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3-METHYL-3-DEAZAADENINE, A STABLE ISOSTERE OF N3-METHYL-ADENINE, IS EFFICIENTLY BYPASSED BY REPLICATION *IN VIVO* AND BY TRANSCRIPTION *IN VITRO*

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Abstract

The goal of the present work was to determine the impact of N3-methyladenine (3-mA), an important lesion generated by many environmental agents and anticancer drugs, on *in vivo* DNA replication and *in vitro* RNA transcription. Due to 3-mA chemical instability, the stable isostere 3-methyl-3-deazaadenine (3-m-c³A) was site specifically positioned into an oligodeoxynucleotide. The oligomer was, then incorporated into a vector system that is rapidly converted to ssDNA inside yeast cells and requires DNA replication opposite the lesion for plasmid clonal selection. For control purposes, an adenine or a stable apurinic/apyrimidinic (AP)-lesion was placed at the same site. The presence of each lesion in the oligonucleotide was confirmed by MALDI-TOF analysis. Plasmids were then transfected into yeast cells. While the AP-site dramatically reduced plasmid replication in all strains, the 3-m-c³A had a slight effect in the rad30 background which significantly increased only in a rev3rad30 background. Considering TLS events opposite 3-m-c³A, the lack of Polη was associated with a substantial increase in AT>GC transitions (p=0.0011), while in the absence of Polζ only events derived from an error free bypass were detected. The 3-m-c³A also did not affect *in vitro* transcription, while the AP-site was a strong block to T7 RNA progression when located in the transcribed strand. We conclude that, in these experimental systems, 3-m-c³A is efficiently bypassed by replication *in vivo* and by transcription *in vitro*.

Keywords

3-methyl-adenine; AP-site; Translesion synthesis; RNA transcription

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CONFLICT OF INTEREST

None

1. INTRODUCTION

DNA is subject to numerous chemical modifications that are induced by exposure to endogenous compounds [1], environmental carcinogens and DNA targeting anticancer drugs [2,3]. Persistence of these chemical changes to DNA may result in mutations, potentially leading to the development of human diseases, including cancer. Methylating agents damage DNA by primarily reacting with the ring nitrogens or exocyclic oxygen atoms of the DNA bases. The reaction mechanism for the alkylation (i.e., S_N1 or S_N2) determines the relative ratio of oxygen to nitrogen modifications [4]. S_N2 type methylating agents, including methyl methanesulfonate (MMS), preferentially react with the N7 of guanine and N3 of adenine, generating N7-methylguanine (7-mG) and N3-methyladenine (3-mA), respectively. MMS has been widely utilized in the past to elucidate the mechanism of DNA replication past 3-mA. However, MMS produces about 70–80% 7-mG and only 10% 3-mA.

We used Me-lex, an alkylating agent that efficiently and selectively generates 3-mA (more than 95%) in A/T-rich regions of double-stranded DNA due to its minor groove binding ability conferred by the lexitropsin dipeptide [5–7]. We showed that in *E.coli* [8] and in *S.cerevisiae* the toxicity and mutagenicity of Me-lex are dependent on the DNA repair background. Yeast strains defective in 3-methyladenine DNA glycosylase (*mag1*) or in both AP endonucleases (*apn1apn2*) were significantly and similarly more sensitive to Me-lex toxicity than the parental strain. However, only the deletion of AP endonuclease activity resulted in a significant increase in mutagenicity. When the mutation spectra obtained in the different DNA repair backgrounds were compared using the Cariello test [9], no significant differences emerged [6]. This suggests that 3-mA induced mutagenicity may be associated with the enzymatic or hydrolytic conversion of the primary 3-mA adduct into an AP-site. Subsequent studies showed that the absence of a single translesion synthesis polymerase (TLS) (*Rev1*, *Polζ* or *Polη*), caused a similar increase in the lethality of Me-lex induced lesions. With regard to mutagenicity, it was found that while *Rev1* and *Polζ* were involved exclusively in an error prone bypass of Me-lex induced lesions [10], *Polη* appears to be involved in an error free bypass [11].

The identification of the lesion responsible for the observed cytotoxicity and mutagenicity of Me-lex in the yeast system described above is still an open question. This is because 3-mA, while relatively stable in double-stranded DNA at physiological conditions ($t_{1/2}$ =24 hours) [12], is 40-fold less stable in single-stranded DNA ($t_{1/2}$ ~35 minutes) [13]. Therefore 3-mA-derived AP-sites may form selectively at the replication fork by non-enzymatic hydrolysis. Thus, some of the biological effects of 3-mA might be ascribed to the formation of AP-sites.

Modification at the 3-position of purines is thought to be a potent block of DNA replication due to the loss of a required contact between this minor groove position and an arginine residue that is faithfully conserved in replicative DNA polymerases. This could provide a possible explanation as to why methyl groups at this position would impede polymerization [14–18].

Plosky et al., [19] synthesized a DNA template containing 3-methyl-3-deazaadenine (3-m- c^3A), a stable isostere of 3-mA, and characterized its ability to act as a blocking lesion for *in vitro* DNA polymerization. While human replicative DNA polymerases α and δ were efficiently blocked by 3-m- c^3A , human TLS DNA polymerases η , ι and κ (as well as *S. cerevisiae* *Polη*) were able to bypass the lesion with varying efficiencies and accuracy.

It was recently reported that 3-m- c^3A significantly destabilizes DNA ($\Delta\Delta G > 4 \text{ kcal} \cdot \text{mol}^{-1}$) due to a drop in the enthalpy (ΔH) term, which is associated with a lower hydration of the duplex relative to the unfolded state [20]. It was proposed that the thermodynamic instability induced by the minor groove methyl group will allow the lesion to populate an

extrahelical conformation that will enhance the ability of DNA repair glycosylase enzymes to rapidly find and remove the 3-mA adduct. Base pair instability may also be, in part, responsible for the *in vitro* inhibition of DNA polymerases when 3-mA [21] or 3-m-c³A is in the template strand.

In order to determine the cytotoxicity and mutagenicity of 3-m-c³A *in vivo*, we adapted a previously described site-specific translesion synthesis assay in yeast cells [22]. The 3-m-c³A and a stable tetrahydrofuran (THF) AP-site were each incorporated at a specific site in a yeast expression vector that was subsequently transfected into yeast cells. The individual lesions replaced a specific site (A) within a sequence context (5'-CAAAC-3') present in the human p53 cDNA. The rationale for this design was based on the observation that this A was heavily alkylated *in vitro* [5] and also found to be a hotspot for AT>GC mutations in different DNA repair backgrounds [5–7,10]. Moreover, among the few hotspots along the p53 cDNA sequence where mutations were recovered using a yeast based p53 functional assay, the chosen sequence was the only one that facilitated the analysis of the events following TLS because, in two cases, a specific TLS event causes the appearance of a specific restriction endonuclease site (see Materials and Methods).

The impact of 3-m-c³A was compared with that of the single (THF) AP-site. The influence of TLS background was also evaluated by transforming plasmids into strains where the TLS polymerases genes REV3, which encodes the catalytic subunit of Polζ, and RAD30, which encodes the polymerase Polη, were individually (rev3 or rad30) or concomitantly deleted (rev3rad30). In parallel, and within the same sequence context, we also determined the influence of 3-m-c³A on *in vitro* transcription. Based upon our results, we conclude that 3-m-c³A, in contrast to an AP-site, does not have a dramatic impact on *in vivo* DNA replication or on *in vitro* transcription.

2. MATERIALS AND METHODS

2.1. Construction of yeast strains

The yPM16, yPM17, yPM18 and yPM19 strains (Table 1) were constructed from yIG397 (WT) [23], yPM6 (rev3), yPM13 (rad30) and yPM14 (rev3rad30) strains, respectively, by genomic deletion of the URA3 locus [10,24]. The deletion of the URA3 locus was necessary in order to use the pELUf1 plasmid (see below) [22].

The URA3 disruption cassette was obtained by PCR (Perfect Taq DNA Polymerase, 5Prime distributed by Eppendorf, Milano, Italy) using the pRS314 plasmid as a template and exploiting its selectable marker TRP1. The following primers were used: URA3-TRP1 dw (5'-tcttaaccacacctgcacagaacaaaaacctgcaggaaacgaagataaat**tttgagttatgacgccaga**-3') and URA3-TRP1 up (5'-attgaagctctaattgtgagtttagtatacatgcatttacttataat**accactcaacctatctcggtc**-3'). The underlined sequence is homologous to the 5'-(or 3'-) end of the URA3 gene, while the sequence in bold is complementary to the 5'-(or 3'-) region of the selectable marker TRP1. The following PCR conditions were used: 95°C for 40 s, 55°C for 60 s and 72°C for 90 s repeated for 35 cycles. The PCR products were transformed into yeast cells, and transformants were selected for tryptophan prototrophy (TRP⁺) and then for disruption of URA3 as FOA^R clones, isolated by replicating on plates containing 5-fluoroorotic acid (FOA) (Toronto Research Chemicals Inc., Toronto, Canada). The deletion of URA3 locus was also confirmed at the genomic level by yeast colony PCR using the primers (a) URA3 forward (5'-atgtcgaaagctacatataaggaacgtgct-3': 1–30 nucleotide positions on URA3 coding sequence) and (b) URA3 reverse (5'-caataaagccgataacaaaatctttgtcgc-3': 538–567 nucleotide positions on URA3 coding sequence) and the following PCR conditions: 94°C for 60 s,

50°C for 60 s and 72°C for 120 s repeated for 35 cycles (yeast colonies were heated at 94°C for 8 min before starting).

2.2. Synthesis of 3-m-c³A and AP-site analog

3-m-c³A is as a stable isostere of 3-mA. The phosphoramidite derivative of 3-m-c³A was prepared as previously described [25]. A modified THF moiety, isosteric with 2'-deoxyribofuranose, serves as a structural analog of the natural AP-site [26]. Both adducts, which are chemically stable, were incorporated into oligonucleotides by standard chemical DNA synthesis. The presence of each lesion was confirmed by MALDI-TOF analyses.

2.3. Vectors

A single 3-m-c³A or THF AP-site was incorporated into a pELUf1 plasmid a 2 micron-based vector that contains the selectable marker LEU2. Modified plasmids were prepared by Enzymax (Lexington, KY, USA). The strategy for the construction of pELUf1 containing a site specific lesion was previously described by Zhao et al [22]. Briefly, after generation of single-stranded pELUf1, a unique doubled-stranded NcoI restriction site located within URA3 gene, generated through the annealing of a single-stranded oligonucleotide, was digested with the restriction enzyme NcoI. The linearized pELUf1 was annealed with: (a) a 57mer bridge oligonucleotide that has complementarity with the original NcoI site (20mer both upstream and downstream, underlined) and the oligonucleotide with site specific lesion (17mer, **in bold**) (5'-gcttaactgtgccctccatgccagatgtttgatcaagaaaaatcagtcagatac-3') and with (b) the 17mer oligo with the single lesion (5'-TTGATC**X**AAACATCTGG, with X = 3-m-c³A or AP-site) (Figure 1). The subsequent steps of ligation and synthesis of the complementary strand of pELUf1-3-m-c³A (or pELUf1-AP) were performed in the presence of dUTP as previously described [22]. The resulting plasmid, pELUf1-3-m-c³A (or pELUf1-AP) is a double-stranded plasmid containing the site-specific 3-m-c³A adduct (or AP-site) in which the undamaged strand contained U in place of T. The pELUf1-A plasmid containing an unmodified adenine at the same position as the lesions was constructed in order to be used as internal control.

2.4. Evaluation of 3-m-c³A cytotoxicity

pELUf1-A, pELUf1-AP or pELUf1-3-m-c³A (2 µg) were transformed into yeast cells by the LiAc/SS-DNA/PEG method [27]. After transformation, yeast cells were plated onto YNB minimal agar plates (0.67% yeast extract nitrogen base without amino acids, 2% dextrose and 2% agar) (Sigma-Aldrich; Diagnostical International Distribution, DID) lacking leucine but supplemented with 5-FOA (0.5 g/L final concentration) to score for colonies containing replicated plasmid. Following the degradation of the U containing strand, plasmid propagation in cells could be achieved only by replicating the remaining strand containing the lesion. Three (in WT and rad30 strains) or two independent (in rev3rad30 strain) experiments were performed; only one experiment was performed in the rev3 strain. Each experiment comprised 2 independent transformations. Transformation efficiency with lesion containing plasmid (3-m-c³A or AP-site) were normalized to that obtained with pELUf1-A vector in the different TLS background, i.e., WT, rev3, rad30 and rev3rad30, (number of transformants scored in transformation relative to that obtained with pELUf1-A vector).

2.5. Evaluation of the molecular events opposite 3-m-c³A

In order to evaluate the 3-m-c³A mutagenicity, yeast colonies recovered from the FOA plates were used as the template for PCR amplification of a fragment of 567 bp encompassing the lesion site using the URA3 forward and reverse primers (see above). After heating the yeast colony PCR mixture (50 µl) at 94°C for 8 min, 35 cycles of amplification were performed according to the following conditions: 40 s of denaturation at 94°C, 60 s of

annealing at 50°C and 120 s of extension at 72°C. After the last cycle, the reaction was continued for 6 min at 72°C. Clones that scored positive by PCR with a correct size band were subjected to different diagnostic restriction enzyme digestions that allowed the identification of specific molecular events. The digestion with the restriction enzyme BclI can occur only in the presence of an error free bypass of the lesion (AT>AT), while the digestion with the BstXI restriction enzyme requires specific error prone bypass of the lesion (AT>CG). An additional digestion with the restriction enzyme NcoI was performed in order to identify colonies containing the empty pELUf1 vector that had escaped selection by the 5-FOA plates (probably due to mutations elsewhere in the *URA3* gene). These clones, were excluded from the analyses. Finally the clones that were not digested by all three restriction enzymes were analyzed, after PCR purification (Qiagen, Milano, Italy), by DNA sequencing (BMR Genomics, Padoa, Italy).

2.6. Evaluation of 3-m-c³A TLS efficiency

To evaluate TLS efficiency, all mutants were considered with the exception of those mutants that after the analyses (PCR, restriction enzymes digestion, sequencing): a) did not yield PCR products (or a PCR product of incorrect size), b) contained the empty vector pELUf1 (presence of NcoI sequence identified by digestion) that escaped selection by the 5-FOA plates (probably due to mutations somewhere in the *URA3* gene); or c) did not contain the inserted oligonucleotide sequence (deletion identified by sequencing).

2.7. Proteins and Reagents

T7 RNA polymerase (T7 RNAP) was purchased from Promega (Milano, Italy) and Proteinase K from Invitrogen (Milano, Italy). Highly purified NTPs and radiolabelled nucleotides were purchased from Amersham Pharmacia Biotech.

2.8. Preparation of DNA templates for T7 transcription

DNA templates used for transcription reactions with T7 RNAP consisted of HindIII-linearized plasmid DNA containing a single 3-m-c³A or AP-site downstream of the T7 promoter. To construct plasmids to receive a single 3-m-c³A or AP-site containing oligonucleotide (5'-TTGATCXAAACATCTGG, with X = 3-m-c³A, AP-site, or unmodified A), oligomers of sequence 5'-gatccctgctcgtcgaccgcttaactgtgccctccatgccagatgtttgatcaagaaaatcagtcagatattctagatctcgattaaagc-3' were annealed to the complementary strands and ligated to a BamHI-XhoI fragment from pUC-GTG-TS to yield pUC-3-m-c³A-TS or pUC-AP-TS, or to a BamHI-XhoI fragment from pUC-GTG-NTS to yield pUC-3-m-c³A-NTS or pUC-AP-NTS [28]. These plasmids were transformed into the F' *E.coli* strain MV1184 to produce single-stranded DNA for primer extension, as previously described [29].

Covalently closed circular plasmids containing a single 3-m-c³A or AP-site in the transcribed (TS) or non-transcribed strand (NTS) were generated by priming 10 µg of plus strand of pUC-3-m-c³A-TS, pUC-AP-TS, pUC-3-m-c³A-NTS, pUC-AP-NTS with a 5-fold molar excess of 3-m-c³A or AP-containing 17mer of sequence 5'-TTGATCXAAACATCTGG-3' (where X = 3-m-c³A or AP-site) phosphorylated at the 5' end. The reaction mixture (300 µL) contained 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM MgCl₂, 1 mM DTT, 600 µM each of dATP, dCTP, dTTP and dGTP, 1 mM ATP, 30 U T4 DNA polymerase and 5 U of T4 DNA ligase [29]. Covalently closed circular molecules were purified after electrophoresis at 4°C in a 1% agarose gel containing 0.3 µg/ml ethidium bromide. Under these conditions, covalently closed circular DNA migrated as supercoiled DNA and could be resolved from single-stranded closed circular and nicked double-stranded plasmids. The 3-m-c³A and AP-containing plasmids were linearized by digestion with Hind III for 30 min at 37°C.

2.9. T7 RNAP transcription reactions

The DNA templates (10 ng) were incubated at 37°C for 5 min in a mixture of 50 units of T7 RNAP, 40 mM Tris-HCl (pH 7.9), 6 mM MgCl₂, 2 mM spermidine, 10 μCi [α -³²P]GTP, 10 mM dithiothreitol, 212 units of RNasin and 100 μM ATP. Elongation proceeds until T7 RNAP reaches the end of the C-less cassette (nucleotide 7), at which the first UTP is necessary for incorporation. Heparin was added to prevent further initiation and 100 μM CTP, UTP, and GTP were added to allow elongation to continue. Incubation continued at 37°C for 30 min. Reactions were stopped by addition of 5 μg of proteinase K, 1% SDS, 100 mM Tris-HCl (pH 7.5), 50 mM EDTA and 150 mM NaCl, followed by incubation for 15 min at room temperature. The nucleic acids were precipitated with ethanol, resuspended in formamide dye, and denatured at 90°C for 3 min. The transcription products were resolved on an 5% denaturing polyacrylamide gel in Tris-borate-EDTA containing 8.3 M urea. Gels were dried and autoradiographed using intensifying screens. Transcripts were quantified using a Typhoon phosphorimager and ImageQuant software from GE Healthcare. The extent of arrest was calculated by dividing the intensity of the band which corresponds to transcription arrest at the lesion by the sum of the intensity of the arrested and runoff RNA bands. All transcripts were labeled up to nucleotide 7, making quantitation independent of substrate length and G-content.

3. RESULTS

3.1. The 3-m-c³A does not impact on plasmid replication efficiency

The goal of the present work was to study for the first time the *in vivo* processing of the stable isostere of 3-mA, namely 3-m-c³A, and compare it with that of a stable THF AP-site (positive control) using a site-specific translesion synthesis assay in yeast cells. The lesion, whose presence was confirmed through MALDI-TOF analysis, was introduced into the URA3 gene and the resulting plasmid, pELUf1-3-m-c³A (or pELUf1-AP) was a double-stranded plasmid containing the site-specific 3-m-c³A (or an AP-site) on one strand [22]. In order to determine whether either of the two lesions impacted on plasmid replication efficiency, the pELUf1-3-m-c³A, pELUf1-AP and the pELUf1-A (used as unmodified control) plasmids were transformed into the yPM16 (WT) strain. The replication efficiency, represented by the number of surviving clones, was utilized as a measurement of the lethal effect associated with the presence of a site specific lesion. The replication efficiency of the 3-m-c³A containing plasmid was comparable to that of the undamaged control (pELUf1-A), suggesting that the presence of this lesion did not significantly impact the ability of the 3-m-c³A containing plasmid to replicate. In comparison, the replication efficiency of the AP-site containing plasmid decreased to 5.3 % (p=0.008) in the WT strain, indicating that the presence of a single AP-site dramatically inhibited DNA synthesis (Figure 2).

Next, we tested the efficiency of TLS polymerases to replicate opposite 3-m-c³A. For this purpose, the lesion containing plasmids pELUf1-3-m-c³A, pELUf1-AP, or the undamaged control plasmid pELUf1-A were transfected into yPM17 (rev3), yPM18 (rad30) and yPM19 (rev3rad30) strains and replication efficiency determined (Figure 2). With respect to the control pELUf1-A, pELUf1-3-m-c³A plasmid replication efficiency was similar in the rev3 yeast background. A decrease (3-m-c³A: 66.6% vs A), although not statistically significant, was observed in the rad30 background. Only in the rev3rad30 background, a statistically significant decrease (3-m-c³A: 61.6% vs A, p=0.015) was observed.

In contrast, the replication efficiency of the pELUf1-AP plasmid we used as control was significantly reduced with respect to the control pELUf1-A plasmid in all strains (rev3, p=0.002; rad30, p<0.0001; rev3rad30, p<0.0001). pELUf1-3-m-c³A replication efficiency was consistently and significantly higher compared to pELUf1-AP in all strains (WT,

$p=0.003$; $rev3$, $p=0.011$; $rad30$, $p<0.0006$; $rev3rad30$, $p=0.0001$). With respect to 3-m-c³A, the results indicate that its lethality significantly increases in cells that are null for Pol ζ and Pol η . It is important to note that during the transformation process in yeast there is no precise control of the plasmid copy number that enters a single cell, while a single replicated plasmid can lead to a selectable clone. Hence, the methodology could underestimate the negative impact on DNA replication of the introduced site-specific lesions. Nevertheless, the results indicate that the AP-site has a much stronger impact on replication as compared to 3-m-c³A.

3.2. 3-m-c³A rarely leads to large deletions

To characterize the base inserted opposite 3-m-c³A, we performed a yeast colony PCR using a pair of primers encompassing the region where the lesion was placed. Approximately a third of pELUf1-A (A clones) ($n=669$), all pELUf1-3-m-c³A (3-m-c³A clones) ($n=1331$) and all pELUf1-AP (AP clones) ($n=60$) clones were studied (a total of 2060 clones examined).

Three types of clones were identified (Table 2): (a) clones yielding no PCR product, (b) clones providing a shorter PCR product and (c) clones yielding a PCR product of the correct size. While almost all ($\geq 98\%$) A- and 3-m-c³A- ($\geq 94\%$) clones were PCR positive, at least half ($\geq 50\%$) of the AP-site clones were PCR negative. None of the control A clones showed shorter PCR products independently from the yeast strain background.

When the proportions of the different types of clones were compared, those observed with pELUf1-3-m-c³A and with the pELUf1-A, were indistinguishable in all backgrounds analyzed. However, they both differed significantly from those observed with pELUf1-AP in all backgrounds, with the exception of the $rev3$ background where the trend was confirmed but statistical significance was not reached.

In order to evaluate whether the TLS yeast background influences the outcome for each lesion, the proportions of the different types of clones were compared in the different strains. No influence was observed for pELUf1-A and pELUf1-AP, while a single, significant ($p=0.04$) decrease in PCR positive clones in yPM19 ($rev3rad30$) with respect to yPM18 ($rad30$) strain was observed for pELUf1-3-m-c³A. At this level of analysis, the results indicate that almost all of the pELUf1-3-m-c³A plasmid contained the correct sequence size within the region where the lesion was located. In contrast, the majority of the pELUf1-AP plasmids contained the incorrect sequence size within the region where the AP-site was placed.

3.3. The 3-m-c³A does not impact on TLS efficiency

In order to better evaluate the TLS process opposite 3-m-c³A and to compare it with the one opposite AP-site, the TLS efficiency was considered investigating only those clones that originated a PCR product of the correct size. Excluded from the calculation were clones, which after restriction enzyme digestions or sequencing contained the empty vector pELUf1 (escaped selection by the 5-FOA plates probably due to mutations elsewhere in the *URA3* gene on the plasmid) or did not contain the inserted oligonucleotide sequence. The comparisons confirmed the results obtained with plasmid replication efficiency (Table 3). TLS efficiency of 3-m-c³A was indistinguishable from that of A except in $rev3rad30$ background where it was significantly lower ($p=0.02$). TLS bypass efficiencies of 3-m-c³A and A were always significantly higher than that observed with the AP-site. Considering TLS efficiency of 3-m-c³A as a function of TLS, we found that it was significantly lower in the $rev3rad30$ than in the $rev3$ background ($p=0.035$). This suggests that only in the absence of both TLS polymerases there is a significant reduction of TLS efficiency.

3.4. Bypass of 3-m-c³A is mostly error free

In order to study the specificity of *in vivo* TLS opposite 3-m-c³A, diagnostic restriction enzyme digestions that allowed the identification of specific molecular events (see Materials and Methods) were performed, followed by direct sequencing. The results are summarized in Table 4. In a WT background, the AP-site showed a significant increase in error-prone bypass with respect to the control (A vs AP: $p=0.0001$) and 3-m-c³A (AP vs 3-m-c³A: $p=0.0001$), while 3-m-c³A did not increase error prone bypass with respect to the control. When REV3 is deleted, either alone (rev3) or in combination with RAD30 (rev3rad30), no error prone bypass was observed opposite 3-m-c³A or AP-site. This suggests that Pol ζ is needed for their error prone bypass. It has to be stressed that, due to the reduction of replication efficiency, the number of events recovered for the AP-site is very small in comparison to 3-m-c³A. In a rad30 background, both the 3-m-c³A and AP-site significantly differ in error prone bypass with respect to the control A (A vs 3-m-c³A: $p=0.0001$; A vs AP-site: $p=0.0002$) and with each other (3-m-c³A vs AP-site $p=0.015$). The proportion of events derived from an insertion of C opposite 3-m-c³A increases in the absence of Pol η with respect to the WT background (WT vs rad30: $p=0.0011$).

3.5. Effect of a single 3-m-c³A or a single AP-site in the transcribed (T) or non-transcribed (NT) strand of template DNA on transcription elongation by T7 RNA polymerase (RNAP)

We extended our analysis to characterize the effect of 3-m-c³A on RNA synthesis utilizing an *in vitro* transcription system in which DNA substrates containing a single 3-m-c³A in the T or NT strand downstream of the T7 promoter where transcribed by T7 RNAP. DNA templates, consisting of HindIII linearized plasmids containing a single 3-m-c³A located downstream of the T7 promoter, were constructed as described in Materials and Methods (Figure 3, panel A). The transcription templates were transcribed by a single molecule of RNAP so that the transcription products represented a single promoter-dependent elongation event [30]. This was accomplished by synthesizing a short ³²P-labeled RNA, followed by T7 RNAP stalling and addition of heparin to prevent further initiation. All NTPs were then added to allow elongation to continue. The effect of 3-m-c³A on transcription elongation was then monitored as recovery of transcripts shorter than those observed with the undamaged template. A negative control, consisting of undamaged DNA was transcribed under the same conditions. To compare the effect on transcription of 3-m-c³A with respect to that of an AP-site, a substrate containing a single AP-site positioned at the same location as the 3-m-c³A was also transcribed. When a single 3-m-c³A was located in the T strand, transcription by T7 RNAP proceeded until the end of the template, as indicated by synthesis of RNAs of similar size as those produced after transcription of the undamaged (A-containing) control (Figure 3, panel B, lanes 1 and 2). Conversely, when a single AP-site was located in the T strand, transcription by T7 RNAP produced transcripts shorter than the full-length transcript present in the control (Figure 3, panel B, lane 3). Comparison of the migration of these RNA products with that of a 10 bp DNA size marker (panel B, lane L) indicated that these transcripts were approximately 284 nt long, as expected from RNA extension up to the site of the lesion. A 3-m-c³A or an AP site located in the NT strand did not block T7 RNAP, as indicated by the production of only full-length runoff transcripts (Figure 2, panel B, lanes 5 and 6).

4. DISCUSSION

We report the first attempt to characterize the consequences of the *in vivo* replication opposite 3-m-c³A, a stable isostere of 3-mA, with the anticipation it will provide insights into the biology of 3-mA, which is enzymatically and/or spontaneously hydrolyzed off the DNA backbone.

The 3-m-c³A was placed at a specific site within the sequence context 5'-CAAAC-3' present in the human p53 cDNA, harbored in a dsDNA plasmid (pELUf1-3-m-c³A). This A was previously observed to be heavily alkylated *in vitro* [5] and also a hotspot for mutations with only AT>GC transitions being observed in different DNA repair backgrounds [5–7,10]. The same mutational pattern was observed in cells deficient in AP endonuclease (apn1apn2) [6] so the question arose whether the mutations induced by 3-mA were indirectly generated by its conversion into an AP-site. To address this issue, an A (pELUf1-A) or a THF AP-site (pELUf1-AP) were placed at the same site. Our data show that the AP-site was able to dramatically reduce the ability of the pELUf1-AP plasmid to replicate (Figure 2, Tables 2 and 3), while the presence of a 3-m-c³A had no significant impact in a WT background. Because the lethality of the 3-m-c³A significantly increased only in a rev3rad30 background, the involvement of Polη and Polζ in the bypass of 3-m-c³A is indicated. Interestingly, when the bypass events opposite 3-m-c³A were considered at the molecular level, the absence of Polη was associated with a specific and significant increase in AT>GC transitions (p=0.0011, Table 4), suggesting that Polη participates to the error free bypass of 3-m-c³A. Interestingly, using the yeast based p53 functional assay and comparing the mutation spectra in a WT vs rad30 strains, AT>GC was the only class of AT targeted mutation significantly more frequent in the rad30 with respect to the WT strain (p<0.05) [24]. The involvement of Polη in the bypass of 3-m-c³A is not surprising. First, Polη, in contrast to the replicative DNA polymerases, does not use a minor groove sensing mechanism involving the N-3 position of A [31,32]. Second, a 3-mA modelled into the active site of a Polη is not expected to be a block at either the insertion step or the subsequent extension step [33]. Third, among the tested eukaryotic DNA polymerases, *S. cerevisiae* Polη appears to be the most effective in bypassing 3-m-c³A.

The role of Polζ in the bypass of 3-m-c³A is less clear. However, in light of the fact that in the absence of Rev3, alone (rev3) or with Polη (rev3rad30), 100% of the recovered clones derived from error free bypass (Table 4), we propose that Polζ is either involved in an error-prone bypass of 3-m-c³A, or in the extension step after another TLS polymerase has incorporated a nucleotide opposite 3-m-c³A.

The absence of a block to DNA replication is paralleled by the inability of 3-m-c³A to block *in vitro* transcription when placed on the T strand of DNA (Figure 3). Under the same transcription conditions, an AP-site was a strong block to T7 progression (Figure 3). In agreement with our findings, previous studies have shown that a natural AP-site located in the T strand affected T7 RNAP transcription [34], and that this effect was modulated by the sequence context around the lesion [34–36].

The difference in behavior of T7 RNAP when transcribing past a 3-m-c³A compared to an AP-site is likely the result of a minor effect of 3-m-c³A on the stability of the RNA-DNA hybrid compared to an AP site. In agreement with our observed lack of transcription inhibition by 3-m-c³A *in vitro*, 3-mA does not elicit transcription-coupled repair *in vivo* [37].

Why does 3-m-c³A not block replication? Recently, 3-m-c³A was thermodynamically characterized (21). It was demonstrated that the lesion destabilizes duplex DNA ($\Delta\Delta G$ was > 4 kcal/mol) due to a significant drop in the enthalpy associated with a decrease in stacking interactions. It may be that the bypass of 3-m-c³A does not block replication because it is extruded out of the base pair stack. In the A₄ sequence studied, this type of extrusion followed by strand realignment could give error free bypass. The hypothesized mechanism(s) by which 3-mA could block replicative DNA polymerases is either by steric hindrance in the active site or because of the involvement of the N3-A atom in hydrogen bonding with the residues in the high-fidelity polymerases used to detect the correct Watson-Crick geometry

of the post-insertion template primer base pair. The fact that we did not observe block of replication *in vivo*, suggests that neither mechanism is operative in our experimental system. Our results suggest that the DNA polymerase(s) involved in the 3-m-c³A bypass is (are) not sensitive to steric hindrance or to the N-3 in hydrogen bonding mechanisms, pointing toward Pol η as the polymerase involved in the 3-m-c³A bypass albeit, other polymerases such as Pol ζ - may play a minor role.

An alternative explanation for the lack of replication block observed with 3-m-c³A, is that this analogous may not be as good a surrogate for 3-mA as expected. While the 3-m-c³A modification is a close isostere of 3-mA, replacement of the minor groove N-CH₃ on 3-mA with a C-CH₃ in 3-m-c³A eliminates the basic nitrogen and the potential formation of positive charge on the aromatic purine ring. This discrepancy is somewhat surprising since both 3-mA [21] and 3-m-c³A [19,21] appear to constitute a strong *in vitro* block to replicative DNA polymerases. However, TLS DNA polymerases in general, and Pol η in particular, appear to have the capacity to replicate through these lesions, albeit with reduced efficiency. For example, human Pol η shows a very similar ability to bypass 3-m-c³A *in vitro* with respect to A [Figure 3 in [19]] that is more than two-orders of magnitude higher than a replicative DNA polymerase (*e.g.*, Pol α).

One way to reconcile the results obtained *in vitro* [19,21] and those obtained *in vivo* (present work) is to hypothesize that what is seen as block *in vitro* (for 3-m-c³A) is actually a pause in the replication fork that is resolved prior to the completion of the cell cycle.

If we assume that the structural difference between 3-m-c³A modification and 3-mA has no influence on the processes we have studied, the results obtained here can shed light on the relative contributions of 3-mA and AP-sites to the mutagenicity and cytotoxicity observed after *in vitro* damaging of a shuttle vector plasmid with Me-lex. From the present results it appears that 3-mA (3-m-c³A) would contribute substantially to lethality *in vivo* only in a rev3rad30 background. In contrast, the AP-site would have the major contribution to lethality in all backgrounds. The toxic effect of 3-mA following Me-lex *in vitro* treatment might be partially ascribed to AP-sites formed at replication forks by the enzymatic or hydrolytic conversion of the primary 3-mA adduct. In terms of mutagenicity both lesions may contribute to this endpoint however, it is interesting to note that besides Pol α , all TLS DNA polymerases tested *in vitro* preferentially inserted the correct T nucleotide opposite 3-m-c³A [19].

In conclusion we found that the impact of 3-m-c³A on plasmid DNA replication is far below that of an AP-site in the same sequence context. Furthermore the bypass of 3-m-c³A is mainly error free, with an error prone mechanism been involved only in the absence of Pol η . 3-m-c³A did not affect *in vitro* transcription. Further studies will be necessary to verify whether these results are specific for the sequence context used.

Highlights

The present work describes for the first time the effect on *in vivo* DNA metabolism of a stable isostere of 3methyladenine, a very biologically relevant DNA lesion generated by several environmental agents and anticancer drugs. We found that this lesion doesn't significantly affect DNA metabolism *in vivo* (and RNA transcription *in vitro*), suggesting that the cytotoxicity previously described as the biological endpoint of this lesion is the likely result of the cellular conversion of 3 methyl adenine to the cytotoxic abasic site lesion. We believe that our results are very relevant to understanding the biology of 3-methyladenine.

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Figure 1.
Sequence of the oligonucleotides containing the different lesions used in this study.

Figure 2.

Influence of TLS background (wild type, rev3, rad30, rev3rad30) on plasmid replication efficiency of pELUf1-A, pELUf1-AP and pELUf1-3-m-c³A. Three (WT and rad30) or two independent (rev3rad30) experiments were performed; only one experiment was performed in rev3 strain. Each experiment comprised 2 independent transformations. Relative replication efficiency was obtained by comparing number of transformants observed in presence of the different adduct (AP or 3-m-c³A) to that observed in control. Number of transformants were expressed per μg of plasmid. Error bars, when present, represent SD.

Figure 3.

Effect of a single 3-m-c³A or a single AP-site on T7 RNA polymerase transcription. Panel (A) DNA template pUC-3-m-c³A-TS for T7 RNAP transcription. DNA templates for T7 transcription, each containing a single 3-m-c³A in the transcribed (T) or in the non-transcribed strand (NT) downstream of the T7 promoter, were constructed as described in Materials and Methods. Runoff RNA (RO) and RNA generated from transcription arrest at 3-m-c³A (or AP site) are marked with dashed lines along with their expected sizes. The transcription start site (+1) is represented by a bent arrow. Panel (B) T7 RNAP transcription of substrates containing a single 3-m-c³A or a single AP site in the transcribed (T) or the non-transcribed (NT) strand. DNA templates were transcribed *in vitro* such that the transcripts were radioactively labeled. Elongation was allowed to proceed for 30 min after addition of NTPs to the reaction. Lanes 1 and 4, unmodified templates (A); lanes 2 and 5, templates containing a single 3-m-c³A in the T (lane 2) or NT strand (lane 5) (3-m-c³A); lanes 3 and 6, templates containing a single AP site in the T (lane 3) or NT strand (lane 6) (AP). RO, full-length runoff transcript; AP, transcripts arrested at the AP site; T, transcribed strand; and NT, non-transcribed strand. L: 10 bp ladder; M: 100 bp ladder.

Table 1

Yeast strains constructed and used in this study

Name	Genotype	TLS background
yPM16	MAT _α ade2-1 leu2-3,112 trp1-1 his3-11,15 can1-100 ura3Δ0::TRP1	WT
yPM17	same as yPM16 but rev3::HYGRO ^R	rev3 (pol _ζ)
yPM18	same as yPM16 but rad30::HYGRO ^R	rad30 (pol _η)
yPM19	same as yPM17 but rad30::KANMX4 ^R	rev3rad30 (pol _ζ pol _η)

Table 2
Type of molecular events identified in analyzed clones by PCR amplification of the lesion containing region.

Lesion	Strain	Clones (N°)	incorrect PCR		correct PCR	correct PCR vs incorrect PCR	(p value)
			negative	shorter			
A	WT	175	3 (2%)	-	172 (98%)	A vs 3-m-c ³ A:	NS
AP	WT	22	15 (68%)	-	7 (32%)	A vs AP:	p=0.0001
3-m-c ³ A	WT	485	14 (3%)	-	471 (97%)	AP vs 3-m-c ³ A:	p=0.0001
A	rev3	50	1 (2%)	-	49 (98%)	A vs 3-m-c ³ A:	NS
AP	rev3	2	1 (50%)	-	1 (50%)	A vs AP:	NS
3-m-c ³ A	rev3	98	4 (4%)	-	94 (96%)	AP vs 3-m-c ³ A:	NS
A	rad30	330	6 (2%)	-	324 (98%)	A vs 3-m-c ³ A:	NS
AP	rad30	19	13 (68%)	1 (5%)	5 (27%)	A vs AP:	p=0.0001
3-m-c ³ A	rad30	567	12 (2%)	-	555 (98%)	AP vs 3-m-c ³ A:	p=0.0001
A	rev3rad30	114	1 (1%)	-	113 (99%)	A vs 3-m-c ³ A:	NS
AP	rev3rad30	17	14 (82%)	1 (6%)	2 (12%)	A vs AP:	p=0.0001
3-m-c ³ A	rev3rad30	181	9 (5%)	1 (1%)	171 (94%)	AP vs 3-m-c ³ A:	p=0.0001

NS: Not Significant
AP: wt vs rev3: NS; wt vs rad30: NS; wt vs rev3rad30: NS; rev3 vs rad30: NS; rev3 vs rev3rad30: NS; rad30 vs rev3rad30: NS.
3-m-c³A: wt vs rev3: NS; wt vs rad30: NS; wt vs rev3rad30: NS; rev3 vs rad30: NS; rev3 vs rev3rad30: NS; rad30 vs rev3rad30: p=0.04;

Table 3

Influence of TLS background (WT, rev3, rad30, rev3rad30) on TLS efficiency.

TLS background	TLS efficiency/ μ g of input plasmid		
	A	AP-site	3-m-c ³ A
WT	100 $\pm$ 50,5	1,3 $\pm$ 1,7	110 $\pm$ 66,8
rev3	100 $\pm$ 17	1 $\pm$ 1,4	89,9 $\pm$ 18,9 *
rad30	100 $\pm$ 32,7	0,7 $\pm$ 1,3	66,8 $\pm$ 31,9
rev3rad30	100 $\pm$ 24	0,8 $\pm$ 1	59,6 $\pm$ 9 *, **

*
p=0.0462**
p=0.02

Table 4

Specificity of TLS opposite 3-m-c³A with respect to the different internal controls (A and AP-sites) in different yeasts TLS backgrounds.

Lesion	Strain	Clones	base inserted opposite the lesion					Complex*	comparison	p value
			Error Free		Error Prone					
		(N°)	T (AT>AT)	G (AT>CG)	C (AT>GC)	A (AT>TA)				
A	WT	172	172 (100%)	-	-	-	-	-	A vs 3-m-c ³ A :	NS
AP	WT	7	2 (29%)	1 (14%)	3 (43%)	1 (14%)	-	-	A vs AP :	0.0001
3-m-c ³ A	WT	471	467 (99.2%)	1 (0.2%)	3 (0.6%)**	-	-	-	AP vs 3-m-c ³ A :	0.0001
A	rev3	49	49 (100%)	-	-	-	-	-	A vs 3-m-c ³ A :	NS
AP	rev3	1	1 (100%)	-	-	-	-	-	A vs AP :	NS
3-m-c ³ A	rev3	94	94 (100%)	-	-	-	-	-	AP vs 3-m-c ³ A :	NS
A	rad30	324	324 (100%)	-	-	-	-	-	A vs 3-m-c ³ A :	0.0001
AP	rad30	5	3 (60%)	-	2 (40%)	-	-	-	A vs AP :	0.0002
3-m-c ³ A	rad30	555	534 (96.2%)	-	20 (3.6%)**	-	1 (0.2%)	-	AP vs 3-m-c ³ A :	0.015
A	rev3rad30	113	113 (100%)	-	-	-	-	-	A vs 3-m-c ³ A :	NS
AP	rev3rad30	2	2 (100%)	-	-	-	-	-	A vs AP :	NS
3-m-c ³ A	rev3rad30	171	171 (100%)	-	-	-	-	-	AP vs 3-m-c ³ A :	NS

* Complex= a GC>AT, 3 nucleotide upstream and GC>TA, 2 nucleotide downstream the 3-m-c³A site was observed.; NS: Not Significant.

**
p=0.0011

AP: wt vs rev3; NS; wt vs rad30; NS; wt vs rev3rad30; NS; rev3 vs rad30; NS; rev3 vs rev3rad30; NS; rad30 vs rev3rad30; NS.

3-m-c³A wt vs rev3; NS; wt vs rad30; p=0.002; wt vs rev3rad30; NS; rev3 vs rad30; p=0.06; rev3 vs rev3rad30; p=0.0067