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Biochemical Pharmacology 70 (2005) 59–69
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Low cytotoxicity of ecteinascidin 743 in yeast lacking the major endonucleolytic enzymes of base and nucleotide excision repair pathways

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Received 22 November 2004; accepted 1 April 2005

Abstract

Ecteinascidin 743 (ET-743) is a promising antitumoral drug for the treatment of soft tissues sarcomas, becoming a good candidate for clinical trials. However, the molecular mechanism of how ET-743 induces cells death is poorly understood. The chemical structure of ET-743 suggests that it can form cytotoxic cross-links with proteins and DNA. Experiments with *Escherichia coli* and mammalian cells indicate that the nucleotide excision repair (NER) pathway promotes ET-743 cytotoxicity. We therefore analyzed cytotoxicity and tolerance to ET-743 in the yeast *Saccharomyces cerevisiae*, defective for NER and/or base excision repair (BER), either in single mutants or in combination with mutant alleles of genes encoding proteins involved in DNA translesion synthesis (TLS) and homologous recombination (HR). Treatment of haploid and diploid *S. cerevisiae* strains with ET-743 led to induced mutagenesis, mitotic gene conversion, and crossing-over. The results indicated that yeast strains lacking endonucleases of the NER and BER pathways are especially resistant for ET-743. The mutagenesis data points to a weak mutagenic activity of ET-743 in both WT and strains lacking BER/NER endonuclease, and that a mutant blocked in both BER and TLS totally lacks induced mutagenesis. The diploid strain shows an increase in the frequencies of crossing-over and mitotic recombination. These data lead us to propose a model for ET-743 action in eukaryotic cells, where the presence of BER and NER endonucleases results in cell death. However, ET-743 damage can be tolerated in BER and/or NER mutants by TLS (error-prone) or in combination with HR (error-free).

Keywords: ET-743; Base excision repair; Nucleotide excision repair; *Saccharomyces cerevisiae*; Apn1p; Rad1p

1. Introduction

The ecteinascidins (ETs) are extremely potent antitumor agents isolated from marine tunicade Caribbean sea *Ecteinascidia turbinate* [1,2], with a good activity in different in vitro and in vivo pre-clinical models [3–6]. Of the numerous ETs that have been isolated, ET-743 is the most promising compound, based on its cytotoxicity and its abundance in the tunicate [2]. ET-743 is an alkaloid composed of three fused tetrahydroisoquinoline rings and is structurally related to the DNA-reactive saframycins [7,8]. The main structural difference between ET-743 and the antibiotic saframycin, which does not posses antitumoral properties, is an extra ring (C subunit). While the A and B subunits of ET-743 provide the scaffold for DNA recognition and binding, the C subunit protrudes out of the minor groove, making only limited contacts with the DNA [9] (Fig. 1A–C).

The precise mechanism of action of ET-743 has yet to be fully understood, but DNA appears to be the primary target [10]. The compound forms a covalent adduct at the N2

Abbreviations: BER, base excision repair; ET-743, ecteinascidin 743; LOG, exponential phase; HR, homologous recombination; NER, nucleotide excision repair; SSBs, single-strand breaks; STSs, soft tissue sarcomas; SC, synthetic complete medium; TC-NER, transcription-coupled NER; TLS, translesion synthesis; WT, wild type

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doi:10.1016/j.bcp.2005.04.013

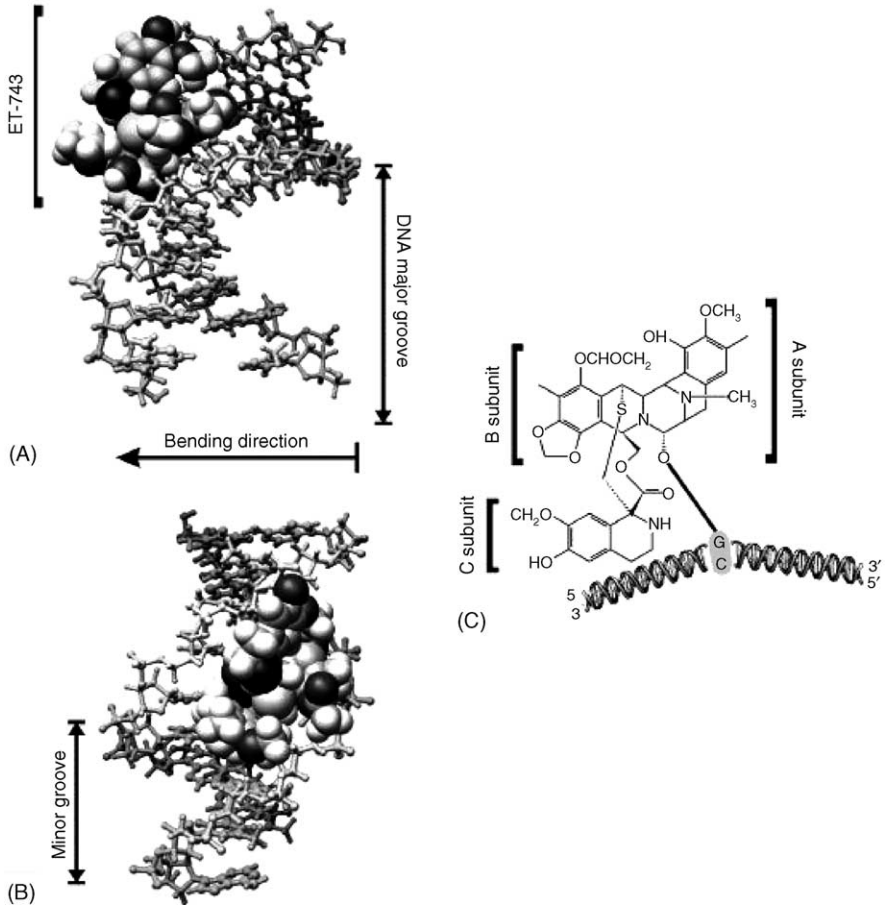

Fig. 1. Stereoisomer view (A and B) and chemical structure of ET-743 (C) alkylated to N2 position of guanine in the DNA minor groove. In (A) and (B) the alkylation produces a bend toward the DNA major groove. The A–C subunits are shown in (C).

position of guanine in the minor groove of duplex DNA and thus bends DNA toward the major groove [3,11–13] (Fig. 1A and B). The sequence specificity of the interaction between ET-743 and DNA occurs at guanines located either in the sequence 5′-PuGC-3′ or 5′-PyGG-3′ [3,14]. The first proposed target to explain the mechanism of action was topoisomerase I, which forms protein-DNA cross-links in the presence of ET-743 [15,16]. The cytotoxic activity of ET-743 was equally on WT and yeast strains deleted for *top1* indicating that DNA-topoisomerase I is not the primary target for this drug [17]. Recent reports have demonstrated a very unique response in mammalian NER-deficient cells. Tumor cells lacking the full complement of NER enzymes XPG and XPF-ERCC1, involved in TC-NER, were found to be significantly less sensitive to ET-743 as compared with NER-proficient cells lines. One possible mechanism to explain the TC-NER mediated cytotoxicity is the formation of DNA SSBs in NER-proficient cells and their relative absence in XPA- and XPD-deficient fibroblasts cells [18], indicating that ET-743 lethality is induced by TC-NER mediated SSBs in transcribed genes. However, not all ET-743-DNA adducts are equally incised by UvrABC proteins [14]. ET-743 adducts at the nonpreferred sequences were incised with the highest efficiency, whereas adducts at the preferred sequences (5′-AGC and 5′-TGC) were incised to a lesser extent. Apparently adducts that are not efficiently removed trap DNA-NER proteins, thus forming cytotoxic complexes. Since is not clear how this alkaloid influences DNA and its repair, we chose the unicellular eukaryotic organism *Saccharomyces cerevisiae* in order to better understand the molecular mechanism of action of this drug. The choice of this organism was based on the fact that biochemical pathways in yeast and mammalian cells are highly conserved. Moreover, yeast cells can be easily genetically manipulated and mutants for specific biochemical pathways can be generated rapidly [19]. In this contribution we will present the ET-743 sensitivity of single, double, triple, and quadruple mutants that are blocked in different mechanisms of DNA repair pathways. Induced mutagenesis was assayed in haploid *S. cerevisiae* strain XV185-14C, where two types of mutations (locus-specific, reversion of the *lys1-1* ochre or *his1-7* missense allele) and frameshift (*hom3-10*) can be detected. Possible recombinogenic effects of ET-743 were investigated in the diploid yeast strain XS2316, which allows the detection of two forms of mitotic recombination

(crossing-over and gene conversion). In addition, we have studied the induction of canavanine forward mutations in WT and different repair mutants after treatment with ET-743.

2. Materials and methods

2.1. Drug, yeast strains, and culture media

ET-743 (PharmaMar S.A.) aqueous stock solution (0.05 mg mL^{-1}) was stored at −20 °C. The relevant genotypes of *S. cerevisiae* strains used in this work are listed in Table 1. Complete liquid medium (YPD) containing 1% (w/v) yeast extract, 2% (w/v) bacto-peptone and 2% (w/v) glucose was used for routine growth. For plates, the medium was solidified with 2% (w/v) bacto-agar. The chemicals were from Merck & Co. and Sigma Co. The minimal medium (MM) contained 0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) glucose and 2% (w/v) bacto-agar. The synthetic complete medium (SC) was MM supplemented with 2 mg adenine, 2 mg arginine, 5 mg lysine, 1 mg histidine, 2 mg leucine, 2 mg methionine, 2 mg uracil, 2 mg tryptophan, and 24 mg threonine per 100 mL MM. For mutagenesis, the omission media, lacking lysine (SC-lys), histidine (SC-his), or homoserine (SC-hom) were sub-supplemented with 0.1 mg lysine, 0.1 mg histidine, or 0.1 mg methionine per 100 mL MM, respectively. In this case, the use of a MM containing the relevant nutritional requirements in a limiting concentration allows: (i) to restrict the cells growth at a titer below the plate's saturation level and (ii) to study the number of yeast revertant colonies from a determined amino acid auxotrophy to its respective prototrophy, which arose due to a mutagenic treatment [20]. The SC-lysine medium contained only 0.5 mg adenine. For canavanine resistance, medium SC-Arg was supplemented with 60 μg mL^{-1} canavanine. For recombinogenesis, leucine was omitted from the synthetic medium (SC-leu), or supplemented with 0.2% (w/v) cycloheximide (SC + cyh). A saline solution [0.9% (w/v) NaCl] was employed for dilution of cell suspensions. Phosphate-buffered saline (PBS; Na_2HPO_4 and NaH_2PO_4; 20 mM; pH 5.8) was used for cell's incubation with ET-743.

2.2. Yeast growth conditions

Exponential phase (LOG) cultures were obtained by inoculation of 5×10^5 cells mL^{-1} of YPD culture in stationary phase (STAT) into 5 mL of fresh YPD medium. After 14 h incubation, at 28 °C, with aeration by shaking, the cultures contained (1–2) $\times 10^7$ cells mL^{-1} with 20–30% budding cells. Cells were harvested and washed twice with saline solution. The number of cells with and without buds was determined by counting in Neubauer chamber.

2.3. Survival assays

To determine sensitivity to ET-743, cell suspensions were prepared containing 2×10^6 LOG cells mL^{-1} with and without ET-743, and incubated in PBS at 28 °C for 5 h with agitation. After incubation, samples were diluted in saline solution, plated onto YEPD agar, and survival was assayed after 48 h at 28 °C. Assays were repeated at least three times and plating was in triplicate for each dose.

Table 1
Saccharomyces cerevisiae strains used in this study

Strains	Relevant genotypes	Reference
BY4742 (WT)	*MAT*α; *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*	[50]
rad1Δ	BY4742; with *rad1::kanMX4*	[50]
rad2Δ	BY4742; with *rad2::kanMX4*	[50]
rad10Δ	BY4742; with *rad10::kanMX4*	[50]
rad14Δ	BY4742; with *rad14::kanMX4*	[50]
rad4Δ	BY4742; with *rad4::kanMX4*	[50]
rad52Δ	BY4742; with *rad52::kanMX4*	[50]
rev3Δ	BY4742; with *rev3::kanMX4*	[50]
ogg1Δ	BY4742; with *ogg1::kanMX4*	[50]
mag1Δ	BY4742; with *mag1::kanMX4*	[50]
apn1Δ	BY4742; with *apn1::kanMX4*	[50]
apn2Δ	BY4742; with *apn2::kanMX4*	[50]
SJR751 (WT)	*MAT*α; *ade2-101$_{oc}$*; *his3Δ200*; *ura3ΔNco*; *lys2ΔBgl*; *leu2-R*	[24]
ntg1Δ	SJR0751; with *ntg1::LEU2*	[44]
ntg2Δ	SJR0751; with *ntg2::hisG*	[24]
ntg1Δntg2Δ	SJR0751; with *ntg1::LEU2 ntg2::hisG*	[24]
ntg1Δntg2Δapn1Δ	SJR0751; with *ntg1::LEU2 ntg2::hisG apn1Δ1::HIS3*	[24]
ntg1Δntg2Δapn1Δrad52Δ	SJR0751; with *ntg1::LEU2 ntg2::hisG apn1Δ1::HIS3 rad52::URA3*	[24]
ntg1Δntg2Δapn1Δrad1Δ	SJR0751; with *ntg1::LEU2 ntg2::hisG rad1::hisG apn1Δ1::HIS3*	[51]
ntg1Δntg2Δapn1Δrev3Δ	SJR0751; with *ntg1::LEU2 ntg2::hisG rad1::hisG apn1Δ1::HIS3 rev3::kanMX4*	[51]
XV185-14C	*MAT*α; *ade2-2*; *arg4-17*; *his1-7*; *lys1-1*; *trp5-48*; *hom3-10*	[20]
XS2316	*MAT*a/α; *+/ade6*; *leu1-12/leu1-12*; *trp5-48/+*; *+/cyh2*; *+/met13*; *+/lys5-1*; *his1-1/his1-1*	[52]

2.4. Measurements of ET-743 induced canavanine resistance

Forward mutation to canavanine resistance was determined in strains proficient and deficient in DNA repair pathways [SJR751 (WT), *ntg1*Δ*ntg2*Δ, *ntg1*Δ*ntg2*Δ*apn1*Δ, *ntg1*Δ*ntg2*Δ*apn1*Δ*rad1*Δ, *ntg1*Δ*ntg2*Δ*apn1*Δ*rev3*Δ and *ntg1*Δ*ntg2*Δ*apn1*Δ*rad52*Δ]. A suspension of 2×10^8 LOG cells mL^{-1} was incubated for 6 h at 28 °C with different concentrations of ET-743. Survival was determined on SC and mutation induction on SC-Arg supplemented with 60 μg mL^{-1} canavanine. Assays were repeated at least three times and plating was in triplicate for each dose.

2.5. Detection of ET-743-induced reverse and frameshift mutation

The haploid strain XV185-14C (Table 1) was used for assaying mutagenicity. A suspension of 2×10^8 LOG cells mL^{-1} was incubated for 6 h at 28 °C with different concentrations of ET-743. The survival was determined on SC and mutation induction (*LYS*$^+$, *HIS*$^+$ or *HOM*$^+$ revertants) on appropriate omission media. Whereas *his1-7* is a non-suppressible missense allele and reversions result from mutations at the locus itself [21], *lys1-1* is a suppressible ochre nonsense mutant allele [22], which can be reverted either by locus-specific or by a forward mutation in a suppressor gene [20,22]. Distinction between true reversions and forward (suppressor) mutations at the *lys1-1* locus was according to Schuller and Von Borstel [23], where the reduced adenine content of the medium SC-lys shows locus reversions as red and suppressor mutations as white colonies. It is believed that *hom3-10* contains a frameshift mutation due to its response to a range of diagnostic mutagens [20]. Assays were repeated at least three times and plating was in triplicate for each dose.

2.6. Detection of induced mitotic recombination

A cell suspension (2×10^6 LOG cells mL^{-1}) was incubated in PBS for 6 h at 28 °C with different concentrations of ET-743. After treatment, the cells were diluted in saline, plated on SC, SC-leu, and SC + cyh, followed by incubation at 28 °C. Colonies grown on SC medium yielded data of cell survival and colonies grown on SC-leu and SC + cyh were scored for intragenic recombination (gene conversion) and intergenic recombination (crossing-over), respectively. Assays were repeated at least three times and plates were done in triplicate for each dose.

3. Results

3.1. Sensitivity of DNA repair-deficient yeast strains for ET-743

The *S. cerevisiae* BY4742 repair-proficient (WT) strain, was sensitive for ET-743 in a dose-dependent manner (Fig. 2A and B). On the other hand, as previously observed for *E. coli* and mammalian cells [14,18], the yeast *rad1*Δ and *rad2*Δ strains, which lack the 5′- and 3′endonucleases of NER, showed an elevated resistance as compared to the isogenic WT strain (BY4742) with 60% of survival at the highest ET-743 dose against ~1.5% of survival of the WT (Fig. 2A). The *rad10*Δ (deficient in the 5′-endonuclease NER complex) and *rev3*Δ (which have a partially functional TLS pathway) mutants displayed both an intermediate sensitivity to high doses of ET-743 (20% and 22%, respectively; Fig. 2A). Mutants *rad4*Δ and *rad14*Δ had the same ET-743 sensitivity as the WT (Fig. 2A). Interestingly, the single mutant *rad52*Δ (HR pathway) also showed an elevated resistance (67%) as compared to the isogenic WT (Fig. 2A).

The single BER mutants had a remarkable resistance to ET-743 when compared to the WT (Fig. 2B). The BER

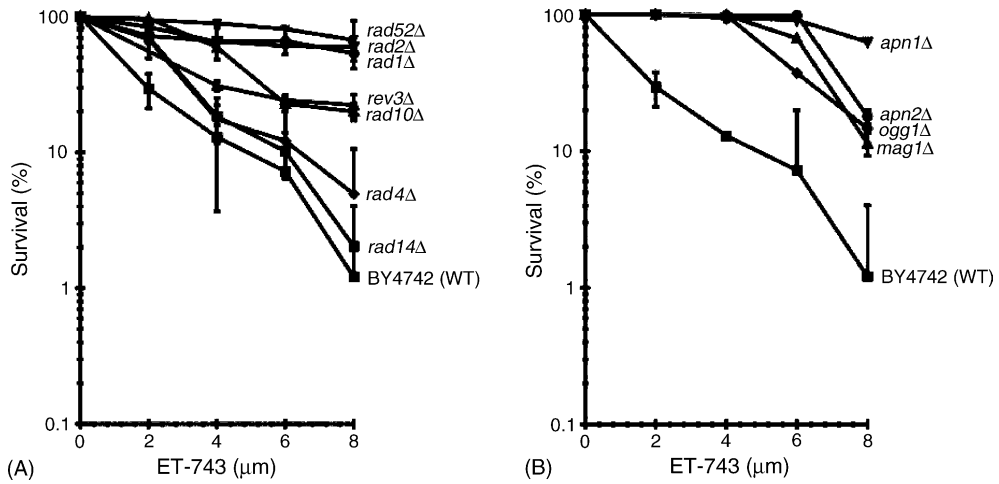

Fig. 2. Survival of *Saccharomyces cerevisiae* strains deficient in (A) NER, HR and TLS, and (B) BER after treatment with different doses of ET-743 (0–8 μM). Error bars represent the standard deviations for three separately treated cultures.

endonuclease-deficient *apn1*Δ strain was the most resistant at all ET-743 doses tested, while the single mutants for DNA N-glycosylases/AP lyases (*ogg1*Δ and *mag1*Δ) and for BER endonuclease *apn2*Δ showed an intermediate sensitivity at the highest ET-743 dose (Fig. 2B).

To examine the overlapping functions of different DNA repair pathways, we tested yeast mutants lacking DNA repair proteins from NER, BER, recombination and/or TLS pathways. The ET-743 sensitivity of single (*ntg1*Δ and *ntg2*Δ), double (*ntg1*Δ*ntg2*Δ), triple (*ntg1*Δ*ntg2*Δ*apn1*Δ) and quadruple (*ntg1*Δ*ntg2*Δ*apn1*Δ*rad1*Δ, *ntg1*Δ*ntg2*Δ*apn1*Δ*rev3*Δ, and *ntg1*Δ*ntg2*Δ*apn1*Δ*rad52*Δ) mutants was tested using strains isogenic to the WT (SJR751). Although the SJR751 WT was more resistant to ET-743 than the BY4742 WT (22% against 1.5% of survival at 8 μM of ET-743, Fig. 4), the resistance pattern of all BER mutants was similar, as all had an elevated resistance to ET-743 (Fig. 3A and B).

Both single- (*ntg1*Δ and *ntg2*Δ) and double (*ntg1*Δ*ntg2*Δ) mutants displayed a similar WT-like sensitivity at highest doses of ET-743 (Fig. 3A), while the triple mutant *ntg1*Δ*ntg2*Δ*apn1*Δ had the same resistance of *apn1*Δ at 6 μM ET-743 (Fig. 4). At 8 μM of ET-743, both single- and triple mutants survived at 71% and 91%, respectively (Figs. 3A and 4). Interestingly, the quadruple mutants were more resistant than the SJR751 WT. Mutant strains *ntg1*Δ*ntg2*Δ*apn1*Δ*rad52*Δ and *ntg1*Δ*ntg2*Δ*apn1*Δ*rev3*Δ showed an intermediate resistance, while the strain *ntg1*Δ*ntg2*Δ*apn1*Δ*rad1*Δ was significantly more ET-743 resistant (Fig. 3B).

3.2. Detection of ET-743 canavanine forward mutation

We investigated how ET-743 affected the induction of canavanine (CAN) forward mutations in the SJR751 (WT), double-, triple-, and quadruple mutants in comparison to the standard mutagen UVC (Table 2). With exception of the quadruple mutant *ntg1*Δ*ntg2*Δ*apn1*Δ*rad1*Δ, all strains tested showed a similar WT-like survival. As expected, the *ntg1*Δ*ntg2*Δ*apn1*Δ*rad1*Δ strain has a strong UVC-induction of canavanine forward mutation. In this case, the elimination of two error-free DNA repair pathways (BER and NER) of the quadruple mutant may lead to an increase in either mutation or recombination rate after exposure to a mutagenic agent [24].

The SJR751 WT and *ntg1*Δ*ntg2*Δ strains showed similar sensitivity to 8 μM of ET-743, with both strains having a two- to five-fold elevated forward mutation induced by ET-743 (Table 2). The *ntg1*Δ*ntg2*Δ*apn1*Δ and *ntg1*Δ*ntg2*Δ*apn1*Δ*rad1*Δ mutants, which are resistant to ET-743 treatment (Table 2), also showed a significantly

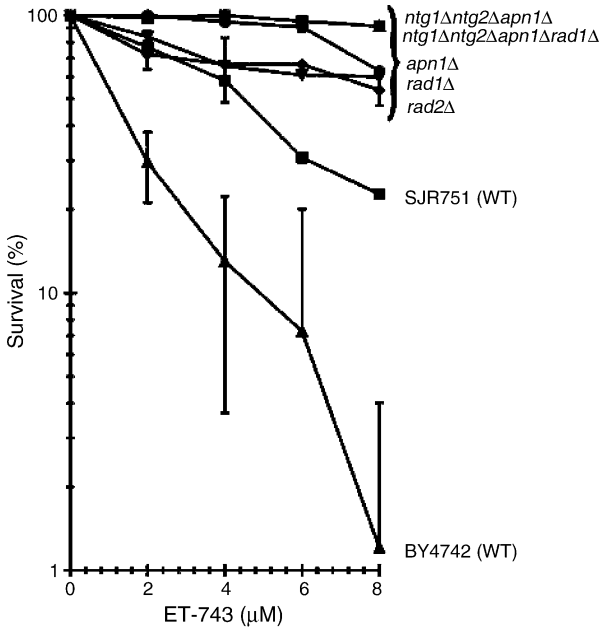

Fig. 4. Survival of *S. cerevisiae* single-, double-, and triple mutants of BER and NER pathways after treatment with different doses of ET-743 (0–8 μM). Error bars represent the standard deviations for three separately treated cultures.

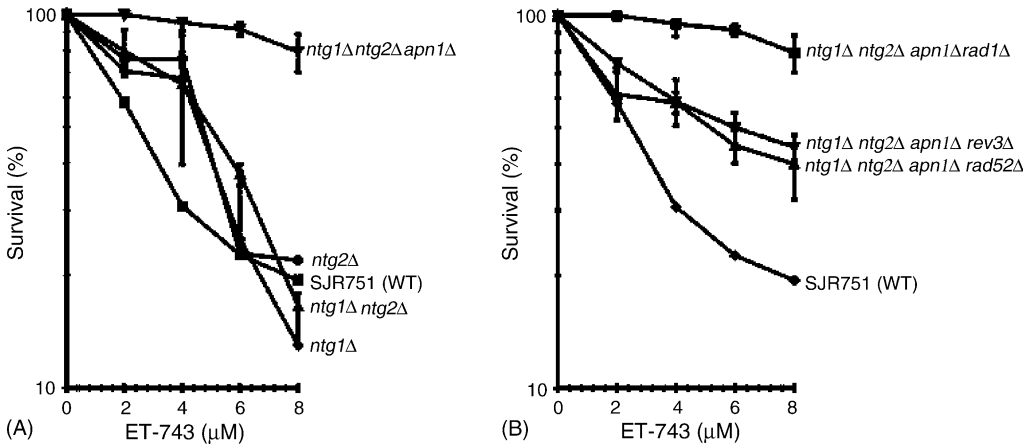

Fig. 3. Survival of *S. cerevisiae* (A) single-, double-, and triple-mutant BER strains and (B) quadruple mutants combining deficiencies in NER, BER, TLS, and HR after treatment with different doses of ET-743 (0–8 μM). Error bars represent the standard deviations for three separately treated cultures.

Table 2
Induction of canavanine resistance (*can1*^{r}) in WT and repair mutants of *S. cerevisiae* after ET-743 treatment

UVC (J/m^2)[a]	Survival (%)	Can$^+$/10^7 survivors	ET-743 (μM)	Survival (%)	Can$^+$/10^7 survivors
SJR751 (WT)					
0	100.00 (704)[b]	2.67 ± 0.70[c] (281)[b]	0	100.00 (704)[b]	2.67 ± 0.70[c] (281)[b]
37	67.47 (475)	18.79 ± 2.60[**] (891)	2	58.24 (410)	2.89 ± 0.8 (200)
56	39.20 (276)	43.43 ± 4.67[**] (1199)	4	30.68 (216)	2.71 ± 1.49 (88)
			8	19.46 (137)	4.85 ± 1.83[*] (99)
*ntg1*Δ*ntg2*Δ					
0	100.00 (459)	4.72 ± 0.38 (217)	0	100.00 (459)	4.72 ± 0.38 (217)
19	76.68 (352)	9.54 ± 2.03[*] (335)	2	100.00 (517)	4.92 ± 0.63 (254)
37	60.13 (276)	16.34 ± 2.73[*] (451)	4	73.85 (339)	5.28 ± 1.50 (179)
56	38.34 (176)	32.02 ± 2.73[**] (563)	8	18.74 (86)	24.59 ± 2.97[**] (211)
*ntg1*Δ*ntg2*Δ*apn1*Δ					
0	100.00 (292)	50.56 ± 8.79 (443)	0	100.00 (536)	50.56 ± 8.79[b] (443)
37	73.62 (215)	186.52 ± 39.51[**] (1202)	2	100.00 (562)	52.51 ± 7.26 (1328)
56	53.43 (156)	259.39 ± 16.53[**] (1214)	4	100.00 (651)	48.34 ± 10.35 (1414)
			8	92.16 (494)	111.29 ± 9.05[**] (2474)
*ntg1*Δ*ntg2*Δ*apn1*Δ*rad1*Δ					
0	100.00 (574)	46.94 ± 5.76 (269)	0	100.00 (505)	56.14 ± 16.89 (283)
6	17.59 (101)	163.69 ± 49.17[*] (165)	2	100.00 (522)	19.73 ± 3.36 (103)
19	4.00 (23)	1,231.85 ± 7.19[*] (284)	4	100.00 (532)	54.80 ± 12.07 (291)
			8	91.08(460)	119.38 ± 10.98[*] (548)
*ntg1*Δ*ntg2*Δ*apn1*Δ*rev3*Δ					
0	100.00 (872)	7.89 ± 2.13 (206)	0	100.00 (872)	7.89 ± 2.13 (206)
19	78.55 (685)	25.00 ± 8.84[*] (513)	2	95.06 (829)	7.60 ± 2.61 (189)
37	66.05 (576)	55.34 ± 6.65[**] (956)	4	58.94 (514)	8.05 ± 1.30 (124)
			8	37.38 (326)	8.13 ± 1.98 (79)
*ntg1*Δ*ntg2*Δ*apn1*Δ*rad52*Δ					
0	100.00 (339)	140.40 ± 8.90 (476)	0	100.00 (339)	140.40 ± 8.90 (476)
19	100.00(354)	230.22 ± 9.44[**] (815)	2	100.00 (362)	75.00 ± 11.21 (270)
37	67.55 (229)	440.35 ± 66.88[*] (1004)	4	75.22 (255)	179.60 ± 38.38 (458)
			8	68.73 (233)	235.38 ± 15.74[**] (548)

[a] Positive control.
[b] Numbers in parenthesis are the actual numbers of colonies scored in three plates for each dose.
[c] Standard deviation.
[*] Significance level obtained in relation to the negative control with $P \leq 0.05$ measured by Student's *t*-test.
[**] Significance level obtained in relation to the negative control with $P \leq 0.01$ measured by Student's *t*-test.

increased (>two-fold) mutation induction at all ET-743 doses tested. The quadruple mutants, containing the *rad52*Δ and *rev3*Δ mutations, responded differently in the CAN assay after treatment with ET-743, despite their comparable survival at all doses used (Table 2). The ET-743 treatment in the *ntg1*Δ*ntg2*Δ*apn1*Δ*rad52*Δ mutant was mutagenic while it was not in the *ntg1*Δ*ntg2*Δ*apn1*Δ*rev3*Δ mutant.

Table 3
Induction of point mutation (*his1-7*) ochre allele (*lys1-1*) and frameshift (*hom3-10*) mutations in haploid XV185-14C strain of *S. cerevisiae* after ET-743 treatment

Agent	Dose (J/m^2)	Concentration (μM)	Survival (%)	Lys1/10^7 survivors[a]	His1/10^7 survivors[b]	Hom3/10^7 survivors[b]
UVC[c]	0		100.0 (915)[d]	0.11 ± 0.00[e] (3)[d]	2.29 ± 0.44[e] (63)[d]	1.34 ± 0.27[e] (37)[d]
	37		38.79 (355)	8.33 ± 2.61[*] (89)	12.11 ± 6.66[*] (129)	6.56 ± 2.07[*] (70)
ET-743		0	100.0 (600)	0.16 ± 0.00 (3)	2.83 ± 1.67 (51)	0.80 ± 0.00 (15)
		1	73.33 (440)	0.45 ± 0.00[**] (6)	4.35 ± 1.49[**] (57)	0.76 ± 0.26 (10)
		2	44.33 (266)	0.59 ± 0.44[**] (5)	10.50 ± 1.98[*] (84)	3.00 ± 1.29 (24)
		4	32.33 (194)	1.33 ± 0.51[**](9)	10.80 ± 4.72[*] (63)	5.46 ± 2.88[*] (33)

[a] Locus non-specific revertants (forward mutations).
[b] Locus-specific revertants.
[c] Positive control.
[d] Numbers in parenthesis are the actual numbers of colonies scored in three plates for each dose.
[e] Standard deviation.
[*] Significance level obtained in relation to the negative control with $P \leq 0.05$ measured by Student's *t*-test.
[**] Significance level obtained in relation to the negative control with $P \leq 0.01$ measured by Student's *t*-test.

Table 4
ET-743-induced crossing-over (+/*cyh*) and gene conversion (*leu1-1*/*leu1-12*) in diploid strain *S. cerevisiae* XS2316 after ET-743 treatment

Agent	Dose (J/m^2)	Concentration(μM)	Survival (%)	Crossing-over/10^5 survivors	Gene conversion/10^5 survivors
UVC[a]	0		100.00 (329)[b]	14.70 ± 2.06[c] (481)[b]	1.95 ± 0.45[c] (64)[b]
	37		83.39 (276)	18.73 ± 1.19[*] (517)	11.74 ± 1.36[**] (324)
	56		27.05 (86)	60.35 ± 4.32[*] (507)	46.89 ± 8.55[**] (394)
ET-743		0	100.00 (295)	16.36 ± 2.293 (481)	2.17 ± 0.50 (64)
		1	100.00 (366)	10.35 ± 2.94[*] (379)	7.13 ± 0.53[**] (261)
		2	21.69 (64)	85.44 ± 10.03[*] (546)	51.48 ± 5.67[**] (329)
		4	11.80 (35)	112.34 ± 22.58[*] (728)	64.90 ± 4.71[**] (331)

[a] Positive control.
[b] Numbers in parenthesis are the actual numbers of colonies scored in three plates for each dose.
[c] Standard deviation.
[*] Significance level obtained in relation to the negative control with $P \leq 0.05$ measured by Student's *t*-test.
[**] Significance level obtained in relation to the negative control with $P \leq 0.01$ measured by Student's *t*-test.

3.3. Detection of ET-743-induced reverse mutation

The capacity of ET-743 to induce point or frameshift mutations was analyzed in *S. cerevisiae* strain XV185-14C (Table 3). Interestingly, ET-743 increased the frequencies of both point (*HIS1*$^+$, *LYS1*$^+$) and frameshift (*HOM3*$^+$) mutations in LOG cells of XV185-14C. The frequency of point mutation for the *his1* locus increased 4 times compared to the control at 4 μM of ET-743. Frameshift mutations, as scored for the *hom3-10* locus, increased seven times after drug treatment. For *lys1-1* only suppressors (forward mutations) could be scored and induction was about eight-fold over the spontaneous level.

3.4. Detection of induced mitotic recombination

The recombinogenic effect of ET-743 was investigated in LOG cells of diploid XS2316 under non-growth (PBS) conditions (Table 4). ET-743 induced statistically significant (Student's *t*-test) recombinogenic events. The frequency of crossing-over (+/*cyh*) and mitotic gene conversion (*leu1-1*/*leu1-12*) increased 29 and 13 times after ET-743 treatment, respectively.

4. Discussion

ET-743 has an unique mechanism of action when compared to the classical antitumoral drugs currently used in therapy. [18,25]. The cytotoxic activities of ET-743 have been largely investigated in all tumor models, especially in adult soft tissue sarcomas (STSs), where it shows a remarkable efficiency. STSs belong to a rare, heterogeneous family of malignancies that can arise from mesenchymal lineages anywhere in the body. Currently, despite adequate control of the primary tumor, more than half of all patients die within 5 years of the primary diagnosis, as a result of widespread metastatic disease [5]. Inoperable or metastatic sarcomas of osseous origin, such as osteogenic and Ewing's sarcomas, are generally fatal, typically showing low responsiveness to chemo- or radio-therapy [5,26–28]. The resistance of STSs to chemotherapy agents is due to the overexpression of several genes related to cell cycle control, cell maintenance, and DNA repair, like *XRCC1* [29–32]. The action of ET-743 on mammalian cells has been studied by several authors in more or less detail. One proposed mechanism of action involved ET-743 interaction with the transcription-coupled (TC) NER machinery [18]. TC-NER acts on the removal of lesions that distort the DNA double helix, interfere in base pairing, and block DNA duplication and transcription. Incision of damaged DNA in both yeast and humans utilizes the function of two endonucleases—Rad1p-Rad10p and Rad2p in yeast, and XPF-ERCC1 and XPG in humans. Rad1p and Rad10p form a tight complex [33,34], and genetic and biochemical studies with a *rad1* mutant allele, whose encoded protein failed to interact with Rad10p, provided strong evidence that complex formation was essential for the biological function of these proteins [33]. The Rad1p-Rad10p complex exhibits single-strand DNA endonuclease activity [35,36] that acts in a structure-specific manner and cleaves 5′-ended single stranded DNA at its junction with the duplex DNA. Yeast Rad2p and its human counterpart XPG also show a single-strand DNA endonuclease activity that cleaves the 3′-end at its junction with the duplex DNA [37]. These activities of Rad1p-Rad10p (XPF-ERCC1), and Rad2p (XPG) nucleases would also correctly incise ET-743 treated DNA on the 5′ and 3′ side of the damage, respectively [38]. Besides providing the endonucleolytic activities for dual incision, Rad1p-Rad10p and Rad2p are also involved in the proper assembly of the NER machinery at the damage site [39]. Our results indicate that the *rad1*Δ, *rad2*Δ and *rad10*Δ yeast mutants show increased resistance in comparison to the BY4742 (WT), *rad4*Δ, and *rad14*Δ strains after ET-743 treatment (Fig. 2A). Interestingly, it has been described that the presence of ET-743 adducts in transcribed genes blocks the TC-NER system by stalling the cleavage intermediates and producing lethal SSBs [18]. However, cells possess DNA repair mechanisms other than NER acting on lesions induced by physicochemical agents. Base excision repair (BER) is one mechanism that, together with NER, removes modified

bases from DNA [40]. BER primarily involves the repair of small, helix non-distorting base lesions and abasic sites [41] and oxidative DNA damage is believed to be primarily repaired by this pathway [42]. There is considerable overlap between the BER and NER with respect to damage processing. For example, similar to BER, NER has been shown to be capable of processing oxidative lesions such as thymine glycol and 8-oxoguanine [40,43]. Also both BER and NER repair abasic sites [24] interact synergistically in repair of endogenous and exogenous oxidative DNA damage [44]. Our data indicate that BER enzymes, together with Rad1p-Rad10p and Rad2p, also recognize the DNA damage induced by ET-743. The ET-743 resistance of single mutant *apn1*Δ when compared to the WT cell demonstrates that this BER endonuclease probably participates in the cytotoxic potential of the drug (Fig. 2B). Apn1p is an enzyme endowed with multiple enzymatic activities that protect nuclear and mitochondrial DNA from the deleterious action of endogenous oxidative and alkylation DNA damage [40]. According to the classic model of BER in yeast, as in the most organisms, a damaged base is removed by a specific *N*-glycosylase and the resulting apurinic/apyrimidinic (AP) site is cleaved by an AP endonuclease [40]. Apn1p cleaves DNA at 5′-side of a regular or reduced AP site [45,46], while Apn2p is a back-up enzyme that accounts for only 10% of cellular AP endonuclease activity [47]. On the other hand, the strains defective for Apn2p, Ogg1p and Mag1p also show an elevated resistance when compared to the BY4742 (WT) strain for ET-743 doses of 2 and 4 μM (Fig. 2B), while the single and double *ntg1*Δ and *ntg2*Δ strains have similar SJR751 (WT)-sensitivity to the same ET-743 treatment (Fig. 3A). Ntg1p, Ntg2p, and Ogg1p are three DNA N-glycosylases endowed with an AP lyase activity [40]. Ntg1p and Ogg1p are both nuclear and mitochondrial proteins, whereas Ntg2p is exclusively nuclear [42]. Ntg1p and Ntg2p excise a variety of oxidized pyrimidines and formamido pyrimidines (FapyA and FapyG) [40], whereas Ogg1p excises 8-oxoG and FapyG [40]. Mag1p initiates BER of DNA alkylation damage by removing 3-MeA and other alkylated purines [40]. We think that Ntg1p and Ntg2p do not recognize the substrate generated by the intercalation of ET-743 into DNA. However, the partial resistance of Ogg1p and Mag1p indicates that these two DNA glycosylases can process ET-743 induced DNA lesions.

The resistance of triple mutant (*ntg1*Δ*ntg2*Δ*apn1*Δ, Fig. 3A) to ET-743, which is a consequence of the Apn1p absence, does not occur in the double mutant *ntg1*Δ*ntg2*Δ (Fig. 3A). On the other hand, the quadruple mutants for BER/NER, BER/TLS and BER/REC show different sensitivities for ET-743 (Fig. 3B), with the strain deficient in both BER and NER having the highest resistance of all strains tested to ET-743. This could indicate that both DNA repair pathways are recruited for repair of ET-743-induced DNA lesions. It should be noted that in all mutants deficient for the major endonucleases of BER and NER an epistatic interaction could be observed after ET-743 treatment (Fig. 4).

The increase of the frequency of forward mutation induced by ET-743 in the quadruple mutant blocked in both BER and NER (*ntg1*Δ*ntg2*Δ*apn1*Δ*rad1*Δ) indicates that an error-prone pathway repairs (some of) the DNA lesions. This was confirmed when the quadruple mutant for BER and TLS, after ET-743 treatment, did not show induction of forward mutations. The *REV3* gene encodes the enzymatically active protein of DNA polymerase ζ (Pol zeta), a translesion enzyme that can bypass thymine dimers during replication in an error-prone manner by introducing any nucleotide in the sister strand [48]. Thus, the results of mutagenesis, also supported by the intermediate sensitivity of the single mutant *rev3*Δ (Fig. 2A) and the quadruple mutant for BER and TLS (Fig. 3B), show that other translesion enzymes, like Rad30p [DNA polymerase η (Pol eta)] contribute to the cell's ET-743 tolerance. The increase in the frequency of crossing-over show that the HR pathway (*RAD52* epistasis group) is necessary to keep the cell's viability after ET-743 treatment in the absence of a functional NER or BER pathways. But it should be noted that the single mutant *rad52*Δ showed a high degree of survival after treatment with different doses of ET-743 (Fig. 2A), corroborating the idea that an error-prone pathway, e.g. TLS, could be responsible for ET-743 tolerance. Moreover, the intermediate sensitivity of the quadruple mutant for BER and HR (*ntg1*Δ*ntg2*Δ*apn1*Δ*rad52*Δ, Fig. 3B), as well as the increase in the frequency of forward mutation suggest an interaction of different DNA repair pathways related to tolerance of ET-743 damage (Table 2).

Our results and those in the literature allow us to propose a most probable model of ET-743 action, which also explains its weakly mutagenic activity (Fig. 5). In *S. cerevisiae* WT cells the NER/BER repair systems recognize the DNA damage after treatment with ET-743, inducing the recruitment of specific endonucleases (e.g. Apn1p, Rad1p-Rad10p, and Rad2p). These complexes are trapped by the C subunit of ET-743, and thus form a covalently attached complex with DNA, stalling the DNA polymerase. The complexes are very cytotoxic and can induce cell death [16,18]. However, when cells lack the endonucleolytic components of BER and NER repair processing of the ET-743 damage is channeled to TLS (Fig. 5). In this case ET-743 induced damage is tolerated by TLS repair that generates insertion of any base or an abasic site directly opposite to the ET-743-induced DNA lesion. This damage could be then direct for two pathways, depending on the presence of proteins of homologous recombination (HR) system and/or TLS components: (1) HR associated to TLS (error-free) or (2) a second TLS, leading to mutant and WT phenotypes (error-prone). It has been described recently that the Mph1p acts in a post-replicative repair pathway in an error-free fashion, characterizing a branch of homologous recombination associated with TLS [49]. In the absence of Rev3p, the ET-743 damage could be tolerated

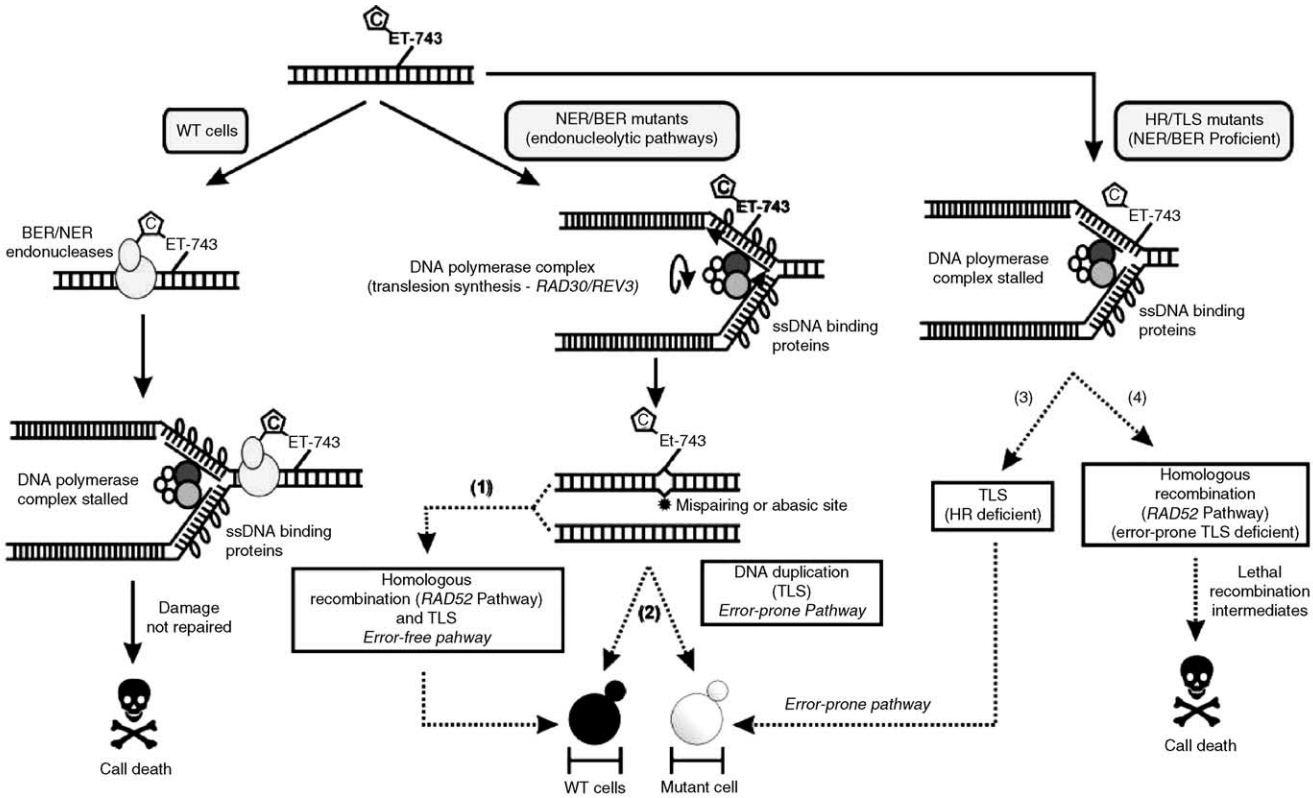

Fig. 5. Model proposed for ET-743 cytotoxicity in WT and mutant cells for DNA repair pathways. In WT, which has all NER and BER functional DNA endonucleases, the ET-743 binds covalently to the endonuclease proteins by means of tetrahydroisoquinoline ring (C subunit), while the A- and B-subunits of ET-743 provide the scaffold for DNA recognition and bonding. This complex represents a permanently bound structure that stalls the DNA polymerase complex, thus inducing cell cycle arrest or even cell death. On the other hand, in the BER and NER endonuclease-lacking mutant strains ET-743 damage can be tolerated by DNA polymerase ζ (Rev3p) and η (Rad30p)-mediated translesion synthesis. In this case, mispairing or abasic sites originating from DNA replication opposite of the ET-743 adduct can be channeled to (1) homologous recombination associated to translesion synthesis (error-free pathway) in the absence of Rev3p or (2) DNA translesion synthesis (error-prone pathway), when Rad52p is not present. Otherwise, in the yeast strains defectives for HR or TLS but proficient for NER/BER pathways, an ET-743-stalled DNA replication fork could be tentatively repaired by (3) TLS in the absence of HR (Rad52p), resulting in cell survival, or (4) by HR when a TLS pathway (Rev3p) is not fully functional. In this last case, the recombinational products resulting from the action of NER/BER endonucleases plus HR proteins together with ET-743 are extremely genotoxic for the cell. Abbreviations: $_{SS}$DNA binding proteins (single strand DNA binding proteins).

by a mechanism that combines HR with TLS catalyzed by Rad30p and Rad52p. This error-free pathway could act as a back-up repair system in absence of a fully functional TLS or when the cell is submitted to treatment with high doses of ET-743. On the other hand, the presence of an ET-743 adduct in DNA could induce the replication fork to stall, generating a signal that recruits proteins of HR or TLS pathways (Fig. 5). Many authors agree that a stalled replication fork in eukaryotes can be repaired by HR or by TLS [53–55], but the choose of which pathway will be used for restoring the replication fork is not fully understood [56]. The use of HR to repair stalled replication forks implies that endonucleases (e.g. Rad1p-Rad10p complex or Mus81p) are needed to resolve the intermediate DNA structures that arise during the recombinational process, like Holliday junctions [56,57]. Interestingly, it has been observed an interplay between HR and NER proteins during the repair of stalled replication forks induced by cisplatin in mammalian cells [58], which reinforces the idea that both pathways act together to deal with recombinational structures. Thus, our model proposes that the repair of a stalled replication fork by NER/BER and HR proteins, in the presence of an ET-743 adduct, probably results in toxic recombinational products that lead to cell death when an error-prone TLS pathway is not functional (Fig. 5). The partial resistance of single mutant *rev3*Δ could be also explained considering the action of error-free TLS polymerases, e.g. Rad30p. Moreover, the absence of a HR protein (e.g. Rad52p) channels the repair of stalled replication forks to a TLS pathway, resulting in cell survival and ET-743 tolerance (Fig. 5).

We are conducing more experiments in order to elucidate the role of BER in the repair of ET-743 induced DNA damage, especially in mammalian cells.

Acknowledgments

The authors thank Dr. Martin Brendel for critical reading of manuscript. This work was supported by research grants

from Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul (FAPERGS), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and GENOTOX (Laboratório de Genotoxicidade, UFRGS).

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