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Influences of Base Excision Repair Defects on the Lethality and Mutagenicity Induced by Me-lex, a Sequence-selective N3-Adenine Methylating Agent*

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Due to its minor groove selectivity, Me-lex preferentially generates N3-methyladenine (3-MeA) adducts in double-stranded DNA. We undertook a genetic approach in yeast to establish the influence of base excision repair (BER) defects on the processing of Me-lex lesions on plasmid DNA that harbors the p53 cDNA as target. We constructed a panel of isogenic strains containing a reporter gene to test p53 function and the following gene deletions: Δmag1, Δapn1apn2, and Δapn1apn2mag1. When compared with the wild-type strain, a decrease in survival was observed in Δmag1, Δapn1apn2, and Δapn1apn2mag1. The Me-lex-induced mutation frequency increased in the following order: wild type < Δmag1< Δapn1apn2 = Δapn1apn2mag1. A total of 77 mutants (23 in wild type, 31 in Δmag1, and 23 in Δapn1apn2) were sequenced. Eighty-one independent mutations (24 in wild type, 34 in Δmag1, and 23 in Δapn1apn2) were detected. The majority of base pair substitutions were AT-targeted in all strains (14/23, 61% in wild type; 20/34, 59%, in Δmag1; and 14/23, 61%, in Δapn1apn2). The Mag1 deletion was associated with a significant decrease of GC > AT transitions when compared with both the wild-type and the AP endonuclease mutants. This is the first time that the impact of Mag1 and/or AP endonuclease defects on the mutational spectra caused by 3-MeA has been determined. The results suggest that 3-MeA is critical for Me-lex cytotoxicity and that its mutagenicity is slightly elevated in the absence of Mag1 glycosylase activity but significantly higher in the absence of AP endonuclease activity.

Most alkylating agents react with nucleophilic sites on DNA, yielding a complex mixture of DNA lesions (1). Thus, quantitative and qualitative analyses of the biological role(s) of individual DNA lesions and their relative contribution to the mutagenicity and/or toxicity are difficult tasks. Many clinically used anticancer drugs are DNA alkylating agents. Hence, an understanding of which biological effect is caused by a specific DNA lesion may lead to a more rational design of antineoplastic agents.

To exercise a significant regulation over the alkylation pattern on DNA, several alkylating agents were recently synthesized, including Me-lex,[1] a methyl sulfonate ester appended to a neutral *N*-methylpyrrolecarboxamide-based dipeptide (lex) (2). The lex dipeptide binds at A/T-rich sequences in the minor groove of DNA (3), and as a result, Me-lex efficiently methylates the N3 position of adenine (3-A), generating almost exclusively N3-methyladenine (3-MeA) (2, 4). This contrasts with the alkylation patterns induced by simple methylating agents such as methyl methanesulfonate ester (MMS), which predominantly yields the major groove N7-methylguanine adduct (1). The significant increase in 3-MeA formation closely parallels the higher cytotoxicity of Me-lex *versus* MMS (4–6). The role of 3-MeA in toxicity is consistent with the observation that Me-lex cytotoxicity is magnified in alkyladenine-DNA glycosylase (*Aag*) null embryonic stem cells (5, 6) and in base excision repair (BER)-deficient bacteria (7, 8). If 3-MeA is mainly a lethal lesion, the 3-MeA specific inducer Me-lex might be expected to combine high cytotoxicity with low mutagenicity, diminishing the undesired carcinogenic property of the different DNA alkylating agents used in cancer therapy (9, 10).

In a previous work, we compared the sequence specificity of lesion formation *in vitro* with the mutation spectrum induced by Me-lex at the human *p53* cDNA using a yeast-based functional assay (11). We observed a general lack of correspondence between adduct formation and mutation hot spots that is consistent with the idea that 3-MeA is a cytotoxic rather than a mutagenic lesion.

3-MeA, or the abasic site generated by its spontaneous breakdown ($t_{1/2}$ of ~24 h at 37 °C), is repaired via the BER pathway(s). In *Saccharomyces cerevisiae*, 3-MeA repair is initiated by a 3-methyladenine-DNA-glycosylase (Mag1) followed by the action of AP endonuclease (Apn1, Apn2), DNA polymer-

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This work is dedicated to Giovanni Battista Fronza with love.

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[1] The abbreviations used are: Me-lex, {1-methyl-4-[1-methyl-4-(3-methoxysulfonylpropanamido)pyrrole-2-carboxamido]-pyrrole-2-carboxamido}propane; lex, lexitropsin; MMS, methyl methanesulfonate ester; 3-MeA, N3-methyladenine; BER, base excision repair; MF, mutant frequencies; ds, double-stranded; ss, single-stranded; AP, apurinic/apyrimidinic.

TABLE I
S. cerevisiae strains constructed for this study

Strain	Genotype	Reference
yIG397	MATa ade2–1 leu2–3,112 trp1–1 his3–11,15 can1–100 ura3–1 URA3 3xRGC::pCYC1::ADE2	(14)
yPM1	as yIG397 and apn1::HIS3	This study
yPM2	as yIG397 and mag1::LEU2	This study
yPC1	as yIG397 and apn2::KAN^R	This study
yPC2	as PM1 and apn2::KAN^R (apn1apn2 HIS3 KANR)	This study
yPM3	as yPM1 and mag1::LEU2 (apn1mag1 HIS3 LEU2)	This study
yPC3	as yPM3 and apn2::KAN^R (apn1apn2mag1 HIS3 KANR LEU2)	This study

ase (δ, ϵ), and DNA ligase (III). APN1 encodes the major AP endonuclease and shares extensive homology with endonuclease IV in *Escherichia coli* (12). A second AP endonuclease, the product of the *APN2* gene, the *S. cerevisiae* homologue of the major human AP endonuclease *HAP1*, accounts for less than 10% of the total AP endonuclease activity in budding yeast (13).

To establish the influence of different BER repair defects on the mutagenic and lethal properties of the specific lesions produced by Me-lex, we constructed, starting from the parental yeast strain yIG397, the following panel of isogenic strains: yPM2 (Δmag1), yPC2 (Δapn1apn2), and yPC3 (Δapn1apn2mag1). All strains contain the *ADE2* gene regulated by a p53-responsive promoter to assess p53 transactivation function. On plates containing limiting adenine, clones expressing wild-type p53 give rise to normal size white colonies, whereas those expressing a non-functional mutant originate small red colonies (14). A yeast expression vector harboring the human wild-type *p53* cDNA was treated *in vitro* with Me-lex and transformed into each strain. Survival levels and *p53* mutant frequencies obtained with wild-type and the BER-defective strains transformed with the exact same Me-lex-damaged DNA were compared. Furthermore, three groups of *p53* mutants induced by Me-lex and isolated in yIG397, yPM2 (Δmag1), and yPC2 (Δapn1apn2) were sequenced and compared in order to verify the influence of both enzymatic activities on the type and distribution of Me-lex-induced mutations along the *p53* cDNA.

MATERIALS AND METHODS

Compounds—Reagents of the highest purity were purchased from Sigma or Aldrich unless otherwise stated. Me-lex was prepared as described previously (2). Restriction enzymes were obtained from New England Biolabs (Beverly, MA).

Vectors, Strain, and Media—The yeast expression vector pTS76 harbors a human wild-type *p53* cDNA under the control of an *ADH1* constitutive promoter and contains the *TRP1* selectable marker. The haploid *S. cerevisiae* strain yIG397 (MATa ade2-1 leu2-3,112 trp1-1 his3-11,15 can1-100 ura3-1 URA3 3xRGC::pCYC1::ADE2) and its isogenic BER-deficient derivatives were used as recipients of pTS76. yIG397 was also used for gap repair with plasmid pRDI-22 as described previously (15). The p53-dependent reporter *ADE2* gene allowed the phenotypic selection of p53 mutants as its recombinant *cyc1* promoter contains three copies of the responsive element *RGC* (14). Standard yeast manipulations were performed as described (16).

DNA Modification, Analysis, and Transformation—Me-lex was dissolved in Me_2SO immediately before the treatment. 3.0 μg of plasmid pTS76 DNA was treated with different Me-lex concentrations (up to 24 mM) in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 50% EtOH for 1 h at 37 °C. DNA was purified by EtOH precipitation, washed with 70% EtOH, and resuspended in sterile water. Damaged or undamaged vectors were then transformed by the LiAc method into the same number of yeast cells (as measured at A_{600}), in the same growth conditions, and transformants were plated on selective synthetic medium plates specific for each strain (see below). After 3 days of incubation at 30 °C, colonies were evaluated. The selection for the plasmid marker (*TRP1*) allowed an indirect determination of the lethal effect of the damaging treatment as the number of transformants scored in transfections with damaged plasmids with respect to that obtained with undamaged vector. As transformation plates contained a minimal amount of adenine, adenine auxotrophs produced small red colonies. The spontaneous and induced mutant frequencies (MF) were defined as the number of small red colonies with respect to the total number of transformants. The fold of mutant induction was defined as the ratio between the MF observed with damaged vector with respect to the spontaneous MF. Phenotypic mutant clones were purified and characterized at the molecular level as described previously (15).

Analysis of Abasic Site Induction—The plasmid pTS76 DNA (3 μg) in 10 mM Tris-HCl (pH 7.0) containing 1 mM EDTA was either directly used for determination of abasic sites or treated with 24 mM Me-lex for 1 h at 37 °C. The DNA was purified by EtOH precipitation, washed with 70% EtOH, and resuspended in sterile water, and abasic sites were quantitated using a DNA Damage Quantification Kit (Kamiya Biomedical Company, Seattle, WA).

Construction of BER Defective Yeast Strains—To inactivate the *MAG1* gene, a mag1 disruption cassette (pUC-mag1-del::LEU2a, a 7159-bp plasmid, generous gift of Leona Samson, Massachusetts Institute of Technology, Cambridge, MA) was used. To inactivate the *APN1* gene, a disruption cassette (YEpApn1, a 6.5-kbp plasmid generous gift of Dr. R.O. Bennett, Harvard School of Public Health, Boston, MA) was used. To inactivate the *APN2* gene, a 2-μm DNA-based marker recycling system for multiple gene disruption was used (pGFKG plasmid, a 11.25-kbp plasmid containing two recombinogenic FRT sequences flanking the *FLP* recombinase gene and the selectable marker KAN^R, a generous gift of Dr. Francesca Storici, NIEHS, National Institutes of Health, Research Triangle Park, NC) (17). The yPC2 (Δapn1apn2) and the yPC3 (Δapn1apn2mag1) strains were obtained by disrupting the *APN2* gene as described above in the yPM1 (Δapn1) and yPM3 (Δapn1mag1) strains, respectively. Each gene disruption was checked by PCR using positive and negative controls (details for primers and PCR conditions used are available upon request).

Statistical Analysis—The Adams and Skopek (18) algorithm uses a Monte Carlo method to simulate a p value of the standard hypergeometric test for a contingency table. Unlike the χ^2 test, which can also be applied to contingency tables and requires that all cells contain five or more events, the hypergeometric test is appropriate when applied to sparse data sets, as are often found in mutational spectra analyses. The Cariello program (19) uses a random number generator to produce a large number of simulated spectra based on the hypergeometric probability of the experimentally observed input spectra. The degree to which the simulated spectra differ from the input spectra is used to estimate the probability that the two input spectra were derived from the same population. A p value equal to 0.05 leads to rejecting the null hypothesis and concluding that the input spectra are different.

RESULTS

Generation of BER-deficient Yeast Strains—To determine the relative role of Mag1 and AP endonucleases (Apn1 and Apn2) on the toxicity and mutagenicity induced by Me-lex, we constructed the set of isogenic BER-deficient mutant yeast strains described in "Materials and Methods" and summarized in Table I. MAG1, APN1, and APN2 were inactivated using disruption cassettes or a PCR-based method (17). The correctness of the gene disruption was verified by a PCR approach. The ability of different mutant strains to grow in the presence of MMS was evaluated using plates containing a gradient of MMS (from 0% up to 0.03%) (data not shown). This type of qualitative/semiquantitative analysis confirmed that disruption of MAG1 (yPM2) was associated with the highest sensitivity to MMS, and the further disruption of *APN1* and *APN2* genes (yPC3) was not accompanied by an appreciable increase in MMS sensitivity. These observations are consistent with previous reports (13, 20). We conclude that the new isogenic

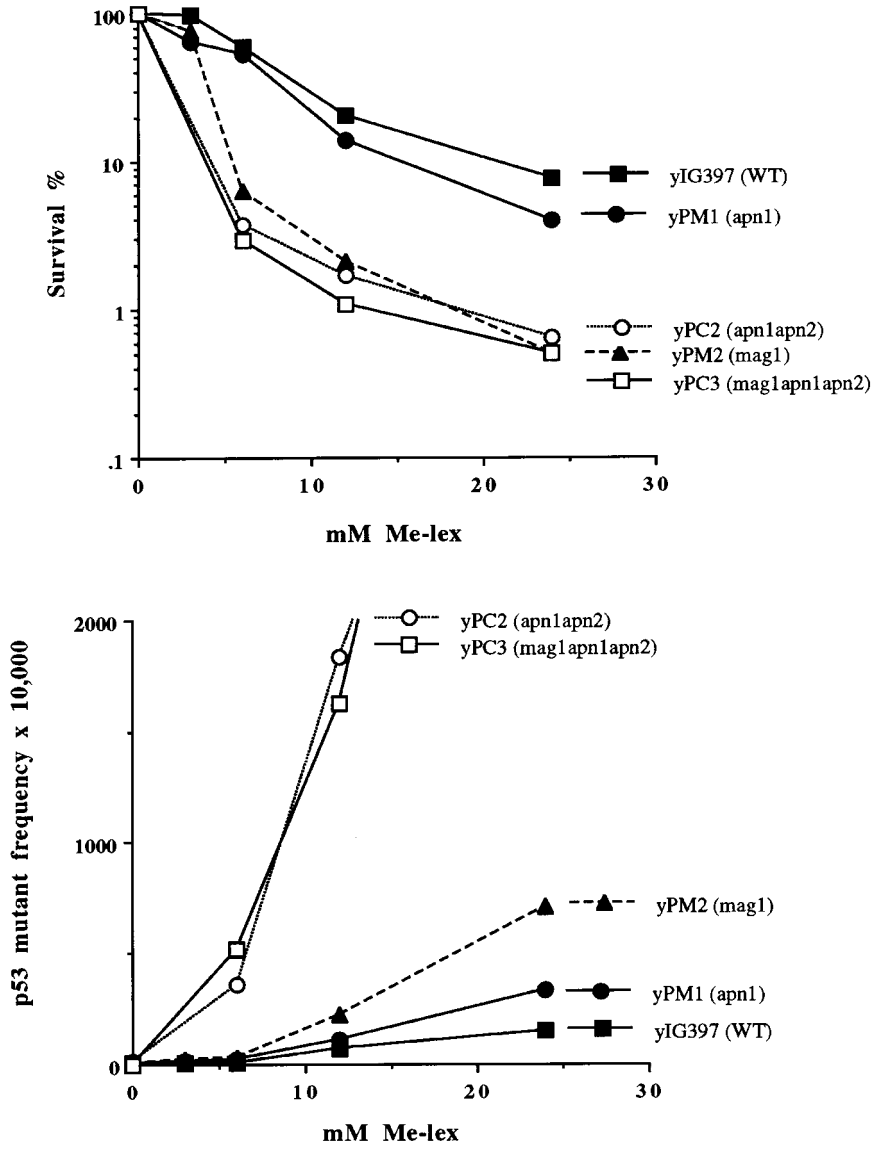

FIG. 1. **Influence of DNA repair capacities on the lethality (*upper panel*) and mutagenicity (*lower panel*) of Me-lex-induced lesions in repair-proficient and repair-deficient yeast strains.**

strains deficient in different and/or multiple steps of the BER pathway had the correct gene disruption and the expected phenotype.

Me-lex Lethality Is Influenced by BER Defects—Plasmid pTS76 was damaged *in vitro* by increasing Me-lex concentrations (Fig. 1, *upper panel*) and transformed into BER-proficient and -deficient strains. Survival showed an Me-lex concentration-dependent decrease in every strain and was influenced by the BER capacity of the recipient yeast strain. All three BER-deficient strains appear to be ~10-fold more sensitive to the lethal effects of Me-lex with respect to wild-type. Confirming the results obtained with MMS gradient plates, the single yPM1 mutant (Δapn1) (Fig. 1, *upper panel*) or yPC1 (Δapn2) (data not shown) showed minimal or no difference in sensitivity to Me-lex, respectively, when compared with yIG397.

Spontaneous MF Is Influenced by BER Defects—To determine spontaneous MF, undamaged pTS76 plasmid was transformed into BER-proficient and -deficient strains. Transformants were selected on plates lacking tryptophan but containing sufficient adenine for adenine auxotrophs to grow and turn red. Although yPM2 (Δmag1) showed an MF indistinguishable from the wild-type (yIG397) (5.77×10^{-4} *versus* 4.35×10^{-4}), yPC2 (Δapn1apn2) revealed a significantly higher MF (13.8×10^{-4} *versus* 4.35×10^{-4}, $p < 0.02$, Chi square test). However, the spontaneous MF reverted back to wild-type level in yPC3 (Δmag1apn1apn2) (2.6×10^{-4}) where the *MAG1* gene was also disrupted. These results are consistent with, and complement, those obtained by Xiao and Samson (20) that support the notion that abasic sites are potent mutagenic lesions. They also suggest that wild-type Mag1 activity partially contributes to the higher MF observed in the yPC2 (Δapn1apn2) strain through the generation of abasic sites by acting on normal undamaged bases.

Me-lex Mutagenicity Is Influenced by BER Defects—MFs increased not only in a concentration-dependent way in each strain (Fig. 1, *lower panel*) but were also strongly influenced by the nature of the BER defects. At every Me-lex concentration, MFs increased in the following order: yIG397 < yPM2 < yPC2 = yPC3. Considering the fold of induction above the spontaneous MF in each strain and at every Me-lex concentration, yPM2 (Δmag1) appears to be roughly 2–3-fold more prone to the induction of Me-lex-induced p53 mutants than the wild-type strain, whereas yPC2 (Δapn1apn2) and yPC3 (Δmag1apn1apn2) are at least 10-fold more prone to the induction of p53 mutants.

Molecular Characterization of Me-lex-induced p53 Mutants in yIG397, yPM2, and yPC2—To verify at the molecular level the influence of Mag1 or AP endonuclease activities on the type

TABLE II
Summary of the molecular features of Me-lex-induced p53 base pair substitution mutations in yIG397, yPM2 (Δmag1), and yPC2 (Δapn1apn2) strains

	yIG397	yPM2	yPC2
AT-targeted	**14 (61)**	**20 (59)**	**14 (61)**
AT > TA	8 (35)	9 (26)	8 (35)
AT > CG	3 (13)	1 (3)	0 (0)
AT > GC	3 (13)	10 (30)	6 (26)
GC-targeted	**9 (39)**	**14 (41)**	**9 (39)**
GC > AT	4 (17)[a]	0 (0)[a]	4 (17)[a]
GC > TA	3 (13)	6 (18)	2 (9)
GC > CG	2 (9)	8 (21)	3 (13)
Total base pair substitutions	**23 (100%)**	**34 (100%)**	**23 (100%)**

[a] $p < 0.03$.

and distribution of Me-lex-induced mutations along the *p53* cDNA, three groups of mutants isolated in yIG397, yPM2 (Δmag1), and yPC2 (Δapn1apn2) strains were characterized. For this purpose, mutants isolated at similar levels of induction (24 mM in yIG397, 12 mM in yPM2, and 6 mM in yPC2) were characterized as described previously (11). At all levels of induction, at least 97% of the mutants were drug-induced.

A total of 77 p53 mutants (23 isolated in yIG397, 31 isolated in yPM2, and 23 isolated in yPC2) were sequenced. At least one molecular alteration was found in each mutant. Seventy-seven mutants evidenced 81 independent mutations (24 in yIG397, 34 in yPM2, and 23 in yPC2). With the exception of one 2-bp deletion (in yIG397), all mutations were base pair substitutions. The molecular features of the base pair substitutions are summarized in Table II. The majority of base pair substitutions were AT-targeted in all three strains (14/23, 61%, in yIG397; 20/34, 59%, in yPM2; 14/23, 61% in yPC2). Among AT-targeted mutations, Mag1 deficiency (yPM2) as well as Apn1Apn2 deficiency (yPC2) were associated with a decrease in AT > CG transversions and an increase in AT > GC transitions (both not quite statistically significant) *versus* yIG397. Among GC-targeted mutations, GC > AT transitions were significantly more frequent in yIG397 and yPC2 (Δapn1apn2) than in yPM2 (Δmag1)(4/9 *versus* 0/14, $p < 0.03$, Fisher's exact test in both cases), whereas GC > CG transversions were instead more abundant (~2-fold but not quite statistically significant) in yPM2 *versus* yIG397 and yPC2. GC > AT transitions were significantly more frequent in yIG397 (and yPC2) than in yPM2 (Δmag1), even considering all base pair substitutions (4/23 *versus* 0/34, $p < 0.03$, Fisher's exact test). At position 602 (codon 201, 5′-^{602}AAATTT^{597}-3′) an Me-lex-induced mutation hot spot ($n = 2$, $p < 0.001$, Poisson's normal distribution), common to all three strains (two, three, and four mutations in yPC2 (Δapn1apn2), yPM2 (Δmag1), and yIG397, respectively) was observed. This sequence corresponds to a known lex binding site that was reported previously as the only site in the *p53* cDNA hot for both Me-lex lesion formation and Me-lex-induced mutation (11).

DISCUSSION

Me-lex Lethality Is Dependent on the Efficiency of Different BER Steps

The results presented in this work demonstrate that lethality of Me-lex-induced lesions is counteracted mainly by Mag1 (Fig. 1, *upper panel*), indicating that 3-MeA is cytotoxic. Minimal survival was also observed in the yPC2 (Δapn1apn2) strain because once 3-MeA is removed by Mag1 in an apn1apn2 background, the newly formed abasic site is also lethal. It is interesting to note that the apn1 mutant showed a similar survival response to Me-lex as wild type. This indicates that Apn2 can compensate for the Apn1 deficiency (Apn1 constitutes ~90% of cellular AP endonuclease activity) and that AP endonuclease activity is not a rate-limiting step in the repair of 3-MeA. For the same reason, the triple mutant yPC3 (Δmag1apn1apn2) is approximately as sensitive as yPM2 (Δmag1) and yPC2 (Δapn1apn2). These results are consistent with the three genes belonging to the same BER pathway and suggest that 3-MeA and the abasic site are equally toxic.

Me-lex Mutagenicity

3-MeA Is Poorly Mutagenic—There is ample evidence that 3-MeA is a lethal lesion in *E. coli* (7, 8, 21) and mouse embryonic cells (5, 6) or based on *in vitro* experiments showing that 3-MeA is a DNA replication blocking lesion (22). A remaining question we tried to address is whether 3-MeA, which so effectively prevents DNA synthesis, can also be a promutagenic lesion. Our data shows that Mag1 marginally protects cells against Me-lex mutagenicity. The difference in mutability between wild-type and the mag1 mutant (yPM2) is rather small (2–3-fold), although not negligible. A relatively weak contribution of 3-MeA to Me-lex mutagenicity emerges also from the comparison of the classes of mutations induced in yIG397 and in yPM2 (Δmag1). This comparison reveals that Mag1 deficiency is significantly associated only with a decrease of GC > AT transitions ($p < 0.03$ Fisher's exact test) (Table II). A decrease in a specific mutation class at GC base pairs in the absence of a DNA repair function is surprising, especially with Me-lex that targets A/T sequences. Moreover, the GC mutation sites are for the most part not adjacent to Me-lex methylation sites. It is known that Mag1, when overexpressed, introduces a strong mutator phenotype in yeast (23), whereas when underexpressed, it decreases spontaneous mutations (20). Furthermore, Berdal *et al.* (24) demonstrated that purified Mag1 protein is able to remove undamaged bases from DNA with G being the best substrate for this undesired activity. Both results (23, 24) predict that Mag1 deficiency will tend not only to exacerbate the mutagenicity of all lesions normally repaired by Mag1 but will also alleviate yeast cells from the "mutagenic stress" derived from "physiological" Mag1 activity. However, the quantitative and qualitative nature of the changes in the Me-lex-induced mutations in the yPM2 mutant strain relative to wild type remain unresolved.

The third evidence supporting a weak contribution of 3-MeA to Me-lex mutagenicity is obtained through the comparison of mutational spectra induced by Me-lex in the presence (yIG397) or in the absence (yPM2) of Mag1 activity using the Cariello test, which considers both mutation type and position (19). The application of this rigorous statistical test, which provides a precise measure of the relatedness of two spectra obtained at the same locus, reveals that the yIG397 and yPM2 spectra are indistinguishable ($p = 0.10$). This is partially due to the fact that the Me-lex-specific mutation hot spot at position 602 is common to both strains, and it does not appear to be "hotter" in the mag1 background. If 3-MeA was directly responsible for the generation of this hot spot, it is reasonable to expect that this hot spot could be hotter in yPM2 than in yIG397. Clearly, this was not the case. This finding strongly suggests that lesions localized at position 602 are not repaired by Mag1 either because this sequence context is a cold spot for BER repair or because the lesion responsible for such mutations is not a substrate of Mag1. Since we showed previously that 3-MeA was formed in abundant amounts at position 602 (11), we favor the former hypothesis. The existence of cold spots for repair is supported by the work of Ye *et al.* (25), which showed that the sequence-dependent rate of 3-MeA repair at the nucleotide level along the *PGK1* gene in normal human fibroblast varied by 6-fold from site to site. An alternative explanation involves an accelerated rate of 3-MeA depurination at that particular

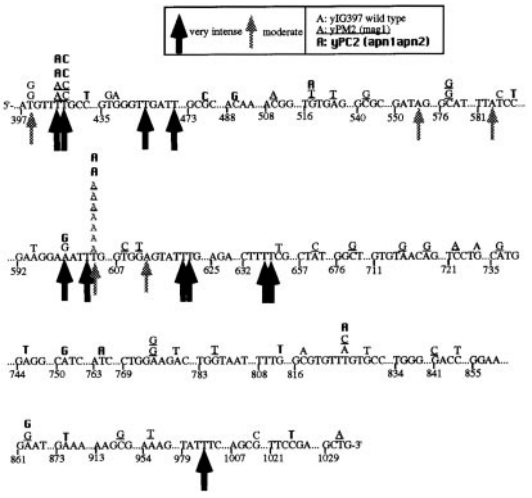

FIG. 2. **Localization and intensity of Me-lex-specific lesions (data from Ref. 11) and induced mutations in yIG397 (wild type), in yPM2 (Δmag1) (*underlined*), and in yPC2 (Δapn1apn2) produced in the PshAI/StuI DNA fragment of the p53 cDNA sequence (non-transcribed (coding) strand), respectively.** Mutations isolated in different strains and not reported in the figure because they were localized outside the sequence reported are as follows. yIG397: C > G, position 293; G > T, position 314; A > G, position 358; A > T, position 392; −GC between positions 846–849. yPM2: C > G, position 292; C > A, position 296; A > T, position 301; G > C, position 350. yPC2: T > C, position 326; G > T, position 350; C > G, position 378. *WT*, wild type.

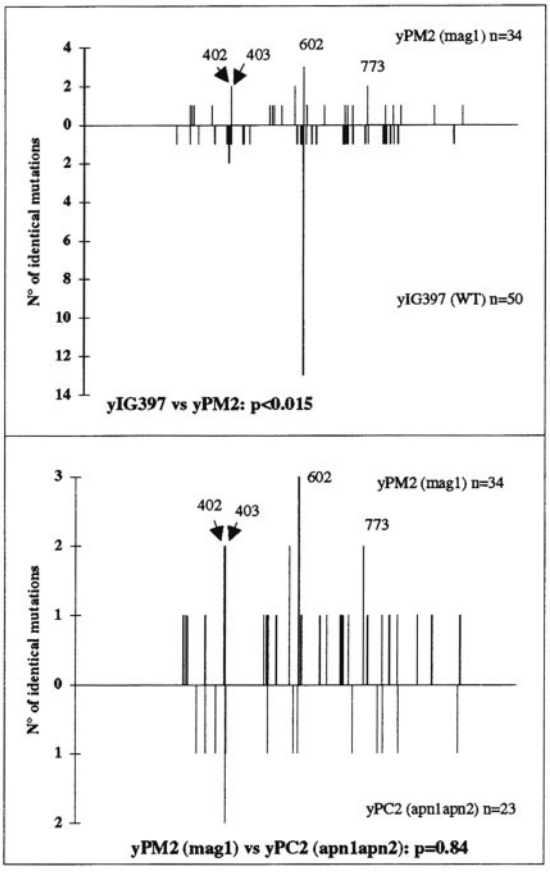

FIG. 3. **Sequence distribution of p53 mutations induced by Me-lex and isolated in yIG397 (11), in yPM2 (Δmag1), and in yPC2 (Δapn1apn2).** Mutation spectra were compared using the Cariello program (19). *Upper panel*, the cumulative mutation spectrum observed in yIG397 is significantly different from the one observed in yPM2 (Δmag1). *Lower panel*, the mutation spectra obtained in yPM2 (Δmag1) and in yPC2 (Δapn1apn2) appear to be indistinguishable. *WT*, wild type.

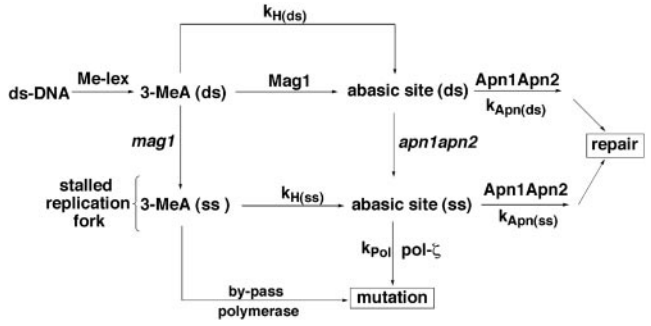

FIG. 4. **Proposed induction of mutagenicity by 3-MeA.** $k_{H(ds)}$, rate of hydrolysis of 3-MeA from dsDNA; $k_{H(ss)}$, rate of hydrolysis of 3-MeA from ssDNA; $k_{Apn(ds)}$, rate of repair of abasic site in dsDNA; $k_{Apn(ss)}$, rate of repair of abasic site in ssDNA; $k_{Pol(ss)}$, rate of polymerization past AP site in ssDNA; $k_{Pol(ds)}$, rate of polymerization past abasic site in dsDNA. If $k_{H(ss)} \gg k_{H(ds)}$ and $k_{Apn(ds)} \gg k_{Apn(ss)}$, the 3-MeA lesion will be converted into an abasic site at a stalled replication fork and qualitatively afford similar mutations in both the yPM2 (Δmag1) and the yPC2 (Δapn1apn2) mutant strains.

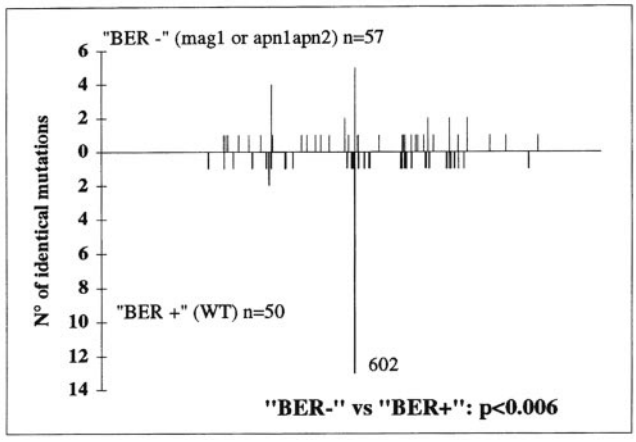

FIG. 5. **Sequence distribution of p53 mutations induced by Me-lex and isolated in BER-deficient (BER−, *i.e.* yPM2 (Δmag1) and yPC2 (Δapn1apn2)) and BER-proficient (BER+, *i.e.* cumulative mutation spectrum observed in yIG397, see Ref. 11) strains.** When the two spectra are compared using the Cariello program (19), they appear to be significantly different. *WT*, wild type.

position. Based on all the evidence, we conclude that 3-MeA is poorly mutagenic.

Analysis of the mutation spectra in more detail shows that there are three additional hot spots ($n = 2$, $p < 0.001$, Poisson's normal distribution) in yPM2 (Δmag1) where AT targeted mutations are observed (Fig. 2). At positions 402 and 403, which are both heavily methylated by Me-lex (11), there are two AT > TA transversions, and two AT > GC transitions, respectively. Clearly, the same lesion induces very different mutations at neighboring bases, and this raises the possibility that phenotypic selection, rather than rates of insertion opposite or near 3-MeA, dominates the observed mutation pattern. For example, the absence of AT > GC transitions at position 402 can be explained since no amino acid substitution would be introduced by this mutation (TT*T*(F) → TT*C*(F)). However, the undetected AT > CG transversion would cause the same amino acid substitution as the observed AT > TA transversion (TT*T*(F) → TT*G*(L)). In addition to the mutations at positions 402 and 403, there is another strong mutation site at position 773 (where no 3-MeA is observed (11)) that shows two AT > GC transitions. Interestingly, in yIG397, no mutation was observed at those positions in the present study, whereas a single AT > GC was observed in the previous study (11).

All together, these results suggest that the mutation spectra

in the presence and absence of Mag1 activity might be distinguished by increasing the number of characterized mutations. Because the two spectra of base pair substitutions obtained in yIG397 (11) appear to be indistinguishable ($p = 0.12$, Cariello test), they can be pooled. The mutation spectrum obtained in yPM2 is distinguishable (Fig. 3, *upper panel*; $p < 0.015$, Cariello test) from the cumulative one in yIG397, whereas it is indistinguishable from the one obtained in yPC2 (Fig. 3, *lower panel*; $p = 0.84$, Cariello test).

Role of Abasic Sites—In contrast to what is observed in yPM2, Me-lex mutagenicity is strongly exacerbated in the absence of both Apn1 and Apn2, independent of the presence (yPC2) or absence (yPC3) of Mag1 and especially at higher Me-lex concentrations. This result suggests that abasic sites or lesions repaired by the activities associated with Apn1 (*i.e.* 3′-phosphodiesterase (26)) and Apn2 (*i.e.* 3′-phosphodiesterase and 3′-5′-exonuclease (27)) are mainly responsible for Me-lex mutagenicity.

As mentioned before (Fig. 3, *lower panel*), the mutation spectra obtained in yPM2 and in yPC2 are indistinguishable ($p = 0.84$, Cariello test), especially with respect to the AT > GC mutations. We propose that the formation of the same secondary lesion is at least partially responsible for this similarity. It is possible that a fraction of 3-MeA is converted into the abasic site due to experimental conditions. We measured the level of abasic sites in the plasmid before and after Me-lex treatment using conditions that mimic those used in the transformation of the plasmid into yeast (chemical exposure, plasmid purification, and transformation conditions) and observed that the level of abasic sites increased by ~5-fold at 24 mM Me-lex (1.5-fold at 12 mM Me-lex). It is also possible that 3-MeA conversion into abasic site occurs *in vivo*. 3-MeA is relatively stable in double-stranded DNA at physiological conditions ($t_{1/2} = 24$ h (28)). However, at the deoxynucleotide level, as would be the case in single-stranded DNA, the stability is dramatically reduced by 40-fold ($t_{1/2}$ of ~35 min (29)). Because of this intrinsic instability, we suggest that at a stalled replication fork, a fraction of 3-MeA lesions will undergo hydrolytic conversion to abasic sites. If such abasic sites, when present in replication intermediates, are not good substrates for AP endonucleases ($k_{Apn(ss)} \ll k_{Apn(ds)}$), and translesion synthesis is preferred at specific A-tract sequences ($k_{Pol} \gg k_{Apn(ss)}$), then the mutation spectra in mag1 and apn1apn2 cells will be similar (Fig. 4). Alternatively, although unlikely, the similarity in the yPM2 and yPC2 spectra might result from the fact that 3-MeA can be bypassed with low efficiency by a specialized polymerase and that bypass of 3-MeA and abasic site will lead to the same mutations.

Since Mag1 and Apn1Apn2 proteins are involved in the same BER pathway and since the two spectra appear to be indistinguishable, it is possible to pool the spectra obtained in yPC2 and yPM2 into a cumulative BER-deficient spectrum and compare it with the cumulative spectrum in yIG397 ("BER-proficient"). Interestingly, the "BER-deficient" and the "BER-proficient" mutation spectra are clearly different (Fig. 5, $p < 0.006$, Cariello test). This analysis shows that a BER defect, irrespective of the specific step, will significantly modify the mutation spectrum.

In conclusion, by combining a chemical and a genetic approach, we characterized the molecular consequences of 3-MeA in wild-type and two strains deficient for different steps in BER repair. The picture that is emerging is consistent with 3-MeA being the critical intermediate for Me-lex cytotoxicity. The mutagenicity of 3-MeA is markedly elevated by the removal of the lesion by Mag1 in strains that cannot repair the resulting abasic sites because of the absence of AP endonuclease activity. The data also suggest that Me-lex may represent a useful model for the design of highly cytotoxic but weakly mutagenic antineoplastic agents.

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Influences of Base Excision Repair Defects on the Lethality and Mutagenicity Induced by Me-lex, a Sequence-selective N3-Adenine Methylating Agent

Paola Monti, Paola Campomenosi, Yari Ciribilli, Raffaella Iannone, Alberto Inga, Dharini Shah, Gina Scott, Philip A. Burns, Paola Menichini, Angelo Abbondandolo, Barry Gold and Gilberto Fronza

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Additions and Corrections

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A 5-HT_4 receptor transmembrane network implicated in the activity of inverse agonists but not agonists.

Lara Joubert, Sylvie Claeysen, Michèle Sebben, Anne-Sophie Bessis, Robin D. Clark, Renee S. Martin, Joël Bockaert, and Aline Dumuis

Page 25504, fifth line from the bottom of the first column and page 25506, right-hand column, line 6: Insert the following reference:

Mialet, J., Dahmoune, Y., Lezoualc'h, F., Berque-Bestel, I., Eftekhari, P., Hoebeke, J., Sicsic, S. S., Langlois, M., and Fischmeister, R. (2000) *Br. J. Pharmacol.* **130,** 527–538

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Influences of base excision repair defects on the lethality and mutagenicity induced by Me-lex, a sequence selective N3-adenine methylating agent.

Paola Monti, Paola Campomenosi, Yari Ciribilli, Raffaella Iannone, Alberto Inga, Dharini Shah, Gina Scott, Philip A. Burns, Paola Menichini, Angelo Abbondandolo, Barry Gold, and Gilberto Fronza

The affiliation for Paola Campomenosi was partially omitted. The additional affiliation should read:

[c]*Human Genetics Laboratory, Department of Structural and Functional Biology (DBSF), University of Insubria, Varese, Italy*

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The insulin receptor catalyzes the tyrosine phosphorylation of caveolin-1.

Akiko Kimura, Silvia Mora, Satoshi Shigematsu, Jeffrey E. Pessin, and Alan R. Saltiel

In this manuscript, we unintentionally failed to cite two studies (Gustavsson, J., Parpal, S., Karlsson, M., Ramsing, C., Thorn, H., Borg, M., Lindroth, M., Peterson, K. H., Magnusson, K. E., and Strålfors, P. (1999) *FASEB J.* **13,** 1961–1971 and Parpal, S., Karlsson, M., Thorn, H., and Strålfors, P. (2001) *J. Biol. Chem.* **276,** 9670–9678) that also demonstrated the presence of the insulin receptor in caveolin-positive structures in 3T3L1 adipocytes. We regret our oversight.

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