

Homologous recombination is a highly conserved determinant of the synergistic cytotoxicity between cisplatin and DNA topoisomerase I poisons

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Abstract

Phase I and II clinical trials are currently investigating the antitumor activity of cisplatin and camptothecins (CPTs; DNA topoisomerase I poisons), based on the dramatic synergistic cytotoxicity of these agents in some preclinical models. However, the mechanistic basis for this synergism is poorly understood. By exploiting the evolutionary conservation of DNA repair pathways from genetically tractable organisms such as budding and fission yeasts to mammalian cells, we demonstrate that the synergism of CPT and cisplatin requires homologous recombination. In yeast and mammalian cell lines defective for *RAD52* and *XRCC2/3*, respectively, the combination of these agents proved antagonistic, while greater than additive activity was evident in isogenic wild-type cells. Homologous recombination appears to mediate a similar interaction of X-rays and CPT, but antagonizes the synergism of cytarabine (Ara-C) with CPT. These findings suggest that homologous recombination comprises an evolutionarily conserved determinant of cellular sensitivity when CPTs are used in combination with other therapeutics. [Mol Cancer Ther. 2004;3(4):393–402]

Received 5/23/03; revised 1/6/04; accepted 1/16/04.

Grant support: Dutch Cancer Society (KWF) grant NKI 97-1440 (J. Schellens and J. Brouwer) and NIH CA23099 (M-A. Bjornsti).

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Introduction

Advanced solid tumors and hematological malignancies are often treated with combination chemotherapy. Along these lines, the translation of basic biological findings to preclinical and clinical investigations of antitumor agents may prove critical to the development of effective therapeutic strategies. Often, preclinical models predict unexpected interactions between agents with known cellular targets, which provides compelling rationale for clinical trials of specific drug combinations. While the synergism of some agents may have a solid mechanistic basis, in other instances, greater than additive activity in preclinical models is more serendipitous. Thus, investigating the mechanism of synergy in well-developed model systems can provide valuable insights into the combined action of specific agents which will ultimately impact the clinical efficacy of combination therapy. For example, both cisplatin (cDDP) and camptothecins (CPTs) induce DNA damage and showed a sequence-dependent synergistic cytotoxicity in a variety of tumor cell lines (1–8). Yet, the synergism of these agents in preclinical studies was surprising and not readily predicted from the mechanism of action of the single agents.

DNA topoisomerase I (Top1) is a highly conserved eukaryotic enzyme that catalyzes changes in the linkage of DNA strands (9). During DNA replication, transcription, and recombination, Top1 appears to act as a swivel to alleviate the overwinding of duplex DNA. CPTs [such as topotecan (TPT) and SN-38, the active metabolite of CPT-11] target Top1 and reversibly stabilize a covalent enzyme-DNA intermediate (10). During S phase, these ternary drug-Top1-DNA complexes are converted into DNA lesions that induce cell cycle arrest and cell death (11). The specific genotoxic lesions and the repair pathways that modulate sensitivity to this class of agent remain largely unknown, although homologous recombination in the budding yeast *Saccharomyces cerevisiae* has been shown to be a major determinant of cell survival (7, 8, 12). In contrast, non-homologous end joining does not appear to play a significant role in the repair of CPT-induced DNA lesions. Several lines of evidence suggest that the formation of double-stranded DNA breaks (DSBs) or stalled replication forks contribute to the cytotoxic action of CPTs (10, 12–16).

Cisplatin is one of the most widely applied anticancer drugs. In contrast to CPTs, cDDP induces intra- and inter-strand DNA adducts (17). The majority of adducts are intra-strand lesions which are repaired by nucleotide excision repair (NER) pathways (4–6). However, the resolution of inter-strand GG Pt-adducts requires the coordinated action of components of the homologous recombination and NER pathways (18–21). Although various DNA adducts have been reported to stabilize Top1-DNA covalent complexes, this has not yet been addressed with Pt-adducts (22–24).

However, even if Pt-adducts act as Top1 poisons, the greater than additive activity of TPT and cDDP suggests that additional cellular processes, such as downstream repair pathways, also function as critical determinants of cell sensitivity.

To investigate the underlying mechanism of synergy between these two agents and define specific genetic alterations that might predict tumor response, we decided to exploit the evolutionary conservation of specific DNA repair pathways in eukaryotes and assess cDDP and CPT activity in well-characterized cell systems. Figure 1 summarizes the conservation of NER and homologous recombination pathways (4–6, 25). Using paired isogenic yeast strains and mammalian cell lines, we report that orthologous gene products mediated cellular responses to each single agent, while a functional homologous recombination pathway was required for the synergistic cytotoxicity of cDDP and CPT (or TPT). Similar results were obtained in mammalian cells treated with X-rays and TPT; however, the converse was observed with Ara-C used in combination with TPT. These findings further suggest that homologous recombination is a major determinant of cellular sensitivity to the distinct lesions induced by cDDP (or X-rays) in combination with TPT, which may be exploited in predicting the clinical outcome of these therapeutic modalities.

Materials and Methods

Chemicals and Reagents

CPT, purchased from Sigma Chemical Co. (St. Louis, MO), was dissolved in DMSO at a concentration of 4 mg/ml and aliquots were stored at -20°C . Stock solutions of Topotecan, from Glaxo Smith Kline (King of Prussia, PA), prepared in 0.1% glacial acetic acid were stored at -20°C . cDDP, either the kind gift of J. Reedijk (Leiden University) or obtained

from TEVA Pharma (Mijdrecht, The Netherlands) and Cytarabine (Ara-C), from Pharmacia Corporation (Kalamazoo, MI), were dissolved in water. Yeast strains and Chinese hamster cell lines were cultured using standard conditions.

Yeast Strains and Chinese Hamster Cell Lines

S. cerevisiae and *Schizosaccharomyces pombe* strains used in this study are listed in Table 1. In each case, all strains were isogenic. *S. cerevisiae* strains MGSC353, MGSC425, and MGSC430 were made by one-step gene disruption using standard techniques. *S. cerevisiae* cells were cultured in YPD medium (10 g/l yeast extract, 20 g/l peptone, 2% dextrose). In homologous recombination proficient strains, CPT sensitivity was achieved by the galactose-induced expression of *TOP1* from the *GAL1* promoter in vector YCpGAL1-TOP1-L (26). YCpGAL1-L served as a vector control. Plasmids were introduced into *S. cerevisiae* cells via LiOAc transformation and individual transformants selected on synthetic complete medium lacking leucine (S.C.-leu media) supplemented with a final 2% dextrose. To induce *TOP1* expression, individual transformants were cultured in S.C.-leu medium supplemented with 2% galactose. In strains defective for homologous recombination (including *rad52* Δ and *rad51* Δ mutants), the endogenous levels of Top1p were sufficient to induce a CPT sensitive phenotype. *S. pombe* strains were cultured in YES medium (5 g/l yeast extract, 3% glucose) using standard techniques.

The Chinese hamster cell lines, listed in Table 1, were cultured in nutrient mixture F-10 (HAM) supplemented with glutamine, 10% FCS, and 25 mM HEPES.

Drug Treatment: Yeast Cells

A single transformant or untransformed cells were grown overnight in selective or rich media, respectively,

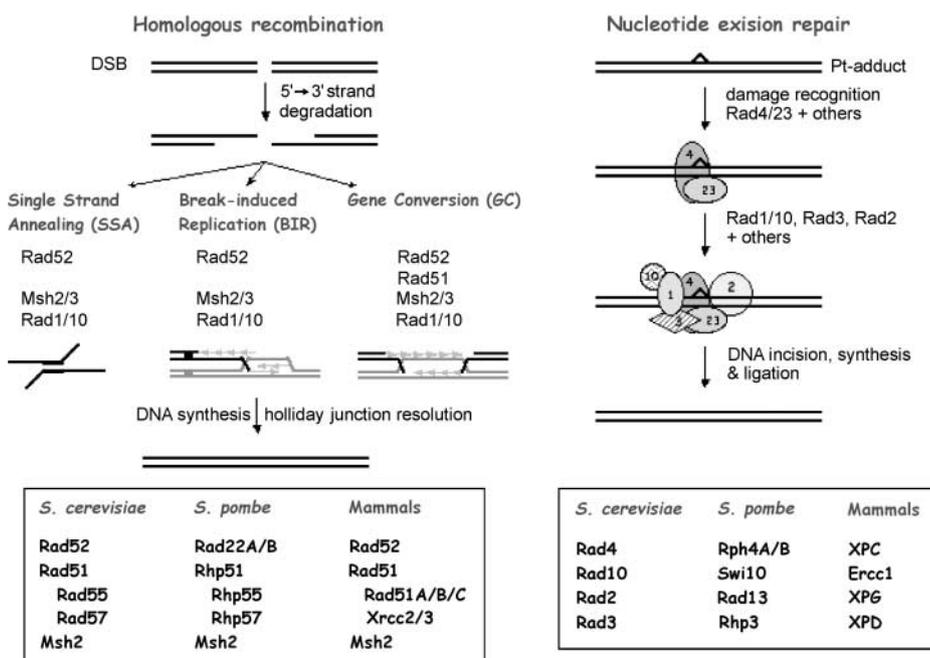


Figure 1. Schematic overview of conserved features of the homologous recombination and the NER pathways. Homologous recombination comprises three interrelated pathways: single-strand annealing, break-induced replication, and gene conversion. Specific common and unique members of these pathways are indicated. Relevant *S. cerevisiae*, *S. pombe*, and mammalian cell orthologues are aligned below each scheme.

Table 1. Yeast strains and mammalian cell lines

Strain/Cell line	Phenotype	Genotype	Ref/Source
<i>S. cerevisiae</i>			
W303-1B	Wild-type* ↑Top1	<i>MATa, ho, can1-100, ade2-1, trp1-1, leu2-3,112, his3-11,15, ura3-1, rad5-535</i> YCpGAL1-TOP1	R. Rothstein
W1588	Wild-type (<i>RAD5</i>) ↑Top1	W303-1B, <i>rad5Δ::RAD5</i> YCpGAL1-TOP1	R. Rothstein
MGSC131	NER- (<i>rad4</i>) ↑Top1	W303-1B, <i>rad4Δ::hisG</i> YCpGAL1-TOP1	(48)
MGSC425	NER-, MMR- (<i>rad4, msh2</i>) ↑Top1	W303-1B, <i>rad4Δ::hisG, msh2Δ::TRP1</i> YCpGAL1-TOP1	This study
MGSC353	NER-, HR- (<i>rad4, rad52</i>)	W303-1B, <i>rad4Δ::hisG, rad52Δ::TRP1</i>	This study
MGSC430	NER-, HR- (<i>rad4, rad51</i>)	W303-1B, <i>rad4Δ::hisG, rad51Δ::TRP1</i>	This study
<i>S. pombe</i>			
Y4	Wild-type	<i>h⁺, ade6-M216, leu1-32, ura4-D18</i>	A. Yasui
Y21	NER- (<i>rad13</i>)	Y4, <i>rad13::ura4⁺</i>	A. Yasui
RGL4	HR- (<i>rad22A</i>)	Y4, <i>rad22A₂::ura4⁺</i>	(40)
RGL11	HR- (<i>rad22A, rad22B</i>)	Y4, <i>rad22A₂::ura4⁺, rad22B::leu2⁺</i>	(40)
<i>Mammalian cell lines</i>			
CHO9	Wild-type	Chinese hamster ovary (CHO)	M. Zdzienicka
EM-C11	BER- (<i>xrcc1</i>)	CHO, <i>xrcc1</i>	M. Zdzienicka
XRC1	NHEJ- (<i>DNA-pkcs</i>)	CHO, <i>DNA-pkcs</i>	M. Zdzienicka
AA8	Wild-type	Lung	M. Zdzienicka
UV5	NER- (<i>xpd</i>)	Lung, <i>xpd (rad3)</i>	M. Zdzienicka
UV20	NER/HR- (<i>ercc1</i>)	Lung, <i>ercc1 (rad10)</i>	M. Zdzienicka
<i>Irs1SF</i>	HR- (<i>xrcc3</i>)	Lung, <i>xrcc3</i>	M. Zdzienicka
V79	Wild-type	Lung	M. Zdzienicka
VH1	NER- (<i>xpd</i>)	Lung, <i>xpd (rad3)</i>	M. Zdzienicka
<i>Irs1TOR</i>	HR- (<i>xrcc2</i>)	Lung, <i>xrcc2</i>	J. Thacker

to an absorbance at 600 nm ($A_{600\text{ nm}}$) of 1.0 (for *S. cerevisiae*) or 0.6 (for *S. pombe*) and aliquots were stored at -80°C . For subsequent experiments, cells were thawed on ice and cultured in appropriate media. Exponentially growing cultures were harvested by centrifugation, resuspended in water and 1 ml fractions were incubated with varying concentrations of cDDP. After 2 h, aliquots were serially 10-fold diluted and plated on medium containing varying concentrations of CPT. For studies of *S. cerevisiae* strains, the untransformed cells were plated onto S.C. medium supplemented with 2% dextrose, 25 mM HEPES (pH 7.2), and the indicated concentrations of CPT. DMSO was adjusted to the same final concentration for each strain examined. Similar conditions were used for plasmid transformed cells, except the medium was S.C.-leu supplemented with 2% galactose. CPT concentrations were as indicated. For *S. pombe* strains, cells were plated on YES medium supplemented with 25 mM HEPES (pH 7.2) and CPT. Viable cells forming colonies were counted following incubation for 5 days. To ensure that the weak hypomorphic *rad5* mutant, in the parental wild-type strain [W303-1B (27)], did not contribute to the drug sensitivity of repair defective strains derived from W303-1B, an isogenic strain corrected for *RAD5* was examined and found to have marginal effects on cell sensitivity to cDDP and CPT (data not shown).

With different strains, equitoxic concentrations of both drugs were empirically determined and used in subsequent experiments. Drug combination data were evaluated

according to the median-effect analysis (28, 29). The combination index (CI) was calculated using the calcsyn 1.1.1. Software (Biosoft, 1996) which uses the median-effect algorithm described by Chou *et al.* (28). The CI for each fraction killed was calculated using the equation:

$$CI = (d_1/D_1) + (d_2/D_2) + \{(d_1d_2)/(D_1D_2)\}$$

where D_1 and D_2 are the concentrations of drug 1 and 2 which alone results in a given fraction killed. d_1 and d_2 are the concentrations producing the same fraction killed in combination. In both cases, mean fraction killed values were defined in a minimum of three independent experiments and subsequently used to derived CI values. Antagonistic effects on drug-induced cytotoxicity are inferred from CI values > 1.2 , CI values between 1.2 and 0.8 ($0.8 < CI < 1.2$) were interpreted as additive, while synergistic cytotoxic effects were indicated by CI values < 0.8 .

Normalized isobologram analysis of the data sets, performed by the same software, confirmed the assignments of antagonistic, additive, and synergistic activity (29).

Drug Treatment: Mammalian Cells

Chinese hamster cell viability following single and combination drug treatment was assessed using the sulforhodamine B assay as described by Ma *et al.* (3). For each experiment, exponentially growing Chinese hamster cells were plated at a density of 1000 cells per 200 μl medium per well in 96-well plates. The cells were allowed to attach for 2 days, then treated with increasing concentrations of cDDP

(or Ara-C). After 2 more days, cells were treated with increasing concentrations of TPT. On the basis of the IC_{50} values of the single agents, a constant molar ratio of cDDP (or Ara-C) with TPT was used in combination. Cells were incubated for an additional 3 days and viability was assessed as described (3). This protracted treatment schedule was used to avoid potential complications of the sulforhodamine B assay attendant with short-term drug exposure. The same schedule was used to assess single agent effects, where cDDP or TPT treatments were omitted on day 2 or 4, respectively. Drug combination data were evaluated according to the median-effect analysis as described above.

Irradiation and TPT Combinations

For combined treatment with irradiation and TPT, or irradiation alone, the same schedule was used as described above. Two days after plating, cells were irradiated using a ^{137}Cs source with a dose rate of ~ 1 Gy/min. Subsequent TPT treatment and viability assays were as mentioned above.

Results

Activity of cDDP and CPT as Single Agents

To explore the relative contribution of specific repair pathways to cellular resistance to cDDP and CPTs, well-characterized, isogenic *S. cerevisiae*, *S. pombe*, and mammalian cell lines (Table 1) were first treated with each drug to define IC_{50} values, which in turn determined the ratio of drug concentrations to be used in combination treatment. Representative examples of survival curves germane to the subsequent discussion of the mechanism of synergy are depicted in Fig. 2. In Table 2, relative IC_{50} values were calculated from such sigmoidal cell survival curves, obtained with the indicated mutant strain/cell line, and corrected for IC_{50} values obtained with the relevant isogenic wild-type parental strain/cell line. Relative to wild-type cells, 4- to 10-fold decreases in cell viability are indicated by an asterisk, while greater than 10-fold effects are indicated by double asterisks. Absolute IC_{50} values are shown parenthetically for wild-type strains/cell lines.

In *S. cerevisiae*, cells proficient for homologous recombination do not exhibit appreciable sensitivity to CPT (12, 30). However, the increased expression of *TOP1* from a galactose-inducible *GAL1* promoter on a low copy vector suffices to induce a CPT-sensitive phenotype without adverse effects on cell viability in the absence of drug (26, 31). This is indicated by \uparrow Top1. In contrast, *TOP1* overexpression was not readily tolerated in cells deleted for *RAD51* or *RAD52* in this strain background. In these mutants, low endogenous levels of *TOP1* sufficed to yield a greater than 3-log drop in cell viability in the presence of CPT (Table 2, Fig. 2A). To eliminate the potential complications of *TOP1* levels on cDDP sensitivity in subsequent drug combination studies, the same \uparrow Top1 strains were also used to determine cDDP IC_{50} values.

Consistent with previous studies (14, 32, 33), deletion of the NER *RAD4* gene, induced about an 8-fold increase in *S. cerevisiae* cell sensitivity to cDDP (Table 2). However,

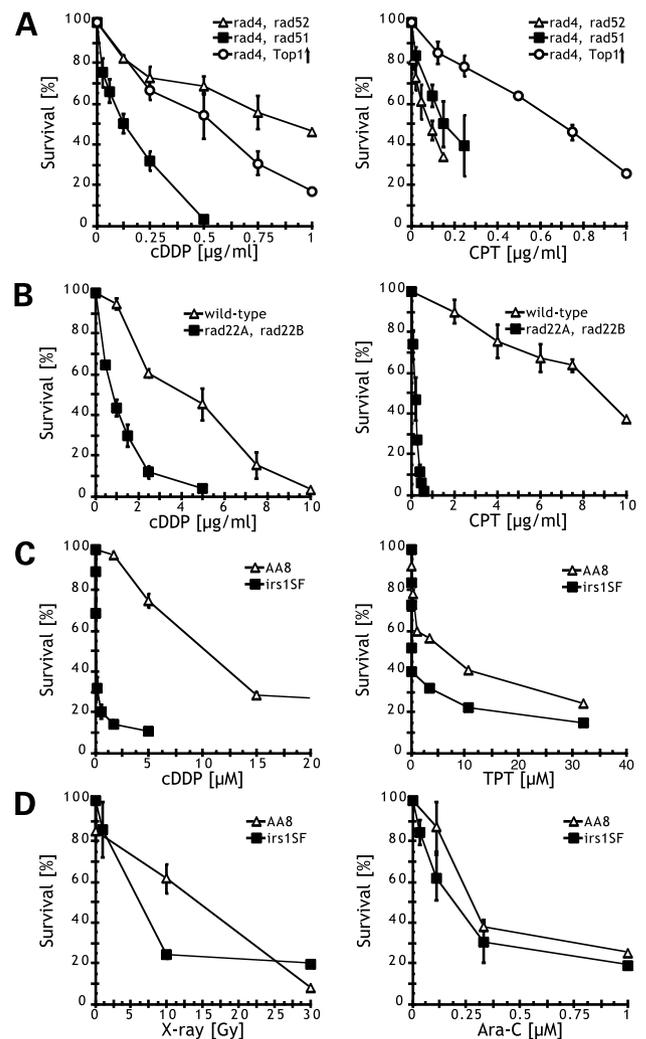


Figure 2. Representative examples of cell survival curves of *S. cerevisiae* strains (A), *S. pombe* strains (B), and Chinese hamster cell lines (C,D) treated with the indicated concentrations/doses of cDDP (left panel in A–C), CPT or TPT (right panel in A–C), X-ray (left panel in D), and Ara-C (right panel in D). Percent survival was determined as described in Materials and Methods in a minimum of three independent experiments.

cDDP cytotoxicity was unaffected by deletion of the mismatch repair gene, *MSH2*, either in the presence or absence of *RAD4* (Table 2, data not shown). In cells defective for both NER and homologous recombination, in double *rad4Δ*, *rad52Δ* mutants or *rad4Δ*, *rad51Δ* mutants, cell sensitivity to cDDP was also enhanced. Yet, in contrast to previous reports of *rad51Δ* cell sensitivity to cDDP (20, 32, 34), a greater than 30-fold decrease in cell viability was observed with cDDP-treated double *rad4Δ*, *rad51Δ* cells (Table 2, Fig. 2A). This supports the coordinated action of NER with gene conversion in the repair of interstrand Pt-adducts (18–22).

As previously reported for other NER mutants, *rad2*, *rad14*, and *rad1* (14, 32), *rad4*-null strains were unaffected by CPT (Table 2). The same was observed with the double *rad4Δ*, *msh2Δ* (NER-, MMR-) mutants. Thus, consistent with

earlier studies, mismatch repair and NER are not major determinants of *S. cerevisiae* cell sensitivity to CPT-induced DNA lesions (14, 32). However, defects in homologous recombination induce ~3-log drop in cell viability when cells are cultured in the presence of CPT [Table 2, Fig. 2A, Refs. (7, 8, 35, 32)].

Similar results were obtained with isogenic strains of *S. pombe* and Chinese hamster cell lines, where orthologues of the relevant NER and homologous recombination genes were mutated (Tables 1 and 2 and Fig. 2B). In *S. pombe*, ~5-fold diminution in cell survival was evident in *rad13* mutant and *rad22A* mutant cells in response to cDDP treatment [Table 2, Refs. (36, 37)]. However, the *rad22B* paralogue (see Fig. 1) did not contribute to cDDP resistance because similar IC₅₀ values were obtained for the single *rad22A* and double *rad22A*, *rad22B* mutant strains [Table 2, Ref. (37)]. As with *S. cerevisiae*, NER did not function to protect cells from CPT (Table 2). Yet, the magnitude of enhanced CPT sensitivity due to deletion of the *S. pombe* homologous recombination gene, *rad22A*, was comparable to that observed with *S. cerevisiae rad52* and *rad51* strains [Table 2 and Ref. (13)]. As with cDDP-induced adducts, deletion of *rad22B* did not appreciably affect the survival of CPT-treated cells.

We have previously reported that NER and homologous recombination, in isogenic cell line pairs, protects Chinese hamster cells from cDDP toxicity (38, 39). Although, with the treatment schedule used here, the effects with the *xrcc3* mutant cell line were more pronounced (Table 2, Fig. 2C). Consistent with these studies (38, 39), base excision repair (BER) and non-homologous end joining pathways do not contribute to cDDP resistance (Table 2). TPT sensitivity was selectively enhanced over 10-fold only in cell lines defective for homologous recombination (*ercc1*, *xrcc3*, and *xrcc2*) (Table 2, Fig. 2C). *ERCC1* functions in both NER and homologous recombination pathways. However, the lack of significant effects of two independent *xpd* mutants on TPT sensitivity argues that the relevant role of *ERCC1* in response to TPT-induced lesions is via homologous recombination (25).

Homologous Recombination Pathways Are Required for cDDP/CPT Synergy

To evaluate the activity of cDDP in combination with CPTs, equitoxic concentrations of both drug were used to treat the various yeast mutant strains and mammalian cell lines. As described in Materials and Methods, the median effect analysis was used to derive CI values for each drug combination, where CI > 1.2 indicates antagonism,

Table 2. Homologous recombination and nucleotide excision repair pathways modulate cell sensitivity to CPT and cDDP

Strain/Cell Line	Phenotype	IC ₅₀ (Relative to Wild-Type Control) ^a			
		cDDP ^b	CPT/TPT ^b	X-ray ^c	Ara-C ^d
<i>S. cerevisiae</i>					
W303-1B	Wild-type ↑Top1	1.0 (4.3)	1.0 (0.71)		
MGSC131	NER- (<i>rad4</i>) ↑Top1	0.12*	0.97		
MGSC425	NER-, MMR- (<i>rad4</i> , <i>msh2</i>) ↑Top1	0.15*	0.99		
MGSC353	NER-, HR- (<i>rad4</i> , <i>rad52</i>)	0.21*	<0.01 ^e **		
MGSC430	NER-, HR- (<i>rad4</i> , <i>rad51</i>)	0.03**	<0.02 ^e **		
<i>S. pombe</i>					
Y4	Wild-type	1.0 (4.2)	1.0 (8.6)		
Y21	NER- (<i>rad13</i>)	0.18*	0.73		
RGL4	HR- (<i>rad22A</i>)	0.24*	0.03**		
RGL11	HR- (<i>rad22A</i> , <i>rad22B</i>)	0.20*	0.02**		
Mammalian cell lines					
CHO9	Wild-type CHO	1.0 (9.3)	1.0 (16.7)		
EM-C11	BER- (<i>xrcc1</i>)	1.3	0.36		
XRC1	NHEJ- (<i>DNA-pkcs</i>)	1.1	0.82		
AA8	Wild-type Lung	1.0 (10.3)	1.0 (1.9)	1.0 (15.7)	1.0 (0.92)
UV5	NER- (<i>xpd</i>)	0.09**	0.46	0.57	0.76
UV20	NER/HR- (<i>ercc1</i>)	0.002**	0.042**	0.45	0.82
<i>irs1SF</i>	HR- (<i>xrcc3</i>)	0.012**	0.039**	0.18*	0.20*
V79	Wild-type	1.0 (3.7)	1.0 (7.9)		
VH1	NER- (<i>xpd</i>)	0.26*	0.41		
<i>irs1TOR</i>	HR- (<i>xrcc2</i>)	0.08**	0.072**		

^aDose necessary to achieve 50% growth inhibition of mutant strain/cells under culture conditions described in Materials and Methods, relative to the isogenic wild-type strain/cells. Relative to wild-type cells: *, 4- to 10-fold decreases in cell viability; **, greater than 10-fold effects.

^bAbsolute IC₅₀ concentrations for wild-type strains/cells are in parentheses. Concentrations of cDDP and CPT were in micrograms per milliliter for *S. cerevisiae* and *S. pombe*, while micromolar concentrations of cDDP and TPT were used with mammalian cell lines.

^cIC₅₀ value (Gy) for wild-type AA8 cells are in parentheses.

^dIC₅₀ value (M) for wild-type AA8 cells are in parentheses.

^eIsogenic wild-type *RAD52* or *RAD51* cells expressing endogenous levels of Top1 are resistant to CPT at the limit of drug solubility (10 μg/ml). Dividing the IC₅₀ values obtained with *rad52Δ* (0.09) and *rad51Δ* (0.15) strains with 10 μg/ml defines relative values with an upper limit of 0.01 and 0.02, respectively.

0.8 < CI < 1.2 indicates additivity, and CI < 0.8 indicates synergism (28, 29). In the yeast experiments, a matrix of cDDP and CPT concentrations was used to define combination interactions, while with Chinese hamster cell lines, a constant molar ratio of drugs was used as described in Ref. (3). The results of the cDDP and CPT analogue combination studies are shown in Figs. 3 and 4. For ease of discussion, cDDP/CPT or cDDP/TPT refers to combination treatment with cDDP and CPT or cDDP and TPT, respectively.

As with the single agents, W303-1B (\uparrow Top1), the corrected wild-type variant W1588 (*RAD5*, \uparrow Top1), and W303-1B (endogenous Top1) gave similar patterns of cDDP/CPT toxicity, with synergy evident only when the fraction killed exceeded 0.7 (Fig. 3, A and B and data not shown). Thus, overexpression of Top1 did not contribute to cDDP/CPT synergy, nor did the hypomorphic *rad5-G535R* mutation. The latter allowed us to use W303-1B isogenic mutants enabling direct comparison with the large body of literature, based mostly on W303-1B-derived strains.

A slight increment in synergy was induced by deletion of the NER gene, *RAD4*. This effect was minor and not reproducibly observed in other model systems (Fig. 3, A and C and B and C). Nevertheless, the modest increase in cDDP/CPT synergy evident in the *S. cerevisiae rad4Δ*, \uparrow Top1 strain was exploited in subsequent studies to accentuate the shift from synergy to additivity or antagonism.

As shown in Fig. 3A, mutation of the mismatch repair gene *MSH2* did not affect the profile of cDDP/CPT drug action. In contrast, deletion of *RAD52* not only suppressed the synergistic activity of cDDP/CPT, but actually antagonized the activity of the two drugs across the entire range of concentrations used. Surprisingly, deletion of *RAD51* did not affect the pattern of drug interactions observed with the wild-type control (Fig. 3B). These data suggest that single-strand annealing and/or break-induced replication, rather than gene conversion [see Fig. 1, Ref. (25)], are required for the synergistic activity of cDDP with CPT. These findings also support the notion that the induction of select homologous recombination pathways converts cDDP/CPT-induced DNA lesions into more severe genotoxic damage, which is not effectively repaired by other pathways. Moreover, because endogenous Top1 levels are sufficient to distinguish antagonism and synergy in *rad52Δ* and *rad51Δ* strains, respectively, this further obviates any concern about TOP1 overexpression in isogenic wild-type strain (Fig. 3A).

These conclusions were also supported by data obtained with isogenic *S. pombe* and mammalian cell lines. In Fig. 3C, abrogation of NER in the *rad13* mutant did not alter the synergistic profile of cDDP/CPT observed with wild-type cells. However, cellular responses to combined drug treatment progressively shifted to antagonism on deletion of *rad22A*, which was accentuated by deletion of *rad22B*. In contrast to the results obtained with single agent treatment (Table 2), deletion of *rad22B* had a marked effect on the combined activity of cDDP with CPT (Fig. 3C). This indicates that the combination of these two agents induces unique DNA damage, recognized by both *S. pombe* *RAD52* homologues. The synergism evident at high drug concen-

trations (fraction killed > 0.9) in the *rad22A*, *rad22B* mutant may derive from the low, yet detectable, levels of homologous recombination reported for this double mutant (40).

As shown for the single agents, abrogation of BER or non-homologous end joining had little effect on the combined activity of cDDP/TPT in isogenic Chinese hamster cell lines (Fig. 4A). Similarly, in two independently

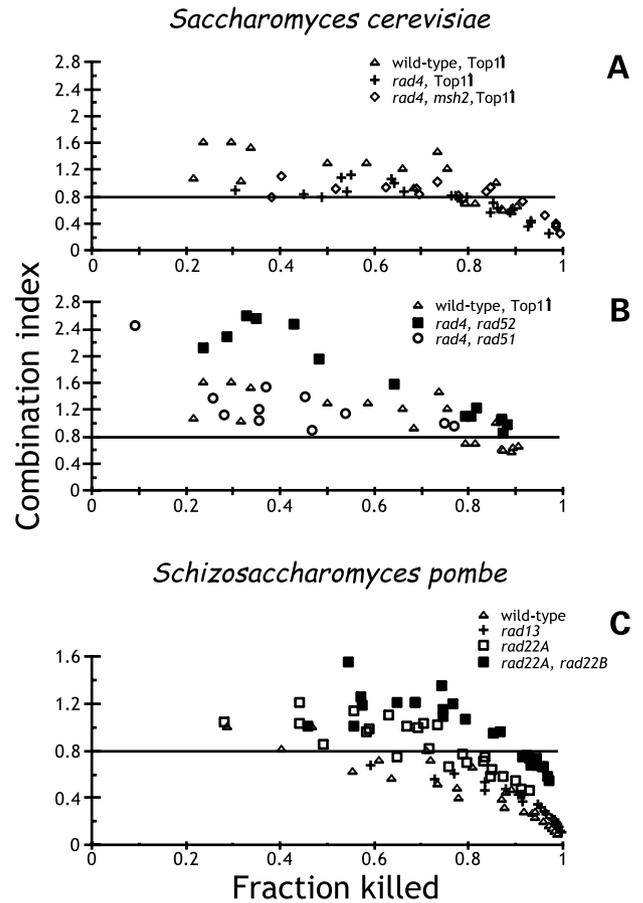


Figure 3. *RAD52* orthologues are required for the synergistic activity of cDDP and CPT in *S. cerevisiae* and *S. pombe*. Congenic strains of *S. cerevisiae* (panels **A** and **B**) or *S. pombe* (panel **C**) were sequentially treated with equitoxic concentrations of cDDP followed by CPT as detailed in Materials and Methods. The number of viable cells forming colonies was determined on agar plates. Clonogenic assay data from a minimum of three independent experiments were averaged and the mean values were subjected to median-effect analysis to define CI values, which were plotted versus fraction of cells killed (a value of 1 represents 100% cell death). CI values > 1.2 indicate antagonistic interactions, additive cytotoxicity is indicated by 0.8 < CI < 1.2, and synergistic cytotoxicity is indicated by CI < 0.8. In **A**, congenic wild-type, *rad4Δ*, and *rad4Δ*, *msh2Δ* *S. cerevisiae* strains were treated with cDDP and CPT in galactose containing selective media to induce *TOP1* expression from the *GAL1* promoter. In **B**, the same wild-type values from **A** were plotted with results obtained from congenic *rad4Δ*, *rad52Δ*, and *rad4Δ*, *rad51Δ* strains in synthetic complete media, where endogenous Top1 levels suffice to induce CPT sensitivity. In **C**, *S. pombe* strains (wild-type or deleted for *rad13*, *rad22A*, or both *rad22A*, *rad22B*) expressing wild-type levels of Top1 were sequentially treated with cDDP and CPT in YES medium. The average error was < 10%.

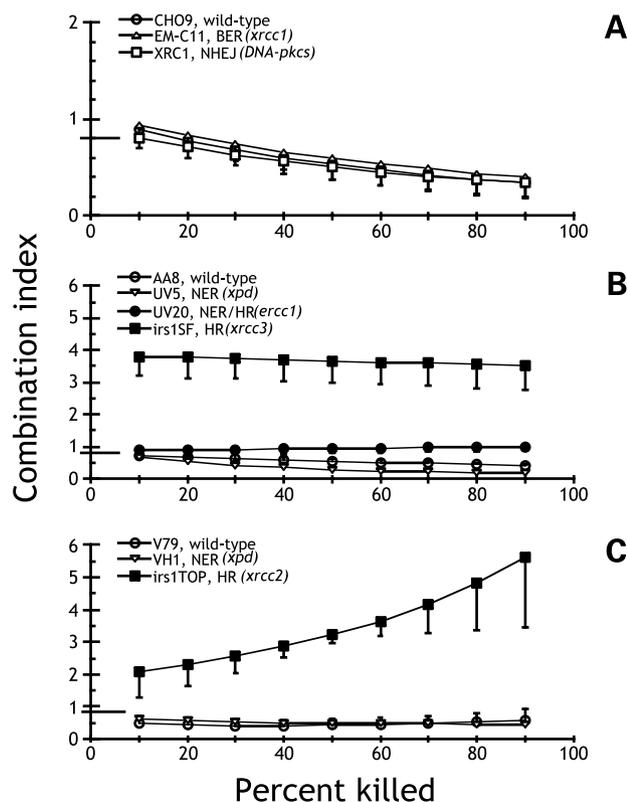


Figure 4. Functional homologous recombination is required to elicit the synergistic activity of cDDP with TPT. In **A–C**, the indicated isogenic parental and repair deficient Chinese hamster cell lines were sequentially treated with equal molar ratios of cDDP and TPT, defined by the IC_{50} values obtained with each single agent, as described in Materials and Methods. In a minimum of three independent experiments, cell viability was determined using the SRB assay and CI values plotted versus the percentage of cells killed. As described in the legend to Fig. 2, $CI < 0.8$ indicates synergy, $0.8 < CI < 1.2$ indicates additivity, and $CI > 1.2$ indicates antagonism.

derived *xpd* mutant cell lines, a functional NER pathway was not linked with cDDP/TPT synergy (Fig. 4, B and C). However, consistent with the results obtained with the yeast models, specific defects in homologous recombination (*XRCC2* and *XRCC3*) completely shifted the pattern of cDDP/TPT activity from one of synergy to highly antagonistic (Fig. 4, B and C). Cell lines deficient in the *RAD51* paralogues *XRCC2* and *XRCC3* were used because *Rad51*-mutant cells are unviable. However, *XRCC2* and *XRCC3* are required for functional homologous recombination given that the central role of yeast *Rad52* in homologous recombination shifts to *Rad51* and *Rad51* paralogues in mammalian cells (25, 41). At drug concentrations sufficient to induce high levels of cell kill, the synergistic activity of cDDP/TPT combinations was abolished in the *ercc1* mutant cell line (Fig. 4B). Given that the magnitude of the shift in CI values did not compare with that observed with *xrcc2* and *xrcc3* mutant cell lines, these data are consistent with the relatively minor role of *ERCC1* in homologous recombination (25).

A Different Combinations of Cytotoxic Agents with TPT Alters the Mechanism of Synergy

The results obtained above clearly indicate that functional homologous recombination pathways, but not NER, are required in yeast and mammalian cells to induce the synergistic activity of cDDP in combination with CPTs. Because homologous recombination also functions to protect cells from TPT, these findings raise the possibility that the synergistic activity of cDDP/TPT is dictated by Top1-mediated DNA damage. Irradiation and the antimitabolite Ara-C have also been shown to have greater than additive activity in combination with CPTs (42, 43). However, the patterns of DNA lesions induced by these treatments are distinct from those induced by cDDP. Thus, we reasoned that the analysis of X-ray/TPT and Ara-C/TPT combinations in isogenic parental AA8, *xpd* (UV5), *ercc1* (UV20), and *xrcc3* (*irs1SF*) cell lines would determine whether similar mechanisms of synergistic drug action apply.

As summarized in Table 2, cell sensitivity to X-ray or Ara-C was largely unaffected by the loss of *XPB* or *ERCC1* function. In contrast, the cytotoxicity of either agent was potentiated in the *xrcc3* mutant cell line. These data are at odds with the enhanced sensitivity of the *ercc1* mutant cell line to cDDP or TPT, which suggests that DNA lesions induced by X-rays or Ara-C are not substrates for the homologous recombination repair pathways requiring *ERCC1* function.

Equitoxic combinations of X-ray with TPT induce similar patterns of synergy and antagonism in this panel of cell lines (Fig. 5A) as defined for cDDP/TPT (Fig. 4B). X-ray/TPT combinations were synergistic in the parental and *xpd*

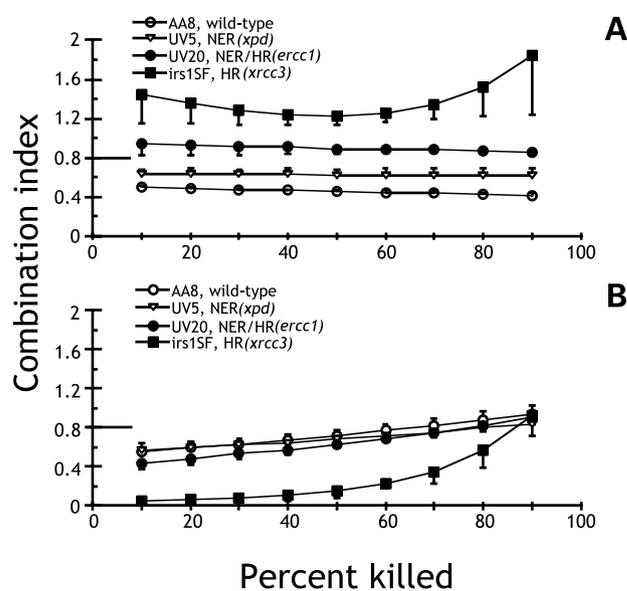


Figure 5. Homologous recombination (*HR*) exerts contrary effects on the synergistic activity of X-rays versus Ara-C, in combination with TPT. Isogenic parental and mutant Chinese hamster cell lines were sequentially treated with equitoxic doses of X-rays (panel **A**) or Ara-C (panel **B**), followed by TPT as described in Materials and Methods ($n \geq 3$). As detailed in the Fig. 4 legend, the CI values were plotted versus the percentage of cells killed.

mutant cell lines, while the *ERCC1* defect induced a slight shift to additive cell kill. Despite the remarkable differences in *ercc1* mutant cell sensitivity to cDDP, TPT, or X-ray as single agents, these cells exhibited similar alterations in response to cDPP/TPT and X-ray/TPT combinations. Moreover, a common mechanistic basis for the synergistic activity of these combinations was evidenced by the shift from synergy to antagonism in X-ray/TPT-treated *xrcc3* mutant cells. Whether TPT is combined with cDDP or X-rays, functional homologous recombination pathways are required to elicit synergistic cell kill.

Remarkably, the exact opposite was observed with Ara-C/TPT. Although the pattern of cell sensitivity to Ara-C, as a single agent, resembled that of X-rays for the four cell lines examined in Table 2, defects in *XRCC3*-mediated homologous recombination dramatically enhanced, rather than suppressed the synergistic activity of this drug combination (Fig. 5B). The CI values obtained with *xpd* and *ercc1* mutant cell lines were identical to those observed with the parental cell line. These data indicate that *XRCC3* functions to protect cells from the DNA lesions induced by Ara-C/TPT, while the same homologous recombination pathway exacerbates the cytotoxic activity of DNA damage induced by cDDP or X-rays in combination with TPT. Thus, the specific repair pathways that determine cell sensitivity to TPT depend on the spectrum of DNA lesions induced by other cytotoxic agents used in combination with this Top1 poison.

Discussion

Determinations of optimal combinations and schedules of chemotherapeutics are often hampered by the lack of insight into drug interactions, and therefore, have to be empirically defined. The remarkable schedule-dependent synergistic activity of platinum agents and Top1 poisons, observed in preclinical models (1–3), prompted the design of several clinical trials to investigate the therapeutic efficacy of Pt-/CPT- analogue combinations. Nevertheless, previous analyses of Top1 protein levels, cDDP accumulation, DNA-adduct formation, or cell cycle effects failed to elucidate the mechanism of synergy (1–3). Thus, the molecular basis for cDDP/TPT synergy was not readily apparent from studies of these drugs as single agents.

Using well-characterized, isogenic yeast strains and cell lines with defects in specific DNA repair pathways, we show that the synergistic activity of cDDP with CPT analogues requires homologous recombination. Moreover, this mechanism of greater than additive cytotoxic drug action is conserved in evolutionarily distinct organisms, including the yeasts, *S. cerevisiae* and *S. pombe*, and mammalian cell lines. As summarized in Fig. 1, the homologous recombination pathway can be divided into three sub-pathways: Gene Conversion, single-strand annealing, and Break-induced Replication (reviewed in 25). The Gene Conversion pathway is dependent on all members of the *RAD52* epistasis group (*RAD50-57*, *MRE11*, and *XRS2*) while single-strand anneal-

ing and Break-induced Replication require *Rad52p* but are *Rad51p*-independent (44, 45). Given the large number of repeated DNA sequences in mammalian cells, about 80% of the double-strand DNA breaks, channeled to homologous recombination repair pathways, are processed by error-prone single-strand annealing while the other 20% are processed by the error-free Gene Conversion pathway (46). Furthermore, in mammalian cells, homologous recombination is not a *RAD52*-dependent pathway as it is in yeast. Rather, *RAD51* plays a more prominent role, as evidenced by the fact that *RAD51* is an essential gene in mice and DT40 chicken cells, while *rad52*^{-/-} mice or DT40 cells are viable (reviewed in 41). The *RAD51* paralogues (*RAD51A/B/C* and *XRCC2/3*) have apparently evolved with functions similar to *RAD52* and support the essential function of *RAD51* (reviewed in 41). This shift in the central function of *RAD52* in homologous recombination in yeast, to that of *RAD51* in mammalian cells, is also evident in our findings. *RAD52*, but not *RAD51*, function is required for the synergistic activity of cDDP/CPT in yeast (Fig. 3), while in mammalian cells, defects in the *RAD51* paralogues, *XRCC2/3*, are major determinants of cellular responses to cDDP/TPT (Fig. 4).

In each model system, the homologous recombination machinery functions to protect cells from the cytotoxic lesions induced by either cDDP or CPT analogues used as single agents (Table 2). This has been reported for *S. cerevisiae* and *S. pombe* (4–8, 12, 14, 22–24, 30, 32) and shown here for Chinese hamster cells. NER functions to protect yeast and mammalian cells from cDDP-induced toxicity (Table 2 and Refs. 4–6, 32, 38); however, NER, BER, mismatch repair, and non-homologous end joining did not affect cellular response to Top1 poisons (Table 2). In contrast to yeast, non-homologous end joining constitutes a significant repair pathway for double-stranded DNA breaks in mammalian cells. The predominant role of homologous recombination appears to be restricted primarily to S phase. Yet, despite these organism differences, non-homologous end joining did not affect cDDP or TPT sensitivity, which supports the view that cytotoxic lesions induced by either agent occur during S phase and is resolved by homologous recombination.

The combined schedule of cDDP with CPT (TPT) revealed a novel function for the homologous recombination machinery in potentiating the cytotoxic action of drug combination. Thus, the repair initiated by *RAD52*-dependent processes in yeast and *XRCC2/3* pathways in mammalian cells converts the initial DNA adducts into genotoxic lesions that are even more lethal. Such a scenario has been demonstrated for temozolomide in cells defective for *O*⁶-methylguanine-DNA methyltransferase (47). In this case, futile cycles of mismatch repair are initiated by temozolomide-induced *O*⁶-methylguanine lesions. Whether this has any functional relationship to the enhanced cytotoxicity of cDDP/TPT induced by the homologous recombination machinery, beyond establishing the basic principle of repair pathways generating genotoxic lesions, has yet to be investigated.

The mechanistic basis of cDDP/CPT synergy appears to be restricted to specific combinations of CPT analogues with other cytotoxic regimens. While a similar role for homologous recombination in exacerbating the toxicity of X-rays with TPT was observed, defects in *XRCC2/3* actually enhanced, rather than suppressed, the synergistic activity of Ara-C/TPT. In the latter case, the homologous recombination machinery acts to resolve potentially lethal DNA lesions. Thus, even if Pt-DNA adducts were to act as endogenous Top1 poisons, as reported for Ara-C (24), this cannot explain the synergy of cDDP/TPT because the results obtained with *xrcc2/3* mutants are completely contrary to those obtained with Ara-C/TPT.

From the data presented for the single agents in Table 2 and the combination of cDDP with CPT or TPT in Figs. 3 and 4, it is clear that the cellular processes contributing to the greater than additive activity of the two drugs may not be inferred from cellular responses of either agent alone. A more thorough investigation of the schedule-dependent activity of cDDP/CPT unveiled a novel mechanism of synergy that was dependent on specific components of the homologous recombination machinery. Moreover, this mechanism is highly conserved through evolution, supporting the use of model organisms to study drug action (30). However, the contrary activity of *XRCC2/3* in potentiating the synergistic activity of cDDP/TPT and X-ray/TPT, while suppressing the cytotoxicity of Ara-C/TPT, underscores the need to define the specific pattern of DNA lesions induced by specific drug combinations to better predict tumor response to combination therapy.

Acknowledgments

Drs. R.A. Verhage, L.E.T. Jansen, J.A. Brandsma, and M. de Ruiter are acknowledged for fruitful discussions and technical assistance. We also thank M. Zdzienicka and J. Thacker for generous gifts of reagents and strains.

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