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High frequency of genomic deletions induced by Me-lex, a sequence selective N3-adenine methylating agent, at the *Hprt* locus in Chinese hamster ovary cells

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Abstract

We have investigated the mutagenicity induced at the *Hprt* locus in Chinese hamster ovary (CHO) cells treated with increasing concentrations of Me-lex, a minor groove selective methylating agent that efficiently generates more than 90-95% of 3-MeA DNA adducts. Me-lex treatment was cytotoxic but weakly mutagenic, resulting in up to 7-fold induction above background in the *Hprt* mutation frequency. The molecular nature of 43 *Hprt* mutations induced by Me-lex was determined by sequence analysis of the *Hprt* cDNA and genomic analysis of the gene locus. Base pair substitutions represented about 25% of Me-lex induced mutations. The mutation spectrum revealed a high percentage of genomic deletions (51%) comprising single/multiple exon(s) and even the loss of the complete locus. When the distribution of mutations among different classes was considered, the difference between the spontaneous and Me-lex induced CHO spectra was statistically significant ($p < 0.012$), indicating that the sites where mutations occurred were Me-lex specific. Based upon these results we hypothesize that a large proportion of mutations may result from the processing of 3-MeA, the main adduct induced by Me-lex, within A/T rich sequences in non-coding regions of the *Hprt* gene. The processing of these lesions by DNA polymerases could result in recombination and genomic deletions, thus representing a severe threat for genome integrity.

Keywords

Me-lex; mutation induction; *Hprt*; hamster cells

1. Introduction

Many antineoplastic agents currently used in cancer therapy generate a wide panel of DNA lesions with different mutagenic and/or toxic potential. One serious complication associated

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Conflict of interest: None

with the use of alkylating chemotherapeutic agents is the induction of pre-mutagenic lesions that could give rise to therapy-related secondary tumors [1].

Me-lex is a methylating agent synthesized to preferentially generate N3-methyladenine (3-MeA). Unlike other methylating agents (*i.e.*, temolozolomide, Streptozotocin, MMS, MNU) that induce highly mutagenic adducts, such as 7-MeG and O6-MeG, Me-lex induces more than 90-95 % of 3-MeA, a DNA lesion that is highly cytotoxic and poorly mutagenic [2,3]. Furthermore, due to its minor groove selectivity conferred by the lexitropsin dipeptide, Me-lex-induced adducts are localized mainly in A-T rich regions [4].

The cytotoxic potential of Me-lex has been demonstrated both in *E. coli* and in mammalian cells, and a correlation between the level of 3-MeA and cell death has been demonstrated [5-7]. Studies in mouse embryonic stem (ES) cells showed that 3-MeA is also the major DNA base adduct formed by Me-lex in cultured cells and that unrepaired 3-MeA lesions induced sister chromatid exchanges, chromosome aberrations, S-phase arrest, p53 induction and apoptosis [8]. More recently, the cytotoxic potential of Me-lex has been demonstrated in mismatch repair deficient leukemic cells and in human glioma cell lines [9-11]. In those reports, the use of 3-MeA inducing compounds was suggested as a promising pharmacological strategy for the treatment of tumors resistant to classical wide-spectrum methylating agents.

Although Me-lex cytotoxicity has been documented in several experimental systems, its mutagenicity has been extensively studied only in yeast [4,12,13]. The Me-lex induced mutation spectrum was determined with a functional assay after *in vitro* treatment of a plasmid expression vector harbouring the human wild type p53 cDNA, followed by transformation in yeast strains containing the ADE2 gene regulated by a p53 response element. In parallel, the Me-lex induced methylation pattern was determined *in vitro* at the same human p53 cDNA sequence in order to make a correlation between sites of methylation and sites of mutation [4]. The results obtained with this combined approach showed that Me-lex with low frequency induced mutations that consisted mainly in AT-targeted base pair changes, with AT>TA transversions being the predominant class of base pair substitutions. The methylation analysis elegantly confirmed the almost exclusive reactivity of Me-lex at adenines within or adjacent A/T rich sequences. However, with the exception of few hot spots, there was minimal overlap between methylated and mutated bases indicating that, in this experimental system, heavily adducted sites are not necessarily converted into base pair substitutions [4]. Other results obtained in repair deficient yeast strains demonstrated that Me-lex toxicity and mutagenicity are dependent on the DNA repair background. Base excision repair (BER) deficient *S. cerevisiae* strains, lacking 3-methyladenine DNA glycosylase or both AP endonucleases, are significantly more sensitive to Me-lex toxicity with respect to the parental strain. However, only the removal of AP endonucleases induced a significant increase in mutagenicity [12]. Furthermore, with the same approach, we have recently demonstrated an involvement of yeast translesions synthesis (TLS) polymerases, such as Pol ζ and REV1, in the mutation fixation process of Me-lex induced lesions [14]. The evidence gathered to date indicates that Me-lex is a molecule with low mutagenic activity that is 3-MeA derived, but there have been no studies addressing its mutagenicity in mammalian cells. Furthermore, the Me-lex mutagenic potential in higher eukaryotic cell system is unknown.

Herein, we investigated the cytotoxicity and mutagenicity of Me-lex at the X-linked hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) locus in a Chinese hamster ovary (CHO) cell line. The *Hprt* gene has been widely used as a selectable genetic marker for studies on chemical- and radiation-induced mutations in a number of mammalian cell systems [15-19]. The large size of the gene, with nine exons dispersed over 34 kb, allows the detection of various types of mutations ranging from single base substitutions to large rearrangements, deletions and insertions [20]. Moreover, mutations arising in splice junctions or intron

sequences that may affect mRNA splicing can be detected [21]. Compared to the analysis conducted on a target gene harboured on a plasmid, the use of an endogenous gene allows a comprehensive evaluation of the *in vivo* activity of the compound.

Our data confirm the low mutagenic potential of Me-lex. However, in contrast with previous yeast-based studies that demonstrated base pair substitutions, the mutation spectrum obtained *in vivo* in mammalian cells revealed a high percentage of genomic deletions. Based upon these data, we hypothesize that a large proportion of the rare mutations induced by Me-lex result from the processing of 3-MeA within A/T rich sequences in non-coding and regulatory regions of the *Hprt* gene. The clustering of these lesions in A-rich sequences could represent a strong impediment to the progression of DNA replication fork, thus promoting genomic rearrangements and/or large deletions leading to cell death.

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 Aldrich (Milano, Italy) unless otherwise stated. Me-lex was prepared as previously described [22].

2.3. Cell culture conditions

The Chinese hamster ovary cell line CHO-9 was 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, CA, USA), 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 10⁻⁴M hypoxanthine, 10⁻⁶M aminopterin and 10⁻⁵M thymidine).

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 dissolving 1 mg of Me-lex in 20.7 µl DMSO and 200 µl ethanol. Survival and mutagenicity were evaluated at 100, 150, 200 and 250 µM Me-lex. Mutagenicity experiments were performed in 25 cm² flasks. For each Me-lex dose, about 3×10⁶ cells were treated for 1 h at 37°C in 3 ml medium without serum. Subsequently, cells were seeded at 200 – 400 cells (depending on Me-lex dose) in 60 mm dish, 5 dishes per dose, for the determination of cell survival measured as colony forming ability. After 8-10 days, the dishes were fixed and colonies counted. In parallel, cells were seeded for expression of mutant phenotype: 5×10⁵ – 1×10⁶ cells per 100 mm dish, 5 dishes per dose. These cells were kept in culture for 8 days, then seeded for determination of mutation frequency: (i) 200 cells per 60 mm dish, 5 dishes per dose, for cloning efficiency; and (ii) 10⁵ cells per 100 mm dish, 10 dishes per dose, in medium containing 10 µg/ml 6-thioguanine (6-TG) for selection of *Hprt* mutants. Eight days after seeding, colonies were fixed and counted. The mutant frequency is expressed as the number of mutants per 10⁵ clone-forming cells. For the isolation of *Hprt* mutants, 12×10⁶ CHO-9 cells were exposed to 150 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 the Me-lex-induced ones.

2.5. Molecular analysis of *Hprt* mutants

Total RNA was isolated from 2×10^6 cells of each mutant (RNeasy Mini Kit, Qiagen, Italy). The *Hprt* cDNA was synthesized in 20 μ l volumes containing 1 μ g RNA (Reverse-iT 1st strand Synthesis kit, AB Gene, Epsom, UK). Approximately 20% of the cDNA product was used as template for PCR. The entire *Hprt* coding sequence was amplified with zee-1/vrl-1 or vrl-8/vrl-16 primers (see Table 1 and Fig. 1A). When the complete *Hprt* cDNA sequence was not obtained, the 5'- and the 3'-ends of the coding sequence were separately amplified with internal primers (vrl-8/vrl-9 for exons 1-4; vrl-6/wies3 for exons 4-8; see Table 1 and Fig. 1A) to verify the presence of shorter cDNAs. 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 (1190/Ex52 for exon5; wies2/wies3 for exon 7 and 8; ham85/ham83 for exon 8; see Table 1 and Fig. 1A). Cell lysate was prepared as described by Menichini et al., 1991. Briefly, 2×10^6 cells were washed twice with phosphate-buffered saline (PBS) and resuspended in 500 μ l of Nonionic Detergent buffer (ND: 50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM MgCl₂, 0.1 mg/ml gelatin, 0.45% Nonidet P40, 0.45% Tween 20, 60 μ g/ml proteinase K). The suspension was incubated for 1 h at 55°C and proteinase K was inactivated by subsequent incubation at 95°C for 10 min. A multiplex PCR-based amplification of all nine exons was carried out with primers described by Xu et al., 1993. Multiplex PCR was performed using 10 μ l of cell lysate and MasterTaq® Kit (Eppendorf, Milano, Italy), with final 3 mM MgCl₂, 10% DMSO and 400 μ M dNTPs. Three units of enzyme were added after a denaturation at 95°C for 5 min. All nine *Hprt* exons were simultaneously amplified to produce eight DNA fragments (exons 7 and 8 are amplified in one fragment). PCR products were displayed by electrophoresis on 4% agarose gel.

3. Results

3.1. Me-lex is cytotoxic but weakly mutagenic in Chinese hamster ovary cells

The DNA repair proficient CHO-9 cells were treated with increasing Me-lex concentrations to determine a survival curve and the mutation frequency at the *Hprt* locus. As shown in Table 2, Me-lex is poorly mutagenic in this mammalian cell system since a drug concentration that reduced cell viability to approximately 10% induced a 7-fold increase over the spontaneous mutation frequency. As a comparison, after exposure of the same cell line to a comparable cytotoxic concentration of methyl methanesulfonate (MMS), the mutation frequency at the *Hprt* locus, was about 40-fold above the spontaneous mutation frequency [23].

3.2. Isolation and molecular analysis of Me-lex induced *Hprt* mutants

Fifty-one *Hprt*-deficient mutants were isolated at 150 μ M Me-lex treatment. This dose led to a 4-fold increase of mutation frequency over the background (Table 2). In the specific experiments performed to isolate independent Me-lex induced mutants, this concentration led to a 5-fold increase of mutation frequency over the background (from 0.3 ± 0.1 to $1.7 \pm 0.1 \times 10^{-5}$). Since we could not exclude the spontaneous origin of a portion of the mutants isolated after Me-lex treatment, 20 spontaneous *Hprt* mutants were isolated in parallel and analyzed for comparison. This allowed the identification of those mutants that either pre-existed in the population before the treatment or were generated spontaneously during the course of the experiment. Accordingly, eight mutants have been excluded from the Me-lex induced spectrum and will be described in the next section.

To determine the Me-lex induced *Hprt* mutation spectrum, the cDNA of each mutant was amplified by PCR using primers that amplify the whole *Hprt* coding sequence [17,18]. The preliminary screening of PCR products by gel electrophoresis revealed a complex mutation pattern. Among the 43 Me-lex induced mutants, only 11 gave rise to a full length *Hprt* product,

6 mutants gave one or two shorter bands, no PCR product was obtained for 18 mutants, a faint band suggestive of low amount of cDNA was observed for 7 mutants of which only one was full-length. Finally, multiple bands were obtained for 1 mutant (see Table 3).

Bands corresponding to full-length PCR products were excised from agarose gel, purified and sequenced to identify the molecular nature of the mutation at the nucleotide level (Table 3). In one case (CL-15A), a frame shift mutation at an AT-run was found, while for the other 10 mutants, cDNA sequence analysis revealed base pair changes that were mainly AT-targeted (C-1A, CL-27C, C-5B, CL-10C3, CL-26B2, CL-3B, CL-19B). Furthermore, all base pair mutations, including those GC-targeted (C-22C, C-6A, CL-6B1) occurred in, or adjacent to, A/T runs.

For mutants with shorter cDNA fragments, probably originating from exon skipping, direct sequencing was performed and the complete loss of one or two exons verified. In these mutants, as well as in those that gave no PCR product, the mutation could be located in non coding regions affecting mRNA splicing (i.e., donor/acceptor splice sites) or in other *Hprt* regulatory sequences affecting RNA transcription and processing. Moreover, shorter cDNAs or complete loss of the *Hprt* coding sequence could result from genomic deletions, encompassing one exon or the entire gene. Thus, for this class of mutants, the mutation can not be identified by cDNA sequencing alone, but must be analyzed at the genomic level. In order to determine the molecular nature of those *Hprt* mutations, multiplex PCRs starting from genomic DNA were performed (Fig. 1). This approach allows all nine *Hprt* exons to be amplified simultaneously in a single reaction [24]. Fig 1 shows an example of multiplex PCR for some of the mutants reported in Table 3. This analysis revealed that genomic deletions, encompassing either one or two exons, had occurred in 4 mutants (C-12A, C-18B, C-20B, C-10B), while in one mutant, CL-8B1, all exons were present. Taking advantage of the fact that the primer pairs used in the multiplex PCR amplified the adjacent intronic regions of each exon, the exon-intron boundaries were sequenced to investigate the occurrence of splice site mutations. Indeed, mutant CL-8B1 presented an AT>GC transition at the donor splice site of intron 4, consistent with the observed skipping of exon 5 in the mRNA. In the mutant C-2C, a complex pattern of mutations, all located in intron 8, was associated with the skipping of exon 8 (Fig 1C). The mutation pattern consisted of a 31 bp deletion accompanied by +1A insertion (in 8 +57) and -1A deletion (in 8 +61) occurred in very AT-rich sequences, a preferential target of Me-lex.

Multiplex PCR was also performed for the 18 mutants that did not give a cDNA amplification product and in 12 cases we found a complete *Hprt* genomic deletion. In one of them (C-23B), all exons were present at the genomic level, while for 5 mutants (C-11B, CL-7A3, CL-25A, C-24C, C-9B) genomic deletions encompassing one or two exons had occurred. Notably, all deletions were localized in the 5' half (before exon 5) of the *Hprt* gene. Thus, we can hypothesise that these mutations resulted from deletions and/or chromosome rearrangements of *Hprt* sequences.

In the 7 mutants producing very low amounts of cDNA (C-3A, C-4A, C-7B, C-10C, C-13C, CL-22B, CL-24B), the amplification products were often shorter than wild type. The poor yield did not allow direct sequencing. However, all *Hprt* exons were found at genomic level. Due to the lack of information regarding non coding sequences in hamster, the precise nature of these mutants could not be defined. However, cDNA amplifications with nested primers were performed in order to identify the region of the cDNA that was missing. As reported in Table 3, this analysis revealed an heterogeneous category of mutations (that we propose to classify as mutations in regulatory regions) that included mutants producing only low amount of short cDNAs encompassing mainly the 5' end of the *Hprt* coding sequence. These data suggest the occurrence of mutations, likely localized at the 5' end of the gene or in other regulatory non coding *Hprt* sequences, may influence mRNA transcription and/or stability.

One mutant, C-5B, gave more than one PCR product with low intensity. The direct sequencing of the longer cDNA fragment, approximately as long as the wild type, revealed a substitution of a 26bp region at the beginning of exon 6 with another 29 base pair sequence not belonging to known *Hprt* sequences (Fig 1D). At genomic level, exon 6 could not be amplified. Since the intronic region flanking exon 6 contains an AT-rich sequence, Me-lex induced lesions could have been clustered in that region inducing a rearrangement that prevented the annealing of the primers used for genomic amplification.

3.3 Isolation and analysis of spontaneous *Hprt* mutants

As stated above, in parallel to the isolation of Me-lex induced *Hprt* mutants, 20 spontaneous mutants were isolated. In the spontaneous mutation spectrum (Table 4), 5 mutants carried base pair changes, 3 mutants revealed genomic deletions, 9 mutants carried mutations, which we hypothesized to be classified as mutations in regulatory regions and 3 mutants had a complex mutation.

Base pair changes included both transitions and transversions at AT, or GC base pairs, localized both at the 5'- and 3'-region of the *Hprt* coding sequence. Of the 3 mutants with genomic deletions, 1 showed a deletion encompassing exons 7/8 and 2 showed a deletion of exon 9. In 9 mutants, no full length PCR products were found after cDNA amplification; multiplex genomic PCR for these mutants revealed that the region encompassing exons 1-6 was amplifiable, while that encompassing exons 7/8 was weakly amplifiable. For these mutants, cDNA amplifications with *Hprt* internal primers revealed the presence of short cDNA encompassing exons 1-4, while the amplification of the 3'-half of the coding sequence (exons 4-8) did not generate PCR products. Taken together, the analyses of cDNA and genomic DNA may suggest that these mutations (at the 3'-end of the gene) may impair/reduce the amplifiability of exons 7/8 at genomic level and also affect the stability of the *Hprt* mRNA. A mutant with the same features had been also isolated after Me-lex treatment (C-10C) and can represent a pre-existing spontaneous mutant. However, since the molecular nature of the mutation could not be defined, this mutant is shown in Table 3. A complex mutation, including a GC>TA in exon 3 and a 66 bp deletion in exons 4-5, was found in three mutants (C-sp12, -sp16, -sp20). This complex mutation has also been found in 8 mutants isolated after Me-lex treatment; being so peculiar, the mutants carrying such a mutation were considered of spontaneous origin and omitted from the Me-lex induced spectrum.

4. Discussion

In the current work, we describe the molecular characterization of mutations induced by Me-lex at the *Hprt* locus in the CHO-9 cell line. Although there was considerable cytotoxicity, likely dependent from the preferential induction (90-95 %) of the lethal 3-MeA adduct, the highest mutation frequency observed after high Me-lex concentrations was only 3 fold over the background. This result is in contrast with the mutation induction of MMS, a methylating agent inducing the mutagenic 7-MeG and the 3-MeA adducts in a 10:1 ratio [25]. Indeed, after treatment with a comparable cytotoxic concentration of MMS, the mutation frequency at the *Hprt* locus in the same CHO cell line was about 40 fold the background [23]. Thus Me-lex showed a low mutagenic potential combined with high cytotoxicity, in agreement with previous data extrapolated from *in vitro* treatment of plasmid DNA and transformation into yeast cells [12].

As summarized in Table 5, the Me-lex induced mutation spectrum revealed 48% of genomic deletions, 23% of base pair changes, 18% of mutants producing low amounts of *Hprt* cDNA (that we classified as "mutations in non-coding regulatory regions), 7% of frameshifts, 2% of splice mutations and 2% of other mutations. The Me-lex induced spectrum significantly differs ($p<0.012$) from the spontaneous mutation spectrum isolated in parallel when compared using

the Cariello's test [26]. The most frequent types of mutation induced by Me-lex were genomic deletions (48%) encompassing the entire *Hprt* locus (27%) and single/multiple exon(s) (21%). In the spontaneous mutation spectrum, genomic deletions represented only 12% of the mutants and no complete *Hprt* deletions were found. In addition, we compared the Me-lex induced spectrum with another spontaneous mutation spectrum at the *Hprt* locus obtained in CHO-K1-H4 cell line by Xu et al. (see Tab 5). The molecular analysis of this spectrum revealed that 20% of mutants had genomic deletions, while single base pair substitutions were found in the 38% of mutants. Furthermore, a low spontaneous deletion of the whole *Hprt* gene (1%) was reported [27]. Thus, even though the number of spontaneous mutants isolated in our experiments is rather small, results from both this study and work reported in the literature [27] consistently support the hypothesis that Me-lex (likely through 3-MeA adducts) was responsible for the high percentage of deletions observed.

The MMS-induced mutation spectrum obtained in CHO-9 cells at the *Hprt* locus [23] is very different from that induced by Me-lex ($p < 0.00001$; Cariello's test; see Table 5 for a comparison). Among the MMS-induced mutants, only 18% carried a deletion mutation while the vast majority of mutants (67%) contained GC>AT base pair substitutions. This difference can be interpreted in light of the methylated products generated by the two compounds. MMS gives rise to a high percentage of guanine adducts (e.g., 7-MeG and O6-MeG), which explains the higher induction of G-targeted base pair mutations [28]. In contrast, Me-lex gives almost exclusively 3-MeA, a lesion known to stall DNA replication in vitro [29] and to induce cell death [2]. Although we did not measure the level of 3-MeA, O6-MeG and 7-MeG in our cells, a preferential *in vivo* induction of 3-MeA over 7-MeG after Me-lex treatment has been confirmed in mouse ES cells [8] and in *E. coli* cells [7]. *In vitro* studies show that Me-lex affords a 3-MeA to O6-MeG ratio of >1000:1, while the ratio for MMS is approximately 20:1 [30].

The mode of processing 3-MeA in AT-rich regions may explain our findings. As already mentioned, the 3-MeA adduct can be converted into AP sites by glycosylases or non-enzymatic hydrolysis, and repaired by the subsequent steps of base excision repair (BER) pathway. However, if encountered during replication, 3-MeA may stall DNA replication. To overcome this block, DNA damage tolerance pathways such as TLS and damage avoidance can be activated, the latter involving also recombination. It has been shown that unrepaired 3-MeA can specifically induce sister chromatid exchanges (SCE), chromatid and chromosome gaps and breaks [8]. SCEs are reciprocal exchanges of DNA between sister chromatids during DNA synthesis and are thought to represent homologous recombination events. Although further experiments are needed to investigate the ability of Me-lex to induce DNA strand breaks, we hypothesize that the processing of 3-MeA adducts in repetitive AT-rich regions, with a high degree of sequence homology, could promote recombination and/or single strand annealing events that lead to deletions. Despite the fact that a small portion of the hamster *Hprt* gene has been sequenced to date [31], the region surrounding the deletions was determined in 2 mutants and the presence of an AT-rich region was found in both cases (Fig 1). Genomic regions containing A/T repeats, such as ALU elements, may act as nucleation points for unequal homologous recombination which can lead to insertion or deletion mutations [32]. Interestingly, the human *HPRT* locus contains a high number of ALU sequences [33] and ALU-mediated deletions and duplications at the *HPRT* locus have been found in patients with Lesh-Nyhan syndrome [34-36].

Me-lex induced exon deletions were all localized in the 5' half of the gene (exons 1, 2 and 3), while in our spontaneous spectrum, they were localized on the 3' half of the gene (3 deletions encompassed exons 7/8 and 9, and 9 mutants presented very weak genomic amplification of exons 7/8). In CHO-K1-H4 cells, 71% of spontaneous deletion breakpoints have been localized in or near exons 4 and 5 [27]. In addition, by using exon-specific probes to map large

spontaneous deletions in the human *HPRT* gene, the 59% of intragenic deletions breakpoints were found between exons 4 and 7 [37]. From this evidence, it has been postulated that the spontaneous deletions are preferentially localized in the 3' half of the *HPRT* gene. The reason for the non-random distribution of spontaneous deletions is not clear. Nevertheless, the localization of Me-lex induced genomic deletions seems different from spontaneous deletions, suggesting the presence of a preferential target for Me-lex in the 5' half of the *HPRT* locus.

Base pair substitutions represented only the 23% of mutations induced by Me-lex and were all located in AT-rich sequences, equally divided between transitions and transversions. Most of them were AT-targeted (7) and only 3 were GC-targeted. The AT>TA and AT>CG base pair changes represented half of the AT-targeted base pair mutations, whereas AT>GC transitions were detected in the other 3 mutants. Furthermore, also the mutation causing the skipping of exon 5 was an AT>GC. In the MMS-induced spectrum, all base pair changes, which represents 67% of mutations, were GC>AT transitions, and are likely the result of mispairing of O6-MeG with thymine during replication [23]. When the distribution of point mutations at defined positions along the *Hprt* gene was taken into consideration (Fig. 2), a highly significant difference between Me-lex and MMS was found ($p < 0.0001$; Cariello's test); notably, we did not find any mutation (considering site and type) in common between the two spectra.

Recently, it has been demonstrated that 3-MeA blocks both human DNA polymerases α and δ [29]. In contrast, Y-family polymerases are able to bypass this modified base *in vitro* with varying efficiencies and accuracy. The three human Y-family polymerases (pol ζ , pol ι and pol κ) are capable of insertion opposite 3-MeA, as well as extension beyond the modified base, with pol κ being the most accurate and pol ζ the most efficient [29]. Although these polymerase activities have been studied *in vitro*, their ability to insert A opposite the lesion and extend the 3-MeA:A mispair could explain the appearance of AT>TA transversions. Me-lex mutagenicity may also depend on the formation of abasic (AP) sites formed either enzymatically by glycosylase during base excision repair or spontaneously through the hydrolytic release of 3-MeA [12]. Recent studies in yeast, using a double-stranded plasmid system that allowed analysis of the mutations arising on the leading and lagging DNA strands, showed that an A is predominantly inserted (66%) opposite an AP site followed by a C (25%) and a G (8%), and that the types and frequency of inserted nucleotides were very similar in both strands [38]. Also in mammalian cells, the preferential misincorporation of dAMP and dCMP opposite an AP site supports the "A-rule" [39] and may explain the AT>GC and AT>TA base pair changes observed in our induced spectrum.

Besides AT-targeted mutations, some mutations at GCs were found in our spectrum. Since they localize in close proximity to AT-rich sequences, we can assume that some rare adducts, such as 7-MeG, or 3-MeG can be produced that enzymatically or spontaneously, are converted into AP sites. The preferential insertion of an A opposite a site of base loss could then give rise to the GC>TA mutations observed. A higher percentage of GC>AT and AT>GC mutations compared to control cells were found after MMS treatment in CHO cells over-expressing N-alkylpurine-DNA glycosylase. This glycosylase removes 3-MeA and 7-MeG and generates abasic sites, suggesting a role of abasic sites in the induction of these types of mutations [40]. However, since the Me-lex mutation induction was low, we can not rule out the possibility that some of these mutations have originated from spontaneous depurination that frequently occurs in mammalian cells [41].

Another class of mutations that we designated as mutations in non-coding regulatory regions included mutants producing weak and/or short *Hprt* cDNAs. A high percentage of this type of mutation was also found in the spontaneous spectrum (Table 5). However, a more detailed molecular analysis allowed us to distinguish among different events that apparently generated the same *Hprt* cDNA. Indeed, in the Me-lex induced spectrum, with the exception of the C-10C

mutant that gave very weak genomic amplification of exons 7/8 (and probably is of spontaneous origin), all other mutants in this class had complete *Hprt* exons at genomic level, but still produced a truncated mRNA. Due to the lack of sequence information on intronic and regulatory regions of the hamster *Hprt* gene, we could not further investigate the origin of these mutations. Nevertheless, our data suggest that the mutations responsible for the formation of short or unstable mRNAs could be localized in non coding regulatory regions located at the 5' end of the *Hprt* gene.

Overall our data confirm that Me-lex weakly induces AT-targeted mutations in mammalian cells. In the endogenous *Hprt* gene, the predominant type of mutation is genomic deletions either of the entire gene or single/multiple exons. The high percentage of genomic deletions was unexpected since the 82% of Me-lex induced mutations previously obtained in yeast were base pair substitutions. This difference stems from the inability to observe large deletions in the yeast shuttle vector system. Indeed, in yeast, clones that phenotypically expressed mutant p53, were picked, plasmid DNA extracted, and the p53 cDNA amplified. In presence of PCR products GAP repair was performed to assure that the mutant phenotype was due to mutation in the p53 coding sequence. In absence of PCR products, further analyses were not performed. In light of the present results, most probably PCR negative p53 cDNA clones had large deletions.

It is worth noting that with the exclusion of base pair changes, the majority of mutations appeared to be generated in the non coding portion of the gene. Although the complete hamster sequence is not available, the analysis of the published 3349 nucleotide encompassing the 9 exons and the adjacent intron bases [31] allows an estimate of the percentage of ATs in AAA, ATT, AAT, TTT, TAT, ATA triplets (and their multiple), which *in vitro* constitute the preferential target of Me-lex [4]. The relative percentage of ATs is comparable in the coding and non coding part of the gene (33% and 38%, respectively). However, the non coding *Hprt* sequences account for 82% of all these AT rich runs. In the human and mouse *Hprt* gene, which have been completely sequenced, one can assume that the non coding part of the *Hprt* gene, representing the 98% of the whole *Hprt* sequence, contains the vast majority of Me-lex targets. Thus, while point mutations induced in non coding *Hprt* by methylating agents such as MMS do not change protein functionality and may pass unnoticed, unless they affect regulatory functions such as splicing or transcription regulation, extensive deletions induced by Me-lex in non coding regions (likely due to formation of replication blocking lesion in highly AT-rich and therefore potentially recombinogenic sequences) would abrogate HPRT function and generate *Hprt* mutants.

The sequence selectivity combined with the ability to generate almost exclusively one single type of lethal lesion make Me-lex a useful tool to study and develop highly cytotoxic alkylating antineoplastic compounds with reduced mutagenic and carcinogenic side-effects. Recently, AT-specific DNA-reactive drugs, such as bizelesin, have been shown to exhibit antitumor activity *in vivo* [42,43]. The interest for these molecules is derived from the fact that sequences important for DNA replication, such as the matrix attachment regions (MARs), are very AT-rich. Thus, it has been speculated that highly proliferating cells, such as tumor cells, may be more sensitive to drugs that target non coding replication-related regions. In addition, the use of molecules that preferentially bind non-coding DNA regions should limit the induction of mutations in regions that are crucial for protein functions. Further studies are needed to explore the possibility of using Me-lex not only as sequence-specific but also as region-specific antiproliferative agent.

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Abbreviations

Me-lex { 1-methyl-4-[1-methyl-4-(3-methoxysulfonylpropanamido) pyrrole-2-carboxamido]-pyrrole-2-carboxamido }propane

Figure 1. Analysis of *Hprt* mutants

A) The positions of the primers used for cDNA and genomic DNA analyses are indicated. Exons (in scale) are represented by open boxes. The primers lying inside exons are in italic. B) Example of multiplex PCR amplification of *Hprt* exons 2-9 starting from genomic DNA. Exon 1 is usually amplified in a separate reaction and was not included in the figure. In case of weak exons 7/8 amplification, also these are checked separately. Exon numbers are indicated on the right. The wild type fragments obtained after PCR amplification of individual exons were pooled and used as additional marker (last lane on the right). Genomic deletion encompassing one *Hprt* exon is documented for C-5B (exon 6), C-9B (exon 4) and C-12A (exon2). In one mutant (C-15B), a deletion of the complete *Hprt* gene has occurred. The sequence analysis of the exon and adjacent regions for the mutant C-2C and C-5B are outlined in C) and D), respectively (see results for description). Exon sequences are shown in upper case and intron sequences in lower case. The positions of the primers used for the amplification are underlined. For the mutant C-2C, the deleted 31 bp region is in bold underlined; the +A (In 8 +57) and -A (In 8 +61) are also indicated. For the mutant C-5B, the 26 bp sequence that replaces the 29 bp sequence at the beginning of exon 6 is represented in bold over the sequence. In this mutant, where the exon 6 was missing from genomic DNA, the wild type intron sequence is reported to show its AT-rich composition.

Figure 2. Comparison between the Me-lex- and MMS-induced mutation spectra in CHO-9 cells
 The Me-lex induced point mutations (11 mutations) significantly differed from the MMS-induced base pair mutations (26 mutations; [23]). Cariello's analysis was performed with base pair changes at defined positions. The difference between the localization of mutations along the *Hprt* gene in the different mutation spectra was highly significant ($p < 0.0001$).

Tab 1

Primers used for molecular analysis of *Hprt* mutants

cDNA amplification	
ZEE-1	5' - GGCTTCCTCCTCACACCGCTC - 3'
VRL-1	5' - GGACTCCTCGTGTTCGAGAT - 3'
VRL-8	5' - CCG CCA GCC GAC CGA TTC CG - 3'
VRL-16	5' - GCA GAT TCA ACT TGA ACT CTC ATC - 3'
VRL-9	5' - TTG AGA GAT CAT CCC CAC CA - 3'
VRL-6	5' - GCT ACT GTA ATG ATC AGT CA - 3'
Splice junctions amplification	
1190	5' - AAC ATA TGG GTC AAA TAT TCT TTC TAA TAG - 3'
Ex52	5' - GCT GAG AAA ATT TAA CAG TAT TTT AG - 3'
wies 2	5' - CAT CTG ATC CAG GTT CCA GGT GG - 3'
wies 3	5' - TTA TAG TCA AGG GCA TAT CC - 3'
ham 85	5' - TGC TTA GAG TTA TTT TAG AGA - 3'
ham 83	5' - CCA TCA GTC TGG TCA AAT GA - 3'

Table 2
Cell survival and *Hprt* mutation frequency induced by Me-lex in CHO-9

Me-lex dose (μ M)	Survival %	Mut Freq ($\times 10^{-5}$) ^a
0	100	0.4 $\pm$ 0.3
100	80.0 $\pm$ 11.7	1.1 $\pm$ 0.7
150	40.8 $\pm$ 18.7	1.6 $\pm$ 0.9
200	13.3 $\pm$ 8.2	2.8 $\pm$ 1.4
250	2.9 $\pm$ 2.3	0.9 $\pm$ 0.9

^aThe *Hprt* mutation frequency is expressed as the number of 6-TG-resistant clones per 10^5 clone-forming cells. Each point represents the mean $\pm$ s.d. of at least 5 independent experiments for survival and 3 experiments for mutation induction.

Unpaired *t*-test was used for statistical analysis.

Table 3

Melex induced *Hprt* mutation spectrum in CHO-9

Mutant	cDNA product [#]	Position (exon)	Type of mutation	Target sequence [§] (5' > 3')	Aminoacid change (or cDNA change)	N° of mutants
Base pair changes						
C-1A	full length	95 (2)	AT>TA	GAG GAT TTG GAA AAG	Leu>STOP	1
CL-27C	full length	95 (2)	AT>CG	GAG GAT TTG GAA AAG	Leu>Trp	1
C-25B	full length	104 (2)	AT>TA	GAA AAG GTG TTT ATT	Val>Glu	1
CL-10C3	full length	221 (3)	AT>GC	TAT AAA TTC TTT GCT	Phe>Ser	1
C-22C	full length	222 (3)	GC>TA	TAT AAA TTC TTT GCT	Phe>Leu	1
CL-26B2	full length	293 (3)	AT>GC	ACT GTA GAT TTT ATC	Asp>Gly	1
CL-3B	full length	424 (6)	AT>GC	GGT AAA ACA ATG CAA	Thr>Ala	1
C-6A	full length	464 (6)	GC>TA	TAC AAC CCC AAA ATG	Pro>His	1
CL-6B1	full length	551 (8)	GC>AT	GAA ATT CCA GAC AAG	Pro>Leu	1
CL-19B	full length	605 (8)	AT>CG	AGG GAT TTG AAT CAT	Leu>Trp	1
Frameshift mutations						
CL-15A	low, full length	466-469 (6)	-A	AAC CCC AAA ATG GTT		1
Splice mutants						
CL-8B1	short cDNA	In 4; -2	AT>GC	ttttctct a gAATGTCCTT	Skipping ex 5	1
Genomic Deletions						
C-11B	no cDNAs		Deletion of exon 1			1
CL-7A3	no cDNAs		Deletion of exon 1			1
CL-25A	no cDNAs		Deletion of exon 1			1
C-24C	no cDNAs		Deletion of exons 1 and 2			1
C-12A	two short cDNAs		Deletion of exon 2*		Skipping ex 2 or 2+3	1
C-18B	two short cDNAs		Deletion of exon 2*		Skipping ex 2 or 2+3	1
C-20B	two short cDNAs		Deletion of exon 2*		Skipping ex 2 or 2+3	1
C-10B	short cDNA		Deletion of exons 2 and 3		Skipping ex 2+3	1
C-9B	no cDNAs		Deletion of exon 4			1
C-14C/C-15B	no cDNAs		Deletion of all <i>Hprt</i> exons			12
C-16A/C-17B						
CL-1C/C-5A2						
C-16A/C-18A1						
CL-20B4/CL-21A2						
CL-23C/CL-29C						
"Mutations in regulatory regions"						
C-4A	very low full length, wt. All exons present on gDNA					1
C-3A/CL-22B	only ex (1-4). All exons present on gDNA					3
CL-24B						
C-7B	low ex (1-4). All exons present on gDNA					1
C-13C	low ex (4-8). All exons present on gDNA					1
C-10C	only ex (1-4). Low gDNA amplification of exons 7/8					1
C-23B	no cDNAs. All exons present on gDNA					1
Other mutations						
C-2C	short cDNA	In 8;+5 In 8;+57 In 8;+61	31 bp deletion +A -A	(see Fig. 1C)	Skipping ex 8	1
C-5B	Multiple and low cDNA products	(6)	Replacement of 29 bp with 26 bp (see Fig. 1D) of unknown origin Ex 6 not amplified from gDNA	(see Fig. 1D)		1

[#] 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.

[§] Non transcribed strand

* In these mutants, the deletion of exon 2 sometimes caused missplicing of exon 3

gDNA = genomic DNA

Table 4

Spontaneous *Hprt* mutation spectrum in CHO-9

Mutant	DNA product [#]	Position (exon)	Type of mutation	Target sequence [§] (5' > 3')	Aminoacid change (or cDNA change)	N° of mutants
CL- sp11	full length	146 (3)	Base pair changes AT>TA GC>CG AT>GC	GAA AGA CTT GCC CGA	Leu>His	1
CL-sp2/CL-sp7	full length	166 (3)		ATG AAA GAG ATG GGA	Glu>Gln	3
CL-sp20	full length	496 (7)		CTG GTG AAA AGG ACC	Lys>Glu	
C-sp14	full length	585 (8)	AT>CG	CTT GAC TAT AAT GAG	Tyr>STOP	1
C-sp8	only ex (1-4)	no full length ex (1-4); ex (1-8)	Genomic Deletions Deletion of exon 7/8 Deletion of exon 9			1
C-sp6/C-sp7						2
C-sp1/C-sp2	only ex (1-4). Low gDNA amplification of exons 7/8		“Mutations in regulatory regions”			9
C-sp3/C-sp4						
C-sp5/C-sp9						
C-sp11/C-sp13						
C-sp17						
C-sp12/C-sp16	short cDNA	287 (3) (4, 5)	Other mutations GC>TA -66 bp (323-388)	CCC ATG ACT GTA GAT	Thr>Asn	3
C-sp20						

[#] 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.

[§] Non transcribed strand

gDNA = genomic DNA

Table 5
Comparison among different *Hprt* mutation spectra induced in CHO cell lines

Type of <i>Hprt</i> mutations in CHO cells	Spontaneous n° (%)	Me-lex induced n° (%)	MMS-induced [#] n° (%)	Spontaneous [§] n° (%)
Base pair changes	9 (38%)	10 (23%)**	26 (67%)**	24 (38%)
AT-targeted	5 (21%)	7 (16%)	0	8 (13%)
AT > TA	1	2	0	2
AT > GC	3	3	0	2
AT > CG	1	2	0	4
GC-targeted	4 (17%)	3 (7%)	26 (67%)	16 (25%)
GC > AT	0	1	25	4
GC > CG	1	0	1	2
GC > TA	3	2	0	10
Splice mutations	0	1 (2%)	6 (15%)[§]	15 (23%)
Frame shifts	0	3 (7%)	0	1 (2%)
Genomic deletions	3 (12%)	21 (48%)	7 (18%)	13 (20%)
Exon deletions	3 (12%)	9 (21%)	4 (10%)	12 (19%)
Complete <i>Hprt</i> deletions	0	12 (27%)	3 (8%)	1 (1%)
Mutations in regulatory regions	9 (38%)	8 (18%)	0	5 (8%)
Other	3 (12%)	1 (2%)	0	6 (9%)
Total n° of mutations	24 (100%)	44 (100%)	39 (100%)	64 (100%)

Base pair changes induced by Me-lex and MMS are highly different** ($p < 0.0001$)
The mutation spectra significantly differ (Cariello's test) also when all types of mutations are considered
Spontaneous vs Me-lex $p < 0.012$
Spontaneous Xu vs Me-lex $p < 0.00001$
Spontaneous vs MMS $p < 0.00001$
Me-lex vs MMS $p < 0.00001$
Spontaneous vs Spontaneous Xu $p < 0.01$
[#] From Op het Veld et al., 1998
[§] From Xu et al., 1995
[§] In these mutants all exons were present at genomic level, but they were not further characterized