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Inhibition of poly(ADP-ribose)polymerase stimulates extrachromosomal homologous recombination in mouse Ltk– fibroblasts

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ABSTRACT

Poly(ADP-ribose)polymerase (PARP) is an abundant nuclear enzyme activated by DNA breaks. PARP is generally believed to play a role in maintaining the integrity of the genome in eukaryote cells via anti-recombinogenic activity by preventing inappropriate homologous recombination reactions at DNA double-strand breaks. While inhibition of PARP reduces non-homologous recombination, at the same time it stimulates sister chromatid exchange and intrachromosomal homologous recombination. Here we report that the inhibition of PARP with 100 μg/ml (0.622 mM) 1,5-isoquinolinediol results in an average 4.6-fold increase in the frequency of extrachromosomal homologous recombination between two linearized plasmids carrying herpes simplex virus thymidine kinase genes inactivated by non-overlapping mutations, in mouse Ltk– fibroblasts. These results are in disagreement with the previously reported observation that PARP inhibition had no effect on extrachromosomal homologous recombination in Ltk– cells.

INTRODUCTION

Homologous recombination is the process by which two DNA molecules exchange homologous sequences. In eukaryotes this process is the basis of a number of important biological events such as recombination between homologous chromosomes during mitosis and meiosis, generation of the antigen recognition molecule repertoire of the immune system, gene amplification and DNA repair (1–5). The complexity of the cellular machinery involved in DNA repair and recombination has now become apparent. In yeast alone, the number of genes whose products are believed to participate in recombination/repair is estimated at over 100 (6).

Poly(ADP-ribose)polymerase (PARP) is an abundant nuclear protein found in all higher eukaryotes (7,8). It recognizes, binds to, and becomes activated by DNA breaks. As its name implies, the enzyme catalyzes the synthesis of polyADP-ribose from NAD^+ on a number of different protein targets. Its substrates include ligases, topoisomerases, histones and PARP itself. Although the biological role of PARP is as yet unclear, it has been recently suggested by Satoh and Lindahl, and others, that one of PARP's overall functions might be the preservation of genomic integrity by decreasing spontaneous homologous recombination at sites of DNA breaks (9–11).

There have been a number of reports indicating that PARP inhibition results in a decrease of random integration of exogenous DNA into the mammalian genome (12,13). PARP inhibition was also reported to increase the frequency of sister chromatid exchange (SCE) in mammalian cells, a process that proceeds via homologous recombination (14). Finally, Waldman and Waldman have reported that PARP inhibition increased the frequency of intrachromosomal homologous recombination in mouse Ltk– fibroblasts (15). Recent studies with PARP knock-out mice have confirmed the anti-recombinogenic function of PARP at DNA strand breaks (16,17).

The above mentioned results suggest that PARP inhibition generally stimulates homologous recombination processes. However, studies of the effect of PARP inhibition on the rate of extrachromosomal homologous recombination in mouse Ltk– fibroblasts and gene targeting in Chinese hamster ovary cells showed that the rate of the former remained unchanged and the frequency of the latter decreased 2-fold in response to PARP inhibition with 3-methoxybenzamide (3-MB) (13,18).

Here, we report the effect of PARP inhibition with 1,5-isoquinolinediol (ISQ) on extrachromosomal recombination in mouse Ltk– fibroblasts. In contrast to the previously published reports, we have observed that PARP inhibition results in a consistent dose-dependent increase in the frequency of extra-chromosomal homologous recombination.

MATERIALS AND METHODS

The recombination system

The recombination system used is similar to the one described by Shapira *et al*. (19). It is based on two plasmids carrying the herpes simplex virus (HSV) thymidine kinase (*tk*) gene with non-overlapping insertion and deletion mutations (Fig. 1). Both mutated *tk* genes are non-functional. Ltk– fibroblasts are deficient in *tk* activity and, hence, cannot survive in hypoxanthine–aminopterin–thymidine (HAT) medium. The appearance of HAT-resistant (HAT^R) colonies, after co-transfection with the two

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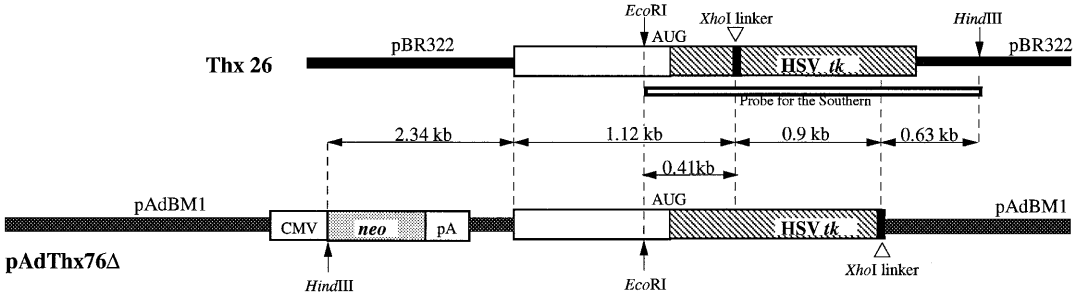

Figure 1. Recombination detection systems. The HSV *tk* gene of the Thx26 vector is inactivated by the *Xho*I linker insertion at position 738. The pAd-Thx76Δ vector contains the HSV *tk* gene of the original Thx76 vector (with the *Xho*I linker insertion at position 1611) deleted 3′ to the *Xho*I site. The homologous sequences between both vectors, surrounding the *Xho*I linker of the Thx26 are 1.12 kb 5′ to the linker and 0.9 kb 3′ to the linker. The pAd-Thx76Δ vector contains the CMV-*neo* cassette which allows the detection of illegitimate recombination events. Recombination between the target and the targeting vector would yield functional *tk*, resulting in HAT^R clones. Prior to transfections both plasmids were linearized at the site of their respective *Xho*I linkers (open triangles). The DNA probe used for the Southern analysis is shown as an open rectangle with thick borders.

plasmids, is diagnostic of an interplasmid homologous recombination event.

The Thx26 vector

The Thx26 vector contains an HSV *tk* gene with an 8 bp *Xho*I insertion mutation at position 738 of the *tk* coding sequence, subcloned into pBR322 (19).

The pAd-Thx76Δ vector

The HSV *tk* gene with a *Xho*I insertion mutation at position 1611 of the *tk* coding sequence (Thx76) was deleted from the sequence 3′ to the *Xho*I linker and subcloned into the commercial pAdBM1 vector (Quantum, Canada). The CMV-*neo* cassette was inserted 5′ to the mutated HSV *tk*.

Cell culture

Ltk– cells were grown and maintained in DMEM (Bio Media, Canada) supplemented with 10% FBS (Gibco BRL, NY) and antibiotic–antimyotic (Gibco BRL) at 37°C in 5% CO_2/95% air.

Transfections and selection

3×10^6 cells were plated on 100 mm dishes (Costar, MA) and allowed to divide once. The cells were transfected with equimolar quantities of Thx26 (4 µg) and of pAd-Thx76Δ (6 µg) plasmids linearized with *Xho*I, using Superfect poly-cationic reagent from Qiagen (Canada) according to the specifications provided by the manufacturer.

At 20 h post-transfection, the cells were trypsinized. One percent of all cells was seeded in an 80 cm^2 TC flask (NUNC, IL) containing complete DMEM supplemented with 250 µg/ml G418. The rest of the cells were plated in four 80 cm^2 TC flasks containing complete DMEM supplemented with HAT.

The cells were then incubated in a CO_2 incubator for 11 days with the selection media being changed every 4 days. Surviving cells were finally fixed with 10% formaldehyde in phosphate-buffered saline and stained with methylene blue. The colonies, formed by at least 20 cells, were counted.

The statistical significance for the differences observed for recombination frequencies was determined by the paired *t*-test. The difference was considered significant for *P*-values <0.05.

PARP inhibition

The competitive inhibitor of PARP, 1,5-isoquinolinediol (Sigma) was dissolved in DMSO at 100 mg/ml (622 mM). The inhibition was performed by direct addition of appropriate amounts of the above solution to the cell culture medium immediately after the completion of the transfection procedure. The PARP inhibition was maintained for 20 h.

Southern analysis of the HAT^R clones

HAT^R colonies were randomly selected and expanded. The genomic DNA (gDNA) from HAT^R clones was extracted by standard proteinase K/phenol/chloroform method. Ten micrograms of the gDNA were then digested with *Eco*RI, *Hin*dIII and *Xho*I enzymes (Pharmacia) and subjected to Southern analysis by standard methods. The *Eco*RI–*Hin*dIII fragment of the HSV *tk* from the Thx26 plasmid, labeled with [^{32}P]dCTP by a random primer oligolabeling kit (Pharmacia), was used as the probe.

RESULTS

The effect of 1,5-isoquinolinediol on cell survival and illegitimate recombination frequency

To assess the toxicity of the ISQ to the Ltk– fibroblasts we have performed a number of standard cell survival clonogenic assays. The results are summarized in Figure 2A. The exposure of cells to the inhibitor for 20 h resulted in a dose-dependent drop in cell survival. At 100 µg/ml (0.622 mM)—the highest drug concentration used—the cell survival was on average only 29% (±2%) of the control.

The effect of ISQ inhibition of PARP on the frequency of random integration (illegitimate recombination) was determined by transfecting the pAd-Thx76Δ plasmid linearized with *Xho*I into Ltk– cells, followed by treatment with different concentrations of ISQ for 20 h and selection of the G418-resistant colonies. The ISQ treatment decreased the frequency of random integration. The maximum decrease of 46.5% (±4.28%) was observed at 100 µg/ml ISQ (Fig. 2B). Given the large variation in the random integration frequencies is observed for the lower doses of the drug, it is unclear whether there is an early dose dependence followed by a plateau or if there is a biphasic response, perhaps

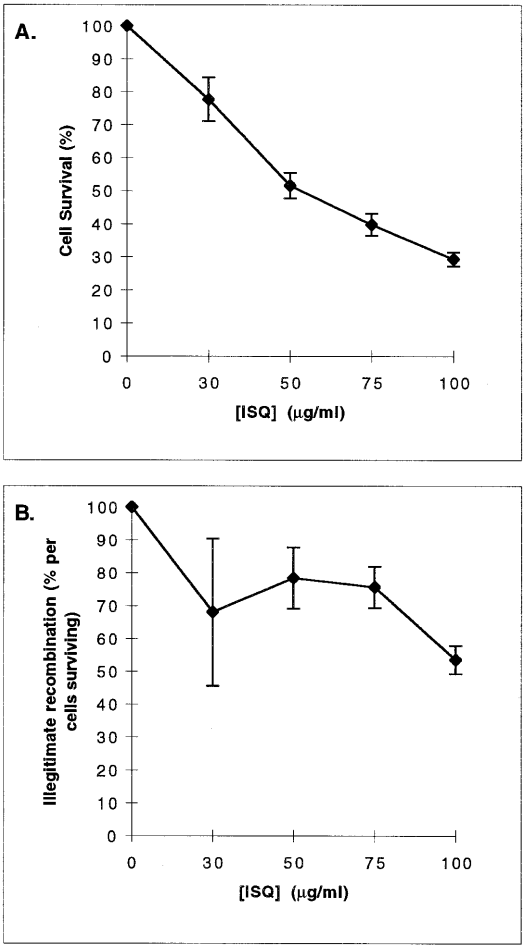

Figure 2. The effect of ISQ on cell survival and on illegitimate recombination in Ltk– cells. (**A**) Ltk– cells (300) were plated in 25 cm^2 TC flasks and treated 5 h after plating with different concentrations of ISQ for 20 h. The cells were then washed twice with phosphate-buffered saline and incubated in DMEM, 10% FBS, antibiotic–antimyotic in a CO_2 incubator for 11 days. The cells were finally fixed with 10% formaldehyde in phosphate-buffered saline, stained with methylene blue and the colonies, formed by at least 20 cells, were counted. For every drug concentration the experiment was carried out in triplicate. An average of 175 colonies per flask were scored in the absence of the inhibitor. The percent survival is calculated as the number of colonies scored for the given concentration of the drug divided by the number of colonies scored in the absence of the drug, multiplied by 100. Each point represents the mean of five separate experiments, and each error bar one standard deviation of that mean. (**B**) Ltk–cells (3×10^5), plated on 60-mm dishes 20 h prior to transfection, were transfected with the *Xho*I-linearized pAd-Thx76Δ plasmid using Superfect poly-cationic reagent from Qiagen and treated with different concentrations of ISQ for 20 h. At 20 h post-transfection, the cells were trypsinized, plated onto 80 cm^2 TC flasks in complete DMEM supplemented with 250 μg/ml G418 and incubated in a CO_2 incubator for 11 days, at which point the cells were fixed, stained and the colonies were counted. An average of 61 $G418^R$ colonies were scored in the absence of the drug. The percentage of illegitimate recombination is calculated as the number of $G418^R$ colonies scored for the given concentration of the drug divided by the number of $G418^R$ colonies scored in the absence of the drug, multiplied by 100. The percentage of illegitimate recombination has been adjusted for the cell death due to the drug toxicity by dividing the observed percentage of illegitimate recombination by the average cell survival rate determined for this drug concentration. The standard deviation of the mean for the percentage of illegitimate recombination has been similarly adjusted by multiplying it by the standard deviation of the mean for the percentage of cell survival for the same drug concentration. Each point represents the mean of five separate experiments.

indicating two different actions of the drug at different dosages.

The effect of ISQ on interplasmid homologous recombination

The appearance of HAT^R colonies subsequent to co-transfection of the Thx26 and pAd-Thx76Δ plasmids is diagnostic of an interplasmid homologous recombination event followed by random integration of the corrected plasmid into the genome. Hence, the frequency of appearance of HAT^R colonies is the product of the frequency of interplasmid homologous recombination and of the frequency of their random integration. It is possible, however, that a functional *tk* gene can be produced by inter or intra-chromosomal recombination between two randomly integrated complementary plasmids, but the frequency of this event should be many-fold lower than that of the event described above: 2×10^{-9} [$(2 \times 10^{-6}) \times 10^{-3}$] versus 10^{-4} events/cell/generation. According to Waldman and Waldman (15), the frequency of intrachromosomal recombination between HSV *tk* sequences in Ltk– cells is 2×10^{-6} events/cell/generation; the average frequency of integration of the pBR322-HSV *tk* vector is ~10^{-3}. The frequency of interplasmid recombination followed by random integration into the Ltk– cell genome, as reported by Shapira *et al.* (19), is ~10^{-4}. Hence, it is reasonable to assume that the vast majority of the HAT^R colonies detected arose by interplasmid homologous recombination followed by random integration.

The CMV-*neo* cassette incorporated into the pAd-Thx76Δ vector allows us to monitor the frequency of random integration of the plasmid by scoring the number of G418-resistant ($G418^R$) colonies produced from an aliquot of the transfected cells. The frequency of interplasmid homologous recombination is then calculated as a ratio of the number of HAT^R colonies to the number of $G418^R$ colonies.

To demonstrate that HAT^R clones arose as a result of a homologous recombination event that gave rise to the wild-type HSV *tk*, the genomic DNA of randomly selected HAT^R clones was subjected to triple digestion with *Eco*RI, *Hin*dIII and *Xho*I restriction enzymes and analyzed by Southern blotting for the presence of the intact *Xho*I-resistant HSV *tk* gene (Fig. 3). *Eco*RI cuts both plasmids once 187 bp 5′ to the initiation codon of the *tk* gene; *Hin*dIII cuts the Thx26 plasmid 0.6 kb 3′ to the stop codon of the *tk* gene, and pAd-Thx76Δ within the CMV-*neo* cassette; *Xho*I cuts both plasmids once at the position of their respective *Xho*I linker insertions (Fig. 1). The presence of the 1.9 kb *Eco*RI–*Hin*dIII restriction fragment resistant to the *Xho*I digestion is characteristic of the reversion of one of the mutated *tk* genes to the wild-type, either by single-strand annealing (SSA; the dominant type of extrachromosomal homologous recombination), by double crossing-over, or by gene conversion of Thx26 at the position of the *Xho*I linker insertion. Although a simple loss of the *Xho*I linker from Thx26 would restore the wild-type *tk* gene, no HAT^R colonies could be detected in the control experiments where the Ltk– cells were transfected with the *Xho*I-linearized Thx26 plasmid alone. The diagnostic 1918 bp *Hin*dIII–*Eco*RI fragment resistant to *Xho*I digestion was detected for every HAT^R clone analyzed with the exception of one clone (Fig. 3, lane 7). In the case of the latter, the band corresponds to a fragment of higher molecular weight. It has probably arisen as a result of a double crossing-over with the resolution of the Holiday junction 5′ to the *Hin*dIII restriction site of Thx26.

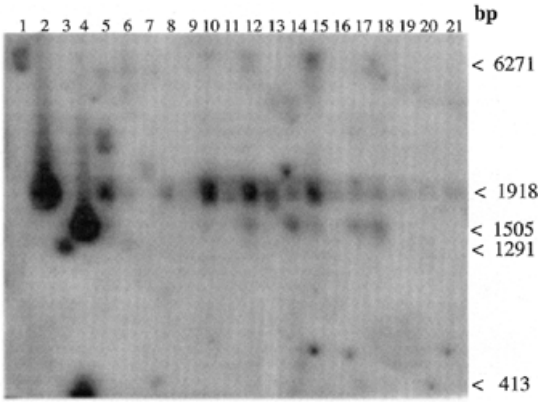

Figure 3. Representative Southern analysis of HAT^R colonies arising after co-transfection of Ltk– cells with the *Xho*I-linearized Thx26 and pAd-Thx76Δ plasmids. The [^{32}P]dCTP-labeled *Eco*RI–*Hin*dIII fragment of the HSV *tk* was used as a probe. Lanes 1–4 feature, respectively, pAd-Thx76Δ digested with *Hin*dIII and *Eco*RI; Thx26 digested with *Hin*dIII and *Eco*RI; pAd-Thx76Δ digested with *Hin*dIII, *Eco*RI and *Xho*I; and Thx26 digested with *Hin*dIII, *Eco*RI and *Xho*I. Lanes 5–21, 10 µg of gDNA isolated from randomly selected HAT^R colonies, digested with *Hin*dIII, *Eco*RI and *Xho*I. The 1918 bp band corresponds to the *Hin*dIII–*Eco*RI fragment of HSV *tk* which is resistant to *Xho*I digestion and can be seen for every HAT^R clone analyzed, with only one exception (lane 7).

The results from all recombination experiments are summarized in Table 1. The statistical significance for the differences observed was determined by the paired *t*-test. Although no significant difference in the rate of extrachromosomal homologous recombination, as compared to the untreated cells, was observed at the lowest concentration of the drug used (30 µg/ml = 0.186 mM), for the ISQ concentrations of 50, 75 and 100 µg/ml (0.310, 0.466 and 0.622 mM), the average increase in homologous recombination was, respectively, 85.6% (P = 0.0066; ranging from 34.4 to 182%), 191% (P = 0.0370; ranging from 150 to 261%) and 463% (P = 0.0030; ranging from 294 to 709%).

DISCUSSION

The precise mechanism of homologous recombination in eukaryotes remains unknown (20). The proposed models, known as the double-strand break (gap) repair model (21–23), the SSA model (24), the one-sided invasion model (25) and the synthesis-dependent strand-annealing model (26), were devised to accommodate the existing data from recombination studies done mainly in lower eukaryotes. Although all of these models differ from each other in several aspects, all postulate that homologous recombination is initiated by a double-strand break (DSB) in one of the recombining molecules.

Two of the major proteins known to recognize, interact with, and become activated by free DNA ends are DNA-dependent protein kinase (DNA-PK) and PARP (27). Whereas DNA-PK activation by free DNA ends most probably leads to the activation of the non-homologous end-joining DSB repair pathway (28), the role of the PARP in DNA repair/recombination remains unclear.

Table 1. Effect of ISQ on recombination between Thx26/*Xho*I and pAd-Thx76Δ/*Xho*I

Exp.	[Inhib.] (µg/ml)	HAT^R colonies per 6×10^6 cells transfected	G418^R colonies per 6×10^6 cells transfected	HAT^R/G418^R ratio	Percentage difference
1	0	80	15×10^2	0.0533	–
	30	63	16×10^2	0.0394	-26.0
	50	73	9×10^2	0.0811	+52.2
	75	80	6×10^2	0.133	+150
	100	63	3×10^2	0.210	+294
2	0	78	57×10^2	0.0137	–
	30	62	26×10^2	0.0238	+74
	50	54	14×10^2	0.0386	+182
	75	49	12×10^2	0.0408	+198
	100	73	8×10^2	0.0912	+567
3	0	33	7×10^2	0.0471	–
	30	40	6×10^2	0.0667	+41.6
	50	19	3×10^2	0.0633	+34.4
	75	17	1×10^2	0.170	+261
	100	18	1×10^2	0.180	+282
4	0	92	60×10^2	0.0153	–
	30	58	36×10^2	0.0161	+5.10
	50	72	27×10^2	0.0267	+73.9
	75	107	27×10^2	0.0396	+158
	100	124	10×10^2	0.124	+709

PARP is an abundant nuclear enzyme present in all higher eukaryotes. Although its structure and enzymatic activities have been extensively studied and are fairly well understood, its biological function remains unclear (7,8,11). It is known that PARP is activated by breaks in chromosomal DNA. It can bind to both single- and double-strand breaks in the DNA and initiate the polymerization of polyADP-ribose on itself and on a number of its protein substrates. The resulting negatively charged branched ADP-ribose polymer is thought to protect the DNA nicks from inappropriate recombination with homologous genomic sequences allowing the former to undergo repair via end-joining reactions. Lindhal *et al*. and others (9–11) have proposed that the role of PARP could be the negative regulation of recombination which ensures the stability of the genome. This hypothesis predicts that PARP inhibition or deactivation should result in an increase of homologous recombination processes. Indeed, PARP inhibition stimulates SCE and increases the frequency of intrachromosomal homologous recombination in mouse cells (14,15). Similarly, knock-out mice that lack PARP feature increased levels of SCE (16). Moreover, increased recombination activity after the loss of PARP anti-recombinogenic function is demonstrated by the fact that the PARP knock-out can rescue V(D)J recombination in SCID mice lacking the DNA-PK (17).

It is generally believed that extrachromosomal recombination is, to a certain extent, similar to meiotic and mitotic recombination and most probably makes use of the same enzymes as the latter. It would be reasonable then to expect that PARP inhibition would be stimulatory to extrachromosomal homologous recombination, as it is for SCE, intrachromosomal and V(D)J recombination.

Using plasmids carrying HSV *tk* genes inactivated by complementary insertion and deletion mutations, we were able to show that treatment of the transfected cells with a PARP inhibitor, ISQ, resulted in a dose-dependent increase in the rate of interplasmid homologous recombination ranging, for the highest ISQ concentration tested, from 2.8- to 7.0-fold.

Given that there is no clear understanding of PARP effects on the cellular processes, the only explanations for the observed increase of extrachromosomal homologous recombination are purely speculative. One possible explanation is that extrachromosomal recombination, similarly to inter- and intrachromosomal recombination, requires a protein whose activity is repressed by PARP. It is also possible that PARP inhibition makes the free ends of the linearized plasmids more readily accessible to the homologous recombination machinery. It might also be that ISQ acts on a protein or proteins other than PARP and that the former affect(s) recombination directly or indirectly. However, in spite of the fact that over the past six years ISQ has been widely used in the studies involving PARP, there have been no reports of ISQs affecting any enzyme other than PARP. Similarly, the suggestion that the observed increase in homologous recombination might be due to ISQs promoting DNA damage and thus stimulating recombination, although plausible is not supported by any reports of ISQ causing any DNA damage directly or indirectly (Guy Poirier, personal communication).

Yet another possible explanation involves the well-established fact that PARP inhibition decreases the frequency of illegitimate recombination (12,13; Fig. 2B and Table 1, this paper). If illegitimate and extrachromosomal recombination represent two competing pathways, then the repression of the former would result in stimulation of the latter.

In light of the recent finding by Ruscetti *et al*. that the protein kinase activity of DNA-PK can be stimulated by PARP in the presence of NAD^+ in a reaction that is blocked by ISQ (27), the following alternative explanation of our results can be offered. PARP might actively promote the functioning of a DNA repair pathway other than the recombination repair pathway, for example the non-homologous end-joining (NHEJ) repair pathway. If NHEJ competes with recombination repair, and if the former is stimulated by PARP, then PARP's inhibition may result in increased homologous recombination.

As mentioned in the introduction, a study similar to ours was conducted by Waldman and Waldman in 1990 (13). They investigated the effect of 3-MB, a competitive inhibitor of PARP, on illegitimate and extrachromosomal recombination in Ltk– cells. In their study cells were transfected with a wild-type HSV *tk* gene or with two defective *tk* gene sequences, circular or linearized, followed by selection for *tk*-positive colonies. While treatment of the cells with 2 mM 3-MB was found to reduce the number of colonies recovered with a wild-type *tk* 10- to 20-fold (numbers non-corrected for the cell survival rates), it reduced the number of colonies recovered with defective *tk* genes only to the same extent as in transfection with a wild-type gene. Hence, the authors have concluded that PARP played a role in illegitimate recombination, but did not play an important role in extrachromosomal homologous recombination.

The two major factors that distinguish our study of the effect of PARP inhibition on the extrachromosomal recombination from the one described above, and which might be responsible for the diverging results of these studies, are the nature of the PARP inhibitor and the method of transfection used.

Our predecessors made use of 3-MB, whereas we used ISQ. ISQ is a more potent and more specific inhibitor of PARP. ISQ was first identified as potent inhibitor of PARP by Banasik and co-workers during a large scale survey using an *in vitro* assay system (29). They showed that its 50% inhibitory concentration was two orders of magnitude lower than that of 3-aminobenzamide. Subsequently, Shah *et al*. reported that ISQ was also a potent inhibitor of PARP *in vivo* and showed that it could completely abolish oxidant-induced activation of PARP in C3H10T1/2 cells (30).

As far as the method of transfection of the plasmids is concerned, we used lipofectin in our study, because in our case it gives more consistent transfection efficiency than the calcium phosphate/DNA coprecipitation or electroporation methods used by Waldman and Waldman in their study (13). The method of transfection can be of importance, since the same authors have reported that an identical concentration of 3-MB resulted in different degrees of inhibition of random integration depending on whether the plasmid DNA had been electroporated or transfected via calcium phosphate/DNA coprecipitation (18).

Also, since the plasmids containing the mutated HSV *tk* genes used by Waldman and Waldman did not have a marker allowing the direct determination of the rate of illegitimate recombination, the determination of the frequency of homologous recombination in their experiments required two separate transfections: one to determine the rate of random integration, and the second to determine the rate of appearance of HAT^R colonies due to homologous recombination followed by

random integration (13). The frequency of homologous recombination was then calculated using the results from the two transfections without taking into account the variability in transfection efficiency from one experiment to another. We believe that our determinations of the frequency of homologous recombination are more accurate, since they derive from the data generated by a single transfection experiment and, hence, are not affected by the variability in the transfection efficiency.

Overall, we believe our finding that PARP inhibition stimulates extrachromosomal homologous recombination while decreasing non-homologous recombination is important First, it appears to solve the conflict in the previously reported results that PARP inhibition stimulates chromosomal but not extrachromosomal homologous recombination. Second, it suggests that PARP inhibition with ISQ may be used to increase gene targeting frequency. We are now investigating this possibility.

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