Inefficient Repair of Tamoxifen-DNA Adducts in Rats and Mice

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Abbreviations:
dN, 2'-deoxynucleoside; dG, 2'-deoxyguanosine; dG_P, 2'-deoxyguanosine 3'-monophosphate; TAM, tamoxifen; N-desTAM, N-desmethyltamoxifen; TAM N-oxide, tamoxifen N-oxide; 4-OHTAM, 4-hydroxytamoxifen; α-OHTAM, α-hydroxytamoxifen; dG-Ν²-TAM, α-(Ν²-deoxyguanosinyl)tamoxifen; dG-Ν²-N-desTAM, α-(Ν²-deoxyguanosinyl)-N-desmethyltamoxifen; NER, nucleotide excision repair. PAGE, polyacrylamide gel electrophoresis.
Abstract

A long-term treatment of tamoxifen (TAM) to women increases the risk of developing endometrial cancer. It may result from genotoxic damage induced by this drug. In fact, TAM-DNA adducts were detected in the liver of rats treated with TAM and initiated to develop hepatocellular carcinomas. To explore the distribution and repair rate of TAM-DNA adducts, the level of TAM-DNA adducts in all tissues of rats and mice was monitored for 28 days and 7 days, respectively, after the termination of TAM treatment, using \(^{32}\)P-postlabeling/polyacrylamide gel and \(^{32}\)P-postlabeling/HPLC analyses. TAM-DNA adducts were formed specifically in the liver of rodents. In rats, the level of hepatic TAM-DNA adducts was decreased only to 43% in 28 days, indicating that the half-life of adducts was approximately 25 days. Among trans- (fr-1 and fr-2) and cis- (fr-3 and fr-4) isoforms of TAM-DNA adducts, a trans-form (fr-1) was removed much slower than other adducts, indicating that the repair rate of TAM-DNA adducts varied depending on the structure of isoforms. The repair rate of TAM-DNA adducts was also compared between the nucleotide excision repair deficient (\(Xpc\) knockout) and the wild mice. Although the level of hepatic TAM-DNA adducts observed with \(Xpc\) knockout mice was slightly higher than that of the wild type, the removal of TAM-DNA adducts in both mice was only 20% in 7 days. Thus, TAM-DNA adducts are not efficiently repaired from the targeted tissue, leading to the development of cancer.
Introduction

Tamoxifen (TAM) is used in standard endocrine therapy for breast cancer patients and as a chemopreventive agent for healthy women at high risk of this disease (Osborne, 1998; Fischer et al., 1998). Beside the significant benefit, long-term treatment of TAM to women increases the risk of developing endometrial cancer (van Leeuwen et al., 1994; Fischer et al., 1998). The development of endometrial cancer may be due to the partial estrogenic effect of TAM through the estrogen receptor (Stygar et al., 2003) and/or genotoxic damage (reviewed by Kim et al., 2004). In rats treated with TAM, a high level of TAM-DNA adducts formed in the liver (Han and Liehr, 1992; Osborne et al., 1996) initiates the development of hepatocellular carcinomas (Hard et al., 1993; Greaves et al., 1993). The formation of TAM-DNA adducts was observed in various tissues including reproductive organs of monkey treated with TAM (Shibutani et al., 2003; Schild et al., 2003). In human, there is a controversy of detecting TAM-DNA adducts. Some research groups including us have detected TAM-DNA adducts in the endometrium of women treated with TAM (Shibutani et al., 2000a; Martin et al., 2003) while other groups did not observed TAM-DNA adducts (Carmichael et al., 1996; Beland et al., 2004).

TAM is metabolized by phase I enzymes to N-desmethyltamoxifen (N-desTAM), 4-hydroxytamoxifen (4-OHTAM) and tamoxifen N-oxide (TAM N-oxide) (reviewed by Kim et al., 2004). α-Hydroxylated TAM metabolites are produced as minor products from TAM and its metabolites in reaction catalyzed by rat CYP 3A2 and human CYP 3A4 (Kim et al., 2004), O-sulfonated by hydroxysteroid sulfotransferase (Shibutani et al., 1998a and 1998b), and react with dG residues in cellular DNA, resulting in the formation of two-trans and two-cis isoforms of α-(N²-Deoxyguanosinyl)tamoxifen (dG-N²-TAM) adducts (Fig. 1) (Osborne et al., 1996; Dasaradhi and Shibutani, 1997). dG-N²-TAM
and $\alpha$-($N^2$-deoxyguanosinyl)-$N$-desmethyrtamoxifen (dG-$N^2$-N-desTAM) were major adducts in the liver of rats and mice treated with TAM (Rajaniemi et al., 1999; Umemoto et al., 2001) and in several tissues including reproductive organs of monkey treated with TAM (Shibutani et al., 2003; Schild et al., 2003). $\alpha$-($N^2$-Deoxyguanosinyl)tamoxifen $N$-oxide (dG-$N^2$-TAM $N$-oxide) was detected as a minor adduct in mouse liver. dG-$N^2$-TAM adducts were also detected in the endometrium of women treated with TAM (Shibutani et al., 2000a). Site-specific mutagenesis studies showed that dG-$N^2$-TAM adducts have highly mutagenic potential, generating mainly G $\rightarrow$ T transversions, accompanied by fewer G $\rightarrow$ A transitions in mammalian cells (Terashima et al., 1999). Similar mutagenic specificity was observed at both lac I and cII genes in the liver of the $\lambda$/lacI transgenic rats treated with TAM (Davies et al., 1999). In breast cancer patients treated with TAM, a high frequency of G $\rightarrow$ T and G $\rightarrow$ A mutations was detected at codon 12 of the K-ras protooncogene in the endometrium (Hachisuga et al., 2005). The mutational spectrum was consistent with that observed in our mutagenesis study (Terashima et al., 1999), suggesting that the mutations occurred at the K-ras gene are due to the genotoxic effect of TAM.

Bulky DNA adducts, including dG-$N^2$-benzo[a]pyrene (Cerutti et al., 1997) and dG-C8-acetylaminofluorene (Howard et al., 1981), are generally removed by nucleotide excision repair (NER) enzymes. In in vitro experimental system using mammalian and human nucleotide excision repair enzymes, we found that TAM-DNA adducts are removed slowly from DNA (Shibutani et al., 2000b). Mutation frequency induced by $\alpha$-acetoxyTAM in NER deficient cell (XPA) was higher than that observed with NER proficient cells, indicating that NER plays an important role in removal of TAM-DNA adducts (McLuckie et al., 2005). If TAM-DNA adducts persist in the tissues of animals...
and women treated with TAM, cancer may be initiated by the cumulative TAM-DNA damage. The repair efficiency of TAM-DNA adducts may thus be a key factor in TAM carcinogenicity.

A few research groups have determined repair of hepatic TAM-DNA adducts in rat using $^{32}$P-postlabeling analysis and chemiluminescence assay (Whilte et al., 1992; Divi et al., 1999; da Costa et al., 2001); however, total amounts of TAM-DNA adducts were only determined. Since each TAM-DNA adduct has different mutagenic potential (Terashima et al., 1999) and repair potential (Shibutani et al., 2000b), the repair rate of each TAM-DNA adduct in rats was determined in the present study using sensitive $^{32}$P-postlabeling/PAGE and $^{32}$P-postlabeling/HPLC analyses. Xpc knockout mice are deficient in both alleles of mouse Xeroderma Pigmentosa Complementation group C, one of factors involved in nucleotide excision repair (Sands et al., 1995). The repair rate of TAM-DNA adducts were also determined in the Xpc knockout mice and the wild type for exploring the contribution of NER to removal of TAM-DNA adducts.

**Materials and Methods**

**Materials.** TAM, calf thymus DNA, micrococcal nuclease, and potato apyrase were purchased from Sigma-Aldrich (St. Louis, MO). Spleen phosphodiesterase was obtained from Worthington Biochemical Corp. (Lakewood, NJ). 3'-Phosphatase-free T4 PNK and nuclease P1 were obtained from Roche Applied Science (Indianapolis, IN). TAM $\alpha$-sulfate and diastereoisomers (fr-1 and fr-2) of trans-forms and diastereoisomers (fr-3 and fr-4) of cis-forms of dG$_{3}'$-monophosphate-$N^2$-tamoxifen (dG$_{3}'$-$N^2$-TAM) were prepared as described previously (Dasaradhi and Shibutani, 1997; Shibutani et al., 1998a). [$^\gamma$-$^{32}$P]-ATP (specific activity, >6000 Ci/mmol) was obtained from Amersham Biosciences Corp. (Piscataway, NJ).
Animal study. Fisher 344 rats (female, 8 weeks-old), Xpc knockout mice and B6129F1 mice (female, 8 weeks-old) were purchased from Taconic (Germantown, NY). The use of animals was in compliance with the guidelines established by the NIH Office of Laboratory Animal Welfare. Animals were acclimated in temperature (22 ± 2 °C) and humidity (55 ± 5%) controlled rooms with a 12 hr light-dark cycle for at least 1 week prior to use. Regular laboratory chow and tap water were allowed ad lib. Rats were treated orally with TAM (20 mg/kg/day) for 7 days. Xpc knockout mice and B6129F1 mice were given TAM (20 mg/kg/day or 120 mg/kg/day) for 7 days by gavage. Control rats and mice were treated with an identical volume of corn oil. The rats and mice were euthanized by CO2 asphyxiation at 5 hr, 7 and/or 28 days after the final treatment, and open thoracotomy. All tissues were removed quickly, frozen, and stored at –80 °C until DNA extraction.

Digestion of DNA samples. The tissue DNA was extracted using a Qiagen DNA isolation kit (Valencia, CA) following the manufacturer’s protocol. The concentration of DNA was determined by UV spectroscopy as 50 µg/ml = O.D. 260 nm 1.0. DNA sample (1.0-5.0 µg) was enzymatically digested at 37 °C for overnight in 100 µl of 17 mM sodium succinate buffer (pH 6.0) containing 8 mM CaCl₂, using micrococcal nuclease (30 units) and spleen phosphodiesterase (0.15 unit) (Terashima et al., 2002). The reaction mixture was incubated for another 1 h with nuclease P1 (1 unit). After the incubation, 150 µl of water was added. The reaction samples were then extracted twice with 200 µl of butanol. The butanol fractions were combined, back-extracted with 50 µl of distilled water, and evaporated to dryness.

³²P-postlabeling/PAGE analysis. The DNA digests were incubated at 37 °C for 40 min with 10 µCi of [γ³²P]-ATP and 3'-phosphatase-free T4 polynucleotide kinase (10 units),
and then incubated with apyrase (50 milliunits) for another 30 min, as described previously (Terashima et al., 2002). Known amounts (0.152 pmol mol, 0.0152 pmol, 0.00152 pmol or 0.000152 pmol) of dG-N²-TAM-modified oligodeoxynucleotide prepared by a phosphoramidite chemical procedure (Laxmi et al., 2002) were mixed with 5 µg of calf thymus DNA (15200 pmol) and served as a standard (1 adduct/10⁵ nucleotides, 1 adduct/10⁶ nucleotides, 1 adduct/10⁷ nucleotides, or 1 adduct/10⁸ nucleotides). The amount of TAM-DNA adducts detected increased linearly depending on the amounts of oligodeoxynucleotide used. A part of the ³²P-labeled sample was electrophoresed for 4-5 h on a non-denaturing 30% polyacrylamide gel (35 x 42 x 0.04 cm) with 1400-1600 V/20-40 mA. The position of ³²P-labeled adducts was established by β-phosphorimager analysis (Molecular Dynamics Inc.). To quantify the level of ³²P-labeled products, integrated values were measured using a β-phosphorimager and compared with the standards. The detection limit for 5 µg DNA was approximately 7 adducts/10⁹ nucleotides.

³²P-Postlabeling/HPLC analysis. After the ³²P-labeled products were developed on the gel as described above, the bands of ³²P-labeled products were cut from the gel and put into 1 ml of distilled water for overnight at the room temperature. The ³²P-labeled products extracted from the gel were evaporated to dryness. Recovery of ³²P-labeled products was ~95%. The ³²P-labeled products were solved in 20 µl of distilled water and subjected to a Hypersil BDS C₁₈ analytical column (0.46 x 25 cm, 5 µm, Shandon), eluted at a flow rate of 1.0 ml/min with a linear gradient of 0.2 M ammonium formate and 20 mM H₃PO₄, pH 4.0, containing 20 to 30 % acetonitrile for 40 min, 30 to 50 % acetonitrile for 5 min, followed by an isocratic condition of 50 % acetonitrile for 15 min. The radioactivity was monitored using a radioisotope detector (Berthold LB506 C-1,
ICON Scientific Inc.) linked to a Waters 990 HPLC instrument. As described above, known amounts (0.152-0.000152 pmol) of dG-N²-TAM-modified oligodeoxynucleotide prepared by a phosphoramidite chemical procedure (Laxmi et al., 2002) were mixed with 5 µg of calf thymus DNA (15200 pmol) and served as a standard. As described previously (Terashima et al., 2002), the amount of TAM-DNA adducts detected increased linearly depending on the amounts of oligodeoxynucleotide used. The detection limit of this assay was ~2 adducts/10⁹ nucleotides for 5 µg DNA.

Statistical analysis. Results were expressed as mean ± SD. Student’s t-test was used to evaluate the difference. Values of p ≤ 0.05 were considered statistically significant.

Results

Rats were treated orally with TAM (20 mg/kg/day) for 7 days and sacrificed at 5 h after the final treatment. ³²P-postlabeling/PAGE was performed to analyze TAM-DNA adducts in each tissues using a standard (5 adducts/10⁸ nucleotides). A high level of TAM-DNA adducts (18.3 adducts/10⁷ nucleotides) was detected in the liver of all rats treated with TAM (Fig. 2A). Since the migration of each trans (fr-1 and fr-2) and cis (fr-3 and fr-4) isoforms of dG₃p-N²-TAM is same to that of dG₃p-N²-N-desTAM (Kim et al., 2005), the major TAM-DNA adduct in the liver was expected to be a mixture of fr-2 of dG₃p-N²-TAM and dG₃p-N²-N-desTAM. Among the extrahepatic tissues, a low level of TAM-DNA adduct (0.5 adducts/10⁷ nucleotides) was detected in one out of three stomach DNA samples (Fig. 2A). The migration of this adduct was similar to that of standard fr-1 of trans-dG₃p-N²-TAM or dG₃p-N²-N-desTAM. When the sample was subjected to HPLC on-lined with a radioisotope monitor, co-migrating with the standard, the retention time of this adduct was identical to that of fr-1 of dG₃p-N²-TAM (data not shown). No DNA adduct was detected in any other tissues.
To determine the ability of repairing TAM-DNA adducts, the level of hepatic TAM-DNA adducts were monitored 7 and 28 days after the final TAM treatment (Fig. 3 and Table 1) using $^{32}$P-postlabeling/PAGE. At day 7 after the cessation, the total amounts of TAM-DNA adduct were 15.8 adducts/10$^7$ nucleotides. No significant change was observed when compared with the value (18.3 adducts/10$^7$ nucleotides) detected at 5 hr. At day 28, the total TAM-DNA adducts (7.8 adducts/10$^7$ nucleotides) was significantly decreased to 43%. $^{32}$P-postlabeling/HPLC analysis was also used to resolve all trans- and cis-forms of dG$_{3p}$-$N^2$-TAM and dG$_{3p}$-$N^2$-desTAM. Typical hepatic DNA samples obtained at 5 hr (Fig. 4A), 7 days (Fig. 4B), and 28 days (Fig. 4C) after the final TAM treatment were shown. By co-migrating with standards (Fig. 4D), the major TAM-DNA adducts were identical to fr-2 of dG$_{3p}$-$N^2$-TAM and dG$_{3p}$-$N^2$-N-desTAM (Fig. 4E). No adducts were observed in rats treated only with vehicle (Fig. 4F). A trans-form (fr-2) and cis-forms (a mixture of fr-3 and fr-4) of both dG$_{3p}$-$N^2$-TAM and dG$_{3p}$-$N^2$-desTAM were removed to 38–47% as compared the values observed at 5 hr (Table 1). The half-lives of fr-2 of dG$_{3p}$-$N^2$-TAM and dG$_{3p}$-$N^2$-desTAM were 22 and 28 days, respectively and the half-lives of cis-forms of dG$_{3p}$-$N^2$-TAM and dG$_{3p}$-$N^2$-desTAM were 23 and 27 days. Another minor trans-forms (fr-1) were reduced to 53-65%, indicating that fr-1 resists the repair more than other isoforms; the half-lives of fr-1 of dG$_{3p}$-$N^2$-TAM and dG$_{3p}$-$N^2$-desTAM were 40 and 29 days, respectively.

Xpc knockout mice and the wild (B6129F1) mice were also given TAM (20 mg/kg/day or 120 mg/kg/day) for 7 days by gavage. The level of TAM-DNA adducts were determined in all tissues using $^{32}$P-postlabeling/PAGE. When mice were treated with TAM (20 mg/kg/day for 7 days) the dose equivalent to that used for rat study, only a trace of TAM-DNA adduct (<1 adduct/10$^8$ nucleotides) was observed in the liver (data not shown). Therefore, mice were treated with six-fold higher dose (120 mg/kg/day) of
TAM, as reported previously (Umamoto et al., 2001). A high level of TAM-DNA adducts were observed in the liver of all Xpc knockout mice (Fig. 2B) and B6129F1 mice (data not shown). In the Xpc knockout mice, no TAM-DNA adduct were detected in extrahepatic tissues (Fig. 2B). Only a trace of TAM-DNA adduct was observed in one spleen of three B6129F1 mice (data not shown). The total amounts of hepatic TAM-DNA adducts in the Xpc knockout and B6129F1 mice were 11.3 adducts/10^7 nucleotides and 7.1 adducts/10^7 nucleotides, respectively (Table 2). The major adducts in both mice were trans-forms (fr-2) of dG3p-N2-TAM and dG3p-N2-desTAM at the level of 10.1 and 6.14 adducts/10^7 nucleotides, respectively. Compared to the wild type mice, the values of TAM-DNA adducts in Xpc knockout mice were slightly higher; 132 % for fr-1, 176 % for fr-2, and 138 % for fr-3 & fr-4. However, no significant difference in the level of TAM-DNA adducts was observed between Xpc knockout mice and the wild type.

On day 7 after the final treatment, the levels of total TAM-DNA adducts were decreased to 71% (8.2 adducts/10^7 nucleotides) for Xpc knockout mice and 80% (5.7 adducts/10^7 nucleotides) for the wild type. No significant difference was observed in the removal of between dG3p-N2-TAM and dG3p-N2-desTAM adducts. Except for cis-forms of Xpc knockout mice, removal of a trans-TAM-DNA (fr-2) and cis-TAM-DNA adducts (fr-3 & fr-4) was slightly faster than that of another trans-TAM-DNA adduct (fr-1).

**Discussion**

Among tissues of rats treated with TAM, TAM-DNA adducts were detected primarily in the liver although a trace of TAM-DNA adduct was observed in some of extrahepatic tissues, as recently reported (Phillips et al., 2005). In contrast, in monkeys treated with TAM, TAM-DNA adducts were observed in brain, ovary and uterus, in addition to liver (Shibutani et al., 2003; Schild et al., 2003). The adduct was also detected in the
endometrium of women treated with TAM (Shibutani et al., 2000a). Thus, the formation of TAM-DNA adducts in rats is species-specific and liver-specific, resulting in developing hepatocellular carcinoma (Hard et al., 1993; Greaves et al., 1993).

Repair rate of hepatic TAM-DNA adducts were determined in rats using \(^{32}\)P-postlabeling/PAGE and \(^{32}\)P-postlabeling/HPLC analyses. After termination of TAM treatment, the level of TAM-DNA adducts was decreased to 86% at day 7 and 43% at day 28 as compared to the values observed at 5 hr after the final treatment. The half-life of total TAM-DNA adducts was approximately 25 days, indicating that TAM-DNA adducts persist for extended period. Our results were consistent with the previous observations determined using chemiluminescence immunoassay (Divi et al., 1999), \(^{32}\)P-postlabeling/TLC (White et al., 1992), and \(^{32}\)P-postlabeling/HPLC (da Costa et al., 2001). Loss of TAM-DNA adducts in the rat liver may be due to repair and/or cell death. Significant cell death was not observed in the livers of F344 rats treated for 26 weeks with a dose of TAM similar to that used in our study (Stanley et al., 2001). Therefore, loss of TAM-DNA adducts probably reflects repair.

The previous papers (White et al., 1992; Divi et al., 1999; da Costa et al., 2001) showed only the fate of total TAM-DNA adducts. In the present study, all isoforms of TAM-DNA adducts were monitored. The half-life (40 days) of minor trans-form (fr-1) of dG\(_{3p}\)-N\(^2\)-TAM was much longer than that of another major trans-form (fr-2) (22 days) and minor cis-forms (fr-3 and dr-4) (23 days), indicating that the fr-1 is more resistant against repair than other isoforms. This result was supported by that fr-1 was not efficiently removed from the DNA by mammalian whole-cell extracts as compared with other isoforms (Shibutani et al., 2000b). On the other hand, half-lives (27-29 days) of dG\(_{3p}\)-N\(^2\)-desTAM isoforms were not significantly different. Thus, each isoform of TAM-DNA adducts may have different repair susceptibility.
When B6129F1 mice were treated with TAM (20 mg/kg/day) the same dose used for rat, only a trace of TAM-DNA adduct was detected. Mice are more resistant to TAM than rats (White et al., 1992; Umemoto et al., 2001); therefore, no liver tumors were developed at doses that were hepatocarcinogenic in rats (Tucker et al., 1984). When a six-fold high dose of TAM (120 mg/kg/day) was given to the mice as reported previously (Umemoto et al., 2001), significant amounts of TAM-DNA adducts were detected in the liver; however, total amounts of TAM-DNA adducts (7.08 adducts/10^7 nucleotides) in B6129F1 mice was 2.6 times lower than that observed in rats treated with TAM (20mg/kg/day). This may be due to that TAM and its metabolites in mice are rapidly excreted into the urine and/or feces and/or that the capability of forming α-hydroxylated and α-sulfated TAM metabolites, precursors of forming TAM-DNA adducts, is low. The level of hepatic TAM-DNA adducts at day 7 after the final TAM treatment was only decreased to 80% as compared to that observed at 5 hr. In contrast to the previous report showing that TAM-DNA adducts were removed in couple days from the mouse liver (Martin et al., 1997), our results indicated that the repair of TAM-DNA adducts in mice was not rapid as observed for rats.

Although the level of hepatic TAM-DNA adducts in the Xpc knockout mice would tend to be higher than that observed with the wild type at 5 h and 7 days after the termination of TAM treatment, the removal of TAM-DNA adducts form the Xpc knockout mice in one week was similar to that observed with the wild type. NER may not efficiently remove TAM-DNA adducts during a period of TAM treatment and after the termination of treatment.

Like trans-dG-N^2-TAM adducts, 3-(deoxyguanosin-N^2-yl)-2-acetylaminofluorene (dG-N^2-AAF) persists in the liver of rats treated with AAF while N-(deoxyguanosin-8-yl)-2-acetylaminofluorene (dG-C8-AAF) and N-(deoxyguanosin-8-yl)-2-aminofluorene (dG-
C8-AF) are rapidly excised by NER (Westra et al., 1976). dG-\textsuperscript{N2}-AAF is accommodated within a minor groove without disruption of the Watson-Crick pair in DNA (Grad et al., 1997) as also observed with cis-dG-\textsuperscript{N2}-TAM (Shimotakahara et al., 2000). Therefore, non-disruptive dG-\textsuperscript{N2} adducts including dG-\textsuperscript{N2}-TAM may not be efficiently recognized by NER enzymes, resulting in the persistence in the rat liver.

There are several evidences showing mutagenic potential of TAM-DNA adducts. Site-specific mutagenesis studies revealed that dG-\textsuperscript{N2}-TAM, a major TAM-DNA adduct detected in liver of rodents treated with TAM and in endometrial tissue of patients treated with TAM, promoted primarily G → T transversions, along with less numbers of G → A transitions (Terashima et al., 1999), as observed at both lac \textit{l} and cll genes in the liver of the \textit{\lambda}/lac\textit{l} transgenic rats treated with TAM (Davies et al., 1999). G → T transversions and G → A transitions were frequently observed at codon 12 of \textit{K-ras} protooncogene in the endometrium of breast cancer patients treated with TAM; the presence of \textit{K-ras} mutation in endometrium was significantly influenced by the duration of TAM treatment and menstrual status of the patient (Hachisuga et al., 2005). The mutational spectrum was consistent with that observed in our mutagenesis study (Terashima et al., 1999) and rodent studies (Davies et al., 1999), suggesting that the mutations occurred at the \textit{K-ras} gene are due to the genotoxic damage induced by TAM.

When DNA adducted plasmid induced by \(\alpha\)-acetoxyTAM were transferred into nucleotide excision proficient or deficient (XPA) human fibroblast, mutation frequency in NER deficient cells was 1.3-3.6 times higher than that observed with NER proficient cell (McLuckie et al., 2005). Unlike the 10-15 fold higher multiple mutations observed in NER proficient cells by damaged UV, the portion of multiple mutations induced by \(\alpha\)-
acetoxyTAM was not significantly different between cell lines. This result may indicate that TAM-DNA adducts are repaired inefficiently. Therefore, if the mutagenic TAM-DNA adducts are not rapidly repaired, they could accumulate over extended periods of time in the specific genes like $K\text{-}ras$, leading to the development of cancers.
 References


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Footnotes

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Figure legends

Figure 1. A mechanism of forming TAM-DNA adducts.

Figure 2. Distribution of TAM-DNA adducts in rat and mouse treated with TAM.
Fischer 344 rat (A) and Xpc knockout mouse (B) were treated orally with TAM (20 mg/kg/day and 120 mg/kg/day, respectively) for 7 days. All tissues were collected at 5 hr after the final TAM treatment. The DNA samples (2.5 µg) extracted from the tissues were used for 32P-postlabeling/PAGE analysis and compared the migration with standards of dG3p-N2-TAM. A known amount of dG-N2-TAM-modified oligodeoxynucleotide was mixed with 2.5 µg of calf thymus DNA and served as a standard (5 adducts/10^8 nucleotides) for determination of the level of TAM-DNA adduct.

Figure 3. Repair rate of hepatic TAM-DNA adducts in rats treated with TAM.
Rats were treated orally with TAM (20 mg/kg/day) for 7 days and euthanized at 5 hr, 7 and 28 days after the final treatment. The level of TAM-DNA adducts in DNA samples (5 µg) extracted from the liver was monitored using 32P-postlabeling analysis. A known amount of dG-N2-TAM-modified oligodeoxynucleotide was mixed with 5 µg of calf thymus DNA and served as a standard (1 adduct/10^6 nucleotides) for determination of the level of TAM-DNA adduct. Total amount of TAM-DNA adduct was represented as the mean ± S.D. from three rats.

Figure 4. 32P-postlabeling/HPLC analysis of hepatic TAM-DNA adducts in rats treated with TAM.
Rats were treated orally with TAM (20 mg/kg/day) or vehicle for 7 days and euthanized at 5 hr, 7 days and 28 days after the final treatment. The hepatic DNA samples (5 µg) extracted from TAM-treated rats euthanized at 5 hr (A), 7 days (B) and 28 days (C) after the final treatment or the control rats (F) were analyzed using $^{32}$P-postlabeling/HPLC and standards (D) containing stereoisomeric trans- and cis-forms of $^{32}$P-labeled dG-N$^2$-TAM and dG-N$^2$-N-desTAM, as described in the Materials and Methods. (E); sample (B) was co-migrated with standards (D).
Table 1  Repair of hepatic TAM-DNA adducts in rats after cessation

<table>
<thead>
<tr>
<th>Duration after cessation</th>
<th>TAM-DNA adducts/10^7 nucleotides</th>
<th>5 hrs</th>
<th>7 days</th>
<th>28 days</th>
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<tbody>
<tr>
<td>dG-N^2-TAM</td>
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<td></td>
<td></td>
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<tr>
<td>trans-form</td>
<td>fr-1</td>
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<td>fr-2</td>
<td>10.6 ± 4.1</td>
<td>8.14 ± 3.97</td>
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<td>0.28 ± 0.21</td>
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<td>dG-N^2-N-desTAM</td>
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<tr>
<td>trans-form</td>
<td>fr-1</td>
<td>0.74 ± 0.30</td>
<td>0.60 ± 0.41</td>
<td>0.39 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>fr-2</td>
<td>5.58 ± 2.16</td>
<td>5.84 ± 3.05</td>
<td>2.61 ± 0.45</td>
</tr>
<tr>
<td>cis-form</td>
<td>fr-3&amp;4</td>
<td>0.18 ± 0.12</td>
<td>0.20 ± 0.16</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>Subtotal</td>
<td></td>
<td>6.49 ± 2.57</td>
<td>6.64 ± 3.60</td>
<td>3.08 ± 0.61</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>18.3 ± 6.6</td>
<td>15.8 ± 7.6</td>
<td>7.84 ± 2.09*</td>
</tr>
</tbody>
</table>

Data are expressed as mean values ± S.D. from three rats. * Significantly difference (p<0.05) was detected between rats sacrificed 5 hrs and 28 days after the last treatment using Student’s t-test.
Table 2  Repair of hepatic TAM-DNA adducts in B6129F1 and its Xpc knockout mice after cessation

<table>
<thead>
<tr>
<th></th>
<th>B6129F1</th>
<th>Xpc knockout</th>
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<tr>
<td></td>
<td>TAM-DNA adducts/10^7 nucleotides</td>
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<tr>
<td><strong>Duration after cessation</strong></td>
<td><strong>5 hrs</strong></td>
<td><strong>7 days</strong></td>
<td><strong>5 hrs</strong></td>
<td><strong>7 days</strong></td>
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<td></td>
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<tr>
<td><em>trans</em>-form</td>
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<tr>
<td>fr-1</td>
<td>0.44 ± 0.10</td>
<td>0.41 ± 0.12</td>
<td>0.46 ± 0.09</td>
<td>0.37 ± 0.21</td>
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<tr>
<td>fr-2</td>
<td>3.75 ± 1.72</td>
<td>3.00 ± 0.91</td>
<td>5.26 ± 2.80</td>
<td>3.83 ± 0.66</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>fr-3&amp;4</td>
<td>0.07 ± 0.03</td>
<td>0.04 ± 0.01</td>
<td>0.07 ± 0.02</td>
<td>0.07 ± 0.03</td>
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<tr>
<td>Subtotal</td>
<td>4.26 ± 1.84</td>
<td>3.45 ± 1.04</td>
<td>5.79 ± 2.91</td>
<td>4.27 ± 0.90</td>
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<tr>
<td><em>trans</em>-form</td>
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<tr>
<td>fr-1</td>
<td>0.38 ± 0.16</td>
<td>0.41 ± 0.12</td>
<td>0.62 ± 0.31</td>
<td>0.51 ± 0.25</td>
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<td>fr-2</td>
<td>2.39 ± 1.52</td>
<td>1.77 ± 0.72</td>
<td>4.82 ± 2.91</td>
<td>3.35 ± 0.68</td>
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</tr>
<tr>
<td>fr-3&amp;4</td>
<td>0.06 ± 0.03</td>
<td>0.03 ± 0.01</td>
<td>0.11 ± 0.06</td>
<td>0.08 ± 0.01</td>
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<td>Subtotal</td>
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<td>2.21 ± 0.85</td>
<td>5.55 ± 3.28</td>
<td>3.94 ± 0.90</td>
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<td><strong>Total</strong></td>
<td>7.08 ± 3.56</td>
<td>5.66 ± 1.89</td>
<td>11.3 ± 6.2</td>
<td>8.21 ± 1.80</td>
</tr>
</tbody>
</table>

Data are expressed as mean values ± S.D. from three mice.
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Fig. 2
Fig. 3

Total TAM-DNA adducts/10^7 nucleotides

Days

0 5 10 15 20 25

0 5 10 15 20 25 30

*