PARP-1−/− defective cells and elicits radiosensitization in rodent, PARP-1−/− proficient cells only. It was earlier reported that PARP inhibitors may suppress potentially lethal damage repair in CHO cells but do not affect the radiosensitivity of human diploid mock treated or exposed to radiation, ANI, or a combination of both (Fig. 8). Maximum incorporation of radioactive thymidine in untreated cells was at 3 to 4 hours after release from the G1–S block. ANI alone did not alter thymidine incorporation and transit to G2. Radiation slowed down DNA synthesis and delayed transit to G2, as expected (Fig. 9). When present, ANI blocked the incorporation of thymidine and progression to G2 in irradiated cells.

De novo Formation of DSB Occurs Long after Radiation in Cells Irradiated in S Phase in the Presence of ANI

To extend these observations, the effect of ANI on the rejoining of radio-induced DSB in the S phase was determined in synchronized HeLa cells that had been prelabeled with [2-14C]thymidine before synchronization. Cells were exposed to radiation without or with ANI and allowed to perform DNA repair for varying times before harvest, and DSB was analyzed by pulsed-field gel electrophoresis. DSB repair took place during the first hour of post-irradiation incubation (Fig. 10), consistent with the fact that DSB rejoining does not require the presence or activity of PARP-1 (17–19). After 3 hours, however, ANI caused accumulation of a large excess of DSB evoking conversion into frank DSB of DNA lesions whose repair was impaired by ANI.

Discussion

ANI Elicits Differential Radiosensitization in Asynchronous Rodent and Human Cells

The results show that in asynchronous cultures, ANI delays SSB repair in all but 3T3 PARP-1−/− defective cells and elicits radiosensitization in rodent, PARP-1−/− proficient cells only. It was earlier reported that PARP inhibitors may suppress potentially lethal damage repair in CHO cells but do not affect the radiosensitivity of human diploid
An extensive analysis of the literature (23–32) confirms that in asynchronous cultures, only rodent cells experience increased radiation susceptibility by PARP inhibitors.

**May Differences in the BER Efficiency Explain Differential Radiation Response in Asynchronous Human versus Rodent Cells?**

Could differences between rodent and human cells in DNA repair pathways account for this observation? Of note, PARP-1 is mandatory to BER at a DNA synthesis step (48). Actually, BER requires physical association between PARP-1 and XRCC1 and DNA ligase III and DNA polymerase $\beta$ (4, 49, 50). Therefore, a defect in any of these components is likely to alter the BER efficiency. It has been recently shown that the repair of a subset of radiation-induced BD is >3-fold as slow in human as in rodent cells (51). Likewise, the expression level of DNA polymerase $\beta$ is 3- to 5-fold as low in human as in rodent cells (51), whereas the activity of polymerase $\beta$ may be a rate-limiting step in the BER process (52). It might thus be proposed that the efficiency of BER is lower in human than in rodent cells in such a way that PARP inhibitors would not impair very much radiation recovery in human cells. However, this explanation does not hold because the analysis of SSB repair by alkaline elution shows that at least from the kinetic point of view, SSB rejoining is as efficient in human as in rodent cells and is nearly equally affected by ANI in PARP-1-proficient cells (Fig. 4). It was also reported that in the absence of DNA polymerase $\beta$, overlapping pathways may allow to compensate this defect (53). Such mechanisms could develop in PARP-1-defective cells. Actually, PARP-1$^{-/-}$ proved able to repair radio-induced SSB more rapidly than PARP-1$^{-/-}$ 3T3s in the presence of ANI and were also slightly sensitive to the inhibitor (Fig. 4). This suggests that in the clonal isolate of PARP-1$^{-/-}$ 3T3s used, another mechanism is favored by the absence of PARP-1 and substitutes in part for the BER defect (54).

**Arrest of Replication Parallels Radiosensitization by ANI**

Using synchronized HeLa cells, we show that radiosensitization by ANI occurs specifically in the S phase and involves profound depression of DNA synthesis. Recently, Horton et al. (55) showed that ANI-induced sensitization to radiation of...
the cytotoxic effect of the SSB inducer, methyl methane sulfonate also requires exposure during the S phase of the cell cycle and leads to arrest in the S phase as long as ANI is present. These observations are fully consistent with the known role of PARP-1 in the assembly of the DNA synthesome and control of the replication forks (5, 6, 56, 57). In agreement with these observations, we tentatively propose that the lethal effect of ANI in cells irradiated during the S phase.
phase results from the collision of unrepaired SSB with uncontrolled replication forks. This hypothesis is supported by three lines of evidence. (a) PARP-1–defective mouse 3T3s are unable to reactivate stalled replication forks and progress into G2 after contact with hydroxyurea (16), yet they exhibit a full DSB repair capacity (16, 19). (b) A role for PARP-1 in triggering recombination for replication restart has recently been suggested (54). (c) Finally, de novo formation of a large amount of DSB is observed long after irradiation (Fig. 10). Whether this occurs through loss of PARP-1–dependent control of the activity of topoisomerase I in response to DNA damage (16, 58, 59) is open to question.

Are PARP Inhibitors Able to Potentiate Antitumor Radiotherapy?

With very few exceptions, radiosensitization proceeds from altered DNA repair, whereas the intrinsic radiosensitivity depends to a large extent on the phase of the cell cycle. To discriminate between these two mechanisms, it is wise to avoid major cell cycle redistribution, hence to adjust the length of drug exposure to the rate of the repair process of interest. We first used a short length of contact with ANI (1 hour) because PARP-1 was known to be involved in BER, and that this process is rapid (1, 60). Under these conditions, the radiosensitizing effect of ANI is specific of the S phase of the cell cycle (Fig. 6). Increasing the length of contact with ANI further leads to almost complete cell death in S-phase synchronized cultures (Fig. 7). In asynchronous cells, the rate of SSB rejoining is reduced by a factor of ca. 7 in the presence of ANI (Fig. 4), but repair is still efficient enough to remove SSBs as cells are irradiated long before the S phase. The cumulated length of the G2-M and G1 phases in human cells [from 71% (HeLa) to 87% (M059K) of the bulk doubling time] reduces the chance that asynchronous growing human cells are irradiated during the S phase and provides enough time for PARP-independent repair to reach completion before entry into S phase. We propose, therefore, that the differential effect of PARP inhibitors in V79 and CHO versus human cells is due to

Figure 9. Cell cycle progression in synchronized HeLa cells irradiated without or with ANI in the S phase. Cells were labeled with bromodeoxyuridine and exposed or not to ANI (30 μmol/L, 1% DMSO) 2 h after release from a thymidine block, irradiated (20 Gy) or not, and returned to the incubator for the time indicated. When present, ANI was maintained for up to harvesting cells. The results show that ANI blocks the transit of cells to G2.
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Figure 10. Time-dependent evolution of the fraction of activity released in synchronized HeLa cells irradiated in the S phase without or with ANI. [2-14C]Thymidine-labeled, synchronized HeLa cells were exposed or not to ANI (30 μmol/L, 1% DMSO) and irradiated (20 and 80 Gy) at the times indicated. ANI was present for the whole length of post-irradiation incubation. Cells were collected at the indicated time points, inserted into agarose plugs, and lysed for DSB analysis by pulsed-field gel electrophoresis. Fraction of activity released is the percentage of DNA migrating out of the wells.

the length of the G1 transit in human cells rather than to a DNA repair defect in the former.

This notwithstanding, the PARP inhibitors nicotinamide and benzamide have been reported to afford increased radiosensitivity in human tumor xenografts in mice (61–63). Nicotinamide has consistently been assayed in clinical trials in combination with carbogen and was found to potentiate the effect of radiotherapy in head and neck carcinoma (64), although its applicability was limited by severe gastrointestinal toxicity (65). Ever since, evidence for a vasoactive effect of nicotinamide has accumulated (63, 66, 67), and it has been proposed that this effect accounts for the increased cure of xenografted tumors by radiotherapy (66). Therefore, it may be questioned whether impaired DNA damage repair in relation to PARP inhibition is able or not to affect significantly on tumor response to radiation in vivo. Inhibition of potentially lethal damage recovery by the novel tricyclic benzimidazole PARP inhibitor AG14361 (66) suggests that although vasodilation plays a major role in radiotherapy, altered DNA damage repair may contribute to enhanced tumor cure in some instances. To discriminate between these issues, we propose to compare the effect of a novel, potent PARP inhibitor on the radiation response of xenografts established from tumors of the same origin (e.g., breast or non–small cell lung carcinoma) and size but with widely differing mitotic index. It is anticipated that PARP inhibitors will enhance radiation cure specifically in tumors with a high S-phase content, without major increase of adverse side effects against surrounding tissues.

Acknowledgments

We thank the Alberta Cancer Board and Dr. Joan Turner for the gift of M059J and M059K cell lines; Drs. C.F. Arlett, D. Averbeck, N. Foray, P.A. Jeggo and J.-P. de Villartay for providing the mutant cell lines used in this study; and Dr. G. de Murcia for providing PARP-1−/− and PARP-1−/− 3T3 fibroblasts and for kind support and helpful discussion.

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Mol Cancer Ther 2006;5:564-574.