

Interactive Competition Between Homologous Recombination and Non-Homologous End Joining

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Abstract

DNA-dependent protein kinase (DNA-PK), composed of Ku70, Ku80, and the catalytic subunit (DNA-PKcs), is involved in double-strand break (DSB) repair by non-homologous end joining (NHEJ). DNA-PKcs defects confer ionizing radiation sensitivity and increase homologous recombination (HR). Increased HR is consistent with passive shunting of DSBs from NHEJ to HR. We therefore predicted that inhibiting the DNA-PKcs kinase would increase HR. A novel DNA-PKcs inhibitor (1-(2-hydroxy-4-morpholin-4-yl-phenyl)-ethanone; designated IC86621) increased ionizing radiation sensitivity but surprisingly decreased spontaneous and DSB-induced HR. Wortmannin also inhibits DNA-PKcs and reduces DSB-induced HR. IC86621 did not affect HR product outcome, indicating that it affects HR initiation. Thus, HR is increased in the absence of DNA-PKcs, but decreased when DNA-PKcs is catalytically inactive, suggesting interactive competition between HR and NHEJ. The effects of IC86621 and wortmannin were proportional to the level of DNA-PKcs, consistent with inhibited DNA-PKcs acting in a dominant negative manner. We propose that inhibition of DNA-PKcs blocks its autophosphorylation, prevents dissociation of DNA-PKcs from DNA ends, and thereby blocks both HR and NHEJ. By blocking the two major DSB repair pathways, DNA-PKcs inhibitors should radiosensitize at all cell-cycle stages and are therefore excellent candidates for augmenting cancer radiotherapy.

Introduction

DNA double-strand breaks (DSBs) are potentially lethal lesions that are efficiently repaired in eukaryotes by homologous recombination (HR) or non-homologous end joining (NHEJ). DSBs can be repaired by gene conversion, a specific type of conservative HR that can occur with or without an associated crossover. DSB-induced gene conversion involves nonreciprocal transfer of information from an unbroken donor locus to a broken (recipient) locus. Single-strand annealing is a

distinct, non-conservative HR process that can result in deletions when interacting regions are arranged as direct repeats (1). Key HR proteins include RAD51, five RAD51 paralogues, RAD52, and RAD54 (2–8). Several other proteins have poorly defined roles in HR, including replication protein A (RPA), p53, ATM, BRCA1, and BRCA2 (9–14). In yeast, Rad50 and Mre11 have been implicated in HR, particularly in an early step involving end processing to 3' single-stranded tails (15). HR typically results in accurate repair, while defects in HR proteins cause genome instability (6, 16–18).

NHEJ often results in imprecise repair, yielding deletions or insertions, yet NHEJ also plays a role in maintaining genome stability (19, 20). NHEJ involves DNA-dependent protein kinase (DNA-PK), XRCC4, Artemis, and DNA ligase IV (21, 22). DNA-PK is a complex of a 469-kDa catalytic subunit (DNA-PKcs), and the DNA end-binding Ku70/Ku80 heterodimer. DNA-PKcs is a nuclear, serine/threonine protein kinase that is activated by association with DNA, and is a member of the phosphatidylinositol-3-(PI3) kinase family. In addition to its roles in NHEJ, DNA-PKcs is important in damage sensing/signaling before checkpoint activation (22). Numerous *in vitro* phosphorylation substrates of DNA-PKcs have been identified, including transcription factors, the eukaryotic single-stranded binding protein RPA, p53, Artemis, and both Ku subunits. Putative *in vivo* substrates include WRN (23) and IRF-3 (24). Importantly, DNA-PKcs is trans-autophosphorylated (25–27). Reversible autophosphorylation appears to regulate DNA-PK kinase activity and its repair functions (28, 29).

A growing body of evidence supports the concept of mechanistic overlap and/or competition between HR and NHEJ (30–35). Previous studies showed DNA-PKcs deficiency increases both DSB-induced and spontaneous HR (30, 34). In this report, we test the effects of a novel DNA-PKcs inhibitory compound on HR. This compound, 1-(2-hydroxy-4-morpholin-4-yl-phenyl)-ethanone (chemical designation IC86621), enhances the sensitivity of human and hamster cells to ionizing radiation (IR). Unexpectedly, and opposite to the effect of DNA-PKcs deficiency, specific inhibition of DNA-PKcs kinase activity by IC86621 reduced both spontaneous HR and HR stimulated by I-SceI nuclease-induced DSBs, with greater effects seen in human than hamster cells. The drug did not affect cell viability, nor did it affect HR product outcome. Wortmannin, another DNA-PKcs inhibitor, similarly reduces DSB-induced HR to a greater extent in human cells than hamster cells. Because DNA-PKcs is expressed at higher levels in human than hamster cells, our results suggest that inhibited DNA-PKcs has dominant negative effects on both NHEJ and HR, and provide new insight into the nature of the competition between these DSB repair pathways.

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Results

A Novel DNA-PKcs Inhibitor, IC86621, Radiosensitizes Cells Expressing DNA-PKcs

To study the effects of DNA-PKcs inhibition on radiosensitivity and HR, we used wild-type and DNA-PKcs-defective Chinese hamster ovary (CHO) cell lines carrying *neo* direct repeat recombination substrates (Fig. 1, A and B; 30, 36, 37); derivatives of the DNA-PKcs-defective CHO cells complemented with a human DNA-PKcs expression vector (30, 38); and human cells carrying inverted repeats of the puromycin acetyl-transferase gene (*pac*; Fig. 1C). Cells with defects in DNA-PKcs are radiosensitive (38, 39), as are wild-type cells treated with the PI3 kinase inhibitor wortmannin (40), OK-1035 (41), or the synthetic peptide HNI-38 (42). IC86621 is a strong inhibitor of DNA-PKcs *in vitro*, and it has limited activity against other PI3-related kinases (43). We tested whether IC86621 would radiosensitize cells by measuring cell survival following IR exposure in the presence or absence of 200 μ M IC86621. Cell lines with functional DNA-PKcs (HT1080-1885, CHO strain 33, and the complemented CHO lines V24-C2 and VD13-C1) showed increased radiosensitivity in the presence of IC86621 (Fig. 2, panels A, B, D, and F). IC86621 had stronger effects in human cells than in CHO cells (Fig. 2, A and B). In contrast, the DNA-PKcs-defective cell lines V24 and VD13 were highly radiosensitive in the absence of IC86621, and there was no further radiosensitization in the presence of IC86621 (Fig. 2, C and E). Thus, radiosensitization by IC86621 is DNA-PKcs dependent, consistent with IC86621 acting as a specific DNA-PKcs inhibitor. The radiosensitivities of IC86621-treated V24-C2 and VD13-C1 are similar to levels seen in untreated V24 and VD13 (Fig. 2, compare panel C to D, and panel E to F), indicating that IC86621 is a potent *in vivo* inhibitor of DNA-PKcs.

Inhibition of DNA-PKcs Reduces DSB-Induced HR

As seen previously (36), DSBs induced by I-SceI nuclease enhanced HR frequencies by >2000-fold in all cell lines in the present study (data not shown). We and others have shown that DSB-induced HR is further enhanced in cell lines with defects

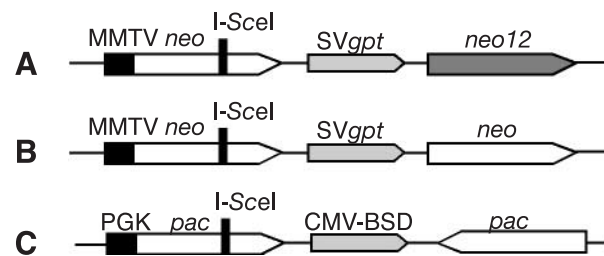


FIGURE 1. HR substrates. **A.** Two copies of a 1.4-kbp fragment carrying the *neo* gene in direct orientation flank an SV40 promoter-driven *gpt* gene. The upstream *neo* is driven by the mouse mammary tumor virus (MMTV) promoter and is inactivated by a frameshift insertion consisting of an I-SceI site. The downstream copy (*neo12*) is inactive because it lacks a promoter. The 12 RFLPs in *neo12* are indicated by shading (for details, see Ref. 36). **B.** As in part **A**, except the downstream *neo* lacks the RFLPs. **C.** Two copies of a 1.7-kbp fragment carrying the *pac* gene arranged as inverted repeats flanking a CMV promoter-driven blasticidin gene (*BSD*). The upstream *pac* is driven by the murine PGK promoter, and is inactivated by an 80-bp deletion of coding sequence replaced by the I-SceI site. The downstream *pac* is inactive because it lacks a promoter.

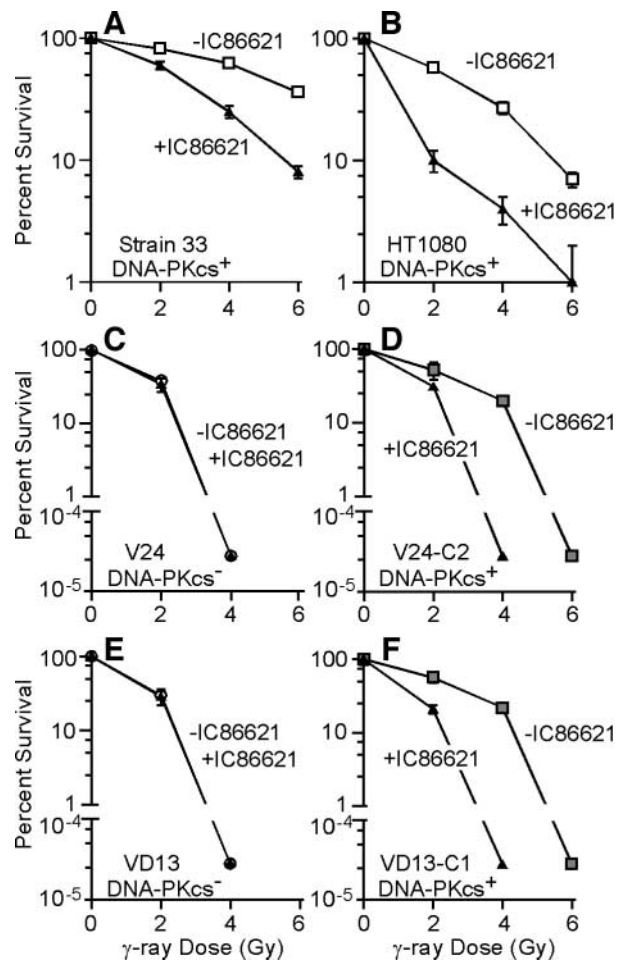


FIGURE 2. IC86621 (200 μ M) radiosensitizes cells expressing DNA-PKcs. Cells were irradiated with ^{137}Cs γ -rays and the resulting colonies with at least 50 cells were counted. Data are averages (\pm SD) of at least three determinations per strain. *Open* and *shaded* symbols indicate untreated cells; *black* symbols indicate cells treated with IC86621.

in NHEJ, including mutants with defects in DNA-PKcs, Ku, and XRCC4, and that complementation of these defects reduces HR (30, 31, 34). In an earlier report (44), it appeared that Ku80 deficiency did not affect DSB-induced HR, but this probably reflects position effects as discussed previously (30). We therefore expected that inhibition of DNA-PKcs with IC86621 would similarly enhance DSB-induced HR. Surprisingly, IC86621 led to dose-dependent reductions in DSB-induced HR in wild-type cells (Fig. 3, A and B). The reduction was greatest in the human cell line HT1080-1885, ranging from 1.5-fold with 25 μ M IC86621 to 19-fold at 200 μ M. In CHO strain 33, DSB-induced HR was reduced \sim 1.5- to 2-fold by 25–200 μ M IC86621. We also tested lower concentrations of IC86621 in CHO strain 33, from 0.1 to 25 μ M, and observed \sim 1.2- to 1.5-fold reductions in DSB-induced HR (data not shown). Thus, IC86621 has modest effects in CHO cells, but these effects are apparent at very low concentrations.

To determine whether the reduction in DSB-induced HR with IC86621 is dependent on DNA-PKcs, we examined HR in

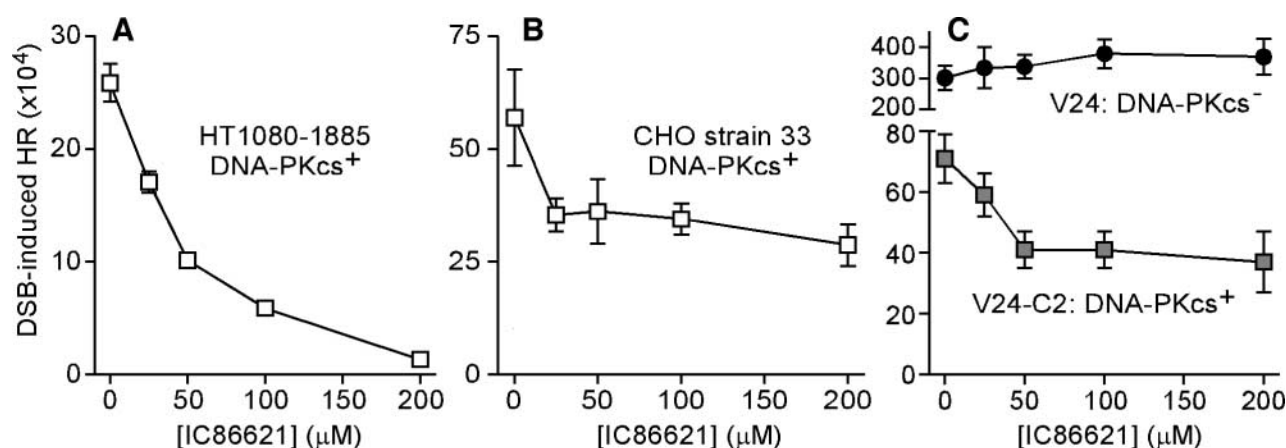


FIGURE 3. DSB-induced HR is reduced by IC86621. Average HR frequencies (\pm SD) with increasing concentrations of IC86621 are shown for DNA-PKcs wild-type (*HT1080-1885* and *CHO strain 33*), mutant (*V24*, *VD13*), and complemented (*V24-C2*, *VD13-C1*) cell lines for three to six determinations per cell line.

CHO V24 cells, which do not express DNA-PKcs (30). In the absence of IC86621, DSB-induced HR was significantly higher in V24 cells than its DNA-PKcs-complemented derivative, V24-C2 (Fig. 3C), consistent with previous results (30). Interestingly, IC86621 had no effect on DSB-induced HR in V24 cells, but V24-C2 showed an IC86621 response that was essentially identical to that of wild-type cells (Fig. 3C). The recombination substrates in CHO strains 33, V24, and V24-C2 all include the multiply marked *neo12* donor allele. To determine whether these effects are influenced by, or dependent on the sequence heterologies present in *neo12*, we examined DSB-induced HR in strains lacking the heterologies (the DNA-PKcs-defective line VD13, and its complemented derivative VD13-C1). Similar to V24/V24-C2, IC86621 did not significantly affect DSB-induced HR in VD13, but it reduced HR by 1.6- to 4.2-fold in VD13-C1 (data not shown). These results indicate that IC86621 affects DSB-induced HR specifically by inhibiting DNA-PKcs. The negative effect of IC86621 on DSB-induced HR is general: IC86621 reduced DSB-induced HR in several assay systems, including human and CHO cells, with inverted and direct repeat recombination substrates present at three different chromosomal loci, and with both fully homologous and highly marked substrates.

Delacote *et al.* (31) reported that 20 μ M wortmannin had no effect on I-*SceI*-induced HR in CHO cells. We therefore tested this concentration of wortmannin in our cell systems. As shown in Fig. 4, wortmannin reduced HR by 3.1-fold in V24-C2 hamster cells ($P = 0.001$, *t* test), which express human DNA-PKcs at low levels (30). Wortmannin also caused a small, but statistically significant reduction in DSB-induced HR in DNA-PKcs-defective V24 cells (1.7-fold; $P = 0.007$, *t* test). However, wortmannin very strongly reduced DSB-induced HR in human HT1080-1885 cells (82-fold; $P < 0.0001$, *t* test). Thus, wortmannin reduces DSB-induced HR in a largely DNA-PKcs-dependent manner. Note that DSB-induced HR is increased in the absence of DNA-PKcs (30, 34), but decreased when DNA-PKcs is present but catalytically inactive (Figs. 3 and 4). These contrasting results provide new insight into the nature of the competition between NHEJ and HR during DSB repair (see "Discussion").

The IC₅₀ values of IC86621 and wortmannin against DNA-PKcs are \sim 100 nM (43). Thus, we presume that there is little or no kinase activity when cells are treated with these inhibitors at concentrations that are 200- to 2000-fold higher than the IC₅₀ (20–200 μ M). For IC86621, this is supported by the observation that DNA-PKcs-complemented cells treated with 200 μ M IC86621 show the same sensitivity to IR as DNA-PKcs null cells in the absence of IC86621 (Fig. 2).

Inhibition of DNA-PKcs Reduces Spontaneous HR

Previously we established that spontaneous HR is enhanced in cells lacking DNA-PKcs (30). To determine whether inhibition of DNA-PKcs would influence spontaneous HR, we measured spontaneous HR frequencies in CHO and human cell lines after cultures were split and expanded in the presence or absence of 100 μ M IC86621. Because parallel cultures were started by dividing a single parent culture, each pair of cultures

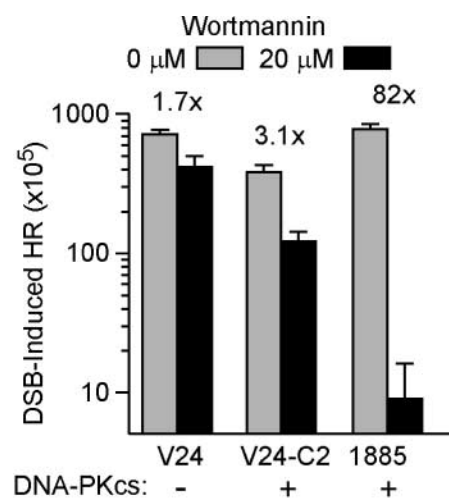


FIGURE 4. DSB-induced HR is reduced by wortmannin. Average HR frequencies (\pm SD) with or without 20 μ M wortmannin are shown for DNA-PKcs wild-type (*HT1080-1885*), mutant (*V24*), and complemented (*V24-C2*) cell lines for three determinations. Fold decreases are shown above bars.

started with same number of pre-existing recombinants. This procedure therefore detects differences in the accumulation of recombinants during culture expansion in the presence and absence of IC86621. Because spontaneous HR is markedly reduced by RFLP markers (Ref. 30, and references therein), we used CHO strains VD13 and VD13-C1 that lack RFLPs in the *neo* donor. At this concentration, IC86621 had a slight negative effect on growth rate (data not shown). We therefore measured spontaneous HR in cultures that were expanded for nearly equivalent numbers of cell generations in the presence or absence of IC86621 (11.1–11.3 generations for CHO strains, and 11.9–12.4 generations for HT1080-1885); this required 8 and 9 days of expansion for untreated and treated cultures, respectively. The slight effect on growth rate is not due to IC86621-induced cytotoxicity: plating efficiencies were similar in the presence or absence of IC86621 (Fig. 5A). As shown in Fig. 5B, IC86621 reduced spontaneous HR in cells expressing DNA-PKcs (VD13-C1 and HT1080-1885), but had no effect in the DNA-PKcs-deficient VD13 cells. Thus, IC86621 decreases spontaneous HR in a DNA-PKcs-dependent manner, similar to its effect on DSB-induced HR, but opposite to the effects of DNA-PKcs deficiency (30). IC86621 reduced spontaneous HR in CHO cells with a *neo* direct repeat, and human cells with a *pac* inverted repeat, again indicating that these effects are general.

Inhibition of DNA-PKcs Does Not Affect HR Product Outcome

In both wild-type and DNA-PKcs-defective cells, DSB-induced HR in the *neo* direct repeat substrate usually results in short-tract gene conversion without associated crossover; conversion tracts average ~200 bp in length (30, 36). To test whether inhibition of DNA-PKcs would affect HR outcome, we characterized 49 G418-resistant products of CHO strain 33 treated with 200 μ M IC86621 by Southern hybridization and found that 48 arose by gene conversion without crossover. In the remaining product, one copy of *neo* and the intervening sequences were deleted. These results are nearly identical to prior results with strain 33 in the absence of IC86621, in which two deletions were detected among 67 products (36). We mapped conversion tracts in 17 products from IC86621-treated cultures; the resulting tract spectrum (not shown) was very similar to that generated previously from CHO strain 33 (36). The average gene conversion tract length (\pm SE) from IC86621-treated cultures was 315 ± 81 bp, which is not significantly different from untreated cells (237 ± 29 bp; $P = 0.37$, *t* test). We conclude that inhibition of DNA-PKcs affects HR initiation, but not late HR stages, such as heteroduplex DNA formation or intermediate resolution.

Discussion

A Novel DNA-PKcs Inhibitor

NHEJ is a key determinant of IR resistance in mammalian cells, and defects in DNA-PKcs greatly increase IR sensitivity (45). Wortmannin, a general inhibitor of PI3 kinases, inhibits DNA-PKcs and sensitizes cells to IR (40). Although DNA-PKcs is inhibited by lower concentrations of wortmannin than ATM *in vitro*, both DNA-PKcs and ATM are strongly inhibited

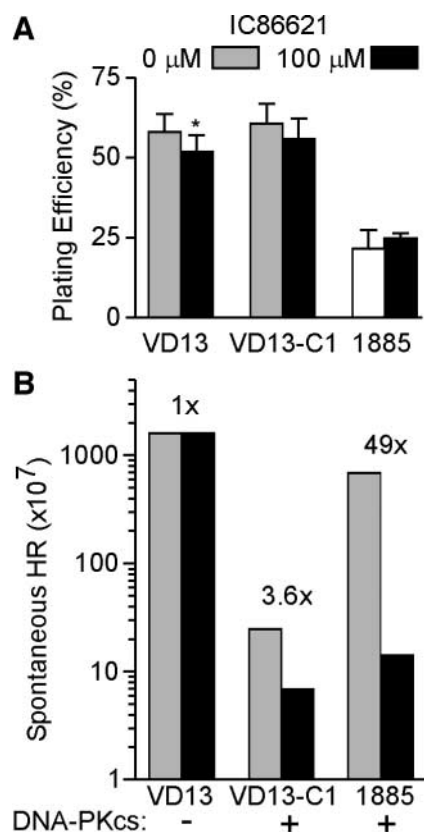


FIGURE 5. Spontaneous HR is suppressed by IC86621. **A.** IC86621 has little or no effect on plating efficiency (* indicates $P = 0.02$, *t* test). **B.** Spontaneous HR frequencies are shown for DNA-PKcs defective (VD13), complemented (VD13-C1), and wild-type (HT1080-1885) cells in the presence or absence of 100 μ M IC86621. In each experiment, 27–6832 G418-resistant colonies were scored. Fold decreases are shown above bars.

in vivo at concentrations required to radiosensitize cells, suggesting that radiosensitization may involve inhibition of both kinases (46, 47). IC86621 strongly inhibits DNA-PKcs, yet unlike wortmannin, even 100 μ M IC86621 has no detectable activity against the closely related protein kinases ATM, ATR, or FRAP (43). As expected for a DNA-PKcs inhibitor, IC86621 inhibits DNA end joining *in vivo* (43) and it sensitizes cells to IR (Fig. 2). We show here that IC86621 reduces radioresistance, DSB-induced HR, and spontaneous HR in a DNA-PKcs-dependent manner (Figs. 2, 3, and 5), indicating that these effects reflect specific inhibition of DNA-PKcs. In contrast, we observed a small, but significant reduction in DSB-induced HR with wortmannin in V24 cells (Fig. 4), which do not express DNA-PKcs (30). This may reflect inhibition of ATM, as ATM-defective cells display several phenotypes consistent with aberrant HR-mediated repair of DSBs (48–50).

Is Spontaneous HR Initiated by DSBs?

Several lines of evidence indicate that spontaneous HR depends on replication, but it is unclear whether DSBs initiate these events. DSBs can arise when replication forks encounter single-strand breaks (reviewed in Ref. 51), or by reversal of stalled or blocked replication forks (52). Alternatively, template

lesions can be bypassed by a recombinational process that is independent of DSBs (53). Spontaneous HR is increased in DNA-PKcs-defective cells (30), but reduced when cells are treated with IC86621 (Fig. 5). Increased spontaneous HR is not a general feature of NHEJ-defective cells as spontaneous HR rates were similar in an *xrcc4* mutant and its complemented derivative, and this probably reflects the fact that XRCC4 acts late in NHEJ (31). We found that both DSB-induced and spontaneous HR are increased in the absence of DNA-PKcs, and that both are decreased when DNA-PKcs is present but catalytically inactive. The parallel effects of altered DNA-PKcs on I-*SceI*-induced and spontaneous HR are consistent with the idea that spontaneous HR is predominantly initiated by spontaneous DSBs.

Interactive Competition Between HR and NHEJ

Competition between HR and NHEJ was suggested by genetic evidence (54), and by DNA end binding functions of HR proteins RAD51 and RAD52, and NHEJ proteins Ku and DNA-PKcs (55, 56). However, the nature of this competition has been obscure. HR and NHEJ may compete passively, with the repair outcome depending, for example, on whether HR or NHEJ proteins bind first to broken ends, and/or the availability of a homologous repair template. If competition is passive, and neither repair pathway is saturated, eliminating one pathway should shunt DSBs toward the other pathway. The observed increases in DSB-induced HR in yeast and mammalian cells with defects in NHEJ proteins, including DNA-PKcs, Ku, and XRCC4 (30, 31, 34, 57) are consistent with passive competition.

Alternatively, HR and NHEJ proteins may interact and influence each others' activities. The idea of interactive competition is supported by several lines of evidence. First, yeast MRX influences both HR and NHEJ (58). Second, although DNA-PKcs has important roles in NHEJ, it phosphorylates several proteins with roles in HR, including p53, ATM, and RPA (reviewed in Ref. 59). Third, WRN, a RecQ helicase family member, has roles in HR (60), but it is also phosphorylated and regulated by DNA-PK (23), and it forms a complex with Ku that regulates end processing (61–63). Fourth, DNA-PKcs and Ku defects both increase DSB-induced HR, but to different degrees (34). Finally, interactions between HR and NHEJ are suggested by several puzzling phenotypes of Ku mutants. Although mammalian Ku mutants show increased sensitivity to DSB damage (64), this is not the case in other cell types. Yeast *yku70* mutants show slightly increased resistance to killing by a single DSB induced by HO nuclease (57, 65), and *yku70 mre11* double mutants are 6-fold more IR-resistant than *mre11* single mutants (66). Similarly, Ku70 knockout in chicken DT40 cells increases IR resistance by 10-fold (67), and suppresses IR sensitivity caused by defects in DNA ligase 4 and DNA-PKcs (67, 68). On the basis of these results, we proposed that Ku increases sensitivity to DSB damage by interfering with HR (57). The fact that DSB-induced HR is increased in the absence of DNA-PKcs (30, 34), yet decreased when DNA-PKcs is present but inactivated by IC86621 or wortmannin, provides new evidence for an interactive competition between HR and NHEJ, in which DNA-PKcs (and by extension, Ku) influences both repair pathways.

The decreases in DSB-induced HR that we observed in cells treated with IC86621 or wortmannin contrast with the study by Delacote *et al.* (31), which indicated that wortmannin has no effect on I-*SceI*-induced HR in CHO cells. In that study, DSB-induced HR was in fact slightly reduced by wortmannin, although the reduction was not statistically significant. It is unlikely that substrate differences or position effects account for these distinct results because we found that IC86621 and wortmannin reduced DSB-induced HR in cells carrying two types of HR substrates present at several different chromosomal loci (Figs. 3 and 4). It is more likely that the minimal effect of wortmannin on I-*SceI*-induced HR in the prior study relates to the low levels of DNA-PKcs in CHO cells, as discussed below. In contrast to the reduction in I-*SceI*-induced HR we observed with both wortmannin and IC86621, Delacote *et al.* (31) found that wortmannin increased both IR-induced HR and IR-induced RAD51 subnuclear foci. It is unclear why DNA-PKcs inhibition differentially affects IR- and I-*SceI*-induced HR, but several factors may be important, including different structures at DSB ends produced by IR and I-*SceI*, differential activation of signaling pathways, and at least for wortmannin, inhibition of both DNA-PKcs and other PI3 kinase family members such as ATM and ATR.

Possible Mechanisms Underlying DNA-PKcs Effects on HR

We considered two types of models to explain why HR is increased in cells lacking DNA-PKcs (30). In the first model, DSBs destined for repair by NHEJ are shunted to HR in the absence of DNA-PKcs. The second posits that in the absence of DNA-PKcs, one or more DNA-PKcs targets that influence HR (such as p53, ATM, RPA, and WRN) would not be phosphorylated, and that this would increase HR. However, neither of these models can account for the reduction of HR when DNA-PKcs is present but catalytically inactive. It is clear that DNA-PKcs kinase activity is important for NHEJ (as evidenced by the radiosensitizing effects of DNA-PKcs inhibitors), but blocking NHEJ by inhibiting DNA-PKcs does not shunt DSBs toward HR, thus ruling out the first model. We can rule out the second model because HR proteins phosphorylated by DNA-PKcs would remain unphosphorylated whether DNA-PKcs were absent or catalytically inactive. Thus, the second model predicts that DNA-PKcs deficiency and inhibition should have similar, not opposite effects on HR, as observed.

We can refine our model of DNA-PKcs influence on HR by drawing from biochemical studies of DNA-PKcs. Chan and Lees-Miller (26) showed that autophosphorylation of DNA-PKcs leads to its dissociation from Ku and the DNA substrate. Calsou *et al.* (69) showed that inhibition of DNA-PKcs with wortmannin prevents dissociation of the DNA-PKcs-Ku holoenzyme from DNA ends, and prevents subsequent processing by DNA polymerase, exonuclease, and DNA ligase. We propose that HR increases in the absence of DNA-PKcs because DSBs are shunted toward HR, but that when DNA-PKcs is present and catalytically inactive, it is not autophosphorylated, it remains bound to DNA ends, and thereby blocks end processing required for both HR and NHEJ (Fig. 6). In this model, DNA-PKcs (and probably Ku) associate with DSBs

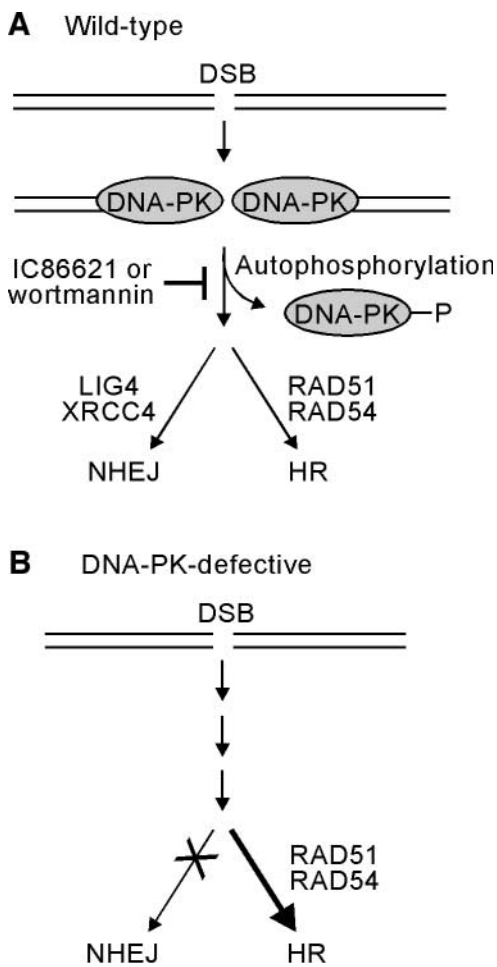


FIGURE 6. Interactive competition between NHEJ and HR. **A.** In wild-type cells, DSBs are bound by DNA-PK, which dissociates from ends following autophosphorylation, allowing NHEJ or HR to proceed. Inhibition of DNA-PK with IC86621 or wortmannin blocks autophosphorylation, preventing DNA-PK dissociation and blocking both NHEJ and HR. **B.** In DNA-PKs defective cells, broken ends cannot be repaired by NHEJ, and HR is increased as DSBs are shunted to this pathway.

earlier than HR proteins, which is consistent with the recent finding that NHEJ precedes HR in yeast (33), and with our finding that HR outcome is not affected when DNA-PKcs is absent (30) or inhibited (this study). This model also explains the seemingly paradoxical observation that IC86621 inhibits HR far more in human (HT1080) cells than in CHO cells, despite the 20-fold higher levels of DNA-PKcs in human cells (70, 71). If inhibited DNA-PKcs actively interferes with end processing, it becomes, in effect, a dominant negative protein, and the high levels of inhibited DNA-PKcs in human cells would therefore produce a strong dominant negative effect. The smaller effects that we and Delacote *et al.* (31) observed in CHO cells would reflect the smaller amount of inhibited DNA-PKcs in these cells.

NHEJ and HR are regulated in part by the cell cycle, with NHEJ more important in G₁, and HR more important in S-G₂ (67, 72). Our results indicate that inhibition of DNA-PKcs impairs both major DSB repair pathways. Thus, DNA-PKcs

inhibitors should sensitize cells to IR at all stages of the cell cycle. Preliminary studies have shown that IC86621 is an effective adjuvant to radiation treatment for control of human tumor xenografts in mice (43), indicating that DNA-PKcs inhibitors like IC86621 may prove useful for cancer radiotherapy in human patients.

Materials and Methods

Plasmid DNAs and Cell Lines

Plasmid pCMV(3xNLS)I-SceI (37) expresses I-SceI nuclease. Plasmid pCMV(I-SceI⁻) (73), with the I-SceI coding sequence in reverse orientation, is a negative control vector. Cell culture and electroporation conditions were as described (36). The CHO K1c derivative strain 33, the CHO V3 derivatives V24, V24-C2, VD13, and VD13-C1, and the human HT1080-1885 cell lines were described previously (30, 36, 37). CHO strains 33, V24, and V24-C2 each carry a single integrated copy of a *neo* direct repeat recombination substrate from plasmid pMSGneo2S12His (Fig. 1A; 36). This substrate includes a MMTV promoter-driven *neo* gene (MMTV*neo*) and a second *neo* with 12 phenotypically silent, single-base changes, called *neo12*. The nucleotide changes in *neo12* create RFLPs that can be used to map gene conversion tracts. MMTV*neo* is inactivated by a 29-bp insertion bearing the I-SceI cleavage site, while *neo12* is inactive because it lacks a promoter. The VD13 and VD13-C1 contain a related construct, pMSGneo2SHis, which lacks the RFLPs (Fig. 1B). In strains V24-C2 and VD13-C1, the DNA-PKcs defect is complemented with a human DNA-PKcs expression vector (38) HT1080-1885 has a single integrated copy of a recombination substrate with inverted repeats of the puromycin acetyl-transferase gene (*pac*; Fig. 1C; 37).

IR Resistance Assays

Cells were seeded at low density into 3.5 cm (diameter) dishes, incubated for 24 h in growth medium \pm 200 μ M IC86621, and irradiated using a ¹³⁷Cs source (Gammacell 40, MDS Nordion, Kanata, Ontario, Canada) at a dose rate of 1.03 Gy/min. Following irradiation, the growth medium was replaced with fresh medium \pm 200 μ M IC86621, incubated for 48 h, then replaced with fresh medium without IC86621. The cells were incubated for an additional 7–10 days before colonies were stained with 1% (w/v) crystal violet in methanol.

Recombination Assays

DSB-induced HR was assayed essentially as described (36). Briefly, 4×10^5 cells were seeded into 3.5 cm wells with specified concentrations of IC86621 or wortmannin, and incubated for 24 h. The cells were then transfected with 2 μ g of pCMV3xNLS(I-SceI) to induce DSBs, or with 2 μ g of the negative control vector pCMV(I-SceI⁻) (73) using Lipofectamine Plus as recommended by Life Technologies, Inc. (Gaithersburg, MD). Twenty-four hours post-transfection, 2×10^4 cells were seeded to each of two 10-cm dishes in medium containing the original concentrations of IC86621 or wortmannin. After an additional 24 h, G418 was added (600 μ g/ml, 100% active) to CHO cell lines, or puromycin (1 μ g/ml) was added to the HT1080-1885 cell line. Cell viability was

determined by plating appropriate dilutions into nonselective medium, and DSB-induced HR frequencies were calculated as the number of G418-resistant colonies per viable cell plated in selective medium. Gross structures of recombinant products were analyzed by Southern blot, and gene conversion tracts were analyzed by restriction mapping of PCR products, as described (17, 36).

Spontaneous HR was assayed as follows: 5×10^4 cells from a single culture were seeded into each of two T-175 flasks with 25 ml of growth medium $\pm 100 \mu\text{M}$ IC86621, and expanded for 11–12 generations. During this 8- to 9-day expansion period, the growth medium was replaced (\pm IC86621) at day 2 and day 4, then again 1 day before cells were replated for selection of recombinants. Before this final media change, cells were briefly treated with trypsin to evenly disperse the cells in the flask. On day 8 or 9, the cells were harvested, counted, and replated in 10-cm dishes with selective media containing either G418 (CHO lines) or puromycin (HT1080-1885) as above. Cell viability was determined at the time of the initial seeding, and after expansion, by plating appropriate dilutions into nonselective medium. Spontaneous HR frequencies were calculated as the number of G418- or puromycin-resistant colonies per viable cell plated in selective media. Cell generations were calculated using the following equation:

$$\text{Generations} = \log_{\text{base}2}(\text{cells recovered}/\text{viable cells plated})$$

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