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Sensitivity to Cisplatin and Platinum-Containing Compounds of *Schizosaccharomyces pombe* Rad Mutants

PAOLA PEREGO, FRANCO ZUNINO, NIVES CARENINI, FERNANDO GIULIANI, SILVANO SPINELLI, and STEPHEN B. HOWELL

Department of Experimental Oncology B, Istituto Nazionale per lo Studio e la Cura dei Tumori, 20133 Milan, Italy (P.P., F.Z., N.C.), Boehringer Mannheim Italia, Monza, Italy (F.G., S.S.) and Department of Medicine and the Cancer Center, University of California, San Diego, La Jolla, CA 92094 (S.B.H.).

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ABSTRACT

The role of genes that affect response to radiation in determining sensitivity to platinum-containing compounds was studied using a panel of 23 strains of the yeast *Schizosaccharomyces pombe*. The radiation-hypersensitive mutants all had the same genetic background and most of them contained mutations that disabled either cell cycle checkpoints or DNA repair. The tested platinum compounds included cisplatin and two complexes containing diaminocyclohexane (oxaliplatin and tetraplatin), two ammine/cyclohexylamine complexes with different orientation of the leaving groups (JM216 and JM335) and a multinuclear platinum complex (BBR 3464). The cytotoxic effect of the selected platinum complexes was evaluated by using a microtiter growth inhibition assay with a 48 hr exposure to drug. The mutants fell into three groups with respect to sensitivity to cisplatin: four mutants (rad2, -7, -11, -15) exhibited minimal change in sensitivity; fifteen mutants (rad4–6, -8–10, -12–14, -16–17, -19–21, and -22) were 5.1–21.7-fold hypersensitive; only rad1 and -3 mutants, defective in checkpoints, and rad18, defective in repair, displayed a marked hypersensitivity. None of the mutants demonstrated appreciable change in sensitivity to JM216 presumably as a consequence of a lack of resistance of the wild-type strain, whereas a moderate increase in sensitivity to JM335 was observed for most of the mutants, and hypersensitivity to BBR3464 was observed only in rad1 and -3. No relevant changes in sensitivity to tetraplatin were observed. Most of the mutants, with the exception of rad2, -7, and -15, were hypersensitive to oxaliplatin. These findings demonstrate that specific mutations have disparate effects on the profile of sensitivity to different members of the same class of cytotoxic agents, which provides genetic evidence that different mechanisms are involved in differential cytotoxicity induced by Pt compounds. The results also demonstrate the utility of such a panel of mutants, constructed on the same genetic background, for detecting specific cellular response; presumably, this reflects the recognition or processing of specific DNA adducts. In conclusion, because the rad1 and rad3 gene products are determinants of cellular response to a large number of platinum-containing compounds, the present results support a critical role of genes involved in cell cycle control in cellular sensitivity to these agents.

Cisplatin is a very effective antitumor agent that is widely used for the clinical management of common solid tumors, such as lung and ovarian cancers (Ozols and Young, 1991; Dancey and Le Chevalier, 1997). A large number of analogs have been synthesized and tested in both preclinical models and clinical trials in an attempt to find agents with a broader range of antitumor activity and less toxicity (Kelland *et al.*, 1992; Farrel, 1993). At least cisplatin seems to kill cells via the process of apoptosis (Eastman, 1990; Ormerod *et al.*, 1996; Perego *et al.*, 1996); for all these drugs, the major molecular determinants of sensitivity are not fully understood. The lesions produced in DNA by cisplatin and related compounds have been widely characterized. Cisplatin produces bifunctional adducts, including DNA intra- and inter cross-links and DNA-protein cross-links (Eastman, 1987); these lesions are thought to be critical to cisplatin cytotoxicity. Fig. 1 shows the structure of the platinum complexes used for this study. The chosen compounds include a) two cisplatin analogs that contain the diaminocyclohexane carrier ligand that differ in the oxidation state (i.e., Pt(IV) for tetraplatin and Pt(II) for oxaliplatin); b) two ammine/cyclohexylamine Pt(IV) complexes in different configuration (i.e., *cis*-oriented JM216 and *trans*-oriented JM335); and c) a novel multinuclear platinum complex (BBR 3464). In particular, JM335 was originally designed to have a compound capable of binding to DNA with a geometry different from cisplatin, which has a symmetric *cis*-ammine carrier ligand (Kelland *et al.*, 1994). BBR 3464 contains two reactive platinum centers; this complex was designed to form different types of DNA adducts (i.e., "long-distance" cross-links). This complex exhibits a promising efficacy against cisplatin-resistant tumors (Pratesi *et al.*, 1997). Although the diaminocyclohexane complexes have been shown to overcome accumulation defects in

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resistant cells, their structure may influence the recognition of DNA lesions (Loh *et al.*, 1992; Mellish *et al.*, 1993). Some of these analogs have different patterns of cross-resistance against panels of human tumor cells (Hills *et al.*, 1989; Farrel *et al.*, 1990; Rixe *et al.*, 1997) and are likely to be recognized differently by repair mechanisms (Fink *et al.*, 1996), which suggests that some of the protective mechanisms and cellular responses are drug-specific. However, the extent to which specific genes are involved in regulating cellular sensitivity to specific members of this class of drugs is not known.

Only a single family of genes has been clearly established as important in modulating the sensitivity of mammalian cells to cisplatin. Cisplatin adducts are removed from DNA by the nucleotide excision repair system (Szymkowski *et al.*, 1992; Huang *et al.*, 1994), and mutations that impair the function of this system cause hypersensitivity to this agent (Bootsma, 1993; Dabholkar *et al.*, 1994). The multifactorial nature of the cisplatin-resistant phenotype and the genetic complexity of mammalian cells are major obstacles to the identification of the role of specific gene products in mediating cisplatin resistance. Based on their importance as determinants of sensitivity to radiation, genes involved in cell cycle control and DNA repair systems, in addition to nucleotide excision repair, are likely to be important regulators of sensitivity to the platinum-containing drugs as well.

In this study, we have taken advantage of the availability of a panel of *Schizosaccharomyces pombe* mutants constructed on the same genetic background to identify genes whose products are particularly important determinants of sensitivity to cisplatin and to determine whether the same genes are important as regulators of sensitivity to selected cisplatin analogs that are currently in clinical development. Yeast strains that are mutants for genes known to be involved in DNA repair and cell cycle control were given preference for inclusion in this panel.

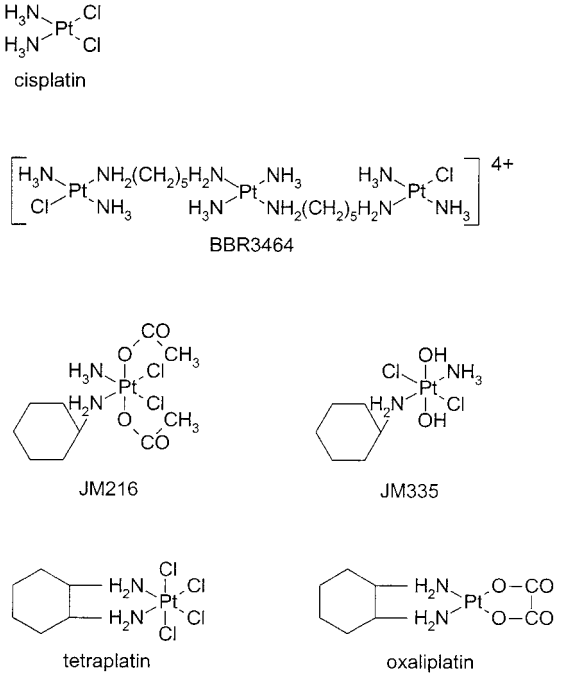

Fig. 1. Structures of the studied platinum-containing drugs.

Materials and Methods

Yeast strains. All the strains used in this study were derived from the parental strain 972h$^-$ by mutagenesis. Complementation analysis showed that rad5 and rad15, as well as rad10/rad16/rad20, and rad1/rad19, are allelic. They were kindly provided by Prof. S. Subramani (University of California, San Diego, USA). Cultures were grown at 30° in YES medium (5 g of Difco yeast extract, 30 g of glucose, 0.075 g of adenine and 0.075 g of of uracil/liter).

Drugs. Cisplatin, tetraplatin, and BBR3464 were kindly provided by Boehringer Mannheim (Milan, Italy). Oxaliplatin was a gift from Debio Pharm (Zurich, Switzerland). JM216 and JM335 were kindly provided by Dr. L. R. Kelland (Institute of Cancer Research, Sutton, U.K.). All drugs were dissolved in 0.9% NaCl.

Cytotoxicity assay. An antiproliferative assay performed in microtiter plates was used to evaluate the cytotoxic effect of cisplatin and the other platinum containing drugs. Preliminary experiments were performed to verify the linearity of the relation between cell number and absorbance at 550 nm. Cell cultures were grown overnight in liquid medium until mid-log phase. Twelve thousand cells were then seeded in 96-well microtiter plates and incubated in drug-containing medium. Alternatively, for measurement of growth after irradiation, exponentially growing cells were resuspended in medium to a final density of 1×10^7 cells, irradiated with a ^{137}Cs source (800 rad/min) and then diluted for seeding in microtiter plates. Plates were incubated for 48 hr at 30°, at which time the absorbance at 550 nm was measured. The IC_{50} was defined as the drug concentration that reduced the absorbance to 50% of the value measured for the non–drug-treated control culture. Each experiment was repeated at least three times using triplicate wells.

Results

A wide panel of *S. pombe* mutants, including strains deficient in functions that we expected to affect sensitivity to

TABLE 1
Pathway defects, known biochemical functions, and homologs of the rad mutants studied
Allelic mutants are indicated in parentheses.

Mutant	Known biochemical function	Homologs (*S. cerevisiae*/human)
Checkpoint mutants		
rad1	Exonuclease	*RAD17*/none
rad3 (rad19)	Lipid kinase domain	*MEC1/ATR, ATM*
rad4	Interaction with chromatides	*DPB11/XRCC1*[a]
rad9	G2 arrest	*DDC1/Hrad9*
rad17	Nucleotide binding site	RAD24/Replication factor C
Nucleotide excision repair mutants		
rad5 (rad15)	Helicase	*RAD3/ERCC2*
rad8	Helicase	*RAD5/ERCC6*[a]
rad10 (rad16, rad20)	Nuclease subunit	*RAD1/ERCC4*
rad13	Nuclease	*RAD2/ERCC5*
Other		
rad2	Chromosome separation	*RAD27/hrad2*
rad6	Error prone repair	*RAD18*/none
rad7		None
rad11	Replication	RPA/hRPA
rad12	Helicase	*SGS1/WRN*
rad14		None
rad18	Chromosome structure	*RHC18*/none
rad21	Double-strand break rejoining	*SCC1/HR21*
rad22	Repair of double-strand breaks	*RAD52*/none

[a] Region(s) of similarity.

TABLE 2

Sensitivity of *S. pombe* mutants to γ-radiation

Sensitivity was evaluated by using a microtiter growth-inhibition assay after exposure to radiation. ID_{50} values are the average (± standard deviation) of at least three independent experiments. When exposed to a dose of γ radiation of 120 Krad wild-type, rad2, rad7, and rad15 exhibited 90%, 66%, 57%, and 64% cell growth, respectively.

Strain	ID_{50} (Krad, mean ± SD)
	K rad
wild-type	>120
rad1	18.2 ± 2.7
rad3	14.2 ± 0.7
rad19	14.8 ± 1.2
rad4	45.3 ± 12.0
rad9	32.0 ± 10.0
rad17	12.0 ± 2.0
rad5	38.0 ± 7.0
rad15	>120
rad8	49.0 ± 12.0
rad10	28.5 ± 7.5
rad16	14.5 ± 7.5
rad20	42.5 ± 7.5
rad13	71.0 ± 4.0
rad2	>120
rad6	21.2 ± 2.7
rad7	>120
rad11	50.6 ± 10.3
rad12	52.5 ± 8.5
rad18	12.7 ± 2.7
rad14	73.0 ± 4.6
rad21	17.5 ± 2.5
rad22	41.0 ± 3.0

cisplatin (checkpoint regulation and DNA repair) was used in this study. In Table 1, the strains have been grouped by pathway defects and the known biochemical defects or the functions mainly affected by each mutation are summarized. Although human homologs have been cloned for some of the studied genes (*ATR*, *hrad9*, *ERCC2*, *ERCC4*, *ERCC5*, *hrad2*, *RPA*, *WRN*, *HR21*), only short regions of similarity have been found for others (*XRCC1, ERCC6*). For some other rad genes, only *Saccharomyces cerevisiae* homologs are known. Rad1, rad3, rad9, and rad17 are required in a complex checkpoint pathway to recognize specific DNA structures, and send signals capable of affecting cell cycle arrest in response to changes in DNA structure (Bentley *et al.*, 1996). For two mutants (rad7 and rad14), no detailed information is available.

The wild-type and each of the mutant strains was tested for its sensitivity to ionizing radiation (Table 2) or to cisplatin (Fig. 2) using a 48-hr continuous exposure to the drug. The sensitivity of the mutants to cisplatin fell into three groups. When the rad2, -7, -11 or -15 genes were disabled, there was no significant (<3-fold) change in cisplatin sensitivity. Mutational inactivation of rad4–6, -8–10, -12–14, -16–17, -19–21, or -22 genes produced an increase in sensitivity that ranged from 5.1- to 21.7-fold, and disruption of rad1, -3, or -18 function resulted in a more marked (27.7–57.7-fold) increase in sensitivity. Thus, among the 22 mutations tested, there was no change in cisplatin sensitivity in three cases, a substantial increase in sensitivity in 15 cases, and a very large increase in sensitivity in three cases.

With the exception of oxaliplatin, all of the analogs were markedly more potent than cisplatin against the wild-type strain 972h$^-$ by a factor that ranged from 9-fold for BBR3464 to 325-fold for JM216. Thus, the cytotoxic potency seems to be a peculiar feature of *cis*-oriented Pt (IV) compounds (JM216 and tetraplatin). In the case of JM216 (Fig. 3), none of the mutations conferred a substantial increase in sensitivity, with the exception of rad1. A completely different pattern of response was found for JM335, because the mutations in most rad genes (with the exception of rad2, -4, -13, -15, and -19) conferred a moderate increase in sensitivity to JM335 (Fig. 4); rad1, rad3 (and, to a lesser extent, rad6), but not rad18, conferred increased sensitivity (>3-fold) to BBR3464 (Fig. 5). In the case of tetraplatin (Fig. 6), no relevant differences in drug sensitivity were detected. Only rad16 conferred appreciable sensitivity to this Pt(IV) compound. Finally, in a completely different pattern, rad1, -3, -9, -17, -18, and -21 all conferred at least 5-fold hypersensitivity to oxaliplatin (Fig. 7). However, a large number of mutations conferred sensitivity to oxaliplatin, with the excep-

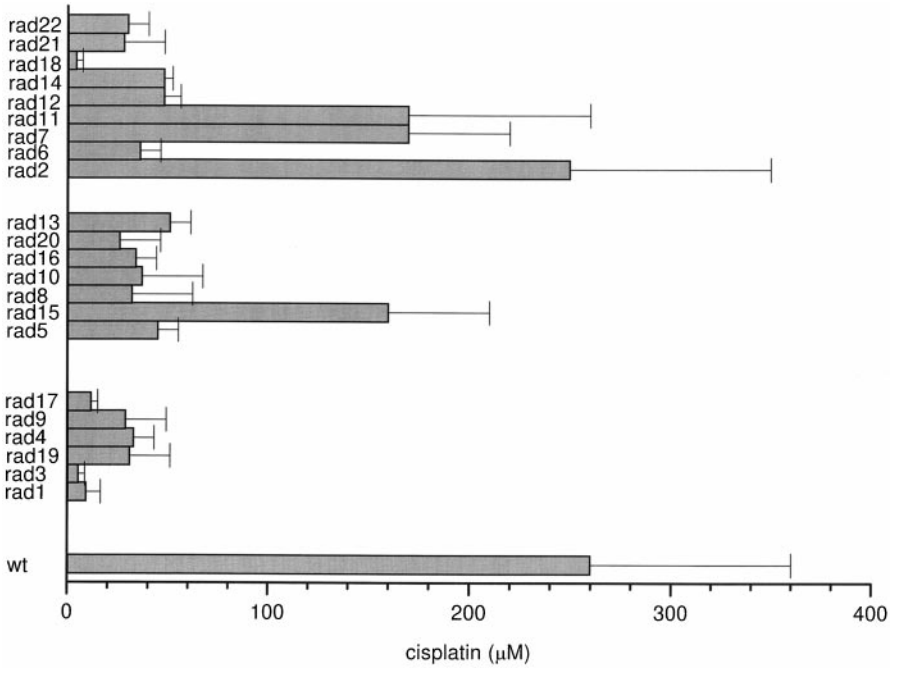

Fig. 2. Sensitivity of *S. pombe* mutants to cisplatin. *wt*, wild-type 972h$^-$ strain. Sensitivity was evaluated by using a microtiter growth inhibition assay with a 48 hr drug exposure. IC_{50} values are the means ± standard deviation of at least three independent experiments.

tion of rad2, -7, and -15. The IC_{50} value of oxaliplatin against the wild-type strain was above the maximum concentration of drug that could be tested in this assay given the limitations of solubility.

Discussion

In this study, the use of a panel of yeast mutants constructed on the same genetic background, all of which were isolated on the basis of their ability to confer hypersensitivity to radiation, permitted rapid identification of genes whose integrity is important to the ability of the cell to withstand the cytotoxic insult delivered by cisplatin. Inclusion in this panel of strains containing mutations in genes whose products are known to have roles in DNA repair and cell cycle control turned out to be a very productive way of identifying genes that influence cisplatin sensitivity. This is unsurprising, perhaps, because at least some of the same DNA repair pathways are involved in repair of both cisplatin and radiation damage. However, the results of this study indicate that some of these genes participate in cellular responses that are quite specific to the type of injury even within a class of drugs that are closely related chemically. For example, the rad1, -3, and -18 mutations produced very large changes in sensitivity to cisplatin, but had marginal or no effect on sensitivity to JM216 or tetraplatin. The rad1 and -3 gene products play

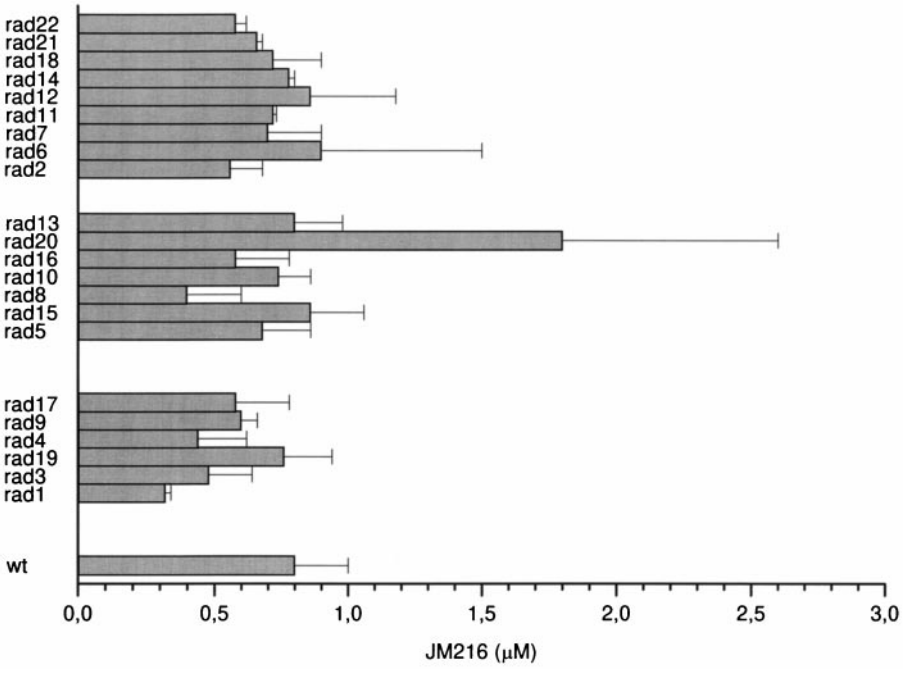

Fig. 3. Sensitivity of *S. pombe* mutants to JM216. *wt*, wild-type 972h$^-$ strain. Sensitivity was evaluated by using a microtiter growth inhibition assay with a 48 hr drug exposure. IC_{50} values are the means ± standard deviation of at least three independent experiments.

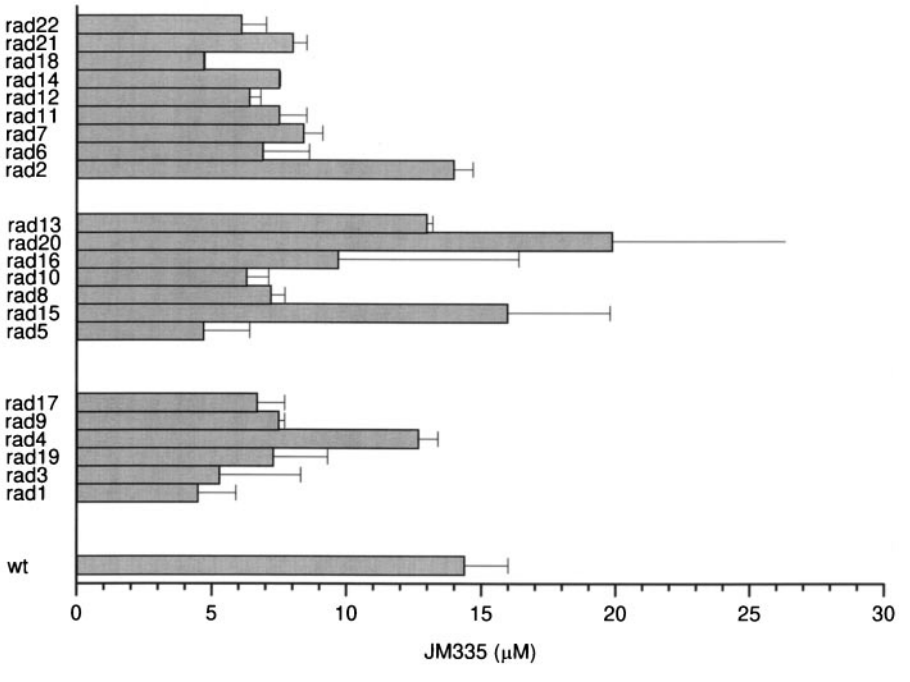

Fig. 4. Sensitivity of *S. pombe* mutants to JM335. *wt*, wild-type 972h$^-$ strain. Sensitivity was evaluated by using a microtiter growth inhibition assay with a 48 hr drug exposure. IC_{50} values are the means ± standard deviation of at least three independent experiments.

central roles in checkpoint pathways preventing inappropriate cell cycle progression when DNA is incompletely replicated or damaged (Al-Khodairy *et al.*, 1994). In particular, rad1, which on the basis of its similarity to Rec1 of *Ustilago maydis* may be a nuclease, seems to be required for regulating G_2/M phase transition (Rowley *et al.*, 1992). Also, rad3, which is a member of the subclass of "lipid kinase" kinases homologous to ataxia-telangiectasia mutated and ataxia and rad-related, seems to be implicated in G_2 arrest (Bentley *et al.*, 1996). Rad1 and rad3 are believed to form a complex with other checkpoint rad proteins, including rad9 and rad17, that function to recognize changes in DNA structure or integrity and transduce signals that result in execution of a cell cycle arrest. In addition, the rad18 gene, which is structurally related to the SMC family of proteins that are involved in modulating chromosome structure in mitosis, is involved in a DNA repair pathway that is different from classical nucleotide excision repair system (Lehmann *et al.*, 1995).

The finding of a similar response to cisplatin of rad10, -16 and -20 was anticipated, because these mutants are allelic (Carr *et al.*, 1994). In particular, these mutations map to the amino-terminal (rad10), carboxyl-terminal (rad16) and central (rad20) domains of the protein that is involved in the initial steps of the excision-repair pathway (Carr *et al.*, 1994). Conversely, although they are allelic and involved in excision repair (Subramani, 1991), rad5 and -15 exhibited different

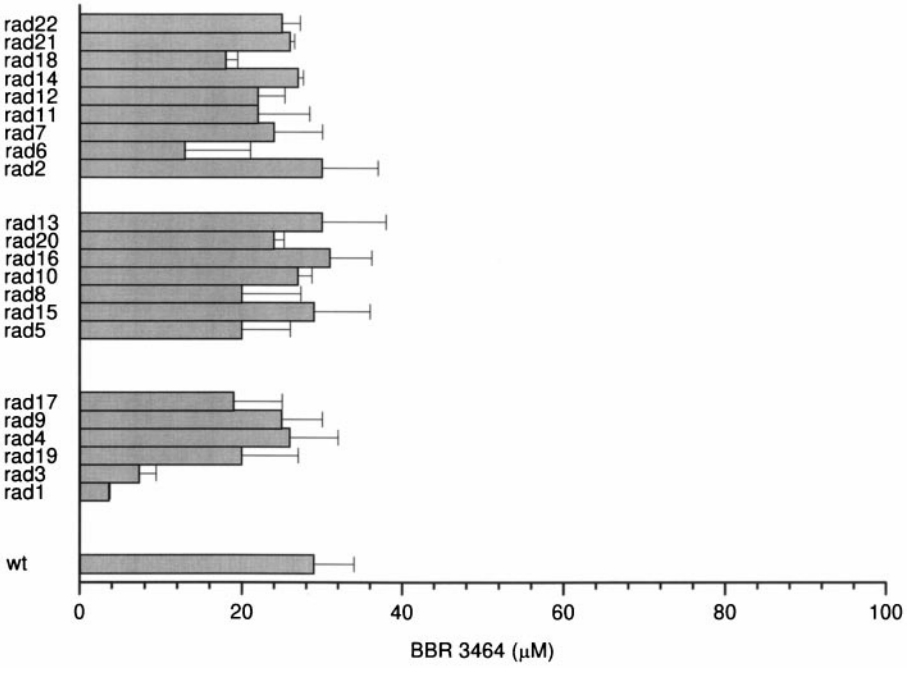

Fig. 5. Sensitivity of *S. pombe* mutants to BBR3464. *wt*, wild-type 972h$^-$ strain. Sensitivity was evaluated by using a microtiter growth inhibition assay with a 48 hr drug exposure. IC_{50} values are the means ± standard deviation of at least three independent experiments.

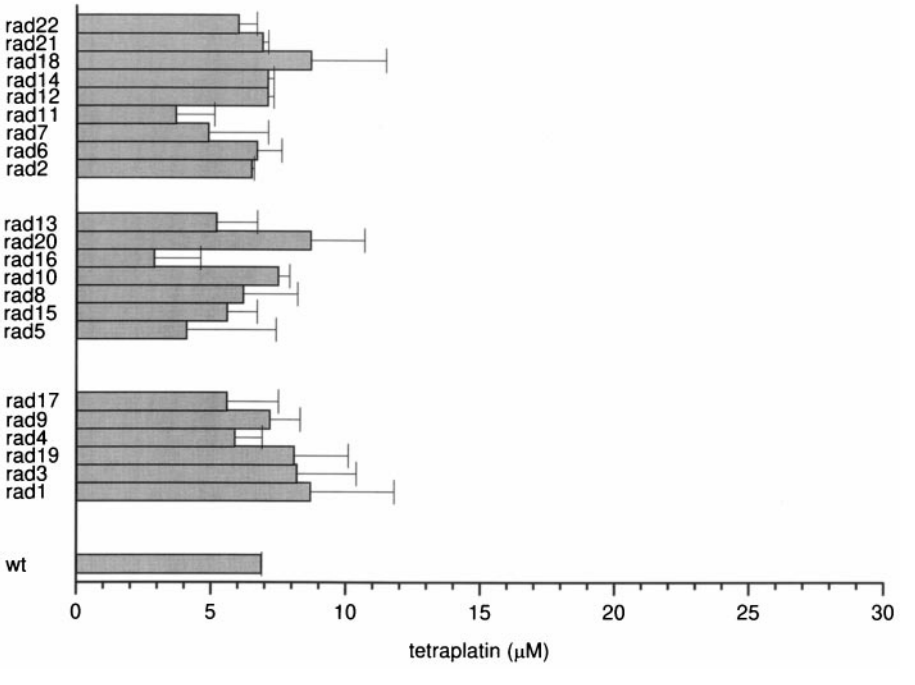

Fig. 6. Sensitivity of *S. pombe* mutants to tetraplatin. *wt*, wild-type 972h$^-$ strain. Sensitivity was evaluated by using a microtiter growth inhibition assay with a 48 hr drug exposure. IC_{50} values are the means ± standard deviation of at least three independent experiments.

degrees of hypersensitivity to cisplatin, which suggests variable importance of different domains of the protein for this phenotype. A similar conclusion could be drawn for two other allelic mutations, rad3 and rad19, involved in checkpoint control.

The increased sensitivity of the wild-type strain to BBR 3464 is somewhat expected. Indeed, in spite of its large molecular size, the multinuclear complex exhibited a potent cytotoxic activity against a number of human tumor cell lines (Giuliani *et al.*, 1997). However, the most potent compounds tested were *cis*-oriented Pt(IV) compounds (JM216 and tetraplatin). In particular, the increased sensitivity of the 972 strain to JM216 over JM335 could be predicted on the basis of the orientation of the leaving groups. The most potent compounds (JM216 and tetraplatin) were characterized by minimal changes in cytotoxic activity against the tested mutants. The most important observation to emerge from this study is that each of the tested mononuclear platinum complexes generated a unique pattern of hypersensitivity in this panel of mutants. In contrast to the pattern of response to cisplatin, with a large number of mutants exhibiting >3-fold hypersensitivity, only a few mutants exhibited a comparable degree of hypersensitivity to the other platinum complexes. The rad1 and -3 mutations had the broadest effect, causing >3-fold hypersensitivity to the tested platinum compounds with the exception of JM216 and tetraplatin. Rad5, involved in excision repair, affected sensitivity only to cisplatin, JM335, and oxaliplatin. Rad6 was involved in sensitization to several Pt compounds, including cisplatin, JM335, oxaliplatin, and BBR3464. Because this mutant, like its *S. cerevisiae* homolog, Rad18, is defective in postreplication repair (Hartwell *et al.*, 1997), it is conceivable that this particular repair process is a critical determinant of cellular sensitivity to a large number of Pt-compounds. Rad18, which conferred marked hypersensitivity to cisplatin, also rendered cells sensitive to JM335 and oxaliplatin but had a lesser effect on sensitivity to BBR 3464. A comparison of the sensitivity pattern of cisplatin and BBR 3464 suggests that defects in genes involved in checkpoints are relevant for cytotoxicity of both compounds, although alterations in nucleotide excision repair (rad5, -10, -16, -20, -13) seem to have a marginal relevance for BBR3464 activity. Indeed, several genes affecting cisplatin sensitivity did not influence cellular response to BBR 3464. For BBR 3464, the unique pattern of sensitivity of the tested mutants could be predicted on the basis of the specific drug-induced DNA lesions (i.e., "long-distance" DNA adducts). A surprising finding of this study was the markedly different pattern of sensitivity between tetraplatin and oxaliplatin. Indeed, the activity of tetraplatin has been ascribed to reduction to Pt(II); thus, one would expect the type of DNA lesions to be similar for these compounds. The profile of sensitivity to oxaliplatin was somewhat comparable with that of cisplatin (rad3 and -18 caused the most evident effects). Minimal changes in sensitivity to tetraplatin were found in all mutants (the one exception involved the rad16 mutant). Although the profile of sensitivity to JM335 was somewhat superimposable upon that of cisplatin, defects in specific genes (rad4 and rad20 implicated in checkpoint and repair) failed to sensitize to JM335. Again, this difference could be related to the type of DNA interaction.

The diversity of determinants of sensitivity uncovered in this study is consistent with studies of cisplatin, tetraplatin, and oxaliplatin made using the NCI 60 cell line panel (Rixe *et al.*, 1997), and with the early clinical evidence that some of the cisplatin analogs have unique patterns of clinical activity. In conclusion, although a number of tumor cell-specific alterations do not allow extrapolations from yeast systems to tumor cell, the use of genetically modified microorganisms is expected to identify the molecular context that allows improvement of tumor response to antitumor agents.

Acknowledgments

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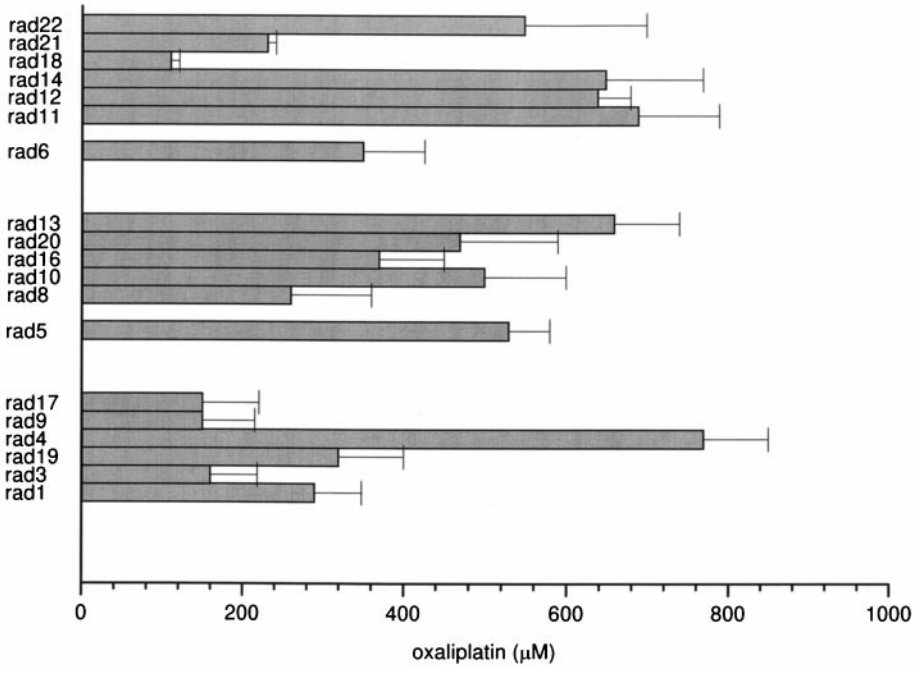

Fig. 7. Sensitivity of *S. pombe* mutants to oxaliplatin. *wt*, wild-type 972h$^-$ strain. Sensitivity was evaluated by using a microtiter growth inhibition assay with a 48 hr drug exposure. IC_{50} values are the means ± standard deviation of at least three independent experiments.

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Send reprint requests to: Dr. Paola Perego, Istituto Nazionale Tumori, Via Venezian 1, 20133 Milan, Italy. E-mail: perego@istitutotumori.mi.it