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XRCC1 deficiency influences the cytotoxicity and the genomic instability induced by Me-lex, a specific inducer of N3-methyladenine

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Abstract

Me-lex is a sequence-specific alkylating agent synthesized to preferentially (> 90%) generate N3-methyladenine (3-mA) in the minor groove of double-strand DNA, in A-T rich regions. In this paper we investigated the effect of XRCC1 deficiency in the processing of 3-mA adducts generated by Me-lex, through the molecular analysis of the *Hprt* mutations and the evaluation of cytogenetic end points such as sister chromatid exchanges (SCEs), micronuclei (MN) and nucleus fragmentation. EM-C11 cells, deficient in XRCC1 activity, showed a 2.5-fold higher sensitivity to the toxicity of Me-lex compared to the DNA repair proficient parental CHO-9 cells, but were not hyper mutable. The spontaneous mutation spectrum at the *Hprt* locus generated in EM-C11 cells revealed a high percentage of genomic deletions. After Me-lex treatment, the percentage of genomic deletions did not increase, but a class of mutations which appeared to target regulatory regions of the gene significantly increased ($p=0.0277$), suggesting that non-coding *Hprt* genomic sequences represent a strong target for the rare mutations induced by Me-lex. The number of SCEs per chromosome increased 3-fold above background in 50 μ M Me-lex treated CHO-9 cells, while at higher Me-lex concentrations a sharp increase in the percentage of MN and fragmented nuclei was observed. In EM-C11 cells the background level of SCEs (0.939 ± 0.182) was approximately 10-fold higher than in CHO-9 (0.129 ± 0.027) and higher levels of multinucleated cells and MN were also found. In EM-C11, even low doses of Me-lex (25 μ M) led to a significant increase in genomic damage. These results indicate that XRCC1 deficiency can lead to genomic instability even in the absence of an exogenous genotoxic insult and low levels of Me-lex-induced lesions, i.e., 3-mA and/or a BER intermediate, can exacerbate this instability.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Keywords

Me-lex; mutation induction; *Hprt*; hamster cells; XRCC1; SCE; micronuclei

1. Introduction

Me-lex is a sequence-specific alkylating agent designed to preferentially (> 90%) generate N3-methyladenine (3-mA) in the minor groove of double-strand DNA mainly in A-T rich regions [1–4]. The interest in this molecule derives from the fact that 3-mA is a very cytotoxic lesion that exerts its action by blocking the progression of the DNA replication machinery [5–10], thus causing cell lethality. This implies that 3-mA generating compounds can be considered promising model molecules for the treatment of actively proliferating cancer cells.

In repair proficient cells, the removal of 3-mA is initiated by alkyladenine DNA glycosylase (AAG), which cleaves the glycoside bond connecting the adducted base to the deoxyribose sugar [11]. The resulting abasic site is recognized by an AP endonuclease that incises the phosphodiester bond 5' to the lesion forming a strand break with a 3' OH and a 5' deoxyribosephosphate terminus. The removal of the abasic residue generates a nucleotide gap, which is filled in by the co-ordinate action of poly (ADP-ribose) polymerase (PARP), DNA polymerase β , XRCC1 and ligase I or III [11]. If encountered during replication, 3-mA may stall DNA replication due to the inability of replicating DNA polymerases to interact with the adducted N3 position of adenine in the DNA [12]. To overcome this block, DNA damage tolerance pathways, such as translesion synthesis (TLS), can be activated to allow survival, albeit with increased mutations [13].

Studies conducted in bacteria, yeast and mammalian cells have demonstrated that Me-lex toxicity is dependent on the DNA repair background. BER deficient *Saccharomyces cerevisiae* strains, lacking 3-methyladenine DNA glycosylase or both Apn1 and Apn2 AP endonucleases, are significantly more sensitive to Me-lex toxicity with respect to the parental strain [14]. Embryonic stem cells derived from *Aag* null mice are hypersensitive to killing by Me-lex, and unrepaired 3-mA induced SCEs, chromosome aberrations, S-phase arrest, p53 induction and apoptosis [15]. The cytotoxic potential of Me-lex has been demonstrated also in tumor cells. In human glioma cells Me-lex is cytotoxic and the deletion of AAG activity increases Me-lex toxicity, indicating that 3-mA is a lethal lesion [16]. In mismatch repair deficient leukemic cells, Me-lex has cytotoxic and clastogenic effects that are increased when PARP is inhibited by 3-aminobenzamide (3-AB) [17]. Interestingly, increased levels of XRCC1 were found both in cells treated with Me-lex and with Me-lex plus 3-AB [17], indicating an involvement of XRCC1 in the processing of 3-mA. Since these cancer cells are resistant to methylating agents inducing O⁶-MeG, such as temozolomide, Me-lex (alone or in combination with PARP inhibitors) could represent a pharmacological strategy for the treatment of tumors resistant to classical methylating agents [18,19].

In the perspective of using 3-mA generating compounds in cancer therapy, it is crucial to define their mutagenic potential, since currently used methylating agents (e.g., temozolomide and procarbazine for gliomas) induce pro-mutagenic DNA adducts and, consequently, may promote the development of secondary tumors derived from the treatment [20]. Me-lex mutagenicity has been addressed in yeast and hamster cells. In yeast, we have shown that Me-lex is poorly mutagenic in a wild-type background and its mutagenicity increases significantly in the absence of AP endonucleases while only slightly in the absence of 3-methyladenine DNA glycosylase [14]. This indicates that a deficiency in the removal of 3-mA is deleterious, but unrepaired AP sites (likely derived from 3-mA) are even more mutagenic. Both in repair proficient and deficient yeast strains, AT-targeted base pair substitutions were the predominant

type of mutation [3,14]. Using the same approach, we have also recently demonstrated an involvement of yeast TLS polymerases Pol ζ , REV1 [13] and Polr η [21] in the fixation of mutations derived from Me-lex induced lesions. Our data indicate that the by-pass of Me-lex induced lesions is a multi DNA-polymerases process that is most effective when all three yeast TLS polymerases are present.

In mammalian cells, Me-lex is cytotoxic but poorly mutagenic, resulting only in a 7-fold increase (relative to background) of the *Hprt* mutation frequency. However, in the mutation spectrum at the *Hprt* locus, there was a high percentage of genomic deletions [22], while base pair substitutions in the *Hprt* coding region represented only 25% of the mutagenic events. This unexpected result led us to hypothesize that a large proportion of the rare mutations in mammalian cells may result from the processing of 3-mA within A/T rich sequences in non-coding regions of the *Hprt* gene. Due to the specific reactivity of Me-lex towards nucleophilic sites in the DNA minor groove, the possible clustering of these lesions in A/T rich sequences could represent a strong impediment to the progression of DNA replication forks, thus promoting genomic rearrangements and/or large deletions [22].

The observation of a high level of genetic deletions in DNA repair proficient mammalian cells suggests that other enzymes (and/or other repair pathways) besides DNA glycosylases may have a role in the mutagenic processing of 3-mA (or a subsequent derivative) in genomic regions with high A/T content. During BER, AP sites arising spontaneously from the action of glycosylases are enzymatically converted into single strand breaks (SSBs). DNA intermediates with a strand break are potentially deleterious since they can lead to double strand breaks (DSBs) and recombination. XRCC1 plays a crucial role in the coordination of BER and SSB repair (SSBR), through its interaction with several key enzymes involved at different steps of these pathways (i.e., Pol β , PARP, Ligase III) [23,24]. In addition to protein-protein interactions, XRCC1 can bind different DNA substrates, such as gapped and nicked DNA [25] and AP sites [26]. For these different activities, it has been proposed that XRCC1 could optimize the passage of DNA substrates from one enzyme to the next in the pathway as a scaffold that co-localizes the proteins through its different interacting domains [27].

XRCC1 deficient cell lines are hypersensitive to monofunctional alkylating agents and exhibited roughly a 10-fold higher level of spontaneous SCEs [28]. The EM-C11 cell line, isolated on the basis of its high sensitivity to killing by ethyl methanesulfonate (EMS), has a defect in the rejoining of SSBs after exposure to X-rays and EMS [28]. The molecular characterization of *Hprt* mutants isolated in EM-C11 cells following EMS or methyl methanesulfonate (MMS) treatments revealed a higher incidence of deletions compared to their parental repair proficient CHO-9 cells, suggesting that XRCC1 deficiency may lead to the formation of genomic deletions following exposure to alkylating agents [29,30].

In order to determine the role of XRCC1 in the cytotoxicity and mutagenicity of Me-lex, the spontaneous and the Me-lex induced mutation spectra at the *Hprt* locus were obtained in the EM-C11 cell line. In addition, cytogenetic assays were conducted in EM-C11 cells, and in its parental CHO-9 cell line, in order to investigate the ability of Me-lex to induce SCEs, micronuclei (MN) and other types of genomic damage.

2. Materials and Methods

2.1. Hazardous procedures

Me-lex should be considered a toxic compound, and was handled accordingly.

2.2. Compounds

Reagents of the highest purity were purchased from Sigma (St. Louis, MO, USA) or Aldrich (Milwaukee, WI, USA) unless otherwise stated. Me-lex was prepared as previously described [1].

2.3. Cell culture conditions

The Chinese hamster ovary CHO-9 and EM-C11 cell lines (obtained from M. Zdzienicka, Leiden University Medical Center, NL) were grown in Ham's F10:D-MEM (GIBCO, Invitrogen, Milano, Italy) 1:1, supplemented with 10% fetal calf serum (Euroclone, Milano, Italy), 100 IU/ml penicillin and 0,1 mg/ml streptomycin (MP Biomedicals, Irvine), in a humidified incubator at 37 °C with 5% CO₂. In order to reduce pre-existing spontaneous mutants, cells were treated with HAT medium (Ham's F10:D-MEM 1:1 supplemented with 10⁻⁴ M hypoxanthine, 10⁻⁶ M aminopterin and 10⁻⁵ M thymidine, Sigma Aldrich, Milano, Italy).

2.4. Mutation experiments

Independent populations starting from 1000 cells were isolated and one population was used for each experiment. For each treatment, Me-lex stock solution (10 mM) was prepared by dissolving 1 mg of Me-lex in 20.7 µl DMSO and 200 µl EtOH. Survival and mutagenicity were evaluated up to 150 µM Me-lex. Mutagenicity experiments were performed as described by Russo et al. [22]. For the isolation of *Hprt* mutants, 12×10⁶ EM-C11 cells were exposed to 75 µM Me-lex. Soon after treatment, the cultures were split into 30 parallel subcultures to ensure the growth of independent mutants. Simultaneously, the spontaneous mutant frequencies were determined in order to calculate the level of mutant induction. Spontaneous *Hprt* mutants were isolated and analyzed in parallel with those induced by Me-lex. The mutant frequency was expressed as the number of mutants per 10⁵ clone-forming cells.

2.5. Molecular analysis of *Hprt* mutants

The molecular analysis of *Hprt* mutants was performed as described [22]. Briefly, total RNA was isolated from 2×10⁶ cells of each mutant (RNeasy Mini Kit, Qiagen, Italy). The *Hprt* cDNA was synthesized in 20 µl volumes containing 1 µg RNA (Reverse-iT 1st strand Synthesis kit, AB Gene). Approximately 20% of the cDNA product was used as template for PCR to amplify the entire *Hprt* coding sequence or, when the complete *Hprt* cDNA sequence was not obtained, the 5' - and the 3' -ends of the coding sequence to verify the presence of shorter cDNAs [22]. PCR products were gel purified and sequenced (BMR Genomics, CRIBI, Padova, Italy). In order to analyze mutants missing one (or more) exon(s) from the *Hprt* cDNA, a crude cell lysate was used as source of genomic DNA for amplification of single exons using primers lying in the introns flanking the missing exons. A multiplex PCR-based amplification of all nine exons was carried out with primers described by Xu et al., [31]. PCR products were displayed by electrophoresis on 4% agarose gel.

2.6. MN assay

The day before treatment, cells were seeded in 35-mm dishes with coverslips. Cells were cultured at densities of 2.5–10×10⁴ cells/coverslip (about 6×10³ cells/cm²), using conditions ensuring that cells were actively growing after 1 h treatment. At the appropriate harvest time, cells were rinsed and exposed to a hypotonic shock *in situ* using Iskandar solution (0.9% NaCl/75 mM KCl; 9:1). Cells were then fixed with MeOH/HOAc (3:1) and stained with Giemsa. All slides including those of positive and solvent controls were coded before analysis and scored blindly for the evaluation of MN by two independent observers. The criteria used for identifying MN fulfil those recommended by the HUMN work [32]: (1) area < 1/3 the main

nucleus area; (2) no overlapping with the nucleus (distinct borders); and (3) same aspect as the chromatin.

2.7. Measurement of SCE levels

Cells seeded onto 20×20 coverslips and treated as above, were cultured in the presence of 50 μ M BUdR for two cell cycle periods in the dark, and pulsed with colcemid 0.1 μ g/mL for the last 2 h. Cells were then treated *in situ* with 75 mM KCl for 20 min and subsequently fixed with MeOH/HOAc (3:1) for 15 min. To visualize SCEs, FPG technique was employed [33]. Briefly, dried slides were incubated with 5 μ g/mL of Hoechst 33258 in Sorensen's buffer (pH 6.8) for 15 min, rinsed with the same buffer and irradiated with black light (UVP Upland UVGL-15 lamp, 365 nm, arranged at a distance of 6 cm above each specimen) for 30 min. After incubation in Sorensen's buffer at 60 °C for 1 h, slides were stained with 2.5 % Giemsa solution in the same buffer. Coded slides were scored blindly by three independent observers. Twenty metaphases per each experimental point were scored.

3. Results

3.1. XRCC1 deficiency increases Me-lex cytotoxicity but not mutagenicity

XRCC1 deficient EM-C11 cells were treated with increasing Me-lex concentrations to determine the survival curve and the mutation frequency (MF) at the *Hprt* locus. Results were compared to those obtained in the CHO-9 parental cell line [22]. As shown in Figure 1, based on LD₅₀ values, EM-C11 cells were 2.5-fold more sensitive to Me-lex compared to the parental CHO-9 cells. A low mutability of EM-C11 cells was found (Table 1) since the MF increased significantly over the background only at 100 μ M Me-lex ($p=0.0357$), a dose that reduced the viability to less than 10%, and did not increase even when cell survival was below 1% (Table 1). When compared to the parental repair proficient CHO-9, the EM-C11 cells did not show a significantly higher mutability following Me-lex exposure (Table 1). Thus, the XRCC1 deficiency affected the cytotoxicity but not the mutation frequency induced by Me-lex.

3.2. XRCC1 deficiency affects the frequency of genomic deletions in untreated EM-C11 cells

A spontaneous mutation spectrum was generated in order to characterize the mutations arising as a result of XRCC1 deficiency *per se*. Ten independent EM-C11 sub-populations starting from 1000 cells were isolated and, for each sub-population, the spontaneous *Hprt* MF (sMF) was determined and *Hprt* mutant clones isolated and characterized (Table 2). Low sMFs were found in every sub-population with an average of $0.13 \times 10^{-5} \pm 0.07$. Two sub-populations were further expanded to simultaneously isolate Me-lex induced and spontaneous mutants.

The analysis of spontaneous or Me-lex induced *Hprt* mutations was first performed on cDNA level by PCR amplification [34,35]. The mutants that generated short cDNA amplicons or that did not give a PCR product were further analyzed by sequencing the intron/exon boundaries and by multiplex PCR [22]. Mutants with all *Hprt* exons present in genomic DNA but which produced either very low amounts of shorter cDNA or no cDNA products were classified as mutants with “mutations in regulatory regions”.

In the spontaneous spectrum (Table 2), 9 mutants carried base pair changes, 6 GC-targeted and 3 AT-targeted. Two mutants had frame shifts and 3 mutants showed the skipping of exons 2 and 2+3. The spontaneous spectrum showed a high number of genomic deletions encompassing at least two *Hprt* exons. Three mutants did not give a cDNA product even though all exons were present at the genomic level and 1 mutant produced a longer cDNA due to a duplication of exons 2+3.

3.3. Molecular analysis of Me-lex induced *Hprt* mutants in EM-C11 cells

Hprt-deficient mutants were isolated at 75 μ M Me-lex, a dose inducing almost a 5-fold increase of the MF over background (see Table 1). Due to the low mutagenic but high cytotoxic potential of Me-lex, we decided not to use higher Me-lex concentrations, which would have greatly reduced the possibility of isolating mutants. Besides, treatments from 75 μ M up to 150 μ M of Me-lex resulted in approximately the same MF. Fifty-six Me-lex induced *Hprt* mutants were isolated at 75 μ M Me-lex.

In the Me-lex induced mutation spectrum (Table 3), 7 mutants carried AT-targeted base pair changes, 2 had frameshift mutations involving A/T bases and 9 carried mutations that generated exon skipping. The mutation responsible for exon skipping was identified for 7 mutants, 6 of which presented a mutated G at the donor or acceptor splice site. In all these cases, a very AT-rich sequence was adjacent to the site of mutation suggesting a Me-lex sequence selective induction. Genomic deletions were found in 11 mutants, including three-base deletions, single exon deletions, and deletion of all *Hprt* exons. In two cases, namely EG-13B and EG-21B, the deletion likely extended far from exons 4 or 5, respectively, since no cDNA products were detected. This result suggests that introns 4 and 5 probably contained A/T rich sequences important for *Hprt* mRNA processing or stability. The largest class of mutations comprised a heterogeneous group of 16 mutants (defined as “mutations in regulatory regions”) that produced low amounts, if any, of shorter length cDNAs that were better characterized using nested primers for the presence of the 5'-end, or the 3'-end of the *Hprt* coding sequence. However, all *Hprt* exons were present at genomic level. One mutant (EN-23A) carried a deletion of 5 base pairs in exon 2 that induced partial skipping of exon 2 resulting in the amplification of both the full-length and the shorter cDNA. Ten out of the 56 Me-lex induced *Hprt* mutants carried mutations that had been already observed in the spontaneous spectrum. Those mutants, being about 18% of all mutants isolated after Me-lex treatment, represent the portion of mutants that are expected to be of spontaneous origin based upon the 5-fold increase of the MF after Me-lex, and were not considered Me-lex induced. Therefore, they were excluded from further analysis.

3.4. Me-lex induces SCEs, MN and nuclear fragmentation

Previous results in CHO-9 cells led us to hypothesize that the processing of 3-mA within A/T rich sequences in non-coding regions of the *Hprt* gene could result in recombination and genomic deletions [22]. To further investigate this issue, we performed a genome-wide analysis of Me-lex genotoxicity through the determination of SCEs and MN in both CHO-9 and EM-C11 cell lines.

In CHO-9 cells, SCE induction was evaluated at a range of 25–100 μ M Me-lex concentrations, which induced low levels of mortality (80–90% of cell survival) [22]. The number of SCEs per chromosome increased 3-fold from mock to 50 μ M Me-lex treated cells (Figure 2A). At 100 μ M Me-lex, a high percentage of fragmented nuclei and a very low number of metaphases were counted (see below), making the evaluation of SCEs unreliable. MMS, an alkylating agent known to induce SCEs and chromosomal aberrations [36,37], was used as positive control at equitoxic (sub-lethal) concentrations [30]. In MMS-treated cells a dose dependent induction of SCE up to 250 μ M was found (Figure 2B), demonstrating the robustness of the assay. Me-lex preferentially (> 90%) generates 3-mA and at equimolar concentrations of Me-lex and MMS, the induction of other SCE-inducing lesions, e.g., 7-mG and 6-mG, is comparable. Since the yield of 3-mA is approximately 40-fold higher with Me-lex treatment than MMS [2], these results are consistent with 3-mA (or its derived BER intermediate) being responsible for the induction of SCEs.

The induction of MN [38,39], as well as the appearance of fragmented and apoptotic nuclei were also investigated. As shown in Figure 2C, 100 μ M Me-lex induced a sharp increase in the percentage of MN and fragmented nuclei, and the appearance of apoptotic nuclei. In MMS-treated cells, the percentage of MN increased at 100 μ M (14.7 %) but did not reach the level detected for Me-lex even at the highest MMS concentration tested (i.e., 500 μ M MMS; 28.2 %) (Figure 2D).

The same end points were also explored in EM-C11 cells. This cell line is known to have a high background level of SCEs and chromosomal aberrations [28]. In agreement with published data [28], in mock-treated EM-C11 cells about a 10-fold higher level of SCEs (0.939 ± 0.182) than in CHO-9 cells (0.129 ± 0.027) was found. In addition, the levels of multinucleated cells and MN were significantly higher in EMC-11 than in CHO-9 cells (Figure 3A). In Me-lex treated EM-C11 cells, aberrant mitosis and a high level of fragmented nuclei were detected at very low doses (Figure 3B). These results indicate that XRCC1 deficiency may lead to genomic instability in the absence of an exogenous genotoxic insult, and that in this cellular environment even low levels of Me-lex induced lesions, i.e., 3-mA (or its derivatives), are sufficient to generate a large amount of genomic damage.

4. Discussion

The effect of XRCC1 deficiency in the processing of 3-mA adducts generated by Me-lex was explored through the molecular analysis of *Hprt* mutations and the determination of cytogenetic end points, including SCEs, MN and nucleus fragmentation in Me-lex treated EM-C11 and the parental DNA repair proficient CHO-9 cells.

EM-C11 cells exposed to Me-lex showed a 2.5-fold higher sensitivity to toxicity than their DNA repair proficient counterpart (CHO-9), but they were not hypermutable. A comparable level of mutability, despite their different sensitivities, has been already reported for these two cell lines following treatment with EMS [29] and MMS [30]. Our results strongly suggest that unrepaired 3-mA (or a derived AP site) is a lethal lesion and that XRCC1 is involved in its repair, probably by facilitating the processing of BER intermediates.

In order to characterize the mutations arising as a result of XRCC1 deficiency *per se*, a spontaneous mutation spectrum at the *Hprt* locus was generated in EM-C11 cells. When compared with the spontaneous mutation spectrum obtained in the parental CHO-9 cell line ([22] and unpublished data), the two spectra appear significantly different ($p=0.0016$; Cariello's test [40]) especially in terms of genomic deletions ($p=0.0054$; Fisher's exact test) (Table 4). These results clearly indicated that XRCC1 deficiency *per se* causes genomic instability leading to deletions. After Me-lex treatment, mutations in regulatory regions reached a level of 35% with respect to the 10.5% in the spontaneous spectrum ($p=0.0277$ Fisher's exact test). This class of mutations was defined in a previous study [22] as mutations that produced low levels and/or short *Hprt* cDNAs without affecting the genomic sequence of *Hprt* exons. The mutations responsible for the formation of short or unstable mRNAs are likely localized in non-coding regulatory regions of the *Hprt* gene [22]. Regulatory regions may contain large stretches of A/T-repeats that constitute the preferential target for Me-lex binding and methylation [3]. Interestingly, within the whole *Hprt* mouse genomic sequence, A/T-runs in introns are abundant (<http://www.ensembl.org>).

The Me-lex induced mutation spectrum in EM-C11 cells differs from the one in CHO-9 cells ($p=0.0157$, Cariello's test) (Table 4). The number of splice mutations in EM-C11 is significantly higher than in CHO-9 ($p=0.0153$; Fisher's exact test). In these mutants, an A/T-rich sequence is adjacent to the site of base pair change suggesting a sequence specific methylation by Me-lex. A-tracts occur often in regulatory regions of many organisms [41] and

it has been postulated that their ability to adopt unusual DNA conformation may be linked to their participation to regulatory processes. An unexpected result from our studies is the low level of genomic deletions found in EM-C11 vs. CHO-9 cells (24% vs 48%). Notably, in EM-C11, a high level of genomic deletions occurred without treatment (38%) and did not increase after Me-lex exposure (24%). Since XRCC1 deficient cells are spontaneously prone to accumulate genomic deletions as well as high levels of SCEs and MN, a Me-lex treatment, which further increases the overall burden of SSBs, may generate large genomic deletions detrimental for cell viability. Besides the presence of multinucleated cells, aberrant mitosis and fragmented nuclei were generated at very low Me-lex doses (25 μ M). At 50 μ M a lower number of multinucleated cells were counted but the mitotic index was also very low (not shown) making the determination of these cytogenetic endpoints not statistically reliable. It is likely that at the 75 μ M Me-lex dose used to isolate mutants, only cells carrying less severe genomic damage could be recovered.

Comparison of the spontaneous and induced mutation spectra in EM-C11 cells and in Me-lex treated EM-C11 and CHO-9 cells indicate that in cells lacking XRCC1, Me-lex promotes the induction of mutations in non-coding *Hprt* regions crucial for the regulation and/or stability of mRNA processing. All together these data suggest that non-coding *Hprt* genomic sequences represent a good target for the rare pro-mutagenic lesions induced by Me-lex, and such a specificity is exacerbated by *Xrcc1* gene dysfunction. These results could also be attributed to an intrinsic instability of these repetitive regions that may become even more unstable in a XRCC1 deficient cell.

The ability of Me-lex to induce recombination and genomic rearrangements [22] was confirmed through the analysis of SCE and MN induction in CHO-9 cells. The formation of SCEs has been correlated with recombination repair and the induction of point mutations, gene amplification and cytotoxicity [42]. Our results clearly show that even in a repair proficient background, i.e., CHO-9 cells, Me-lex induced SCEs in a dose-dependent manner. A comparable level of basal SCEs was found in both AAG proficient and AAG deficient ES cells [15], indicating that AAG deficiency *per se* does not affect spontaneous recombination. After Me-lex exposure, a significant increase of SCEs was found in *Aag*^{-/-} cells providing evidence that 3-mA was the lesion responsible for SCE induction [15]. The appearance of MN, fragmented and apoptotic nuclei was also analysed. MN assay has emerged as one of the preferred methods for assessing chromosome damage because it enables both chromosome loss and chromosome breakage to be measured reliably [38,39]. Our results showed that sub-lethal Me-lex concentrations were able to induce a significant increase of MN, fragmented nuclei and apoptotic cells. When cells were treated with MMS, a lower induction of the same endpoints was observed, suggesting a direct role of A/T-targeted 3-mA lesions in chromosome damage induction. In a recent study conducted in yeast, a large number of unselected chromosomal aberrations arising from randomly induced DSBs in the entire *Saccharomyces cerevisiae* genome has been analysed. Interestingly, only repeat-associated DSBs could efficiently lead to chromosome aberrations, while DSBs in unique sequences were efficiently repaired through homologous recombination [43]. Because MN can only be expressed in cells that complete nuclear division, Me-lex concentrations that poorly affect the overall survival have to be used to perform this assay. Nevertheless, the cytogenetic analyses revealed a significant genotoxic effect of Me-lex that can explain its high cytotoxicity.

To reconcile all our data, we propose a model for Me-lex cytotoxicity and mutagenicity in mammalian cells. We emphasize that any consideration should take into account the specific reactivity of Me-lex towards A/T-rich sequences. In repair proficient cells, 3-mA is efficiently removed by AAG and processed through the subsequent steps of the BER pathway. If 3-mA (or one of its BER derivatives) is encountered during DNA replication it may stall DNA replication; in these circumstances, TLS can be involved and the lesion bypassed with the

enhanced induction of base pair mutations (mainly A/T-targeted). However, the processing of 3-mA specifically localized in A/T-rich regions may promote the formation of clustered SSBs and even generate an irreversible block of DNA replication that stimulates homologous recombination. If we assume that regulatory regions are characterized by the presence of A/T-rich sequences, the presence of 3-mA could originate a high percentage of mutations that affect mRNA stability (mutations at regulatory regions and splice mutations). At low Me-lex doses (25–50 μM) these events will promote DNA recombination, visualized as SCEs. As the level of 3-mA increases, double strand breaks may be generated, which would promote deletions, MN, fragmented nuclei and apoptosis. In a XRCC1 deficient background, cells have to manage the burden of SSBs spontaneously generated genome-wide. The presence of 3-mA or other repair intermediates from Me-lex exposure, in addition to spontaneous SSBs, would cause DNA replication forks to collapse resulting in extensive genomic damage even at a low Me-lex concentrations.

In summary, we have shown that XRCC1 deficiency increases Me-lex cytotoxicity and affects the type of mutations induced at the *Hprt* locus without increasing the Me-lex induced MF. Our results indicate that the processing of 3-mA, or one of its BER derivatives, is a complex process where DNA repair processes, besides BER, may participate. With this perspective, it will be important to explore other repair pathways, such as homologous recombination and nonhomologous end joining, that may contribute to the error-free repair of 3-mA and the modulation of Me-lex cytotoxicity.

Abbreviations

Me-lex	{ 1-methyl-4-[1-methyl-4-(3-methoxysulfonylpropanamido) pyrrole-2-carboxamido]-pyrrole-2-carboxamido}propane
XRCC1	(X-ray repair cross-complementing 1).

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Figure 1. Survival curve in EMC-11 and CHO-9 cell lines after Me-lex treatment
The survival curve in EMC-11 (♦) and the parental CHO-9 (□) cells after Me-lex treatment are reported. Each point represents the mean $\pm$ s.d. of 5 independent experiments.

Figure 2. SCE, MN and nuclear fragmentation induced by Me-lex and MMS in CHO-9 cells
The number of SCE per chromosome induced in CHO-9 cells is reported for Me-lex (A) and MMS (B). The number of MN, fragmented nuclei and apoptotic cells, over 1000 scored cells, are reported for Me-lex (C) and MMS (D) treated CHO-9 cells.

Figure 3. Cytogenetic analysis of Me-lex treated EMC -11 cells

A) Multinucleated cells and MN spontaneously induced in CHO-9 and EM-C11 cells. B) Multinucleated cells, aberrant mitosis and fragmented nuclei in EM-C11 cells before and after Me-lex treatment. Insert in the top right of panel B shows a magnification of a micronucleated cell. MultiN = multinucleated cells; AbMi = aberrant mitoses; AbMe = aberrant metaphase; MN = micronucleus; FN = fragmented nucleus; G = ghost.

Table 1 Cell survival and *Hprt* mutation frequency induced by Me-lex in EM-C11 and CHO-9

Me-lex dose	Survival	Mut Freq	
	Survival CHO-9 ^a	Mut	
Freq CHO-9 ^a			
(μ M)	%	($\times 10^{-5}$) ^b	
	%	($\times 10^{-5}$) ^b	
0	100	0.5 $\pm$ 0.2	
	100	0.4 $\pm$ 0.3	
25 ^c	89.2 $\pm$ 11.4	1.6 $\pm$ 0.4	<i>N.S.</i>
50 ^c	57.8 $\pm$ 10.1	1.8 $\pm$ 0.8	<i>N.S.</i>
75 ^c	19.2 $\pm$ 8.7	2.7 $\pm$ 0.9	<i>N.S.</i>
100	6.1 $\pm$ 3.1	2.8 $\pm$ 1.5	
<i>p</i> = 0.0357	80.0 $\pm$ 11.7	1.1 $\pm$	
0.7			
150	0.4 $\pm$ 0.5	2.8 $\pm$ 2.2	<i>p</i> =
0.0357	40.8 $\pm$ 18.7	1.6 $\pm$ 0.9	
200			
	13.3 $\pm$ 8.2	2.5 $\pm$ 1.4	
250			
	2.9 $\pm$ 2.3	0.9 $\pm$ 0.9	

^aFrom Russo et al., 2009^bThe *Hprt* mutation frequency is expressed as the number of 6-TG-resistant clones per 10⁵ clone-forming cells. Each point represents the mean $\pm$ s.d. of 5 independent experiments for survival and 3 experiments for mutation induction. Non-parametric Mann-Whitney test was used for statistical analysis.^cFor these doses, only two repetitions could be used for statistical analysis and the comparison was not statistically significant (*N.S.*).

Table 2

Spontaneous *Hprt* mutation spectrum in EM-C11

Mutant/(s) sequence ^b	cDNA product ^a Aminoacid change	Position (exon) mutants	N° of	Type of mutation	Target
>3')	(or cDNA change)				(5'
Base pair changes					
EG-sp2/3	full length	118 (2)		GC>TA	CCT
CAT GGA GTG ATT	Gly>STOP		2		
EG-8/-10	full length	118 (2)		GC>TA	CCT CAT
GGA GTG ATT	Gly>STOP	2 ^c			
EG-spl A/1B	full length	139 (3)		GC>TA	AGG
ACT GAA AGA CTT	Glu>STOP		2		
EB-17	full length	533 (8)		AT>GC	CAG ACT
TTT GTT GG	Phe>Ser	1 ^c			
EC-7	full length	595 (8)		AT>TA	GAG TAC
TTC AGG GAT	Phe>Ser	1 ^c			
EN-13	full length	614 (9)		AT>GC	AAT CAT
ATT TGT GTC	Ile>Thr	1 ^c			
Frameshift mutations					
EN-sp17	very low, full length	207-212 (3)		+G	GTG CTG
AAG GGG GGC TAT		1			
EC-13		112/113		-C	TTT ATT
CCT CAT GGA		1 ^c			
Splice mutations					
EN-sp9	very low, short cDNA				Splice junctions not sequenced
	Skipping ex 2+3	1			
EA-6/-9	short cDNAs				All exons present on gDNA
	Skipping exon 2/2+3	2 ^c			
Genomic Deletions					
EN-sp1/5/10	short cDNA				Deletion of exon 2+3

Mutant/(s) sequence ^b	cDNA product ^a Aminoacid change	Position (exon) mutants	Type of mutation	Target (5')
>3'	(or cDNA change)	3		
EH-5			Deletion exs 6–8 on gDNA	
	Skipping ex 6–8/Ins 58 bp	1 ^c		
EG-sp4/13	no cDNA		Deletion of all <i>Hprt</i> exons	
EF-8/–10	no cDNA	2		
			Deletion of all <i>Hprt</i> exons	
EH-3		3 ^c		
EL-1 other <i>Hprt</i> exons	no cDNA		Deletion ex 7–8 on gDNA. Low amplification of	
		1 ^c		
EL-6	no cDNA		Deletion ex 7–8 on gDNA	
Mutations in regulatory regions				
EN-sp 15/19/20	no cDNA		All exons present on gDNA	
		3		
Other mutations				
EN-sp13	longer cDNA		Duplication of exons 2+3	
		1		

^a cDNA amplifications of the full lenght, the 5'- (ex 1–4) and the 3'- (ex 4–8) half of the *Hprt* coding sequence were performed for each mutant (see M&M). Short cDNA refers to PCR fragments lacking the internal part of the *hprt* coding sequence.

^b Non transcribed strand gDNA = genomic DNA

^c Spontaneous mutants isolated from EM-C11 independent populations before treatment

Table 3

Melex induced *Hprt* mutation spectrum in EM-C11

Mutant(s) sequence ^b	cDNA product ^a Aminoacid change	Position N° of	Type of mutation (5' > 3')	Target
cDNA change				
Base pair changes				
EN-15A	full length	110 (2)	TA>CG	GTG TTT
ATT CCT CAT	Ile>Thr	1		
EN-11A	full length	296 (3)	TA>GC	GTA GAT
TTT ATC AGA	Phe>Cys	1		
EN-20C	full length	437 (6)	TA>GC	CAA ACT
CTG CTT TCC	Leu>Arg	1		
EN-25C	full length	466 (6)	AT>TA	AAC CCC
AAA ATG GTT	Lys>STOP	1		
EN-5A	full length	479 (6)	TA>AT	GTT AAG
GTT GCA AGC	Val>Asp	1		
EN-6B	full length	543 (8)	TA>GC	GTT GGA
TTT GAA ATT	Phe>Leu	1		
EG-18B	full length	545 (8)	AT>GC	GGA TTT GAA
ATT CCA	Glu>Gly	1		
Frameshift mutations				
EN-29A	low, full length	294-297 (3)	-T	CT GTA GAT
TTT ATC AG		1		
EN-19B	low, full length	408-409 (6)	+AA	AG GAC ATA
ATT GAC AC		1		
Splice mutations				
EN-28A	short cDNA	In 3: +1	GC>AT	TACTGT g
taagataatt	Skipping ex 3	1		
EN-2B/-14C	short cDNA	In 3: -1	GC>CG	tttttaacta g
AATGAT		Skipping ex 4		
/-17B/-21A		5		

Mutant(s)	cDNA product ^a	Position	Type of mutation	Target
sequence ^b	Aminoacid change	N° of (exon)	(5' > 3')	(or)
cDNA change)				
<i>/-27C</i>				
EN-30B	short cDNA	In6: +2	TA>GC	TGCAAGg <u>t</u>
atgatgcc	Skipping ex 6	1		
EN-3C/-7B	short cDNA		Splice junctions not sequenced	
	Skipping ex 2	2	All exons present in gDNA	
Genomic deletions				
EN-1C	full length	81-83 (2)	-CTA	AAT CAC
<u>TAT</u> GTC GAG	-Tyr	1		
EG-3A/-30A	full length	565-567 (6)	-GTT	TTT GTT <u>GTT</u>
GGA TAT	-Val	2		
EN-10A	low, two short cDNAs		Deletion of exon 3	
EG-13B	no cDNAs	1	Deletion of exon 4	
EG-21B	no cDNAs	1	Deletion of exon 5	
EG-2B/-8B	no cDNAs	1	Deletion of all <i>Hprt</i> exons	
<i>/-24C/-27B</i>		5		
<i>/-28C</i>				
Mutations in regulatory regions				
EN-12A/-24A	no cDNAs		All exons present on gDNA	
EN-22B	very low, short cDNA	2	All exons present on gDNA	
	Skipping ex 4	1	No mutations found at splice junctions	
EG-4A/-5C	low ex (1-4)		All exons present on gDNA	

Mutant/(s) sequence ^b	cDNA product ^a Aminoacid change	N° of	Position (exon)	Type of mutation	Target
mutants					
8					
/-16A/-20B					
/-21A/-22C					
/-25A/-26C					
EG-6B					Low gDNA ex 1. All other exons present on
gDNA		1			
EG-9A	low cDNA ex (3-9)			All exons present on gDNA	
EG-12A/-19B	no cDNA s	1		All exons present on gDNA	
/-29A		3			
Other mutations					
EN-23A	two cDNA: full length,			Deletion (28-32) in exon 2	tttttcag
ATTAGT GAT		1			
		short			
		Skipping ex 2			

^a cDNA amplifications of the full length, the 5'- (ex 1-4) and the 3'- (ex 4-8) half of the Hprt coding sequence were performed for each mutant (see M&M). Short cDNA refers to PCR fragments lacking the internal part of the hprt coding sequence.

^b Non transcribed strand gDNA = genomic DNA

Table 4 Comparison among *Hprt* mutation spectra in EM-C11 and CHO-9 cell lines

		EM-C11	
		CHO-9 ^a	
Type of <i>Hprt</i> mutations induced	Spontaneous	Spontaneous Me-lex induced	Me-lex
	n° (%)	n° (%)	n° (%)
Base pair changes		9 (31%)	7
(15%)	16 (43%)	10 (23%)	
AT-targeted		3 (10%)	7
(15%)	12 (32%)	7 (16%)	
AT>TA		1	2
	1	2	
AT>GC		2	2
	9	4	
AT > CG		0	3
	2	2	
GC-targeted		6 (21%)	0
	4 (11%)	3 (7%)	
GC > AT		0	0
	0	1	
GC > CG		0	0
	1	0	
GC > TA		6	0
	3	2	
Splice mutations^b		3 (10%)	9
(19%) ^c	1 (3%)	1(2%)^c	
Frame shifts		2 (7%)	2
(4.3%)	0	3 (7%)	
Genomic deletions		11 (38%)^d	11
(24%)	3 (8%)^d	21 (48%)	
Exon deletions		6 (21%)	6
(13%)	3	9 (21%)	
Complete <i>Hprt</i> deletions		5 (17%)	5
(11%)	0	12 (27%)	
Mutations in regulatory regions		3 (10.5%)^e	16
(35%) ^e	12 (32%)	8 (18%)	
Other		1 (3.5%)	1
(2%)	5 (14%)	1 (2%)	
Total n° of mutations		29 (100%)	46

		EM-C11	
		CHO-9 ^a	
Type of <i>Hprt</i> mutations induced	Spontaneous	Spontaneous	Me-lex
		Me-lex induced	
	n° (%)	n° (%)	n° (%)
	n° (%)	n° (%)	
(100%)	37 (100%)	44 (100%)	

^aFrom Russo et al., 2009 and data unpublished

^bThis category includes splice mutants for which the mutation at splice junction was found

^c $p=0.0153$ (Fisher's exact test)

^d $p=0.0054$ (Fisher's exact test)

^e $p=0.0277$ (Fisher's exact test)