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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.

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 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.*, temozolomide, 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 *Escherichia coli* and in mammalian cells, and a correlation between the level of 3-MeA and cell death has been shown [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,

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

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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 *Saccharomyces 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 Polζ 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 the induction of 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 CHO 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% CO_2. 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).

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 cm^2 flasks. For each Me-lex dose, about 3×10^6 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: 5×10^5 to 1×10^6 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) 10^5 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 10^5 clone-forming cells. For the isolation of *Hprt* mutants, 12×10^6 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 μl volume containing 1 μg 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/wies 3 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 exon 5; wies 2/wies 3 for exon 7 and 8; ham 85/ham 83 for exon 8; see Table 1 and Fig. 1A). Cell lysate was prepared as described by Menichini et al. [17]. Briefly, 2×10^6 cells were washed twice with phosphate-buffered saline (PBS) and resuspended in 500 μl of Nonionic Detergent buffer (ND: 50 mM KCl, 10 mM Tris–HCl pH 8.3, 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% Nonidet P40, 0.45% Tween 20, 60 μg/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. [24]. Multiplex PCR was performed using 10 μl of cell lysate and MasterTaq® Kit (Eppendorf, Milano, Italy), with final 3 mM $MgCl_2$, 10% DMSO and 400 μM dNTPs. Three units of enzyme were

Table 1
Primers used for molecular analysis of *Hprt* mutants.

cDNA amplification	
zee-1	5′-GGCTTCCTCCTCACACCGCTC-3′
vrl-1	5′-GGACTCCTCGTGTTTGCAGAT-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′