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DNA repair pathways involved in repair of lesions induced by 5-fluorouracil and its active metabolite FdUMP

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ABSTRACT

5-Fluorouracil (5-FU) is an antitumor antimetabolite that can be converted into fluoronucleotides and FdUMP. Fluoronucleotides are incorporated into DNA and RNA, while FdUMP results in nucleotide pool imbalance. *Saccharomyces cerevisiae* is unable to convert 5-FU into FdUMP, making yeast a unique model system to study the cellular effects of 5-FU and FdUMP independently. A panel of repair-deficient yeast strains was used to identify the DNA repair pathways needed for repair of lesions generated by 5-FU or FdUMP. This included yeast deficient in base excision repair (BER), nucleotide excision repair (NER), translesion synthesis (TLS), mismatch repair (MMR), post-replication repair (PRR), homologous recombination (HR) and non-homologous end-joining (NHEJ). The results revealed an important role of BER, since BER-mutants (ntg1, ntg2, apn1, apn2) showed pronounced sensitivity to both 5-FU and FdUMP. MMR mutants also showed high sensitivity to both compounds. In contrast, deficiencies in NER, NHEJ and TLS repair had only minor influence on the sensitivity to FU and FdUMP. Interestingly, deficiencies in HR (rad52) and PPR (rad6, rad18) were associated with increased sensitivity to 5-FU, but not to FdUMP. Taken together, our study reveals an important contribution of DNA repair pathways on the sensitivity to 5-FU and its active metabolite FdUMP. Importantly, the repair mechanisms differed for the 2 antimetabolites since lesions induced by 5-FU were repaired by BER, MMR, HR and PRR, while only BER and MMR were required for repair of FdUMP-induced lesions.

1. Introduction

5-Fluorouracil (5-FU) is a pyrimidine analogue widely used as a chemotherapeutic agent, in particular for colorectal cancer [1,2]. 5-FU is an uracil analog with a fluorine atom at the fifth position and is metabolized like uracil [3].

For cytotoxic activity, 5-FU requires uptake and metabolic activation by cellular phosphorylases and kinases [1,4]. As outlined in Fig. 1, 5-FU activation involves its conversion into fluoronucleotides and to FdUMP. The fluoronucleotides are incorporated into DNA and RNA during macromolecular synthesis [5,6] leading to a wide range of biological effects which can act as a trigger for apoptotic cell death [7]. This includes DNA mutations and protein miscoding [8], inhibition of pre-rRNA processing [9], inhibition of post-transcriptional modification of tRNAs [10] as well as polyadenylation and splicing of mRNA [1]. The FdUMP metabolite inhibits thymidylate synthase (TS) resulting in nucleotide pool imbalance with decreased levels of dTMP and increased concentrations of dUMP. Since most DNA polymerases have poor discrimination for dTTP and dUTP, a strong increase in dUMP concentrations may result in a significant incorporation of uracil into DNA [1,3,11,12]. It has also been reported that FdUMP can be further phosphorylated to FdUTP followed by DNA incorporation [13]. For these reasons, FdUMP is believed to be the major active metabolite of 5-FU [14,15].

During DNA repair, mismatched bases are removed from DNA by uracil glycosylase and the DNA backbone is nicked by an abasic endonuclease. Then, the 5′-deoxyribose phosphate is removed by a flap endonuclease and the resulting gap is filled with another trinucleotide through the action of a repair DNA polymerase

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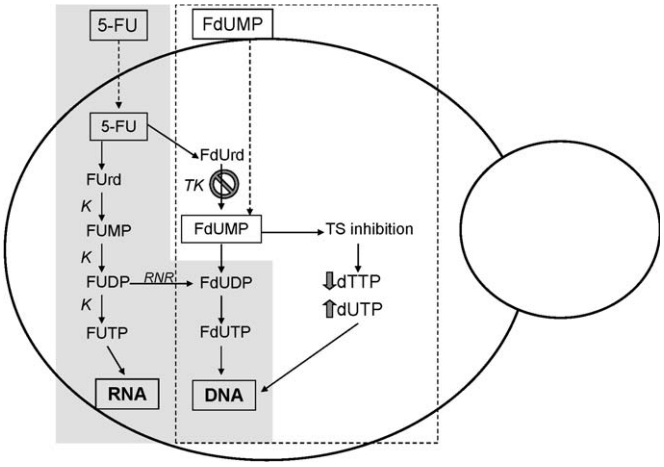

Fig. 1. 5-FU metabolism in *Saccharomyces cerevisiae*. 5-FU shares the same facilitated transport system as uracil, adenine and hypoxanthine, whereas derivatives of 5-fluoro-2′-deoxyuridine enters the cell by a distinct facilitated membrane transport mechanism used by purine and pyrimidine nucleosides [40]. Fluoropyrimidines are converted into fluorinated ribonucleotides and deoxyribonucleotides by the same pathways as uracil and thymine. The absence of thymidine kinase in yeast does not allow the direct conversion of 5-FU into FdUMP, suggesting that the toxicity of 5-FU treatment is due to misincorporation of fluoropyrimidines into DNA and RNA (grey box), while the FdUMP-mediated toxicity is principally related to TS inhibition (box outlined by dashed lines). FUrd = 5-fluorouridine; FUMP, FUDP and FUTPs = 5-fluorouridine-5′-mono-, di- and triphosphate; FdUDP = 5′-fluoro-2′-deoxyuridine diphosphate; FdUTP = 5′-fluoro-2′-deoxyuridine triphosphate; dUTP = deoxyuridine triphosphate. Enzymes: TK = thymidine kinase; K = kinases; RNR: ribonucleotide reductase.

followed by DNA ligation [16]. Although uracil excision is not essential for the cytotoxic activity of 5-FU [16,17], an incomplete/futile repair process may lead to accumulation of toxic repair intermediates including abasic sites and DNA strand breaks which can provoke cell death [18]. Accordingly, studies with repair-deficient yeast strains showed that deletion of *UNG1*, which codes for uracil DNA glycosylase had a protective effect against 5-FU in contrast to deletion of *APN1*, the major abasic site endonuclease in yeast, which was accompanied by pronounced sensitivity to the drug [16].

Yeast is unable to convert 5-FU into FdUMP due to the absence of thymidine kinase [19], making yeast a unique cellular model to study the cellular effects of 5-FU and FdUMP independently. Specifically, treatment with 5-FU will lead to its conversion into fluoronucleotides (but not into FdUMP) that can be incorporated into DNA during replication, while treatment with FdUMP allows us to study the impact of TS inhibition and the nucleotide pool imbalance (Fig. 1).

In the present study, we have compared the sensitivity of *Saccharomyces cerevisiae* strains deficient in the major repair proteins and repair pathways to 5-FU and FdUMP. Our results show that base excision repair and mismatch repair play an important role in the sensitivity to both 5-FU and FdUMP. Homologous recombination and post-replication repair are also needed for repair of lesions induced by 5-FU, but seem to play a minor role for FdUMP. These data emphasize the importance of DNA repair in the response to 5-FU and identify differences in the biological response to the major 5-FU metabolites.

2. Materials and methods

2.1. Chemicals

5-Fluorouracil (5-FU) was purchased from ICN Pharmaceutics (Valeant Pharmaceuticals International, USA). 5-Fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP) was bought from Sigma–Aldrich (St. Louis, MO, USA). Reagents for culture medium (yeast extract, bacto-peptone, bacto-agar and glucose) were acquired from Merck.

2.2. Yeast strains and media

The relevant genotypes of the *S. cerevisiae* strains used in this work are indicated in Table 1. Mutants strains were obtained from

Table 1
Saccharomyces cerevisiae strains used in this study. BER: base excision repair; NER: nucleotide excision repair; MMR: mismatch repair; HR: homologous recombination; NHEJ: non-homologous end-joining; PRR: post-replication repair; TLS: translesion synthesis.

Strains	Relevant genotypes	DNA repair pathway affected	Source
BY4742 (WT)	*MATα*; *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*	–	Euroscarf
ung1Δ	BY4742; with *ung1::kanMX4*	BER	Euroscarf
apn1Δ	BY4742; with *apn1::kanMX4*	BER	Euroscarf
rad27Δ	BY4742; with *rad27::kanMX4*	BER	Euroscarf
rad1Δ	BY4742; with *rad1::kanMX4*	NER	Euroscarf
rad10Δ	BY4742; with *rad10::kanMX4*	NER	Euroscarf
rad6Δ	BY4742; with *rad6::kanMX4*	PRR	Euroscarf
rad18Δ	BY4742; with *rad18::kanMX4*	PRR	Euroscarf
rad50Δ	BY4742; with *rad50::kanMX4*	NHEJ	Euroscarf
rad52Δ	BY4742; with *rad52::kanMX4*	HR	Euroscarf
rev1Δ	BY4742; with *rev1::kanMX4*	TLS	Euroscarf
rev3Δ	BY4742; with *rev3::kanMX4*	TLS	Euroscarf
BY4741 (WT)	*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*	–	Euroscarf
mlh1Δ	BY4741; with *mlh1::kanMX4*	MMR	L Meira
pms1Δ	BY4741; with *pms1::kanMX4*	MMR	L Meira
FF18733 (WT)	MATa; *leu2-1*, *trp1-289*; *his7-3*; *ura3-52*; *lys1-1*	–	R Medina-Silva
apn1Δ	FF18733; with *apn1::URA*	BER	R Medina-Silva
apn2Δ	FF18733; with *apn2::kanMX*	BER	R Medina-Silva
apn1Δ apn2Δ	FF18733, with *apn1::URA apn2::kanMX*	BER	R Medina-Silva
SJR751 (WT)	*MATα*; *ade2-101$_{oc}$*; *his3Δ200*; *ura3ΔNco*; *lys2ΔBgl*; *leu2-R*	–	RL Swanson[a]
ntg1Δ	SJR751; with *ntg1::LEU2*	BER	RL Swanson[a]
ntg2Δ	SJR751; with *ntg2::hisG*	BER	RL Swanson[a]
ntg1Δ ntg2Δ	SJR751; with *ntg1::LEU2 ntg2::hisG*	BER	RL Swanson[a]
ntg1Δ ntg2Δ apn1Δ	SJR751; with *ntg1::LEU2 ntg2::hisG apn1::HIS3*	BER	RL Swanson[a]
ntg1Δ ntg2Δ apn1Δ rad1Δ	SJR751; with *ntg1::LEU2 ntg2::hisG apn1::HIS3 rad1::hisG*	BER/NER	RL Swanson[a]
ntg1Δ ntg2Δ apn1Δ rev3Δ	SJR751; with *ntg1::LEU2 ntg2::hisG apn1::HIS3 rev3::kanMX4*	BER/TLS	RL Swanson[a]
ntg1Δ ntg2Δ apn1Δ rad52Δ	SJR751; with *ntg1::LEU2 ntg2::hisG apn1::HIS3 rad52::URA3*	BER/HR	RL Swanson[a]

[a] Swanson et al. (1999).

the parental wild-type strains BY4741, BY4742, FF18733 and SJR751 by gene disruption. Yeast strains deficient in MMR were kindly provided from Dr. Lisiane Meira (Biological Engineering Division, MIT, Cambridge, USA) and BER pathway mutants were kind gifts from Dr. Renata Medina-Silva (Pontifícia Universidade Católica, PUC, Brazil). Complete liquid medium (YPD) containing 1% (w/v) yeast extract, 2% (w/v) bacto-peptone and 2% (w/v) glucose was employed for routine growth. Medium containing 2% (w/v) bacto-agar was used for plates.

2.3. Yeast growth conditions

Exponential phase (Log) cultures were obtained by inoculation of 5×10^5 cells/mL of YPD culture in stationary phase into 5 mL of fresh YPD medium. After 14 h incubation, at 30 °C with aeration, the cultures contained $1–2 \times 10^7$ cells/mL with 20–30% budding cells. The number of cells was determined by counting in Neubauer chamber.

2.4. Survival assays

The survival after treatment with 5-FU and FdUMP was measured by preparing cell suspensions containing 5×10^6 Log - cells/mL and incubated in culture medium at 30 °C for 4 h with agitation. After incubation, samples were diluted in saline solution, plated onto YPD agar, and incubated at 30 °C for 2–3 days. 5-FU concentrations employed were 18.75, 37.5, 75 and 150 μM; and FdUMP concentrations were 37.5, 75, 150 and 300 μM. All assays were performed at least twice with each dose in triplicate.

2.5. Drop tests

Logarithmic cultures were serially diluted by 1:10 steps and 6 μL aliquots spotted onto rich media plates with or without 5-FU (150 μM) or FdUMP (300 μM). Plates were incubated at 30 °C for 2 days. Experiments were performed at least twice with each dose in triplicate.

3. Results and discussion

Understanding the repair of genotoxic anticancer agents should facilitate the identification of predictive markers for response prediction and help to identify tumors with intrinsic or acquired drug resistance.

5-FU is widely used for the treatment of solid tumors and many studies have been conduced to elucidate its mechanism of action [1,6–10,20]. In mammalian cells, 5-FU is converted into fluoronucleotides, that are incorporated into nucleic acids, thereby altering their function and stability. Alternatively, 5-FU may be converted into FdUMP, a thymidylate synthase (TS) inhibitor leading to nucleotide pool imbalance and uridine incorporation into DNA [1,21]. Both fluoronucleotides and uracil are recognized by DNA repair proteins such as glycosylases, followed by the recruitment of other enzymes in order to eliminate the lesion and restore the integrity of the DNA. However, many DNA repair-related intermediates are in themselves toxic, including the AP sites, which are generated by glycosylases [16,22].

In the present work, all cytotoxicity experiments were carried out with cells in the logarithmic growth phase because 5-FU and FdUMP depend upon ongoing DNA synthesis for incorporation into

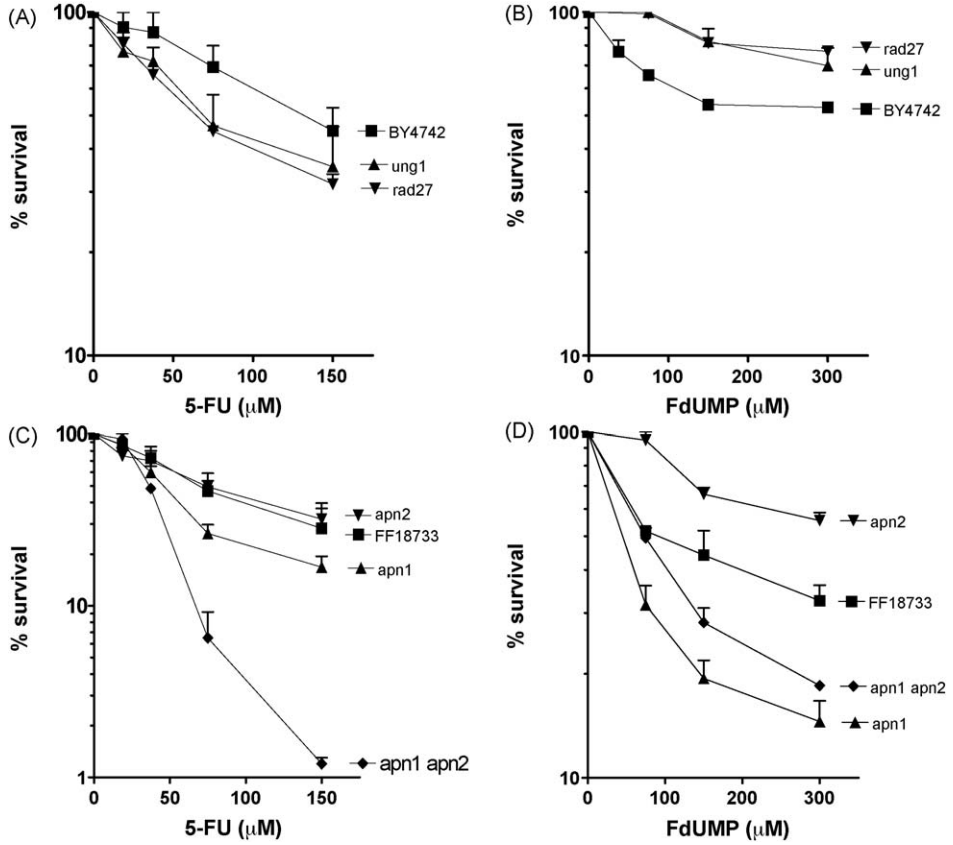

Fig. 2. Survival of *S. cerevisiae* strains single (A and B) and double (C and D) mutants deficient in BER proteins after treatment with 5-FU and FdUMP. The survival of parental strains (BY4742 and FF18733) is compared with that of variants defective in the Ung1 glycoysylase, the Rad27 structure-specific flap endonuclease, and the Apn1 and Apn2 AP endonucleases.

DNA [6]. *S. cerevisiae* strains deficient in the BER proteins *ung1* and *rad27* showed basically unchanged sensitivity to 5-FU, and modest resistance to FdUMP, in comparison with wild-type cells (compare Fig. 2A and B). Our findings are in agreement with previous studies suggesting that deletion of Ung1 is accompanied by either unchanged [17,23] or decreased [16] sensitivity to 5-FU. Ung1 is a glycosylase involved in the removal of uracil bases in DNA resulting in the formation of an AP site [16]. It is believed that Ung1 removes uracil from U:G, 5-FU:G, U:A and 5-FU:A mispairs, as well as uracil from single-stranded DNA [3]. In the absence of Ung1, uracil remains in the DNA. Uracil lesions are better tolerated by the cells than the Ung1 repair intermediates, the AP sites, which can be converted into DNA strand breaks [16,18]. Rad27, a structure-specific flap endonuclease in long-patch base excision repair, is needed to remove 5′deoxyribose phosphate (dRP)-blocked ends, thereby generating a nucleotide gap. Loss of Rad27 is accompanied by accumulation of 5′dRP-blocked sites in the DNA which, apparently, are less toxic than a nucleotide gap [16].

5-FU exposure was accompanied by pronounced sensitivity of *apn1*Δ, *apn1*Δ*apn2*Δ double-, *ntg2*Δ, *ntg1*Δ*ntg2*Δ double-, *ntg1*Δ*ntg2*Δ*apn1*Δ triple- and *ntg1*Δ*ntg2*Δ*apn1*Δ*rad52*Δ quadruple mutants (Figs. 2C, 3A and C). In comparison, FdUMP only showed enhanced toxicity in *apn1*Δ, *apn1*Δ*apn2*Δ double-, *ntg1*Δ*ntg2*Δ double-, and *ntg1*Δ*ntg2*Δ*apn1*Δ triple mutants (Figs. 2D and 3B). Apn1, Ntg1 and Ntg2 are important BER components. Apn1 is the major AP endonuclease in *S. cerevisiae* while Ntg1 and Ntg2 are N-glycosylase/AP lyases. The absence of Apn1 will lead to an accumulation of AP sites, which are potentially deleterious lesions that can be converted into toxic DNA strand breaks. Ntg1 and Ntg2 recognize and remove damaged bases, generating AP sites that can be repaired either by the lyase activity of glycosylases or by the Apn1 endonuclease [22].

Our results showed that the triple mutant *ntg1*Δ*ntg2*Δ*apn1*Δ is very sensitive to 5-FU and FdUMP (Fig. 3A and B). It is important to note that the single mutant *apn1*Δ shows also significant sensitivity to both compounds (Figs. 2C, D, 3A and B). For 5-FU, the sensitivity of the double mutant *ntg1*Δ*ntg2*Δ *is due to* the deletion of *NTG2*, since the double mutant shows the same sensitivity as the *ntg2*Δ single mutant (Fig. 3A). Ntg1 and Ntg2 remove oxidized purines and pyrimidines with different substrate specificities. Furthermore, they have different localization, since Ntg1 is present in both the nucleus and the mitochondria while Ntg2 only is present in the nucleus [22,24,25]. In addition, *NTG1* is damage-inducible, whereas *NTG2* is expressed constitutively [24]. The important differences in survival suggest that Ntg2 may be more important than Ntg1 for the removal of fluoronucleotides. In contrast, only the *ntg1*Δ*ntg2*Δ double mutant was sensitive to FdUMP (Fig. 3B) indicating that both Ntg1 and Ntg2 proteins recognize FdUMP-induced lesions and can replace each other. Deletion of apn1 was more important for the sensitivity than deletion of apn2, while the double mutant *apn1*Δ*apn2*Δ was very sensitive to both drugs (Fig. 2C and D).

Both *mlh1*Δ and *pms1* showed increased sensitivity toward 5-FU and FdUMP (Fig. 4A and B), indicating a role for MMR in lesion processing of the two drugs. MMR is also important in the response of mammalian cells to 5-FU. However, in mammalian cancer cells, as well as in colon cancer patients, MMR deficiency is associated with increased resistance, rather than increased sensitivity, to 5-FU [26–29]. This difference may be explained by the additional cellular functions of the MMR system in mammalian cells, in particular with respect to induction of apoptotic signaling [30]. Interestingly, the MED1/MBD4 protein is absent in *Saccharomyces cerivisiae*. MED1 is a BER protein that is required for integrity of the MMR system [31] and which also influence the induction of

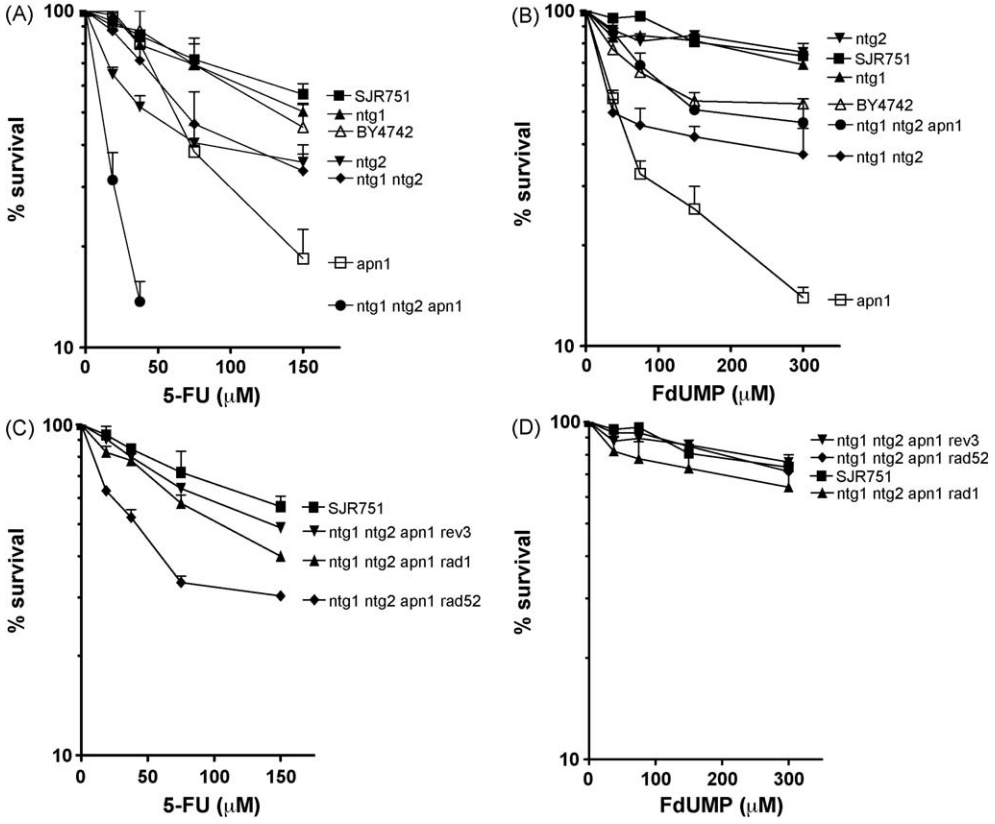

Fig. 3. Survival of *S. cerevisiae* strains single-, double-, triple- and quadruple mutants deficient in BER, HR, NER and TLS, after treatment with 5-FU and FdUMP. The survival of parental strains (BY4742 and SJR751) is compared with that of variants defective in the BER proteins Ntg1, Ntg2 and Apn1, the HR protein Rad52, the NER endonuclease Rad1 and the TLS protein Rev3.

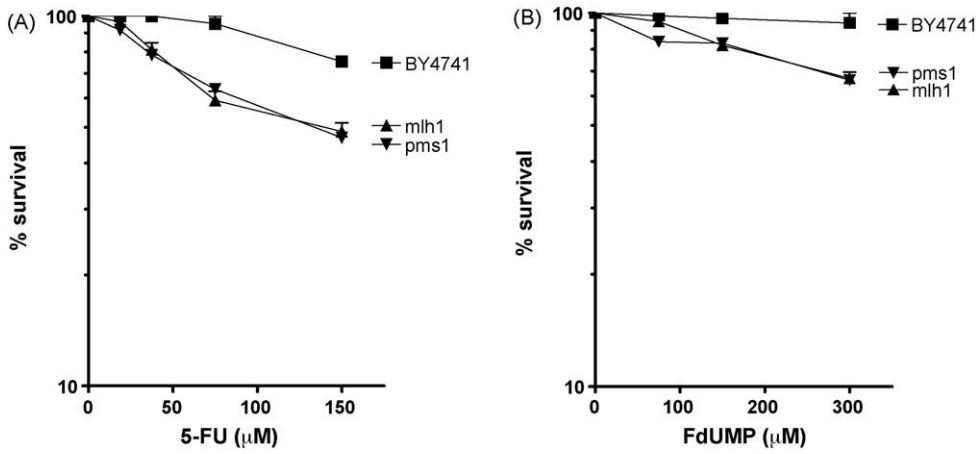

Fig. 4. Survival of *S. cerevisiae* strains deficient in MMR after treatment with 5-FU and FdUMP. The survival of the parental strain (BY4741) is compared to that of variants defective in the MMR proteins Mlh1 and Pms1.

apoptotic signaling [32]. Therefore, differential expression of important MMR-related proteins in yeast and human may explain the observed discrepancy in the sensitivity to 5-FU.

Next, the influence of proteins involved in NER, TLS and NHEJ was determined. Deletion of the NER endonucleases Rad1 and Rad10 had marginal influence on the sensitivity to 5-FU and FdUMP (Fig. 5A and B). The same result was observed for *rev3*Δ, which is deficient in the catalytic subunit of the TLS protein DNA polymerase zeta and for *rev1*Δ, which is deficient in the deoxycytidyl transferase, that forms a complex with DNA polymerase ζ [33] (Fig. 5C and D). In addition, deletion of *rad50*, that is deficient for a subunit of the MRX complex needed for NHEJ [34], had also little effect on the sensitivity to the two drugs (Fig. 6). These data suggest that deficiencies in the NER, NHEJ and TLS pathways have little influence on the sensitivity to 5-FU and FdUMP.

Interestingly, mutants deficient in *rad52*Δ, *rad6*Δ and *rad18*Δ showed increased sensitivity to 5-FU, but not to FdUMP (Fig. 6). Rad52 is the major protein involved in double-strand break repair by HR in *S. cerevisiae* [35–37] while Rad6 and Rad18 act in PRR.

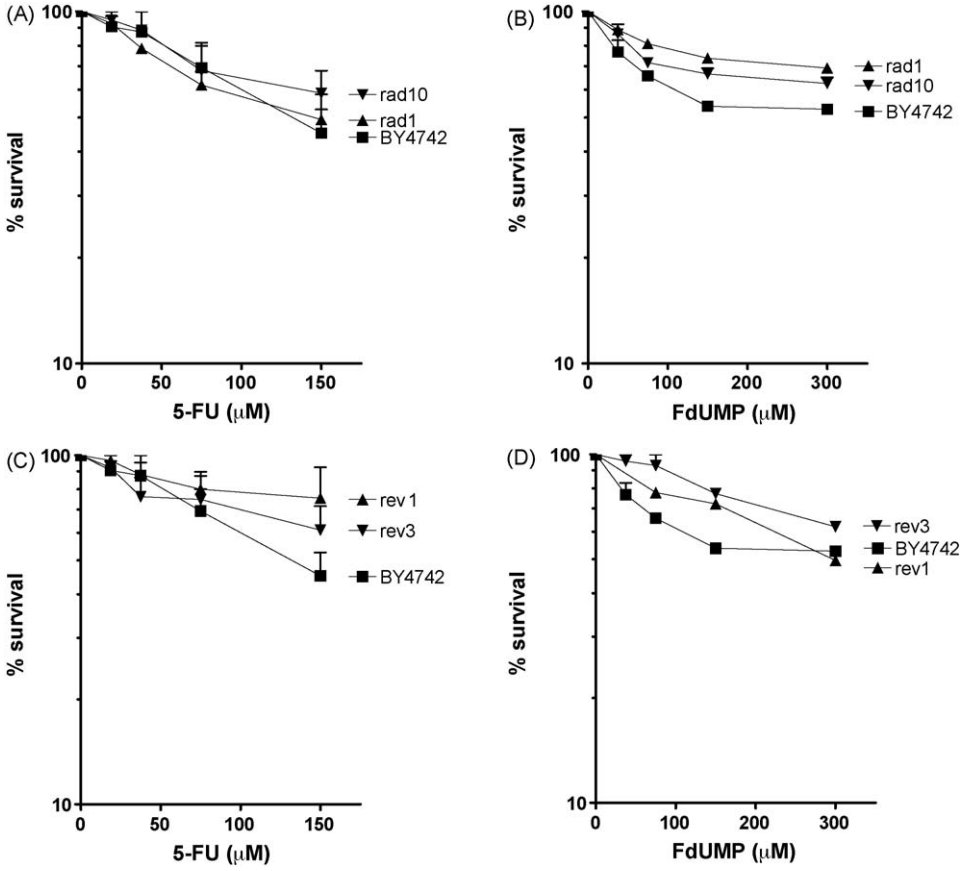

Fig. 5. Survival of *S. cerevisiae* strains deficient in NER (A and B), and TLS (C and D) after treatment with 5-FU and FdUMP. The survival of the parental strain (BY4742) is compared to that of variants defective for the NER endonucleases Rad1 and Rad10 and the TLS proteins Rev1 and Rev3.

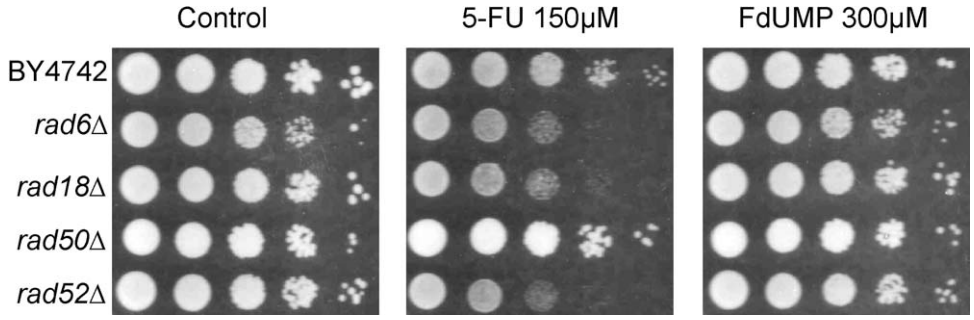

Fig. 6. Sensitivity of *S. cerevisiae* strains deficient in PRR and recombination repair to 5-FU and FdUMP Logarithmic cultures were serially diluted 10-fold, and were spotted onto YPD media plates with 5-FU (150 μM) or FdUMP (300 μM). The growth of the parental strain (BY4742) is compared to that of variants defective for the PPR proteins Rad6 and Rad18, the NHEJ protein Rad50, and the HR protein Rad 52.

Rad6 is an ubiquitin-conjugating enzyme needed for replication across DNA lesions [38], while Rad18 is maintaining the integrity of single-stranded DNA [39].

In conclusion, the BER pathway would initiate the repair of fluoronucleotide lesions, recognizing and removing the mismatched bases derived from 5-FU lesions mainly by the Apn1, Ntg1 and Ntg2, glycosylases and endonucleases. Although the Ung1 glycosylase and the Rad27 flap endonuclease likely participate in the processing of 5-FU lesions, their absence have no strong impact on the cellular survival. Failure of the BER process could result in the formation of DNA single (SSBs) and double (DSBs) strands breaks, which are recognized by the HR pathway (*RAD52*). Alternatively, MMR or PRR could be involved in processing the fluoronucleotide lesions that were not removed during replication (Fig. 7).

In contrast, for FdUMP, only BER and MMR play an important role (Fig. 7). DNA damage caused by FdUMP is processed mainly by Ntg1, Ntg2 and Apn1. Failure of this process could generate toxic single-strand breaks while mispairs, that were not repaired by BER, might be repaired by MMR (Mlh1 and Pms1). FdUMP cytotoxicity is principally attributed to TS inhibition, suggesting that the proportion of uracil misincorporation would be higher than for the FdUTPs. This is in agreement with recent results showing the formation of double-strand breaks after treatment with 5-FU, but not with FdUMP in human adenocarcinoma cells [20].

Taken together, the availability of a large panel of isogenic *S. cerevisiae* strains differing in defined repair proteins provides a powerful tool for identification of relevant repair processes. Since some repair pathways have additional functions in higher eukaryotes, major findings would need confirmation in mammalian models. A better understanding of the relevant repair processes is needed for personalized treatment with genotoxic anticancer agents and for the design of novel therapeutic reagents and strategies with better efficacy and/or less toxicity.

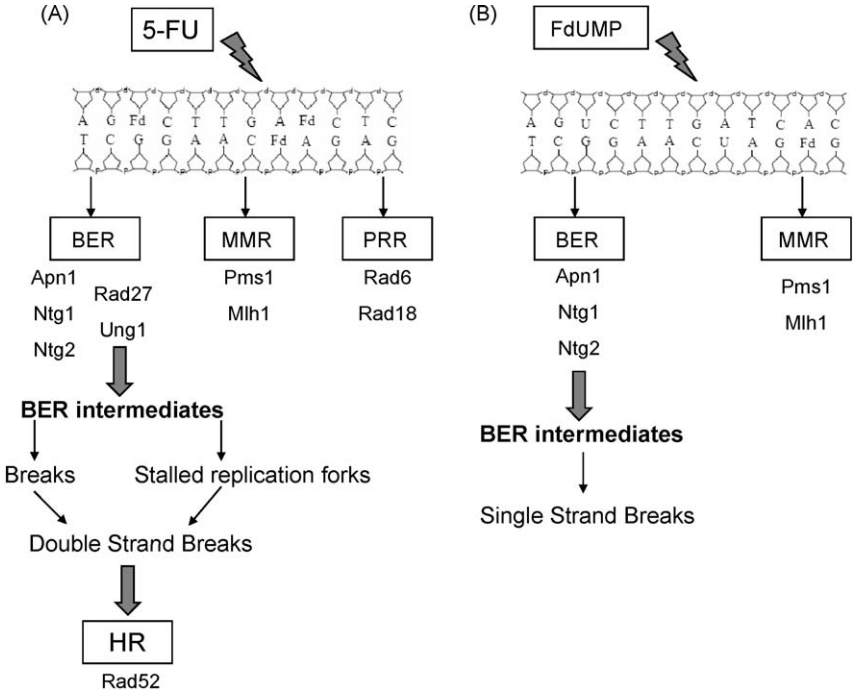

Fig. 7. Processing of 5-FU and FdUMP-induced-induced DNA lesions. (A) Fluoronucleotides misincorporated into DNA during S phase can be repaired by the BER, NER, HR or PRR. BER glycosylases and endonucleases would start repairing the damage and failure in this process could lead to formation of DNA single and double-strand breaks. Double-strands breaks are substrates for HR, that repairs the DNA with high fidelity. An other possibility is the involvement of MMR in the removal of fluoronucleotides from the DNA. The lesions that persisted in the DNA after replication can be processed by PPR. (B) Processing of FdUMP-induced DNA lesions. Misincorporation of uracil into DNA as a consequence of TS inhibition is mainly repaired by BER. If FdUMP has undergone further phosphorylation, FdUTP might be incorporated into DNA followed by subsequent removal by the MMR pathway. Fd = fluoronucleotides.

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