ABSTRACT

Most cytotoxic anticancer agents damage DNA directly, interfere with DNA metabolism or chromosome segregation, and are particularly toxic in dividing cells. Although a considerable amount of information on the mechanisms of action of these agents is available, the molecular bases for selective tumor cell killing by chemotherapy are largely unknown. Many genetic alterations found in sporadic and hereditary cancers affect functions in DNA repair and cell cycle control and result in sensitivity to DNA damaging agents. We have therefore set out to determine the effects of these cancer mutations on sensitivity or resistance to various chemotherapeutic agents. Because most of the affected genes are well conserved among eukaryotes, we have carried out a comprehensive analysis of a panel of isogenic yeast strains, each defective in a particular DNA repair or cell cycle checkpoint function, for sensitivity to the Food and Drug Administration-approved cytotoxic anticancer agents. Widely different toxicity profiles were observed for 23 agents and X-rays, indicating that the type of DNA repair and cell cycle checkpoint mutations in individual tumors could strongly influence the outcome of a particular chemotherapeutic regimen.

INTRODUCTION

The goal of chemotherapy is to selectively eliminate tumor cells. Identifying the molecular differences that exist between tumor and normal cells will aid in achieving this selectivity. Cancers accumulate a large number of genetic changes during progression toward malignancy due to an intrinsic genetic instability that should provide distinguishing characteristics to exploit therapeutically (1). However, because methods do not yet exist to comprehensively genotype mammalian cells, it has not yet been possible to catalogue these changes and correlate them with chemotherapeutic responses. However, comprehensive genotyping may not be necessary if specific lesions could be identified that create a vulnerability to chemotherapeutic agents. The very mutations that promote genetic instability would be expected to confer such vulnerability because they cripple DNA repair functions or cell cycle checkpoints (2).

Contrary to current clinical practice, there is no reason to expect that the type of lesion creating genetic instability would necessarily correlate with the tissue of origin of a tumor (3). Indeed, lesions creating a genetic instability (for example, mismatch repair defects) occur in a wide variety of tumors from different tissues of origin (1). We propose, therefore, that identification of the particular mutations creating genetic instability in a tumor will be more predictive of the chemotherapeutic sensitivity of that tumor than will be its tissue of origin. In cases in which chemotherapy is effective, a conjunction of chemotherapeutic agent and tumor vulnerability may have been achieved fortuitously. Additionally, by using model organisms, defects that lead to genetic instability could be used to identify new selective agents.

For this approach to work, one would need to correlate vulnerability to chemotherapeutic agents with particular DNA damage response defects. Here, we used yeast cells with defined genetic defects in DNA damage response elements to see whether currently used chemotherapeutic agents have specificity for individual DNA damage response defects. The agents for which such a specificity exists are candidates for correlating effectiveness in human cancer with defects in DNA damage response elements in those same tumors.

MATERIALS AND METHODS

 Yeast Strains. All strains used are haploid and in the A364a background. For treatment with X-rays and drugs, except pentostatin, the following strains were used (with the relevant genotype indicated in parentheses): yMP10381 (wild type), yMP10636 (rad1Δ), yMP10691 (rad14Δ:His3), SP50248 (mnh1::TRP1), yMP11082 (pms1::LEU2), yMP10467 (mag1::URA3), yMP10590 (apn1::LEU2), yMP10612 (mgt1::LEU2), 9170-Tx1a (rad5::LEU2), yMP10425 (rad8::LEU2), SP50280 (rev1::LEU2), yMP10382 (rev3::LEU2), yMP11406 (rad50::hisG), yMP10383 (rad51::LEU2), yMP10630 (rad52-1), yMP10535 (rad9::LEU2), YMP10485 (rad18::LEU2), yMP10913 ( mec1-1), yMP10605 ( mec2-1), SP50262 ( mad1::LEU2), yMP11372 ( mad3::LEU2), and SP50265 ( sgk1::LEU2). For treatment with pentostatin, all strains were as above except for SP50646 (mgt1::kan). All strains are of the genotype MATa ade2 ade3–130 leu2 trpl ura3–52 cyh2 SCR::URA3, except for yMP10485 (rad7::LEU2) and yMP10636 (rad1Δ), which are CYH2+, yMP10630 (rad52-1) and yMP11406 (rad50::hisG), which are MATa; and SP50646 (mgt1::kan), which is MATa and lacks SCR::URA3. SCR::URA3 is a cassette consisting of two halves of ADE3 separated by URA3 for measuring sister chromatid recombination (4). The alleles rev1::LEU2, mad1::LEU2, sgk1::LEU2, and mgt1::kan were generated by PCR fragment-directed gene replacement (5) using plasmids pJF252 or pJF250 (6) for amplification of the LEU2 marker gene and puCkanMX (7) for amplification of the kan gene, and they generally lacked greater than 90% of the open reading frame. Strains harboring the PCR fragment-directed gene replacements apn1::LEU2 and mad3::LEU2 were obtained from Amanda Paulovich, and mnh1::TRP1 was obtained from Brian Thornton. Strains harboring the point mutations rad52-1 (8), mec1-1 (9), and mec2-1 (9) and the gene replacements pms1::LEU2, rad18::LEU2, mgt1::LEU2, mag1::URA3, rev3::LEU2, rad50::hisG, rad51::LEU2, rad14::His3, rad6::LEU2, rad1Δ, rad9::LEU2, and rad17::LEU2 were obtained from Amanda Paulovich.

Drugs. Chemotherapeutic drugs were obtained from the Developmental Therapeutics Program at the National Cancer Institute; cytarabine monophosphate was also obtained from Sigma. Drugs were stored at −80°C in aliquots and used only once. Camptothecin sodium salt and idarubicin solutions were prepared for each experiment.

Toxicity Assays. In preliminary experiments, appropriate concentration ranges were determined for each drug and each strain. Exponentially growing yeast cultures in complete minimal medium were diluted to 7.4 × 10^6 cells/ml, and 135 μl were dispensed into each well of flat-bottomed 96-well plates. Drugs were dispensed in seven 2-fold serial dilutions in 5% DMSO or 5% ethanol (thiotepa and lomustine) or in H₂O (cisplatin, mitomycin, cytarabine, cytarabine phosphate, hydroxyurea, and fluorodeoxyuridine), and 15-μl aliquots were added in triplicate to the yeast-containing wells. Fifteen μl of the appropriate solvent were also added in triplicate in control wells. Plates were incubated for 18 h at 30°C, and the Abs₅₀ of the cultures was read in a Bio-Tek Instruments EL340 microplate reader. Slowly growing strains, especially rad6

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and rad52 mutants, were incubated until the A_{600} of control wells read at least 0.4 but no more than 0.8. For three agents, IC_{50} values were determined by measuring colony-forming units in the case of losustine and cytarabine phosphate because this method resulted in greater sensitivity or in the case of mitoxantrone because the color of the drug interfered with the A_{600} reading. In this case, after incubation, aliquots from each well were plated onto rich medium agar plates. Sulfanilamide (0.1 mg/ml) was added to the growth medium for methotrexate and trimetrexate assays. Each experiment was carried out three times with yeast strains freshly streaked out from –80°C stocks.

**RESULTS**

We have assembled a panel of isogenic yeast strains, each defective in a particular DNA damage repair or cell cycle checkpoint pathway. The damage repair pathways include nucleotide excision repair (rad1 and rad14), mismatch repair (mhl1 and pms1), base excision repair (mag1 and apn1), direct reversal of O-alkylguanヌine (mgi1), error-free (rad18), error-prone (rev1, rev3), and error-free and error-prone damage (rad6) tolerance during DNA replication (also called postreplication repair or daughter-strand gap repair) and recombinational repair (rad50, rad51, and rad52). The roles of these genes in DNA repair have been reviewed extensively (10). Cell cycle checkpoints include the DNA damage checkpoint (rad9 and rad17; Ref. 11), DNA damage and S-phase checkpoint (mec1 and mec2-rad53; Ref. 9), spindle assembly checkpoint (mad1 and mad3; Ref. 12), and homologue of the genes mutated in Bloom’s and Werner’s syndromes (sgs1; Ref. 13).

There are 76 FDA-approved anticancer agents, and of these, 44 are expected to be toxic to yeast. In preliminary experiments, we determined that 31 of these agents showed at least minimal toxicity in yeast. In two cases, cytarabine (NSC-63878) and topotecan (NSC-609699)/irinotecan (NSC-616348), we used analogues that were active yeast. The initial step in the activation of cytarabine in human cells is its conversion to cytarabine monophosphate. Because yeast lack the required deoxycytidine kinase, we used cytarabine monophosphate (NSC-99445), which is taken up efficiently and shows activity. Likewise, we used camptothecin sodium salt (camptothecin phosphate (NSC-99445), which is taken up efficiently and shows activity. Likewise, we used camptothecin sodium salt (camptothecin phosphate, NSC-100880) in place of topotecan and irinotecan. Here we report the IC_{50} data for these agents in the damage response-deficient yeast strains. Fig. 1 shows the profiles of the alkylating and cross-linking agents. Fig. 2 shows the antimetabolites and nucleotide analogues. Finally, Fig. 3 shows topoisomerase poisons, X-rays, bleomycin, and actinomycin D. Most of the DNA repair and DNA damage checkpoint pathways are represented by more than one mutant in the strain panel. In a majority of the profiles (e.g., cisplatin: Fig. 1a), the various mutants representing a particular pathway show a similar sensitivity. This coherent response indicates that the observed sensitivity is a property of a pathway defect rather than a property of the individual mutant strain. Although many of the drugs have been studied previously in various yeast mutants (14, 15), this report is the first comprehensive examination of the relative sensitivities of damage response-defective yeast strains to anticancer agents.

The profiles are of three general types. First, there are agents showing selective toxicity, primarily in strains containing one pathway defect. We define selective toxicity in a particular strain or pathway as a 10-fold or greater decrease in the IC_{50} relative to the next, most sensitive strain. We conclude that the selective agents cause a specific type of damage only requiring a subset of damage response functions for survival (e.g., mitoxantrone: Fig. 3b). Second, there are agents that show toxicity in many pathway defects indicating the introduction of multiple types of DNA damage (e.g., thiopeta, mitomycin C, hydroxyurea, and X-rays: Figs. 1, b and e, 2c, and 3c). Third, there are agents that lack selective toxicity between the various mutant strains and wild type. Such profiles indicate that although the agents are toxic, cell killing occurs by a mechanism that is probably independent of DNA damage (e.g., methotrexate: Fig. 2e).

**Selective Agents.** Five agents (cisplatin, cytarabine phosphate, camptothecin sodium, mitoxantrone, and idarubicin) showed a high degree of specificity.

Cisplatin (Fig. 1a) is specifically toxic to strains defective for the poorly understood Rad6/Rad18-controlled pathway of damage tolerance during S-phase. It is known, however, that Rad6 and Rad18 are required for the completion of S-phase when a damaged DNA template is present (i.e., daughter-strand gap repair; Ref. 16). Rad6- and Rad18-mediated survival does not require Rad50, Rad51, Rad52, Rad1, Rad14, Rev1, or Rev3 (17). The sensitivity of these strains reflects the additional role of nucleotide excision repair (Rad1 and Rad14), recombinational repair (Rad50, Rad51, and Rad52) and error-prone damage tolerance (Rev1, Rev3, and Rad6) in the repair of inter- and intrastrand DNA cross-links as well as mono-adducts formed by cisplatin. The importance of the Rad6 and Rad18 function is evidenced by the greater than 10-fold increase in sensitivity in rad6 and rad18 strains relative to the other sensitive strains (e.g., rad50). With the exception of the magnitude of the rad6 and rad18 sensitivity, the profiles for cisplatin and mecloatreline are highly similar, indicating a common response to cross-links induced by platinum adduct formation and alkylative cross-linking. Thiopeta, which is not generally thought to create DNA cross-links, is also similar.

The nucleotide analogue cytarabine monophosphate (Fig. 2a) shows highly selective toxicity in the sgs1 mutant strain. This profile does not resemble those of agents causing known types of DNA damage. Cytarabine inhibits DNA synthesis, is incorporated into DNA, and has also been reported to be recombinnogenic in yeast (18). The sgs1 mutant and the homologous Bloom’s and Werner’s syndrome defects in human cells cause hyperrecombination (19). It is therefore possible that the observed selectivity of cytarabine phosphate is due to an effect on recombination and that the Sgs1 protein is required for tolerance. We found that a sgs1 rad52 double mutant is less sensitive to cytarabine phosphate compared to the sgs1 single mutant, corroborating a possible role for recombination in the mechanism of toxicity (data not shown). We have observed differential growth inhibition of the sgs1 mutant by ademen arubinoside phosphate and fludarabine phosphate, indicating that the arabinose moiety of these agents may be important for selectivity (data not shown). The profile of fludarabine (Fig. 2b) shows a greater sensitivity of the rad50 and mec2 mutants than that of cytarabine phosphate.

The selective profile includes, in addition to cisplatin and cytarabine monophosphate, three topoisomerase poisons. These agents stabilize the covalent complex of topoisomerase and DNA ends during DNA relaxation and lead to single-strand breaks and DSBs for topo I and topo II poisons, respectively. The single-strand breaks caused by topo I poisons are converted into DSBS during DNA replication (20, 21). The camptothecin analogue, a topo I poison, kills DSBS repair mutants with high specificity. The DNA damage checkpoint mutants also contribute to survival, albeit to a lesser degree (Fig. 3a).

The topo II poisons mitoxantrone (Fig. 3b) and idarubicin (Fig. 3c) also showed selectivity for DSBS repair defects. It is noteworthy that
cells deficient in the DNA damage checkpoint are not sensitized to these topo II poisons. This can be explained by the observation that topo I poison-induced damage requires ongoing DNA synthesis to create DSBs, thus largely limiting the damage to S-phase when DNA damage-induced and DNA synthesis checkpoints can contribute to cell survival. topo II poisons, in contrast, create DSBs throughout the cell cycle, with the majority occurring in mitosis, when the DNA damage-induced and synthesis checkpoints are no longer capable of protecting cells. Interestingly, two other topoisomerase poisons, daunorubicin and doxorubicin, show profiles that are nonselective, suggesting the presence of non-topo II-mediated cellular damage (Fig. 3, f and g).

Broadly Selective Agents. Nine agents (mitomycin C, thiotepa, lomustine, carmustine, streptozotocin, mechlorethamine, bleomycin, hydroxyurea, and X-rays) show selective toxicity in a large number of DNA damage response-defective strains. Most of these responses are consistent with the known mechanisms of action of the agents. Mag1 (3-methyladenine glycosylase) is involved in the removal of damage created by alkylation of adenine (22), and as expected, the mag1 mutant is sensitive to thiotepa (Fig. 1b). The rad1 and rad14 strains are sensitive to agents that introduce DNA cross-links (e.g., cisplatin and mechlorethamine: Fig. 1, a and c). Thiotepa, however, is a non-cross-linking alkylating agent, and the sensitivity of these strains was unexpected (23). Lomustine (Fig. 1f) and carmustine (Fig. 1g) produce broad profiles; although both are capable of forming DNA cross-links (24), nucleotide excision repair-defective strains (e.g., rad1 and rad14) were not sensitive. These differences demonstrate a continuum of pathway responses to cytotoxic drugs and underscore the importance of identifying the critical toxic lesions for agents that are capable of inducing different forms of DNA damage. Streptozocin (Fig. 1d), a methylating agent, also yielded a broad profile, although the mgt1 strain was particularly sensitive. This was expected, because the Mgt1 protein, the yeast homologue of human MGMT, is required for O6-methylguanine reversal (25, 26).

The ribonucleotide reductase inhibitor hydroxyurea (Fig. 2c) blocks DNA replication by depleting the dNTP pool. This is consistent with the observed sensitivity of the intra-S phase checkpoint-deficient mec1 and mec2 strains (9, 27). Additional DNA damage in the form of DSBs is indicated by the sensitivity of rad50, rad51, and rad52 strains. This result is consistent with the observation that stalled replication forks result in DNA breaks (28). Since these breaks occur exclusively during S phase, the Rad9 and Rad17 functions may not be required, because the cell cycle would already be slowed down in a MEC1-dependent fashion due to a decreased level of dNTPs. X-ray treatment (Fig. 3c) results in a profile similar to the topo I poison camptothecin sodium (Fig. 3a), except for the strong requirement of the Rad6 and Rad18 proteins for survival. X-rays are often referred to as a DSB-causing agent, which is consistent with the strong sensitivity of the recombinational repair mutants. The sensitivity of rad6 and rad18 strains indicates that other forms of DNA damage are also present in irradiated cells as expected. The profiles of camptothecin
sodium (Fig. 3a) and mitoxantrone (Fig. 3b) suggest that DSBs do not necessarily require Rad6 and Rad18 functions for repair or tolerance.

**Nonselective Agents.** Nine agents (methotrexate, trimetrexate, fluorouracil, fluorodeoxyuridine, pentostatin, dacarbazine, actinomycin D, daunorubicin, and doxorubicin), although toxic, showed only minor differences in sensitivity among the various mutant strains and the wild-type strain. These nonselective profiles, especially in the case of agents that block key steps in DNA synthesis (i.e., methotrexate, trimetrexate, fluorouracil, and fluorodeoxyuridine), are surprising because the induced damage must not require a DNA damage response for survival. Methotrexate (Fig. 2e) inhibits dihydrofolate reductase, resulting in a block in the biosynthesis of purines and thymidine. Our results suggest that the purine deficiency might affect RNA synthesis more strongly than DNA replication. Interestingly, the profile of the methotrexate analogue, trimetrexate (Fig. 1f), does show a moderate sensitivity of mec1 and mec2 strains, as would be expected for a block in DNA synthesis. Fluorouracil (Fig. 2g) and fluorodeoxyuridine (Fig. 2h) inhibit thymidilate synthase, resulting in a block in the synthesis of dTMP from dUMP. In this case, only DNA synthesis would be affected, and it is surprising that mec1 and mec2 strains are not more sensitive. Pentostatin (Fig. 2d) inhibits adenine deaminase, which is involved in posttranscriptional modification of RNA and is not expected to cause DNA damage (29). Dacarbazine, doxorubicin, daunorubicin, and actinomycin D (Fig. 1h and Fig. 3, f–h) showed very similar, virtually nonselective profiles. It is likely that these agents kill yeast cells by a mechanism that does not require a DNA damage response for survival. Daunorubicin, doxorubicin, and actinomycin D, for example, are capable of generating free radicals, which target membranes in addition to DNA (30).

**DISCUSSION**

The goal of this study has been to determine the cellular contexts that contribute to the sensitivity of cells to cytotoxic anticancer drugs. Although there is extensive information on the mechanisms of action for cancer drugs, there has been little progress in determining whether there are specific contexts (that is, molecular defects) that determine the selective killing of tumor cells over normal cells. To achieve a constant genetic context in which to assess various molecular defects, we chose to use a panel of isogenic yeast strains. The yeast mutants tested here were chosen because they affect the major pathways known to be required for survival in response to DNA damage and because they represent most of the pathway defects identified thus far that lead to genetic instability in cancer cells. It is possible that additional pathways responsible for genetic stability may be identified in the future. This may include Sgs1, the DNA damage response function of which is poorly understood.

We chose the nonbiological, nonhormonal, FDA-approved agents to assess the relative sensitivity of the yeast mutants and were able to obtain IC50 values for 23 of the 44 agents tested. The profiles shown...
Differential Toxicities of Anticancer Agents

The analysis of the FDA-approved cancer drugs using this isogenic panel of yeast addresses an important question in cancer therapy: do we need new drugs or better diagnostics? Agents, such as mitoxantrone, that selectively target a single pathway defect suggest that improved diagnosis may be efficacious. Cancers defective in DNA DSB repair may be hypersensitive to topo II poisons. The availability of better diagnostics that would allow the routine determination of pathway defects in individual tumors might greatly improve benefits from current chemotherapeutic agent. Conversely, the agents resulting in broad toxicities in multiple genetic backgrounds may lead to higher toxicity in noncancer cells. It is therefore important to identify agents with high specificity for unique genetic changes associated with cancers. Primary drug screens carried out in the context of a single genetic alteration may provide a valuable route to new selective drugs. Therapeutic agents could be screened for high toxicity in human cells carrying a particular damage response defect but low toxicity in isogenic wild-type control cells. This preliminary study has shown that many of the current cancer drugs (X-rays etc.) are relatively nonspecific and suggests that developing more specific therapeutics may be beneficial. At the same time some of the commonly used cancer drugs, such as cisplatin and the topoisomerase poisons, have significant specificity in their killing, and this provides strong evi-
dence that new molecular diagnostics could improve their utility. The results described in this communication pertain to the activity of anticancer agents in yeast, and it should be noted that the biology of yeast and mammalian cells differs in several important aspects. Foremost is the absence of programmed cell death in yeast. Although programmed cell death or apoptosis may not alter the intrinsic sensitivity of cells harboring specific defects to particular agents, it may alter the response of the cell to damage (i.e., survival). In addition, the relative importance of alternative pathways for cellular response to cytotoxins can vary between the two systems. This is true for DNA DSB repair pathways, the major pathway of which in yeast is homologous recombination (represented by rad50, rad51, and rad52 in this study), whereas in mammalian cells, nonhomologous end joining is a more important repair pathway (31). We expect, however, that sensitivity differences seen in yeast can, in many cases, be translated to mammalian cells with the same fundamental defect, such as a deficiency in DSB repair, regardless of the precise causative genetic defect. Finally, the examination of anticancer agents with non-DNA sensitivity differences seen in yeast can, in many cases, be translated to cytotoxins can vary between the two systems. This is true for DNA relative importance of alternative pathways for cellular response to generating a sizable panel (rodent panel of matched pair cell lines). Although current limitations in to include the spindle poisons in this study. Theoretically, the studies agents are not active against yeast tubulin; hence, it was not possible to respond to various cancer treatments.

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