Multiple pathways of recombination define cellular responses to cisplatin

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Background: Cisplatin is a DNA-damaging drug used for treatment of testicular tumors. The toxicity of cisplatin probably results from its ability to form DNA adducts that inhibit polymerases. Blocked replication represents a particular challenge for tumor cells, which are committed to unremitting division. Recombination provides a mechanism by which replication can proceed despite the presence of lesions and therefore could be significant for managing cisplatin toxicity.

Results: Recombination-deficient *Escherichia coli* mutants were strikingly sensitive to cisplatin when compared with the parental strain. Our data identified both daughter-strand gap and double-strand break recombination pathways as critical for survival following treatment with cisplatin. Although it is established that nucleotide excision repair (NER) significantly protects against cisplatin toxicity, most recombination-deficient strains were as sensitive to the drug as the NER-deficient *uvrA* mutant. Recombination/NER deficient double mutants were more sensitive to cisplatin than the corresponding single mutants, suggesting that recombination and NER pathways play independent roles in countering cisplatin toxicity. Cisplatin was a potent recombinogen in comparison with the *trans* isomer and canonical alkylating agents. Mitomycin C, which like cisplatin, forms DNA cross-links, was also recombinogenic at minimally toxic doses.

Conclusions: We have demonstrated that all of the major recombination pathways are critical for *E. coli* survival following treatment with cisplatin. Moreover, recombination pathways act independently of NER and are of equal importance to NER as genoprotective systems against cisplatin toxicity. Taken together, these results shed new light on how cells survive and succumb to this widely used anticancer drug.

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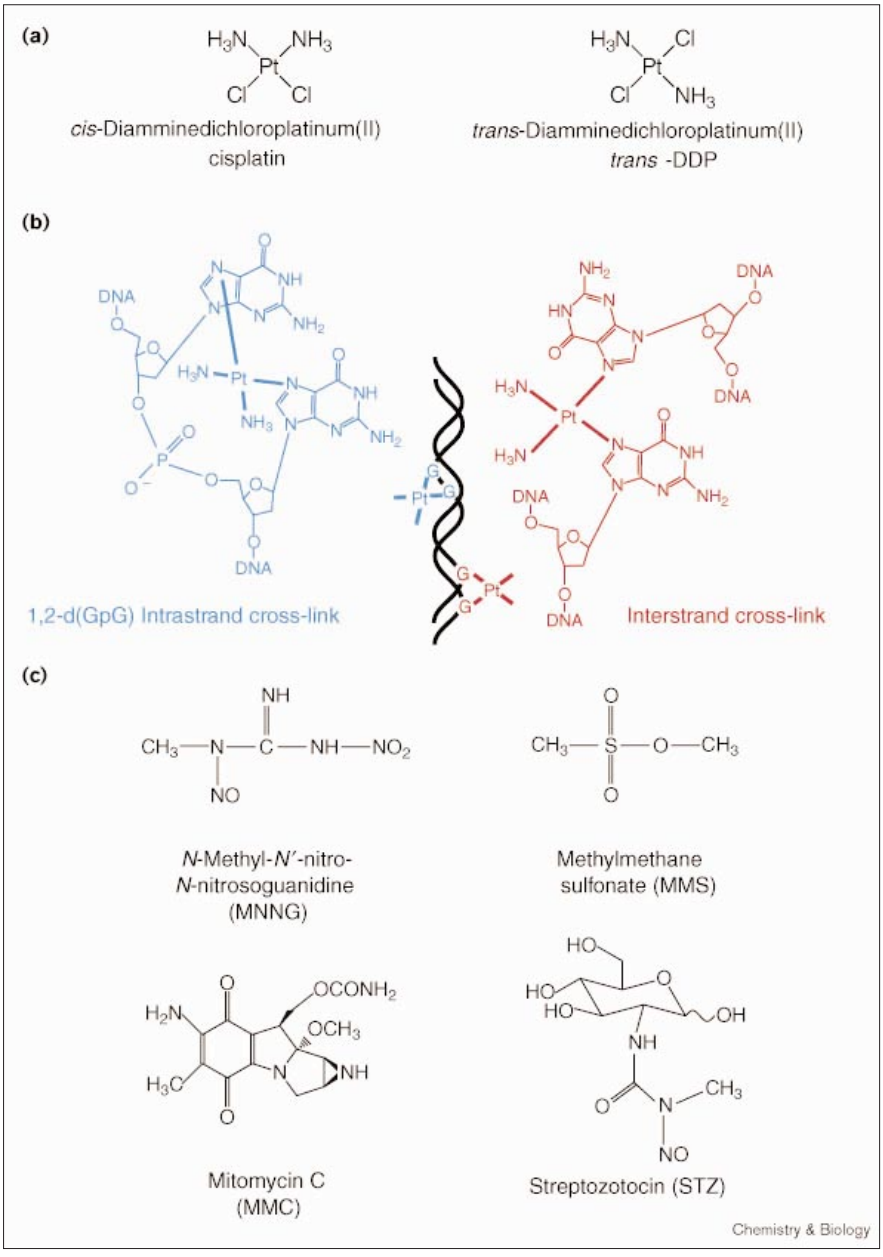
Introduction

The cytotoxicity of many DNA-damaging agents is believed to result from the formation of lesions that block the processivity of DNA polymerases and cause replication arrest [1]. Replication arrest, in turn, leads to the formation of secondary DNA damage such as daughter-strand gaps and double-strand breaks [2]. If uncorrected, such damage can be lethal because of both the loss of essential genes and faulty chromosomal segregation; all organisms, therefore, have developed strategies for repair of these types of damage. In *Escherichia coli*, the principal mechanism for repair of daughter-strand gaps and double-strand breaks is recombination, in which the injured DNA strand is paired with an intact homologous strand that provides a template for repair of the secondary lesion (for reviews see [1–3]).

The widely used chemotherapeutic drug cisplatin (*cis*-diamminedichloroplatinum(II); Figure 1a) is toxic to cells and is strikingly effective against testicular tumors [4]. Although the cytotoxicity of cisplatin is attributed to its capacity to damage DNA, the detailed molecular mechanism to account for the therapeutic efficacy and organotropic specificity of this drug remains elusive. Cisplatin binds to the N7 atom of purine bases in DNA to form predominantly 1,2-d(GpG) (Figure 1b), 1,2-d(ApG) and 1,3-d(GpNpG) (in which N is any nucleotide) intrastrand cross-links, and a small percentage of interstrand crosslinks (between two guanines in complementary strands; Figure 1b) [5,6]. These DNA adducts elicit a variety of cellular responses, including inhibition of DNA synthesis. The 1,2-intrastrand cross-links, in particular, are strong blocks to replication *in vitro* and *in vivo* [7,8]. Cisplatin induces recombination in *Candida albicans* [9] and *Drosophila melanogaster* [10], and it induces meiotic crossing-over in germ cells of mice [11]. Recombination deficient mutants, such as *recA* and *recBC* in *E. coli* [12–14], *RAD52* in *Saccharomyces cerevisae* [15,16], and *RAD21* and *RAD22* in *Schizosaccharomyces pombe* [17], show sensitivity to cisplatin. Remarkably, despite these observations, there has been no systematic analysis to date of recombination as a strategy for managing DNA damage caused by cisplatin. To address this gap in

Figure 1



Structures of DNA-damaging agents used in this study. **(a)** Structures of diamminedichloroplatinum(II) isomers. **(b)** Structures of cisplatin–DNA cross-links. **(c)** Chemical structures of other DNA-damaging agents used in this study.

understanding, we assembled a series of *E. coli* strains that were deficient in the major pathways of recombination and studied their responses to treatment with the drug. We report that recombination-deficient mutants showed exceptionally high sensitivity to cisplatin in comparison to their parental strain. Indeed, most recombination-deficient mutants were as sensitive to cisplatin as were mutants lacking nucleotide excision repair (NER). Recombination/NER- deficient double mutants produced increased sensitivity to cisplatin indicating that these two pathways act independently in the cellular defenses against the drug. In addition, we found that even modestly toxic doses of cisplatin were potently recombinogenic when compared with other DNA-damaging agents. The results suggest a model for cisplatin cytotoxicity that can accommodate the currently known cellular effects of the drug and might account for the therapeutic specificity of cisplatin.

Results

Mutants deficient in the initiation of recombination are hypersensitive to cisplatin

Two pathways can initiate recombinational repair in *E. coli*: the RecFOR pathway for the repair of daughter

strand gaps and the RecBCD pathway for the repair of double-strand breaks. Daughter strand gaps are formed when the processivity of the replication fork is interrupted by a noncoding DNA lesion, such as a UV-induced dimer, in the template strand, and the lesion is left opposite a single-stranded (ss) gap in the nascent strand [18]. Genetic evidence implicates proteins of the RecFOR pathway in the recombinational repair of UV-induced daughter strand gaps [19]. Biochemical studies demonstrate that the RecOR complex promotes the binding of RecA protein to ss DNA (in the presence of ss DNA binding protein), and it facilitates the homologous pairing by RecA [20]. The RecFR complex is thought to interact directly with the stalled replication fork, and it might function in fork disassembly or reassembly during recombination and repair [21,22]. To assess the importance of the RecFOR pathway in the cellular response to cisplatin DNA damage, we examined the survival of *recF*, *recO* and *recR* mutants after treatment with cisplatin (Figure 2a). At the highest cisplatin dose (100 μM), the surviving fraction for each mutant was approximately three orders of magnitude lower than that for the isogenic wild-type strain. The high sensitivity of these mutants is consistent with a role of the RecFOR gene products in recombinational repair of daughter strand gaps produced as a consequence of replication blockage by cisplatin adducts.

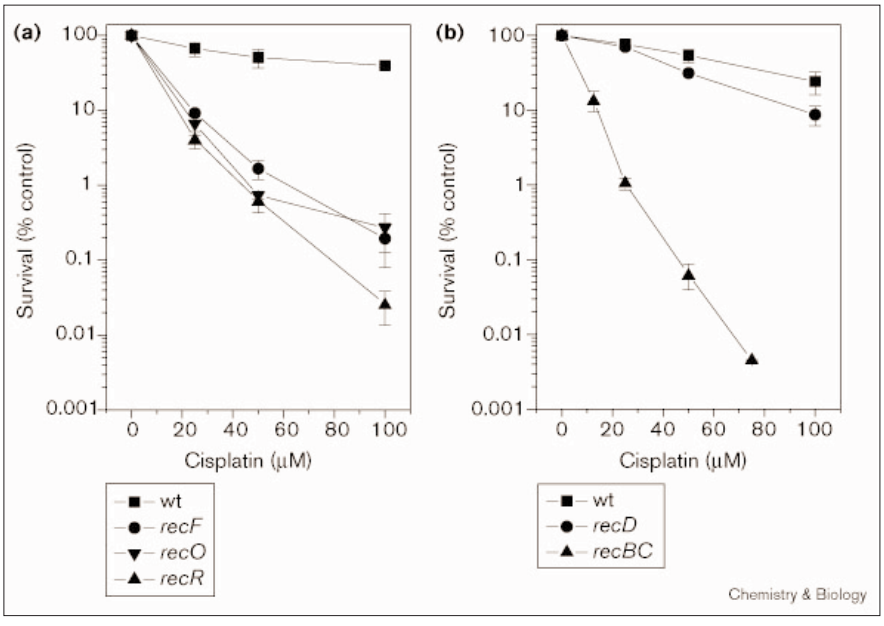
In *E. coli* the RecBCD pathway is essential for recombinational repair of X-ray-induced double-strand breaks [23]. The RecBCD complex combines helicase and nuclease functions that simultaneously unwind and asymmetrically degrade double-strand breaks (the strand with the 3′ terminus is nicked more frequently than the strand with the 5′ end). Once the enzyme complex encounters a χ sequence (5′-GCTGGTGG-3′) from the 3′ direction, it pauses and nicks the DNA to generate a 3′ ss DNA tail that serves as a substrate for RecA polymerization and initiation of recombination [24,25] (reviewed in [26,27]). To determine if the RecBCD pathway participates in the cellular processing of cisplatin-induced DNA damage, we examined the two major phenotypes shown by *recBCD* mutants. The *recBC* mutant is deficient in normal helicase and nuclease activities and it is sensitive to DNA-damaging treatments [28]. In agreement with previous reports [12], the *recBC* mutant showed high sensitivity to cisplatin (Figure 2b). The surviving fraction for the *recBC* strain at a cisplatin dose of 75 μM was approximately four orders of magnitude lower than for the parental wild-type strain. The *recD* mutant is defective for normal nuclease activity, and it exhibits wild-type sensitivity to DNA-damaging agents [29]. In contrast to the results with the *recBC* strain, the *recD* mutant showed little or no sensitivity to cisplatin. These data provided genetic evidence that cisplatin-damaged DNA resulted in the formation of double-strand breaks.

Mutants deficient in resolution of recombination intermediates are also hypersensitive to cisplatin

Both the RecFOR and RecBCD pathways mediate the formation of RecA nucleoprotein filaments on ss DNA. These filaments catalyze the pairing and the strand exchange reactions between the damaged DNA molecule and an intact homologous duplex. The cisplatin hypersensitivity of *recA* mutants is well documented [13,14] and

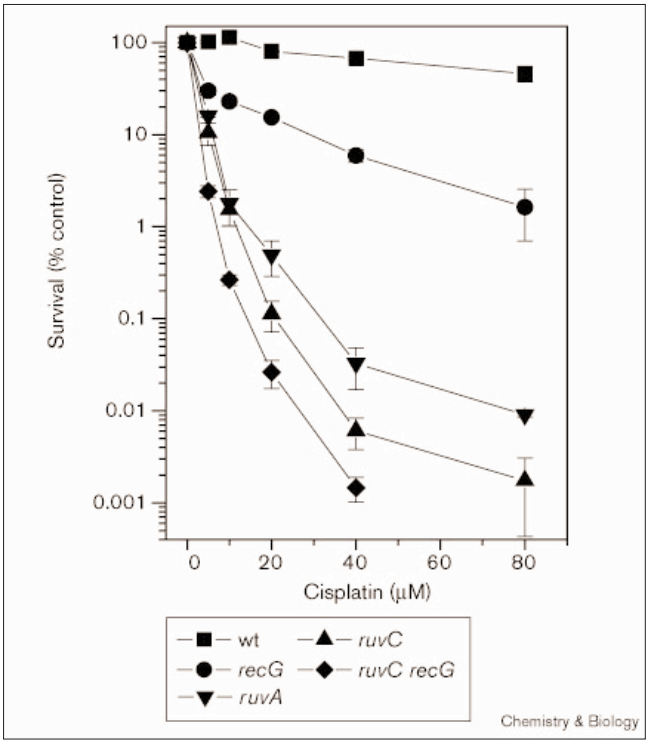
Figure 2

Survival of *E. coli* strains treated with cisplatin. For each data point, results shown are the mean of at least three independent experiments plated in duplicate, ± SEM. **(a)** Effects of *recF*, *recO* and *recR* mutations on cisplatin sensitivity. **(b)** Effects of *recBC* and *recD* mutations on cisplatin sensitivity.



was confirmed in this investigation (data not shown). The ensuing cross-over converts the damage-containing strand into duplex DNA and results in the formation of a four-way Holliday junction. In the late steps of recombination, the Holliday junction is subjected to the branch migration activities of either the RuvAB complex or the RecG protein [30,31], and it is cleaved by the RuvC resolvase [32]. Accordingly, we tested individual *ruvA*, *ruvC* and *recG* mutants, as well as a *ruvC recG* double mutant, for sensitivity to cisplatin. It should be noted that the transposon insertion in the *ruvA60* mutant has a polar effect on *ruvB* expression [33]. As shown in Figure 3, the individual *ruvA* and *ruvC* mutants showed a striking sensitivity to cisplatin that was equal or greater in magnitude to that observed for the mutants deficient in the RecBCD and RecFOR pathways of recombination. At a cisplatin concentration of 80 μM, the *ruvA* and *ruvC* strains exhibited a decreased survival of approximately four orders of magnitude in comparison with the wild-type strain. The sensitivities of these mutants indicated that branch migration and resolution of Holliday junctions by the RuvABC pathway were of critical importance, along with the earlier stages of recombination, for the post-replicative repair of cisplatin DNA damage. In contrast to the RuvABC-deficient strains, the *recG* mutant was found to be only tenfold more sensitive to cisplatin than the parental strain at a cisplatin dose of 80 μM. The *ruvC recG* double mutant, deficient for both pathways of branch migration and resolution, showed an additive effect, exhibiting higher sensitivity than either individual mutant strain. This observation is consistent with previous suggestions that the RecG and RuvABC pathways do not overlap significantly [34]. Taken together, these results indicate that RuvABC function is as important as RecBCD function for cell survival following cisplatin DNA damage, and that the RecG pathway plays a comparatively minor role that is independent of RuvABC in the processing of cisplatin damage.

Figure 3



Effects of *recG*, *ruvA*, *ruvC* and *ruvC recG* mutations on cisplatin sensitivity in *E. coli*. For each data point, results shown are the mean of at least three independent experiments plated in duplicate, ± SEM.

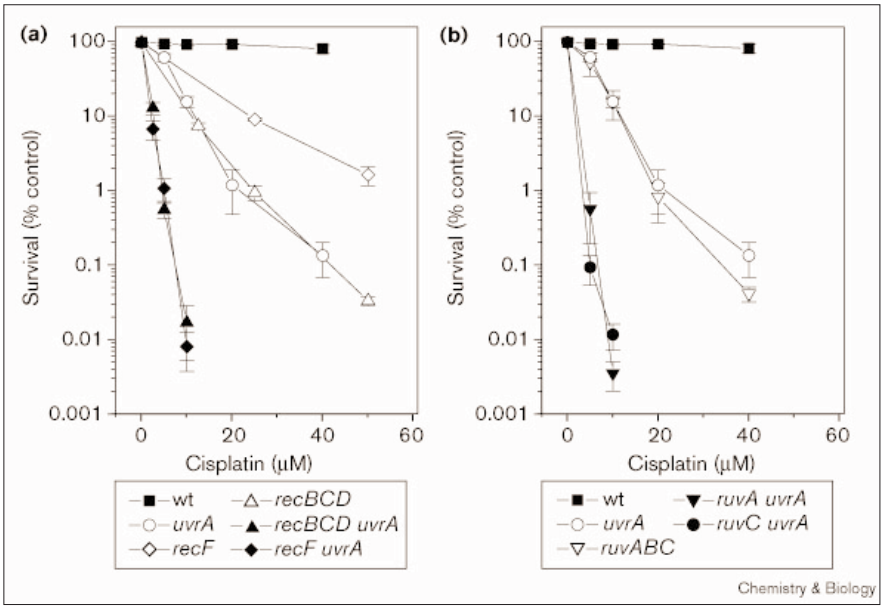
Recombination deficient and nucleotide excision repair (NER) deficient strains are equally sensitive to cisplatin

In order to appraise the significance of the results of the previous experiments, we compared the cisplatin sensitivity of recombination deficient mutants with a strain deficient in NER. NER acts on a broad range of DNA damages and has been assigned the central role in modulating the sensitivity of eukaryotic and prokaryotic cells to cisplatin. Cisplatin intrastrand adducts are removed from DNA by the NER repair system *in vivo* and by a reconstituted NER system *in vitro* [35]. Mutations that impair the function of this system cause hypersensitivity to cisplatin that is held as a benchmark for mutant susceptibility to the drug. Accordingly, we compared survival following cisplatin treatment of the NER deficient strain *uvrA* with that for representative recombination deficient strains: *recF*, *recBCD* and *ruvABC* (Figure 4). The NER-deficient strain showed hypersensitivity to cisplatin as previously described [12,36] but, interestingly, not higher than mutants deficient in the RecBCD or RuvABC functions. The survival curves for *uvrA*, *recBCD* and *ruvABC* strains essentially overlapped, whereas only the *recF* mutant was slightly less sensitive than *uvrA*. The comparable sensitivities of these mutants establish a crucial role for recombination alongside NER in determining cell survival following cisplatin DNA damage.

Recombination/nucleotide excision repair (NER) deficient double mutants show increased sensitivity to cisplatin

The comparable sensitivity of the recombination and NER-deficient single mutants presented in Figure 4 posed the question of whether or not the two pathways (recombination and NER) act independently in the processing of cisplatin-induced DNA damage. We addressed this question by comparing the effects of cisplatin on the survival of recombination and NER single and double mutants. If a double mutant showed an increased sensitivity to cisplatin in comparison to the parental single mutants, this would suggest that recombination and NER are non-overlapping pathways for the repair of cisplatin damage. We constructed a series of recombination/NER deficient double mutants: *recF uvrA*, *recBCD uvrA*, *ruvA uvrA* and *ruvC uvrA*, and tested them for sensitivity to cisplatin. As shown in Figure 4, all of the tested double

Figure 4



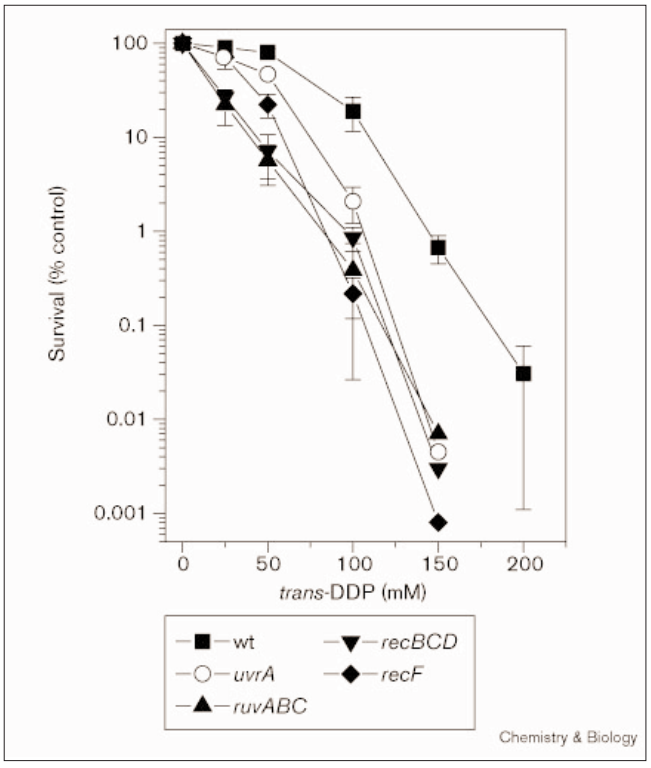
Comparison of cisplatin sensitivities in *E. coli* recombination and NER single mutants and recombination/NER double mutants. For each data point, results shown are the mean of at least three independent experiments plated in duplicate, ± SEM. **(a)** Effects of *uvrA*, *recF, recBCD*, *recF uvrA* and *recBCD uvrA* mutations on cisplatin sensitivity (*recF* survival profile from Figure 2a is shown for comparison). **(b)** Effects of *uvrA*, *ruvABC*, *ruvA uvrA* and *ruvC uvrA* mutations on cisplatin sensitivity.

mutants showed decreased survival. The *recF uvrA* and the *recBCD uvrA* double mutants showed comparable sensitivities, and both showed higher sensitivity than the corresponding single mutants (Figure 4a). In the same manner, *ruvA uvrA* and *ruvC uvrA* double mutants also showed increased sensitivities in comparison with the analogous single mutants (Figure 4b). At the relatively low cisplatin dose of 10 μM, all of the recombination/NER double mutants tested showed a striking reduction in survival of approximately four orders of magnitude in comparison to the parental wild-type strain. Taken together, these results suggest that the recombination and the NER pathways act independently of each other in protecting the cell from cisplatin-induced damage.

Recombination deficient mutants show low sensitivity to *trans*-DDP

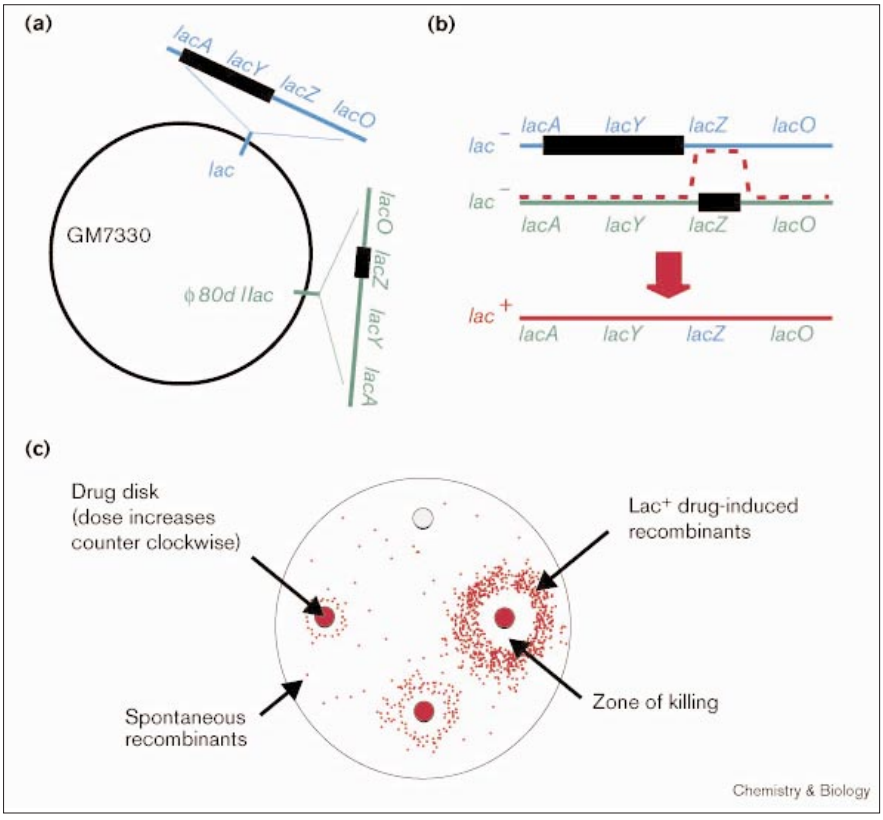
The *trans* isomer of cisplatin, *trans*-diamminedichloroplatinum(II) (*trans*-DDP; Figure 1a), also reacts with DNA to generate a spectrum of N7 intrastrand and interstrand cross-links [37,38], but it is far less cytotoxic than cisplatin and it is ineffective against tumors. Consequently, *trans*-DDP is a useful reference compound for calibrating the relative significance of various cellular responses to cisplatin. To determine whether the extreme sensitivities of recombination mutants were unique to the therapeutically active *cis* isomer, we tested the same panel of isogenic mutants (*recF*, *recBCD*, *ruvABC* and *uvrA*) for survival after *trans*-DDP treatment (Figure 5). The *uvrA* strain showed slightly higher sensitivity to *trans*-DDP in comparison with the wild type, as previously reported [13]. The recombination-deficient mutants *recF*, *recBCD* and *ruvABC* showed similarly modest sensitivity (again, in comparison to the wild type), even at *trans*-DDP concentrations of 150 μM. The lack of sensitivity of these mutants suggested that, in contrast to

Figure 5



Survival of *uvrA, ruvABC, recBCD* and *recF E. coli* strains treated with *trans*-DDP. For each data point, results shown are the mean of at least three independent experiments plated in duplicate, ± SEM.

Figure 6



Assay for drug-induced recombination. **(a)** Schematic of the chromosome of the *lac* diploid strain GM7330 showing the normally present *lac* locus (blue) and the inserted duplicate but reverse, ϕ*80d*II*lac* locus (green). The black boxes represent the deletions that render the strain *lac*⁻. **(b)** A recombination event between the two incomplete *lac* loci yields a functional *lac*⁺ product. **(c)** Schematic of the experimental set up: drug was applied to filter disks, in increasing amounts counter clockwise, to a lawn of GM7330 on MacConkey agar plates. The clear zone surrounding the disks is the zone of killing by the drug. The *lac*⁺ recombinants grow as concentrated zones of red colonies around the drug disks (or the zone of killing). The sporadic red colonies over the entire plate are spontaneous recombinants.

cisplatin, *trans*-DDP did not result in significant levels of either daughter strand gaps or double-strand breaks that require homologous recombination for their repair.

Cisplatin is highly recombinogenic

The extreme cisplatin sensitivity of recombination deficient strains underscores the importance of recombination strategies for cell survival following cisplatin exposure. These observations suggested that cisplatin might induce high levels of recombination events in surviving populations. We next, therefore, examined the relative amounts of drug-induced recombination for a panel of compounds including cisplatin, the alkylating agents *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), methylmethane sulfonate (MMS), streptozotocin (STZ) and the bifunctional cross-linking agents mitomycin C (MMC) and *trans*-DDP (for chemical structures of the compounds see Figure 1c). An assay was used in which inverted inactive *lac* operons could be made functional only through a recombination event (Figure 6). Induced Lac⁺ recombinants appeared within a lawn of Lac⁻ cells as concentrated zones of red colonies around a drug-containing disk, whereas spontaneous recombinants appeared as sparse red colonies over the entire plate. The cytotoxicity of the drug was visible as a clear cell-free zone surrounding the disk. As shown in Figure 7, at approximately equal cytotoxic doses (determined by the approximate radius of the zone of killing) cisplatin stimulated an extremely high number of Lac⁺ recombinants as compared with other DNA-damaging agents. The alkylating agents MNNG, MMS and STZ induced some recombinants, but they showed high levels of cytotoxicity without stimulating correspondingly high levels of recombination. The therapeutically inactive *trans* isomer of cisplatin showed almost no induction of Lac⁺ recombinants, even at the highest dose tested (120 nmol; 3 o'clock in Figure 7). At this *trans*-DDP dose the level of toxicity achieved was roughly equal to that of the lowest dose of cisplatin (30 nmol; 9 o'clock in Figure 7). This result correlated with our observation that recombination deficient mutants showed little sensitivity to the *trans* isomer. Only MMC, which forms covalent adducts with the exocyclic amines of guanines and abundant interstrand cross-links [39], showed comparable recombinogenicity to cisplatin. This result poses the possibility that the interstrand cross-links of cisplatin and MMC might be the lesions that induce recombinogenicity. It must be noted, however, that the levels of interstrand cross-links in our experiments were not measured and therefore we cannot make a correlation between recombinogenicity and interstrand cross-links at this time. We also note that *trans*-DDP

forms interstrand cross-links *in vitro* [37], but this compound did not induce a significant number of recombinants in our assay. This result possibly reflects the fact that the interstrand cross-links of *trans*-DDP might form less frequently *in vivo* than they do *in vitro* (i.e., few interstrand lesions might have been formed at the concentrations of *trans*-DDP used) [40,41]. Regardless of which lesion induces recombination, the high ratio of recombination to cytotoxicity for cisplatin and MMC, when compared with that found for the other DNA-damaging drugs and *trans*-DDP, might provide an important key to understanding the therapeutic activities of these two compounds.

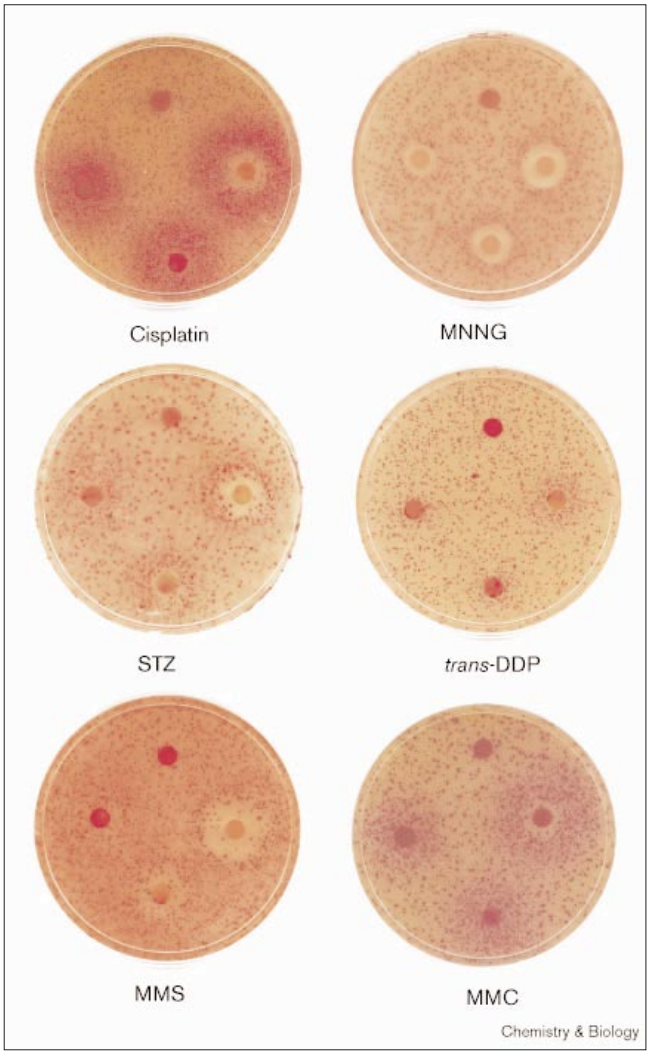
Discussion

This study was an analysis of the role of recombination as a cellular defense against cisplatin. The results showed that *E. coli* recombination-deficient mutants *recF*, *recO*, *recR*, *recBC*, *recBCD*, *ruvA*, *ruvC*, *ruvABC*, *recG* and *ruvC recG*, were strikingly sensitive to the drug. The sensitivities of the recombination-deficient mutants were comparable to the cisplatin sensitivity of the NER deficient strain (*uvrA*). This result is significant because, until this work, NER was considered the pathway of greatest importance as a cellular defense against cisplatin-induced damage. Recombination/NER deficient double mutants (*recF uvrA*, *recBCD uvrA*, *ruvA uvrA* and *ruvC uvrA)* produced increased sensitivity to cisplatin, underscoring the possibility that recombination and excision repair pathways might be independent strategies for managing the DNA damage induced by this drug.

Our results indicated that recombination proteins are required for survival following cisplatin-induced DNA damage. On the basis of current models, there are two major pathways for recombinational repair and homologous recombination in bacteria [1–3]. The daughter strand gap repair pathway requires the RecFOR and the RecA, RuvABC and/or RecG gene products (Figure 8a), and the double-strand break repair pathway requires the RecBCD and RecA, RuvABC and/or RecG gene products (Figure 8b). Given that mutants deficient in the gene products involved in both pathways showed high sensitivity to cisplatin we can make the conclusion that cisplatin-induced DNA damage led to the formation of both daughter strand gaps and double-strand breaks. Although the formation of daughter strand gaps (Figure 8a) as a result of replication blocks is consistent with the knowledge that cisplatin inhibits DNA synthesis, the induction of double-strand breaks is not widely associated with the activities of the drug. Cisplatin does not react with DNA in a manner that would lead directly to strand breaks or abasic sites, and therefore double-strand breaks must arise following exposure to cisplatin as indirect, secondary DNA lesions. Cisplatin DNA damage could lead to the formation of double-strand breaks following the encounter of a replication fork with an unrepaired daughter strand gap caused by a cisplatin adduct in the previous round of replication (Figure 8b). An unrepaired daughter strand gap could also lead to the formation of double-strand breaks because of the activities of single strand endonucleases [1]. Alternatively, double-strand breaks could be formed by cisplatin adducts via a recently proposed model in which induced replication arrest results in a double-strand break through the annealing of the ends of the complementary, newly synthesized daughter strands [42].

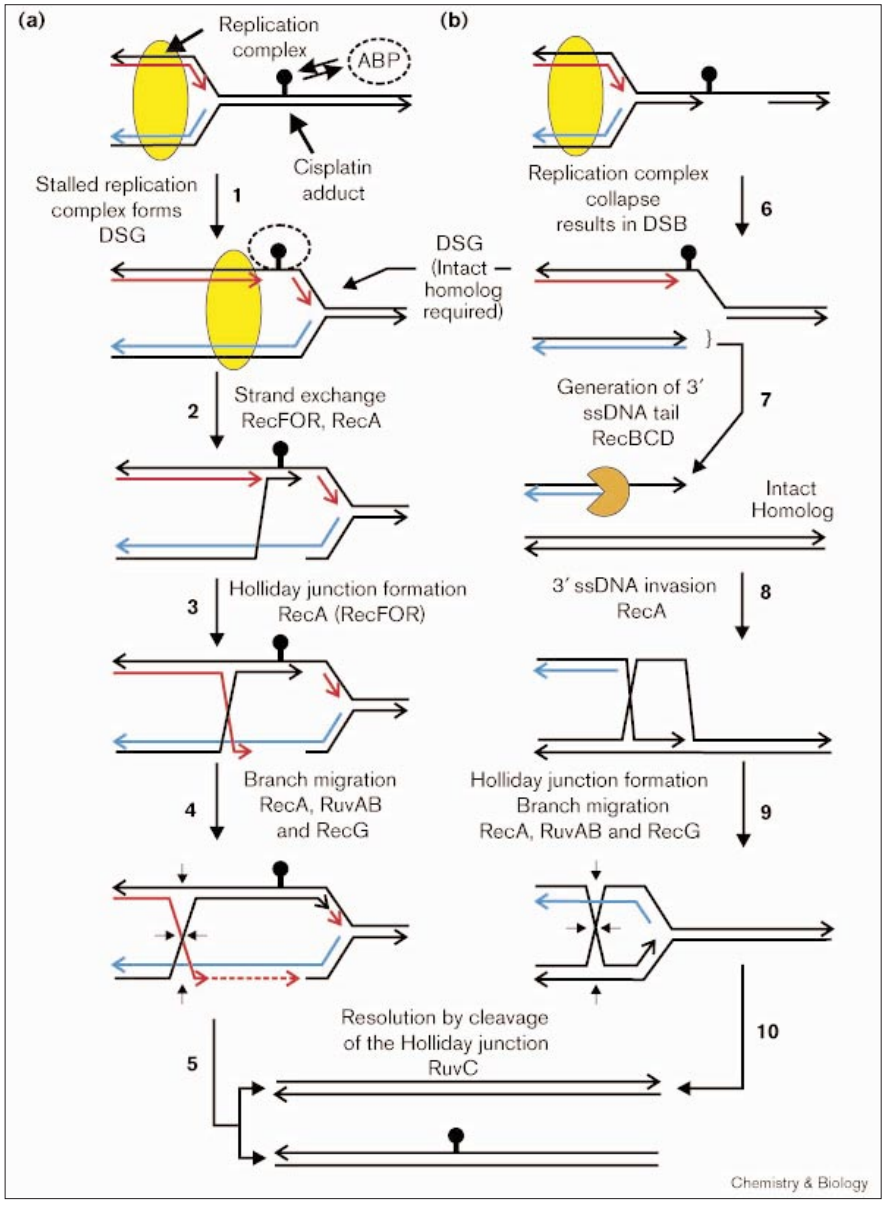
It has been discovered that cisplatin–DNA cross-links are uniquely recognized by a variety of cellular proteins (adduct

Figure 7



Lac+ recombinants induced by DNA-damaging agents in the *E. coli* strain GM7330. Doses applied to filter disks increase counter clockwise from 12 o'clock position for all compounds: cisplatin: 0, 30 60, 120 nmoles; *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG): 0, 5, 10, 20 μg; streptozotocin (STZ): 0, 10, 20, 40 μg; *trans*-DDP: 0, 30, 60, 120 nmoles; methylmethane sulfonate (MMS): 0, 0.65, 1.3, 2.6 μg; and mitomycin C (MMC): 0, 0.05, 0.1, 0.2 μg.

Figure 8



Models for recombinational repair of secondary DNA lesions (defined here as daughter-strand gaps and double-strand breaks) induced by cisplatin damage. Models are based upon [64,65]. **(a)** daughter-strand gap pathway. Step 1: The replication complex encounters persistent cisplatin–DNA adducts (perhaps because of poor NER of the 1,2 intrastrand cross-link). Stalled replication results in the formation of a daughter-strand gap opposite the adduct. The presence of an adduct binding protein (ABP) might present an even stronger block to replication than the adduct alone. Step 2: Interactions between the proteins of the RecFOR pathway and the replication fork initiate RecA nucleation and strand exchange. Step 3: The ensuing RecA catalyzed strand exchange (with the aid of the RecFOR accessory proteins) results in the formation of a Holliday junction. Step 4: Branch migration of the Holliday junction catalyzed by the RecA, RuvAB or RecG proteins results in the repair of the daughter-strand gap and restoration of the replication fork. Step 5: Resolution of the Holliday junction by RuvC restores two double-stranded DNA molecules. This could be a mechanism of damage tolerance as the cisplatin adduct is bypassed by recombinational repair and persists in the DNA. **(b)** double-strand break pathway. Step 6: The replication complex encounters an unrepaired daughter-strand gap or a nick opposite the adduct. Collapse of the replication fork forms a double-strand break and a daughter-strand gap; the daughter-strand gap portion of the collapsed replication fork is processed by the daughter-strand gap pathway (a). There are other mechanisms by which the double-strand breaks could arise, and we have presented only one scenario. By the proposed scheme, repair of the double-strand breaks requires an intact homolog of the damaged duplex. Step 7: The RecBCD complex (shown in red) binds the free end of the double-strand break and generates ss DNA that is a substrate for RecA nucleation. Step 8: RecA nucleoprotein filaments catalyze the invasion of the RecBCD generated ss tail into the homologous duplex. Step 9: RecA catalyzed strand exchange and branch migration results in the formation of a Holliday junction and restoration of the replication fork. Step 10: Resolution of the Holliday junction by RuvC yields two intact duplexes (only one molecule is shown).

binding proteins, or ABPs in Figure 8), and many of these interactions have been proposed to play a key role in the mechanism of action of the drug [35,43]. In the context of our present findings, protein recognition of cisplatin cross-links might contribute to the formation of daughter strand gaps and double-strand breaks. For example, an ABP could contribute to a replication arrest by providing an exceptionally strong block to the processivity of DNA polymerases, as has been shown for the rat high mobility group protein HMG-1 [44] (Figure 8, step 1). Alternatively, specialized ABPs could introduce strand breaks via enzymatic nicking activities at sites of cisplatin cross-links. For example, T4 endonuclease VII nicks DNA site-specifically at a cisplatin 1,2-d(GpG) cross-link [45], and it is possible that other proteins possess similar activities. It has also been proposed that mismatch repair proteins, which selectively recognize cisplatin–DNA adducts [46–48], could initiate repair events targeted to the newly synthesized strand opposite a cisplatin cross-link, leaving the offending lesion intact and leading to an iterative process of excision and resynthesis.

In these cases, such errant nucleolytic activities would result in direct or post-replicative formation of double-strand breaks that would require recombination for their repair. We do not know if there are ABPs that recognize MMC adducts but, given the similarities in recombinogenicity of cisplatin and MMC, it would be interesting to examine cellular extracts for such proteins.

Cisplatin forms a variety of DNA adducts and the specific lesion responsible for cisplatin cytotoxicity remains undefined. In the context of this study, it is reasonable to speculate that the highly abundant 1,2-intrastrand cross-links (~90% of all adducts formed *in vitro*) led to frequent replication arrests and contributed significantly to formation of daughter strand gaps and double-strand breaks. This interpretation is based on several lines of reasoning (reviewed in [8,35]): first, the 1,2-intrastrand cisplatin–DNA cross-links are inefficiently repaired by NER compared with the minor 1,3-d(GpNpG) cross-link (6–8% of all adducts) and are therefore persistent; second, the 1,2-intrastrand cross-links inhibit phage and *E. coli* polymerases *in vitro* and *in vivo* more strongly than the 1,3-intrastrand cross-links; and third, the recombination mutants in our study exhibited low sensitivity to *trans*-DDP, which does not form 1,2-intrastrand cross-links because of geometric constraints. It is noteworthy that a single 1,2-d(GpG) intrastrand cross-link does not inhibit the DNA unwinding or the ATPase activities of RecA, but it inhibits both the helicase and DNA-dependent ATPase activities of the RecB protein [49]. It would be interesting to further investigate the effect of cisplatin DNA cross-links on the *in vitro* activities of the RecBCD holoenzyme and the other recombinases (RuvABC and RecG).

We must note that the interstrand cross-link, although a minor adduct formed by cisplatin (~2% of all adducts), is also a viable candidate for the lethal lesion. The precise mechanism for repair of interstrand cross-links is not, as yet, understood, but it is believed to involve recombination and excision repair [1]. It is possible, therefore, that the interstrand cross-links also contributed to the cisplatin sensitivity of the various recombination mutants. Although we observed increased sensitivity to cisplatin by the recombination/NER double mutants, we cannot exclude the possibility that there is partial overlap in activities of the two pathways in repair of this (or other minor) subset of cisplatin adducts. A further understanding of how the individual cisplatin cross-links are processed by specific recombination and repair strategies could provide insights into identification of the specific adduct(s) responsible for the therapeutic activity of the drug.

Although our study is a genetic analysis of recombinational pathways of tolerance/repair of cisplatin-induced DNA damage in bacteria, it is useful to bear in mind the question that underlies most research on cisplatin — namely, why are tumors of the testis so singularly susceptible to the drug? The detailed molecular mechanism that can account for the striking organotropism of cisplatin is yet to be discovered. From the perspective of our present study, several observations of possible relevance have been made. Firstly, the majority of testicular tumors (95%) derive from germ cells [50], which are unique in that they require recombination for meiotic crossing-over and proper chromosome segregation during cell division. In addition to our findings that cisplatin is highly recombinogenic in bacteria, it has been shown that cisplatin can induce high levels of recombination in mouse testicular germ cells [11]. Cisplatin also causes abnormal homologue pairing, and it disrupts the proper formation and resolution of recombination intermediates during testicular germ cell meiosis [51,52]. Secondly, testicular tissues have been found to overexpress several mismatch repair (MMR) proteins including MSH2, MSH4, MLH1 and MSH5 [47,53–56]. One function of MMR proteins is to ensure the fidelity and regulate the levels of recombination, and to enable completion of meiotic cell division. Thirdly, resistance to a number of DNA-damaging agents, including cisplatin and alkylators, correlates with the loss of MMR proteins, both in *E. coli* and in eukaryotes [8,57]. Thus, MMR proteins paradoxically seem to sensitize rather than protect cells from cisplatin and some other DNA damaging agents. How MMR contributes to cisplatin toxicity is not understood, but it has been proposed that MMR proteins initiate abortive repair opposite cisplatin adducts [57], or inhibit recombination-dependent bypass of the adducts during replication [58]. At least one apoptotic pathway for cisplatin-induced cell death (that involving p73) requires an MMR protein, MLH1 [59]. Moreover, some MMR proteins specifically recognize cisplatin-damaged DNA [46–48]. We propose that the relationships among these observations provide a framework within which we might begin to understand the molecular mechanism for the organotropic action of this drug. For example, high levels of cisplatin-induced recombination could lead to cell death by triggering MMR-mediated damage signaling pathways that are specific to germ cells. The abundant MMR proteins could also sensitize germ cells by interfering with the required high level of recombinational repair of cisplatin damage. Further exploration of the relationships among recombination, repair of DNA damage, and the roles of MMR proteins in both of these processes are warranted.

Finally, in our study of DNA-damaging agents that induce recombination (Figure 7), only MMC rivaled cisplatin as a recombinogen. It is noteworthy that this drug, like cisplatin, is differentially toxic to testicular cancer cells *in vitro* [60]. It is tempting to speculate that induced recombination might be the common denominator in the mechanism of action of a specific class of anticancer agents. Understanding the role of recombination in genome maintenance could, therefore, be of great significance for future tissue-specific drug design efforts.

Significance

Many antitumor drugs extend life but do not completely cure cancer. The DNA-damaging anticancer compound cisplatin is one exception; therapeutic regimens including cisplatin as the principal chemotherapeutic agent afford an essential cure for testicular cancer and delay the progression of ovarian and several other cancers. In the past decade, several different and non-mutually exclusive hypotheses have been put forth to explain the cytotoxic and organotropic effects of this compound. It is well established that the cytotoxicity of cisplatin derives from its ability to form covalent DNA adducts and, because these adducts can be removed from DNA by nucleotide excision repair (NER), NER has long been considered the central player in countering cisplatin cytotoxicity. The present study shows that recombination is an equally critical genoprotective system as NER against cisplatin-induced DNA damage. Using a battery of single and double mutants, we have determined firstly, that cisplatin-damaged DNA leads to the formation of multiple replication (or repair) intermediates that utilize recombination strategies for their resolution, secondly, that these recombination pathways appear to be non-overlapping with NER in the processing of cisplatin DNA damage, and thirdly, that such recombination strategies are quantitatively of equal importance as NER for cell survival following cisplatin DNA damage. Moreover, the work shows that cisplatin and mitomycin C (another clinically used drug), in comparison to a panel of other DNA-damaging agents, were able to induce recombination at doses that were minimally toxic. It is possible that there is an important connection between the capacity of cisplatin to induce robust levels of recombination and the therapeutic specificity of this drug for testicular tumors. Testicular tumors derive from germ cells, which are cells that are unique in that they undergo meiotic recombination as an essential step during cell division. Meiotic recombination is a highly regulated and a precise event, and if disrupted, germ cells enter apoptosis. Cancer cells derived from germ cells might inherit such regulatory mechanisms specific for meiotic recombination, which might be triggered by cisplatin-induced recombination events. Thus, although recombination is a powerful protective pathway against cisplatin-induced damage, it might actually selectively sensitize germ cells and germ cell tumors to the drug.

Materials and methods

Chemicals

Methylmethane sulfonate was obtained from Eastman–Kodak, and mitomycin C and streptozotocin were from Sigma. *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine, *cis*- and *trans*-diamminedichloroplatinum(II) were from Aldrich.

Bacterial strains

The mutant strains that were used in this study are listed in Table 1. All the strains used for cytotoxicity studies are derivatives of AB1157. The auxotrophic phenotype of all mutant strains was confirmed by growth on the appropriate supplemented minimal medium.

Table 1

Genotypes of *E. coli* K-12 strains.

Strain	Genotype	Source
AB1157	*thr-1 ara-14 leuB6–(gpt-proA)*62 *lacY1 tsx-33 glnV44*(AS) *galK2*(Oc) *hisG4*(Oc) *rfbD1 mgl-51 rpoS396*(Am) *rpsL31*(Str^R) *kdgK51 xylA5 mtl-1 argE3*(Oc) *thi-1*	E.A. Adelberg
AB2500	As AB1157 but *uvrA6 deoB16 thyA12*	W.D. Rupp
AM207	As AB1157 but *recR252*::mTn*10*	R.G. Lloyd
AM547	As AB1157 but Δ*ruvABC65*	R.G. Lloyd
C266	As AB1157 but *recG258*::Kan	F. Stahl
CS85	As AB1157 but *ruvC53 eda51*::Tn*10*	R.G. Lloyd
GM5560	As AB1157 but *recA56 srl300*::Tn*10*	Lab stock
GM5593	As AB1157 but *uvrA6 ruvA60*::Tn*10*	Lab stock
GM5598	As AB1157 but *uvrA6 ruvC64*::Kan	Lab stock
GM7330	Δ*lacMS286*φ*80d*II*lacBK1 ara thi*(?)	Lab stock
GM7522	As AB1157 but *uvrA6 recBCD*::Kan	Lab stock
JC5519	As AB1157 but *recB21 recC22*	A.J. Clark
JC3913	As AB1157 but *uvrA6 recF143*	M. Volkert
JC9239	As AB1157 but *recF143*	A.J. Clark
KM21	As AB1157 but Δ*recBCD*::Kan	K.M. Murphy
KM353	As AB1157 but *recD1901*::Tn*10*	K.M. Murphy
N2057	As AB1157 but *ruvA60*::Tn*10*	R.G. Lloyd
N2445	As AB1157 but *recO1504*::Tn*5*	R.G. Lloyd
N3398	As AB1157 but *recG258*::Kan *ruvC53 eda51*::Tn*10*	R.G. Lloyd

All strains are F$^-$. Am, *amber* mutation; AS, *amber* suppressor; Δ, deletion; Oc, ochre mutation; Str, streptomycin; Kan, kanamycin; Tn*5* and Tn*10* encode kanamycin and tetracycline resistance respectively; mTn*10*, miniTn*10*.

Cytotoxicity assay

Overnight cultures were diluted 1000-fold and grown in Luria-Bertani (LB) medium [61] until the density of the populations reached 2×10^8 cells/ml as determined by OD_{600}. The exponentially growing cells were resuspended in M9 minimal medium [61] and treated with drug dissolved in H_2O for 2 hr at 37°C. Appropriate dilutions in M9 medium were plated on LB plates and incubated at 37°C until colonies could be scored. Results from three to six independent experiments plated in duplicate were averaged and plotted against drug concentration.

Drug-induced recombination assay

Strain GM7330 carries a specially constructed non-tandem duplication of partially deleted *lac* operons (*lacMS286*φ*80d*II*lacBK1*). φ*80d*II*lacBK1* has a small deletion in the proximal portion of the *lacZ* gene, whereas *lacMS286* contains a distal deletion. The deletions are non-overlapping, so functional Lac$^+$ revertants result only after a recombination event. The construction and properties of *lac* duplication strains have been described elsewhere [62].

Strain GM7330 was grown overnight in LB medium and diluted ten-fold in minimal salts without glucose [63]. Diluted cells (1.5 ml) were added to MacConkey agar plates (Difco; supplemented with 1% lactose), the cells were allowed to settle for 10 min, and then the excess medium was removed by aspiration. This procedure produced a uniform lawn of cells on the plate. Sterile 6.35 mm disks (Difco) were placed on each dry plate and aliquots of drug were added to the disks. Not more than 10 µl were spotted on the disks at one time and the disks were allowed to dry before further addition of drug. In this manner, the drug was delivered by diffusion from the disk, yielding a gradient of drug concentration that decreased with distance from the disk. The low solubility of *trans*-diamminedichloroplatinum(II) (*trans*-DDP) precluded testing at higher doses than those presented. The plates were then incubated for 48 hr at 37°C. Plates were scanned (bottom down) with a Umax 1220 scanner and CorelDraw software.

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