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Comparison of the biological effects of MMS and Me-lex, a minor groove methylating agent: Clarifying the role of N3-methyladenine

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ABSTRACT

N3-methyladenine (3-mA), generated by the reaction of methylating agents with DNA, is considered a highly toxic but weakly mutagenic lesion. However, due to its intrinsic instability, some of the biological effects of the adduct can result from the formation of the corresponding depurination product [an apurinic (AP)-site]. Previously, we exploited Me-lex, *i.e.* {1-methyl-4-[1-methyl-4-(3-methoxysulfonylpropanamido)pyrrole-2-carboxamido]-pyrrole-2 carboxamido}propane, a minor groove equilibrium binder with selectivity for A/T rich sequences that efficiently reacts with DNA to afford 3-mA as the dominant product, to probe the biology of this lesion. Using human p53 cDNA as a target in a yeast system, a weak increase in mutagenicity was observed in the absence of Mag1 (3-methyladenine-DNA glycosylase 1, mag1), the enzyme devoted to remove 3-mA from DNA. Moreover, a significant increase in mutagenicity occurred in the absence of the enzymes involved in the repair of AP-sites (AP endonucleases 1 and 2, apn1apn2). Since methyl methanesulfonate (MMS) has been extensively used to explore the biological effects of 3-mA, even though it produces 3-mA in low relative yield, we compared the toxicity and mutagenicity induced by MMS and Me-lex in yeast. A mutagenesis reporter plasmid was damaged *in vitro* by MMS and then transformed into wild-type and Translesion Synthesis (TLS) Polζ (*REV3*) and Polη (*RAD30*) deficient strains. Furthermore, a mag1rad30 double mutant strain was constructed and transformed with the DNA plasmid damaged *in vitro* by Me-lex. The results confirm the important role of Polζ in the mutagenic bypass of MMS and Me-lex induced lesions, with Polη contributing only towards the bypass of Me-lex induced lesions, mainly in an error-free way. Previous and present results point towards the involvement of AP-sites, derived from the depurination of 3-mA, in the observed toxicity and mutagenicity.

1. Introduction

3-mA is a common DNA lesion formed from the reaction of many methylating agents, including antineoplastic drugs, as well as environmental and endogenous compounds [1–3]. 3-mA has been implicated as a replication blocking lesion based on *in vitro* observations that *E.coli* DNA Polymerase I or the AMV reverse transcriptase stop at adenine in dimethyl sulfate or N-methyl-N-nitroso-N′-nitroguanidine treated DNA templates [4,5]. The ability to block DNA replication is associated with the toxicity of these agents that produce an array of DNA adducts, among them 3-mA in low relative yield. Further structural studies on replicative DNA polymerases provided the possible explanation for the inhibitory effect of 3-mA as being due to alterations of key contacts occurring in the catalytic site between specific residues and the N3 atom of purines in the template strand [6–9].

There is limited direct evidence on the blocking action of a replicative DNA polymerase by 3-mA. In a prior study, the selective generation of a 3-mA adducted template was accomplished using Me-lex, which efficiently and selectively generates 3-mA (>90% of total lesions) at A/T-rich sequences due to its DNA equilibrium binding properties [10–12]. This approach using a DNA sequence

Abbreviations: AP, apurinic/apyrimidinic; apn1apn2, AP endonucleases 1 and 2 defective; BER, base excision repair; 3-mA, N3-methyladenine; 3-m-c3A, 3-methyl-3-deazaadenine; mag1, 3-methyladenine-DNA glycosylase 1 defective; Me-lex, {1-methyl-4-[1-methyl-4-(3-methoxysulfonylpropanamido)pyrrole-2-carboxamido]-pyrrole-2-carboxamido}propane; MF, mutation frequency; MMS, methyl methanesulfonate; TLS, Translesion Synthesis; sMF, spontaneous mutation frequency.

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containing a single Me-lex binding site, *i.e.* 5′-CA_4C, made it possible to know exactly where the 3-mA lesions were located on the DNA [13]. Only the two 5′-A's were methylated and these caused the halting of polymerization by T7 Sequenase and Klenow fragment (exo-). It was also demonstrated in glioma cells treated with Me-lex that replication blocks were produced in a dose-response manner using a microfluidic assisted replication tract analysis [14]. However, it was not possible to determine whether 3-mA and/or a repair intermediate were responsible for the block.

Two *in vitro* studies reported on the inability of human DNA polymerases α and δ, T7 Sequenase and Klenow fragment polymerases to bypass a stable isostere of 3-mA *i.e.* 3-methyl-3-deazaadenine (3-m-c3A) [13,15] which lacks the positive charge of 3-mA that can result in the formation of a stable imino tautomer via deprotonation. More recently, 3-m-c3A, placed at a specific site within the 5′-CA_4C p53 sequence context, which is a hotspot for 3-mA lesion formation and induced mutations, was shown *in vitro* to be efficiently bypassed by transcription. Moreover, once incorporated into a double stranded DNA plasmid, it was also efficiently bypassed *in vivo* in yeast by DNA replication [16]. The results with the 3-m-c3-A re-opened the question whether 3-mA is *per se* a lethal lesion.

We previously exploited a yeast-based p53 functional assay to study the toxicity and mutagenicity of Me-lex, being a specific inducer of 3-mA. The Me-lex induced mutation spectrum in the human p53 cDNA was determined and compared with methylation pattern determined *in vitro* at the same locus, showing minimal overlap: few methylation sites were revealed to be also mutation sites and many of the strongest sites of alkylation were cold spots for base pair substitutions. Those results were consistent with the hypothesis that 3-mA is lethal and only weakly mutagenic [12].

Subsequently, the toxicity and mutagenicity of Me-lex were shown to be dependent on the nature of the DNA repair background [17]: mag1 and apn1apn2 base excision repair (BER) defective *S. cerevisiae* strains were both significantly more sensitive to Me-lex toxicity, but only in apn1apn2 cells did Me-lex treatment result in a significant increase in mutagenicity. The higher toxicity observed in mag1 strain with respect to wild-type supports the hypothesis of 3-mA being a lethal lesion, presumably because it can block replication. Moreover, the mutation spectra induced by Me-lex were not affected by the status of *MAG1* or by defects in different downstream BER steps. Those observations suggested that 3-mA mutagenicity might be actually associated with the conversion of 3-mA into an AP-site through the enzymatic activity of Mag1 or from spontaneous hydrolytic depurination of the 3-mA adduct.

The contribution of yeast TLS polymerases to Me-lex mutagenicity was also determined. While Rev1 and Polζ play a role in error prone bypass, Polη appears to be involved in error free bypass [18,19]. Consistent with these observations, Polη was shown *in vitro* to preferentially insert a T opposite 3-m-c3A and to efficiently extend this primer terminus [15]. Also, the structural modelling of 3-mA in the active site of Polη reveals sufficient room to accommodate the methyl group when positioned at either the templating base or at the post-insertion site. Therefore, 3-mA is not expected to block Polη [20]. In a different experimental approach, we recently confirmed that Polη (in association with Polζ) is partially involved in the error-free bypass of 3-m-c3A *in vivo* [16].

In many related studies on 3-mA, MMS has been used *in vivo* to produce 3-mA [15,20]. However, MMS generates many different lesions: 1-methyladenine (1-mA), 7-methyladenine (7-mA), 3-methylguanine (3-mG), 7-methylguanine (7-mG), O^6-methylguanine (6-mG), 3-methylcytosine (3-mC) and methylphosphotriesters. In combination, these lesions constitute ∼90% of the total adducts in double stranded DNA (3.5; 1.8; 0.6; 83.0; 0.3;<1.0 and 0.8%, respectively), and 3-mA represents only ∼10% of total adducts. All these lesions have different stabilities and are processed with different efficiencies by a number of DNA repair proteins [21].

Table 1
Yeast strains used in this study.

Background	Strain	References
wild-type	yIG397	[12]
rev3	Same as yIG397 but rev3::HYGRO^R	[18]
rad30	Same as yIG397 but rad30:: HYGRO^R	[19]
mag1	Same as yIG397 but mag1::LEU2	[17]
mag1rad30	Same as mag1 but rad30:: HYGRO^R	This work

In order to clarify the role of 3-mA, we determined and compared the toxicity and mutagenicity induced by MMS with those induced by Me-lex using our well-established yeast system. The effects of Polζ and Polη deletion on the toxicity and mutagenicity of the two compounds were also evaluated. The results confirm the important role of Polζ in the mutagenic bypass of lesions produced by MMS and Me-lex, with Polη being partially involved in the bypass of Me-lex induced lesions, and mainly in an error-free way. Previous and present results point towards a key role of AP-sites derived from the non-enzymatic hydrolytic depurination of 3-mA in the observed toxicity and mutagenicity of molecules that generate this DNA adduct.

2. Material and methods

2.1. Vectors and yeast strains

The yeast expression vector pTS76 [22], harbouring the human wild-type p53 cDNA (under the control of an *ADH1* constitutive promoter) and the selectable marker *TRP1*, was used for the *in vitro* damaging treatments. The haploid *S. cerevisiae* strain yIG397 (wild-type) and its isogenic Polζ and Polη defective derivatives (rev3 and rad30 strains, respectively) [18,19] were employed as recipients for pTS76 transformation (Table 1). The mag1 derivative [17] and its isogenic Polη deficient derivative (mag1rad30) (present work) were also used for *in vitro* Me-lex treatment (Table 1). The mag1rad30 strain was constructed by disrupting the *RAD30* gene in the mag1 strain. The *RAD30* disruption cassette and yeast manipulations were performed as previously described [19].

By exploiting the p53-dependent expression of the *ADE2* reporter gene, transformants were selected on plates lacking tryptophan but containing sufficient adenine for adenine auxotrophs (*i.e.* clones with a p53 mutation induced by the damaging treatment) to grow and turn red [19 and references therein].

2.2. DNA modification, transformation and analysis

The plasmid pTS76 DNA was treated *in vitro* in 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 50% EtOH with MMS (40 min) or Me-lex (1 h) at 37 °C. MMS was used at the 0.8% dose, while Me-lex was used at 3 and 6 mM concentrations. The MMS concentration selected corresponds to a level of plasmid survival in the different yeast backgrounds comparable with that previously observed after Me-lex treatment [19 and references therein]. Higher MMS doses were associated with the degradation of pTS76 plasmid after *in vitro* treatment (identified as a smear in agarose gel electrophoresis) and therefore were not exploitable. DNA was purified by EtOH precipitation, washed with 70% EtOH and resuspended in sterile water. DNA modification, transformation and analysis were performed as previously described [19 and references therein].

At least three independent experiments were performed. The mutation frequency (MF) is reported and defined as the number of red colonies with respect to the total number of transformants