Dependence on *RAD52* and *RAD1* for anticancer drug resistance mediated by inactivation of mismatch repair genes

Stephen T. Durant, Melanie M. Morris, Maureen Illand, Helen J. McKay, Carol McCormick, Gillian L. Hirst, Rhona H. Borts* and Robert Brown

Mismatch repair (MMR) proteins repair mispaired DNA bases and have an important role in maintaining the integrity of the genome [1]. Loss of MMR has been correlated with resistance to a variety of DNA-damaging agents, including many anticancer drugs [2]. How loss of MMR leads to resistance is not understood, but is proposed to be due to loss of futile MMR activity and/or replication stalling [3,4]. We report that inactivation of MMR genes (*MLH1*, *MLH2*, *MSH2*, *MSH3*, *MSH6*, but not *PMS1*) in isogenic strains of *Saccharomyces cerevisiae* led to increased resistance to the anticancer drugs cisplatin, carboplatin and doxorubicin, but had no effect on sensitivity to ultraviolet C (UVC) radiation. Sensitivity to cisplatin and doxorubicin was increased in *mlh1* mutant strains when the *MLH1* gene was reintroduced, demonstrating a direct involvement of MMR proteins in sensitivity to these DNA-damaging agents. Inactivation of *MLH1*, *MLH2* or *MSH2* had no significant effect, however, on drug sensitivities in the *rad52* or *rad1* mutant strains that are defective in mitotic recombination and removing unpaired DNA single strands. We propose a model whereby MMR proteins – in addition to their role in DNA-damage recognition – decrease adduct tolerance during DNA replication by modulating the levels of recombination-dependent bypass. This hypothesis is supported by the finding that, in human ovarian tumour cells, loss of *hMLH1* correlated with acquisition of cisplatin resistance and increased cisplatin-induced sister chromatid exchange, both of which were reversed by restoration of *hMLH1* expression.

Addresses: CRC Department of Medical Oncology, CRC Beatson Laboratories, Glasgow G61 1BD, UK. *Yeast Genetics, Institute for Molecular Medicine, John Radcliffe Hospital, Oxford, UK.

Correspondence: Robert Brown
E-mail: gpma61@udcf.gla.ac.uk

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Results and discussion

We examined drug sensitivities of isogenic haploid strains of *S. cerevisiae* that had specific MMR genes disrupted (Table 1; for a review of the biochemistry and genetics of eukaryotic MMR, see [5]). Disruption of the MutS homologues *MSH2*, *MSH3* and *MSH6* and the MutL homologues *MLH1* and *MLH2* (but not *PMS1*) conferred a significant increase in resistance to cisplatin and carboplatin compared with the wild type (Table 1, Figure 1a). Genetic inactivation of *MLH1*, *MSH2*, *MSH3* and *MSH6* also led to increased resistance to doxorubicin. None of the MMR mutants, however, showed any significant increase in resistance or sensitivity to UVC radiation (Table 1). Transformation of the *MLH1* gene back into the *mlh1* mutant strain (*mlh1* + p*MLH1*) led to increased sensitivity to cisplatin and doxorubicin compared with vector-alone controls (*mlh1* + vector; Table 1). Together, these results demonstrate a direct role of MMR proteins in cisplatin, carboplatin and doxorubicin sensitivity.

Loss of MMR usually leads to increased gene mutation rates, leading to a mutator phenotype [6,7]. We examined the MMR mutant strains used for mutant frequency (Table 1) and mutation rate [8]. The wild-type strain had a mutation rate of 3.7×10^{-8} per viable cell, *mlh2* mutants a rate of 6.7×10^{-8} per viable cell and *pms1* mutants a rate of 8.7×10^{-6} per viable cell. The drug-resistance phenotype of *mlh2* mutants and absence of it in *pms1* mutants suggests that Mlh2p, but not Pms1p, has a role in processing of the type of damage induced by these agents. The mutator phenotype in *pms1* but not in *mlh2* mutants argues that loss of MMR activity *per se* (or at least MMR activity requiring Pms1p) need not lead to resistance, and that acquisition of drug resistance is not due to the mutator phenotype of these strains.

The *S. cerevisiae RAD52* gene is involved in meiotic and mitotic recombination [9]. *RAD52* inactivation led to increased sensitivity to cisplatin and UV radiation (Table 1). Inactivation of *MLH1*, *MLH2* or *MSH2* in a *rad52* mutant strain had no significant effect on sensitivity to cisplatin, carboplatin or UV radiation (Table 1, Figure 1b). Thus, inactivation of *RAD52* leads to loss of the resistance mediated by MMR gene inactivation and a sensitisation of the yeast to these agents. This suggests that drug resistance mediated by loss of MMR is dependent on Rad52p activity and implicates a recombination-dependent process in damage tolerance. Possible models for recombinational bypass of lesions during DNA replication, that would be Rad52p dependent, have been proposed previously [10].

Inactivation of *RAD1* also led to increased sensitivity to cisplatin, carboplatin and UV radiation (Table 1). The

Table 1

Sensitivities of MMR mutants to anti-cancer drugs and UV radiation.

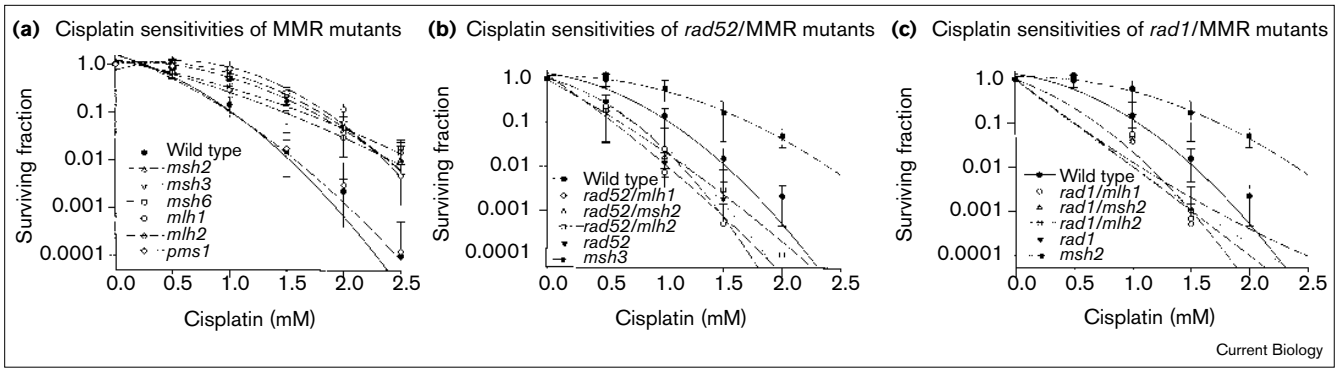
Genotype	Strain	IC90 Cisplatin (mM)	RF	IC90 Carboplatin (mM)	RF	IC90 Doxorubicin (mM)	RF	IC90 UV (J/m^2)	RF	Mutant frequency
Wild type	2096-1B	1	1	13	1	70	1	170	1	1.6×10^{-6}
msh2	RHB 2348	1.7	1.7*	20	1.5*	370	5.3*	210	1.2	2.5×10^{-5}
msh3	RHB 2347	1.8	1.7*	28	2.2*	310	4.4*	130	0.8	n.d.
msh6	NHT 173	1.7	1.6*	29	2.2*	420	6*	190	1.1	n.d.
mlh1	RBT 311	1.5	1.4*	36	2.8*	150	2.1*	150	0.9	4.7×10^{-5}
mlh2	RBT 324	2	2*	40	3.1*	n.d.	–	150	0.9	9.6×10^{-7}
pms1	RBT 269	1.1	1.1	10	0.7	n.d.	–	140	0.8	8.7×10^{-5}
mlh1 + vector	RBT311:v	1.6	1.6*	29	2.2*	120	1.7*	n.d.	–	n.d.
mlh1 + p*MLH1*	RBT311:mlh1	0.5	0.5*	10	0.8	30	0.4*	n.d.	–	n.d.
rad52	RHB 2692	0.7	0.7*	13	1	n.d.	–	70	0.4*	n.d.
rad52/msh2	RHB 2700	0.7	0.7	13	1	n.d.	–	70	0.4	n.d.
rad52/mlh1	RHB 2698	0.5	0.5	12	0.9	n.d.	–	80	0.5	n.d.
rad52/mlh2	RHB 2699	0.6	0.6	13	1	n.d.	–	100	0.6	n.d.
rad1	RBT 302	0.6	0.6*	7	0.5*	n.d.	–	10	0.06*	n.d.
rad1/msh2	RHB 2694	0.6	0.6	7.5	0.6	n.d.	–	13	0.08	n.d.
rad1/mlh1	RHB 2693	0.7	0.7	8	0.6	n.d.	–	11	0.06	n.d.
rad1/mlh2	RHB 2695	0.6	0.6	8	0.6	n.d.	–	11	0.06	n.d.

All strains are isogenic derivatives of a *Matα* wild-type strain. The construction of all of the mismatch-repair-deficient strains except *mlh2* has been described [22]. *MLH2* was deleted using an oligonucleotide-based KANMX disruption cassette. *RAD52* was disrupted with *LEU2* using plasmid pMS20 obtained from D. Schild [23]. *RAD1* was deleted using a *LEU2* disruption/deletion plasmid obtained from R. Keil [24]. IC90, concentration of drug inducing a 90% reduction in surviving fraction. RF, resistance factor relative to the wild-type strain. Those marked with an asterisk are significantly different in drug sensitivity from the wild-type strain or the corresponding single mutant, as assessed by a two-tailed Student's *t*-test at 1.5 mM cisplatin or 15 mM carboplatin. Mutant frequency, the number of L-canavanine-resistant colonies per 10^6 colony forming units; n.d., not determined.

RAD1 gene in *S. cerevisiae* is involved in nucleotide excision repair (NER), but has also been implicated in mitotic recombination [11]. Purified Rad1p interacts with DNA bubble structures, is required for the endonucleolytic cleavage that removes 3′ single-stranded DNA ends [11], and is also required for removal of non-homologous ends during recombination [12]. These are activities that could be required for recombination-dependent DNA-damage bypass by removing bases to allow initiation of replication after bypass [13]. Inactivation of *MLH1*, *MLH2* or *MSH2* had no significant effect on cisplatin or carboplatin sensitivities in a *rad1* mutant strain (Table 1, Figure 1c). These observations are consistent with Rad1p being necessary for increased damage bypass.

We propose a model whereby loss of MMR proteins can increase *RAD52*/*RAD1*-dependent recombinational bypass of adducts (see Figure 2). Treatment of cells with cisplatin and carboplatin induces predominantly 1,2 intrastrand DNA crosslinks which is believed to be the major cytotoxic lesion [14], although a role for the minor lesions cannot be excluded. The 1,2 intrastrand crosslink induced by cisplatin and carboplatin is poorly repaired, either due to not being recognised by NER [15] or by inhibition of repair, for instance by damage-recognition proteins [16]. Consistent with this, inactivation of *RAD1* in *S. cerevisiae* did not give the extreme hypersensitivity to cisplatin observed for UV radiation (Table 1). Persistent or non-repaired DNA lesions could lead to a cytotoxic signal being generated during DNA replication. The heterodimer of Msh2p and Msh6p — hMutSα —recognises 1,2 cisplatin crosslinks in a complementary duplex DNA and with greater affinity if the platinated guanine residues are opposite non-complementary bases [17]. Bypass of cisplatin DNA lesions during DNA replication has been shown in cisplatin-resistant human cells which correlates with defects in MMR [18]. The mechanisms leading to bypass are largely unknown, but possibilities include recombinational mechanisms, as well as trans-lesion DNA synthesis. It has been shown that MMR proteins can affect levels of homologous recombination in yeast [19,20]. Alternatively, MMR proteins recognising cisplatin adducts may lead to inefficient MMR, which competes with recombinational bypass of the lesion. If *RAD52*/*RAD1*-dependent recombinational bypass of DNA adducts occurs during replication, then inhibition of, or competition with, either initiation or progression of this process by MMR proteins will lead to sensitivity. Conversely, loss of MMR proteins will reduce the probability of lethal signals being generated during replication by increasing adduct bypass, leading to resistance. Consistent with DNA replication being necessary for MMR-dependent drug sensitivity, we observed a significantly increased resistance of cells exposed to cisplatin during the stationary phase of growth compared with exponentially growing cells — for an exponentially growing wild-type strain of *S. cerevisiae*, the concentration of cisplatin that results in 90%

Figure 1



Drug toxicity was measured by exposing exponentially growing cells in liquid culture for 24 h to cisplatin, plating out 400 cells onto YPD medium and allowing colony formation. Values shown are the means of multiple independent experiments representing at least 15 repeat values at each drug concentration and using independent clonal isolates. Curves through data points represent second order linear regression. Error bars represent 99% confidence limits.

inhibition of clonal growth (ID90) is 1.0 mM, whereas that of stationary cells is 1.8 mM. On the other hand, the cisplatin sensitivity of *msh2* mutants (ID90 = 1.7 mM) is not affected by growth phase.

To examine the potential relevance of the observations made in *S. cerevisiae* to how tumours may acquire resistance to these chemotherapeutic drugs, we examined cisplatin sensitivities in a matched set of human ovarian tumour cell lines. If recombinational bypass of DNA adducts during DNA replication occurred, this would be manifested by a sister chromatid exchange (SCE). A2780 is a human ovarian carcinoma cell line derived from an untreated patient, whereas A2780/cp70 is an *in-vitro*-derived cisplatin-resistant derivative that has lost MMR activity due to loss of *hMLH1* expression [4]. We introduced a human chromosome 3 containing a wild-type *hMLH1* gene into the A2780/cp70 line and showed restoration of Mlh1p expression, MMR activity and partial restoration of cisplatin sensitivity (Table 2). We also observed increased resistance of A2780/cp70 to doxorubicin and the methylating agent N-nitrosomethylurea (MNU) and restoration of sensitivity of the chromosome 3 transfers (Table 2). The restoration of drug sensitivities in the A2780/cp70/ch3 line supports a direct role for *MLH1* in cell death induced by these drugs in these ovarian cells. Furthermore, as shown in Table 2, A2780/cp70 cells, which

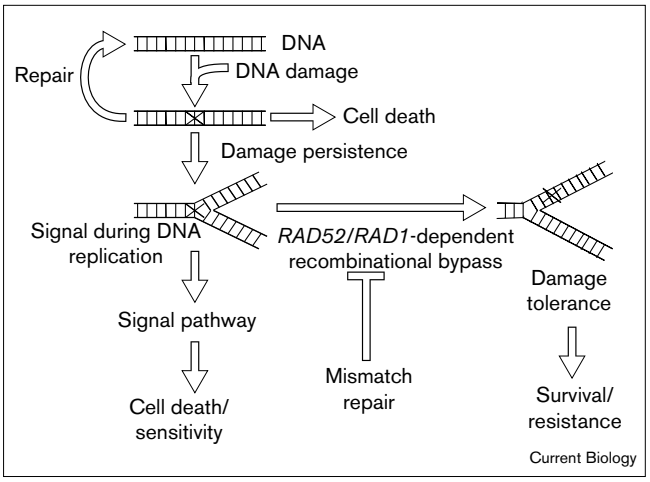
Table 2

Drug sensitivities and SCE induction in ovarian tumour cell lines.

Cell line	MMR status	IC50 MNU (mM)	IC50 Cisplatin (μM)	IC50 Doxorubicin (nM)	Untreated*		Cisplatin-treated*	
					Number of SCEs per cell	per chromosome	per cell	per chromosome
A2780	+	0.2	10	15	1.2	0.026	6.3	0.14
A2780/cp70	–	0.79	65	25	1.5	0.034	16.2	0.37
A2780/cp70/ch2	–	0.65	56	25	2.0	0.044	18.2	0.4
A2780/cp70/ch3	+	0.21	12	16	1.0	0.022	9.6	0.21

A2780/cp70/ch3 and A2780/cp70/ch2 are derivatives of A2780/cp70 that have, respectively, chromosome 3 or 2 introduced by microcell-mediated chromosome transfer. MMR status was determined by *in vitro* assays using plasmid substrates with defined mismatches (P. Karran and O. Humphries, personal communication). Western analysis with hMlh1-specific antibodies (data not shown) indicated that MMR+ lines express hMlh1, whereas MMR– lines do not. IC50 values are the concentrations of MNU, cisplatin or doxorubicin necessary to reduce the surviving fraction of cells by 50%. Cells were treated with MNU after depletion of O^6-alkyltransferase activity using O^6-benzylguanine, as previously described [4]. Values are the mean of multiple independent clonogenic assays. A2780 and A2780/cp70/ch3 are significantly more sensitive to MNU, cisplatin and doxorubicin than A2780/cp70 and A2780/cp70/ch2 as determined by two-tailed Student's *t*-test ($p < 0.01$). *The number of SCEs in exponentially growing cells that were either untreated or treated with 10 μM cisplatin for 1 h. SCEs were quantified by Hoechst staining, followed by Giemsa staining of 5-bromo-2′-deoxyuridine (BrdU)-labelled metaphase spreads. At least 40 metaphases were scored. A2780 and A2780/cp70/ch3 have significantly less SCEs after cisplatin treatment than A2780/cp70 and A2780/cp70/ch2, as determined by two-tailed Student's *t*-test ($p < 0.01$).

Figure 2



Model of MMR modulation of recombination bypass affecting drug sensitivity. Certain types of DNA damage induced by chemotherapeutic drugs such as cisplatin are poorly repaired and may persist in the genome. We propose that signals are generated during DNA replication of this unrepaired damage that could lead to cell death, but have the potential to be bypassed in a *RAD52*/*RAD1*-dependent manner that will lead to damage tolerance and cell survival. This recombinational bypass can be inhibited by MMR expression. Thus, loss of MMR leads to increased drug resistance because of increased bypass.

have lost Mlh1p expression, have a higher level of SCEs induced by cisplatin than the MMR-proficient parental A2780 line. Restoration of Mlh1p expression in the A2780/cp70/ch3 line reduced the level of SCEs induced, whereas introduction of chromosome 2 (A2780/cp70/ch2), which does not restore MMR activity, had no effect. These observations are consistent with a chromatid exchange mechanism being modulated by MMR and with the hypothesis that recombination bypass of cisplatin adducts leads to damage tolerance in MMR-defective tumours.

The data presented in yeast and mammalian cells provide evidence for MMR proteins directly affecting cytotoxicity induced by cisplatin, carboplatin and doxorubicin. Although loss of MMR is associated with methylation tolerance in mammalian cells, increased tolerance to methylating agents has not been observed in yeast strains defective in MMR genes, except for *msh5* mutants [21]. This may imply that O^6-methyl guanine induces toxicity by a different mechanism or that this lesion is processed by other repair pathways in yeast masking any effects of MMR on sensitivity to methylating agents. The yeast strains described in the present study will provide a means to examine other anticancer agents and platinum analogues for MMR-dependent cytotoxicity.

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