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ARTICLE *in* MUTATION RESEARCH/FUNDAMENTAL AND MOLECULAR MECHANISMS OF MUTAGENESIS · NOVEMBER 2008

Impact Factor: 3.68 · DOI: 10.1016/j.mrfmmm.2008.10.004 · Source: PubMed

Mutation Research 660 (2009) 33–39

Contents lists available at ScienceDirect

Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis

journal homepage: www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

Time course evaluation of *N*-nitrosodialkylamines-induced DNA alkylation and oxidation in liver of mosquito fish

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ARTICLE INFO

Article history:
Received 17 July 2008
Received in revised form 3 October 2008
Accepted 8 October 2008
Available online 21 October 2008

Keywords:
N-Nitrosamines
Oxidatively damaged DNA
DNA alkylation
LC–MS/MS
Small fish model
Gambusia
Mosquitofish
DNA adducts

ABSTRACT

Here we simultaneously measured N7-alkylguanines and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) in liver of small fish, respectively, to assess the time course of the formation and removal of alkylation and oxidative damage to DNA caused by *N*-nitrosodialkylamines. Mosquito fish (*Gambusia affinis*) were killed at various times during (4 days) and post-exposure (16 days) to *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine (NDEA) alone or their combination with concentrations of 10 and 50 mg/l. The modified guanine adducts were sensitively and selectively quantitated by isotope-dilution LC–MS/MS methods. During exposure, N7-methylguanine (N7-MeG) and N7-ethylguanine (N7-EtG) in liver DNA increased with the duration and dose of *N*-nitrosodialkylamine exposure, while 8-oxodG was dose-dependently induced within 1 day. It was found that NDMA formed substantially more N7-alkylated guanines and 8-oxodG than NDEA on the basis of adducts formed per micromolar concentration, suggesting that NDMA can be more easily bioactivated than NDEA to form reactive alkylating agents with the concomitant formation of oxygen radicals. After cessation of exposure, N7-alkylguanines remained elevated for 1 day and then gradually decreased over time but still higher than the background levels, even at day 16 (half-lives of 7–8 days). However, 8-oxodG was excised quickly from liver DNA and returned to the background level within 4 days post-exposure (half-lives less than 2 days). Taken together, this study firstly demonstrated that in addition to alkylation, *N*-nitrosodialkylamines can concurrently cause oxidative damage to DNA *in vivo*.

1. Introduction

N-Nitrosodialkylamines such as *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine (NDEA) are carcinogenic compounds occurring widely in the environment. The carcinogenic and cytotoxic effects produced by *N*-nitrosodialkylamines are mainly due to their metabolic conversion to reactive alkylating intermediates [1], which can react with nucleophilic nitrogen and oxygen atoms in DNA. A number of DNA adducts could be formed including those alkylated at the O^6, N^2, N-7 and N-3 positions of guanine, the N-7 and N-3 positions of adenine, the O^2 position of cytosine and the O^2 and O^4 position of thymine as well as the phosphodiester backbone [2,3]. Among these adducts, alkylation at the N-7 position of guanine in DNA represents a good biomarker for determining exposure to alkylating agents since it is the predominant reaction site and is slowly repaired by a base excision repair pathway involving DNA glycosylases [4]. Although N7-alkylguanine adducts are not considered to be promutagenic, they are hydrolytically unstable, undergoing spontaneous depurination to produce apurinic sites and single strand breaks in DNA. If not repaired, apurinic sites can potentially cause G to T transversions [5].

In addition to the damage due to alkylation, oxidative stress has been suggested to participate in both the metabolism (activation and detoxification) and the carcinogenic actions of *N*-nitrosodialkylamines [6,7]. It was shown that metabolism of NDMA and NDEA can lead to an increase in the formation of reactive oxygen species (ROSs) which cause oxidative damage to DNA [8,9]. 8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG, also called 8-OHdG) is a major product of oxidatively damaged DNA resulting from the reaction of the hydroxyl radical with guanine residues in DNA. The detection of this lesion is considered important due to its abundance and mutagenic potential, and its concentration could be

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doi:10.1016/j.mrfmmm.2008.10.004

a good indicator of ROS and a potential biomarker of carcinogenesis *in vivo* [10,11].

Over the past decade, small fish species have been proven useful both as environmental sentinels and as versatile animals in toxicity and carcinogenicity bioassays [12,13]. The use of small fish models in cancer research has the obvious advantages of economy, rapid response, low background incidence of tumors, and the opportunity to work with large numbers of specimens when compared with the more traditional rodent models [14]. However, only few studies have attempted to measure specific adduct levels in small fish species exposed to alkylating carcinogens, that are widely utilized for liver tumor induction. This paucity is perhaps due to the fact that most of the existing analytical methods were not sensitive and reliable enough to utilize such small amounts of tissue available from these species [15]. Nowadays liquid chromatography–tandem-mass spectrometry (LC–MS/MS) is a powerful technology that can overcome the sensitivity and selectivity issues in analysis of DNA adducts [16,17]. We have previously reported the development of a sensitive and selective method based on LC–MS/MS to simultaneously measure N7-methylguanine (N7-MeG) and N7-ethylguanine (N7-EtG) in DNA hydrolysates [18]. This new LC–MS/MS method makes it possible to measure alkylated DNA in small fish species treated with low concentrations of alkylating agents (as low as 1 mg/l). Moreover, very recently, we have also developed a quantitative LC–MS/MS method for the determination of cellular 8-oxodG [19], that is capable of quantifying background 8-oxodG in small quantities of DNA and would be a useful tool for monitoring oxidative stress in small fish.

Though exposure to *N*-nitrosamines could result not only in DNA alkylation but also oxidative damage to DNA, to the best of our knowledge no attempts have been made to simultaneously examine these two classes of DNA modifications in *N*-nitrosamine-treated animals. In the present study, we investigated the time course of the formation and removal of N7-alkylated guanines and 8-oxodG in liver of small fish exposed to NDMA and NDEA alone or their combination under controlled laboratory conditions. Mosquito fish (*Gambusia affinis*) was chosen as a test species because it is sensitive to chemical induction of liver neoplasia and can be cultured easily in the laboratory [20].

2. Materials and methods

2.1. Chemicals

Solvents and salts were of analytical grade. Reagents were purchased from the indicated sources: Triton X-100, desferrioxamine mesylate salt (DFO), sodium dodecyl sulfate (SDS), proteinase K, nuclease P1, 8-oxodG, 2′-deoxyguanosine (dG), NDMA and NDEA (Sigma–Aldrich, St. Louis, MO); RNase, RNase T1 and alkaline phosphatase (Roche Diagnostic, Mannheim, Germany); guanine (Gua), N7-MeG and N7-EtG (Merck, Darmstadt, Germany); $^{15}N_5$-2′-deoxyguanosine ($^{15}N_5$-dG) and $^{15}N_5$-8-oxo-7,8-dihydro-2′-deoxyguanosine ($^{15}N_5$-8-oxodG) (Cambridge Isotope Laboratories, Andover, MA). The internal standards $^{15}N_5$-guanine ($^{15}N_5$-Gua), $^{15}N_5$-N7-methylguanine ($^{15}N_5$-N7-MeG) and $^{15}N_5$-N7-ethylguanine ($^{15}N_5$-N7-EtG) were synthesized as described previously [18,21].

2.2. Fish treatment

Mosquito fish (*Gambusia affinis*) were purchased from a commercial freshwater fish farm (Tainan, Taiwan) 3 months before experiments were performed. After arrival, fish were reared in aquaria with flow-through tap water, which was filtered with activated charcoal and aerated. At least 2 weeks before exposure, adult male and female fish, 30- to 35-mm long, were further selected and maintained in flow-through aquaria at 25 ± 1 °C with a 12-h light–dark cycle. Water quality was monitored daily by examining pH, dissolved oxygen and ammonia to ensure adequate conditions. Fish were fed commercial flake food twice a day, except for the 24 h before treatment. Fish were randomly divided into 6 groups of 120 fish each and then respectively exposed to 20 l of 0 (control), 10 and 50 mg/l NDMA or NDEA alone and a mixture containing equal concentration of NDMA and NDEA (10 mg/l of each). Exposures were static and conducted in 25 l glass tanks for 4 days with daily renewal of test medium. Glass tanks were placed in a water bath that was maintained at 25 ± 1 °C and fish were not fed during the exposure period. Three replicates of four fish in each treatment were killed at days 0, 1, 2, 3 and 4 during exposure. The remaining exposed fish were rinsed and transferred to tanks holding fresh water without *N*-nitrosamines, and then three replicates of four fish from each treatment were killed at 1, 2, 4, 8 and 16 days post-exposure. Immediately after killing the fish, their livers were removed, pooled at four per sample, slowly frozen and stored at −80 °C until DNA isolation. The total wet weight of the four pooled mosquito fish livers ranged from 50 to 80 mg.

2.3. Isolation and preparation of DNA

Fish liver DNA isolation was performed according to the procedure recommended by the European Standard Committee on Oxidative DNA Damage (ESCODD) [22]. Briefly, approximately 50 mg of liver tissue was homogenized with 3 ml of buffer A (320 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris/HCl, pH 7.5, 0.1 mM DFO and 1% (v/v) Triton X-100). After homogenization, the sample was centrifuged at 1500 × *g* for 10 min. The resulting pellet was washed with 1.5 ml of buffer A and recovered by centrifugation (1500 × *g* for 10 min). A total of 600 μl of buffer B (10 mM Tris/HCl, pH 8, 5 mM EDTA-Na_2, 0.15 mM DFO) and 35 μl of 10% (w/v) SDS was added, and the sample agitated vigorously. After 30 μl of RNase A (1 mg/ml) in RNase buffers (10 mM Tris/HCl, pH 7.4, 1 mM EDTA, and 2.5 mM DFO) and 8 μl of RNase T1 (1 U/μl in RNase buffer) were added, the samples were incubated at 37 °C for 1 h to remove contaminating RNA from the DNA. After the removal of RNA, 30 μl of proteinase K (20 mg/ml) was added and the samples were incubated at 37 °C for 1 h. Subsequently, 1.2 ml of NaI solution (7.6 M NaI, 40 mM Tris/HCl, pH 8.0, 20 mM EDTA-Na_2, 0.3 mM DFO) and 2 ml of 2-propanol were added. The sample was gently shaken until the DNA had precipitated completely and then centrifuged at 5000 × *g* for 15 min. The DNA pellet was washed with 1 ml of 40% (v/v) 2-propanol. After centrifugation (5000 × *g* for 15 min) the DNA pellet was washed with 1 ml of 70% (v/v) ethanol. Finally, DNA pellet was collected by centrifugation and dissolved in 200 μl of 0.1 mM DFO overnight. DNA concentration was measured by the absorbance at 260 nm. Protein contamination was checked using the absorbance ratio A260/A280; an absorbance ratio over 1.6 was acceptable. RNA contamination was checked by HPLC with UV detection after digestion to nucleosides [23] and was found to be less than 0.1%. Routinely, 50–100 μg DNA was obtained per 50 mg of liver tissue.

2.4. Simultaneous analysis of N7-MeG and N7-EtG in liver DNA

DNA samples (10 μg) were spiked with 376 fmol of $^{15}N_5$-N7-MeG and 240 fmol of $^{15}N_5$-N7-EtG. The DNA solutions were subjected to neutral thermal hydrolysis at 100 °C for 30 min to release N7-alkylguanines by the cleavage of the N-glycosidic bond. The partially depurinated DNA backbone was precipitated by the addition of 2 volumes of ice-cold ethanol and was centrifuged at 5000 × *g* for 15 min. The supernatant was dried under vacuum and redissolved in 200 μl of 96% (v/v) acetonitrile with 0.1% formic acid for N7-alkylguanine adduct analysis. N7-MeG and N7-EtG were quantified by using LC-MS/MS with on-line solid phase extraction (SPE) as described previously by Chao et al. [18]. Briefly, after automatic sample cleanup, LC–MS/MS analysis was performed using a PE Series 200 HPLC system interfaced with a PE Sciex API 3000 triple quadrupole mass spectrometer with electrospray ion source (ESI). The samples were analyzed in the positive ion multiple reaction monitoring (MRM) mode and the transitions of the precursors to the product ions were as follows: m/z 166 → 149 (quantifier ion) and 166 → 124 (qualifier ion) for N7-MeG, m/z 171 → 153 for $^{15}N_5$-N7-MeG, m/z 180 → 152 (quantifier ion) and 180 → 135 (qualifier ion) for N7-EtG, and m/z 185 → 157 for $^{15}N_5$-N7-EtG. With the use of isotope internal standards and on-line SPE, this method had a high sensitivity with limits of detection (LODs) of 0.4 fmol and 0.2 fmol for N7-MeG and N7-EtG, respectively, which corresponds to 0.05 and 0.02 μmol adducts/mol Gua when using 10 μg of DNA per analysis.

The guanine content was determined by an isotope-dilution LC–MS/MS method [18]. Briefly, portions of the DNA samples (<1 μg) were hydrolyzed using 0.1 N HCl at 80 °C for 30 min to release total guanine. 20-μl aliquot of the guanine samples were diluted 50 times with 5% (v/v) methanol containing 20 mM ammonium acetate. A 100-μl aliquot of diluted guanine sample was spiked with 4.64 pmol of $^{15}N_5$-Gua as internal standard. The mass spectrometer was operated in the positive ion MRM mode. The transition of m/z 152–135 was chosen for guanine and the corresponding transition of m/z 157–139 for $^{15}N_5$-Gua.

2.5. Analysis of 8-oxodG in liver DNA

8-OxodG concentrations in liver DNA were measured using a validated method of LC–MS/MS with on-line SPE as recently reported by Chao et al. [19]. Briefly, DNA samples (20 μg) were spiked with 2.82 pmol of $^{15}N_5$-8-oxodG and 84.3 pmol of $^{15}N_5$-dG. The DNA solutions were then enzymatically digested to nucleosides with nuclease P1 and alkaline phosphatase. The resulting DNA hydrolysates were analyzed using the same LC–MS/MS as described above. Detection was performed in the positive ion MRM mode for simultaneous quantitation of 8-oxodG and dG. Optimal MRM conditions were obtained for four channels: 8-oxodG (m/z 284 → 168), $^{15}N_5$-8-oxodG (m/z 289 → 173), dG (m/z 268 → 152), and $^{15}N_5$-dG (m/z 273 → 157). With the use of isotopic internal standards and on-line SPE, this method exhibited

a low LOD of 1.8 fmol for 8-oxodG, which corresponds to 0.13 μmol adducts/mol dG when using 20 μg of DNA per analysis.

3. Results

3.1. Formation and persistence of N7-MeG and N7-EtG in liver DNA of mosquito fish treated with N-nitrosodialkylamines

To investigate the formation and persistence of DNA damages in fish liver DNA, fish were killed at various times during (4 days) and post-exposure (16 days) to NDMA and/or NDEA. In this study, the background levels of N7-alkyguanines in liver of the control fish were stable during the 20 days of the study period (data not shown). The mean level of N7-MeG in liver of control fish was 7.35 ± 1.33 μmol/mol Gua, while the amount of N7-EtG was extremely low and only detectable in about half of the control fish, with a range of 0.05–0.16 μmol/mol Gua. Fig. 1 demonstrates the rapid formation of N7-MeG in liver DNA when fish treated with NDMA. The levels of N7-MeG were elevated ~2.5- and 5.4-fold over basal levels, respectively, after exposure to 10 and 50 mg/l NDMA within 1 day, and continuously increased with the duration of exposure. The formation of N7-MeG in the 50 mg/l NDMA treated group was significantly faster than that in the 10 mg/l NDMA treated group. Compared with the 10 mg/l NDMA group (Fig. 1a; from 7.25 to 41.4), there was a 3.6-fold increase in N7-MeG in the 50 mg/l NDMA group (Fig. 1b; from 7.25 to 130.3) at the end of 4-day exposure. After cessation of exposure, the level of N7-MeG in the 10 mg/l NDMA group remained at about the same level for 1 day and decreased gradually, while that in the 50 mg/l NDMA group remained elevated for 1 day and decreased thereafter. On the other hand, as expected, the amounts of N7-EtG were unaffected by NDMA treatment and consistently very low or undetectable.

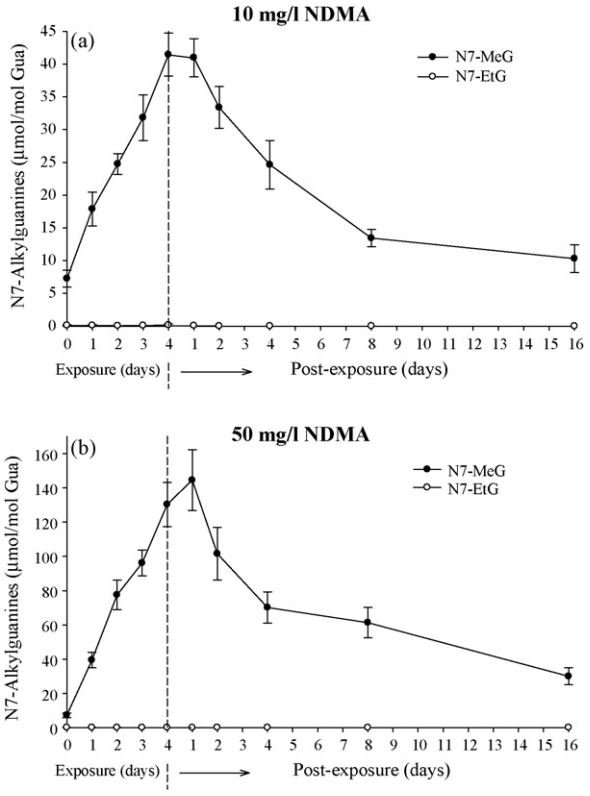

Fig. 1. Levels of N7-MeG and N7-EtG in fish liver DNA at various times during and post-exposure to (a) 10 mg/l and (b) 50 mg/l NDMA. The results are presented as mean ± S.D. (n = 3 at each time point).

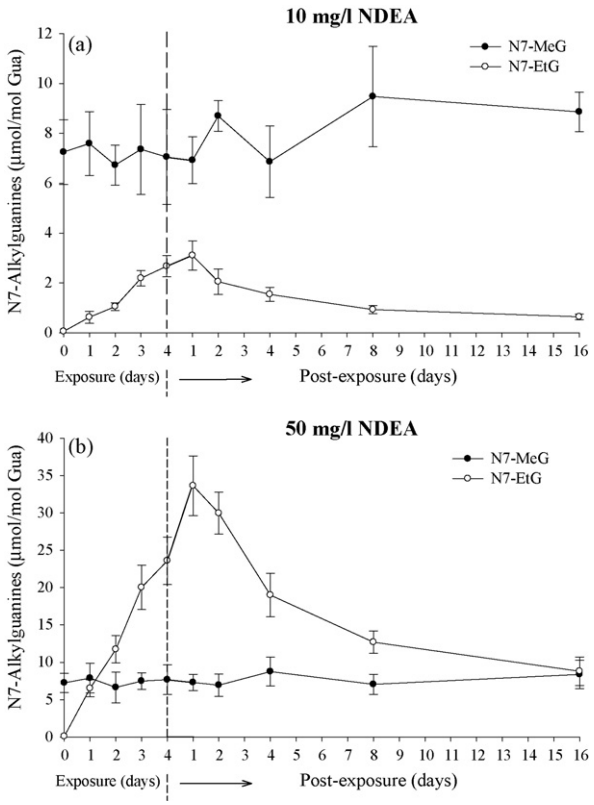

Fig. 2. Levels of N7-MeG and N7-EtG in fish liver DNA at various times during and post-exposure to (a) 10 mg/l and (b) 50 mg/l NDEA. The results are presented as mean ± S.D. (n = 3 at each time point).

The time courses for the formation and persistence of N7-EtG in liver DNA of NDEA-treated fish were shown in Fig. 2. As compared to the extremely low values of N7-EtG for fish not receiving NDEA, the levels of N7-EtG were significantly elevated at the very beginning of NDEA exposure (day 1). Accumulation of N7-EtG was clearly proportional to the duration of NDEA exposure and remained elevated for 1 day post-exposure, then declined gradually. Likewise, the formation rate of N7-EtG was also found to increase with the dose of NDEA. Compared with the 10 mg/l treated NDEA group (Fig. 2a; from 0 to 2.67), there was a 9-fold increase in N7-EtG in the 50 mg/l NDEA treated group (Fig. 2b; from 0 to 23.6) at the end of 4-day exposure. Meanwhile, the background level of N7-MeG remained unaffected by NDEA treatment.

As shown in Fig. 3, the concentrations of N7-alkylguanines in liver DNA were plotted as a function of time when fish treated with a mixture of NDMA and NDEA (10 mg/l of each). Both N7-MeG and N7-EtG were apparently formed concurrently after 1-day exposure. The levels of N7-MeG and N7-EtG increased with the duration of exposure, and interestingly both remained elevated for 1 day post-exposure, then declined with the time of post-exposure incubation. Furthermore, it was noted that the combined treatment of NDMA and NDEA at 10 mg/l each did not generate significantly different levels of N7-MeG and N7-EtG than either treatment alone at 10 mg/l (see Figs. 1 and 2) at the end of 4-day exposure.

Taken together, after cessation of exposure, over 50% of N7-alkylguanine was removed at day 8 post-exposure as compared with maximum hepatic DNA alkylation found at day 1 post-exposure in each treatment (Figs. 1–3). The rates of removal in N7-alkylguanine levels in liver DNA were further estimated by linear regression analysis, assuming first-order kinetics; i.e., the decline in N7-alkylguanine content in DNA was analyzed as a func-

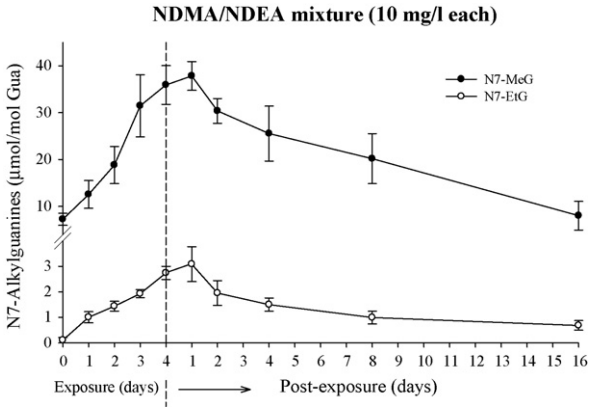

Fig. 3. Levels of N7-MeG and N7-EtG in fish liver DNA at various times during and post-exposure to a mixture of 10 mg/l NDMA and 10 mg/l NDEA. The results are presented as mean ± S.D. (n = 3 at each time point).

tion of time, assuming that a constant fraction of N7-alkylguanine was removed from DNA in each equal time interval [24]. First order rate constants and half-lives were calculated using the amounts from days 1 to 16 post-exposure. The data are summarized in Table 1. The half-life of N7-MeG in fish liver DNA ranged from 7.1 to 7.5 days. Correlation coefficients in the regression analysis for N7-MeG ranged from 0.94 to 0.99, indicating that the apparent elimination of this adduct could be approximated by first-order kinetics analysis. The half-life of N7-EtG was similar to that of N7-MeG with a range from 6.8 to 7.8 days, and its good correlation coefficients (R = 0.94–0.95) also indicated a reasonable fit to first-order kinetics. Moreover, the rates of decline of N7-MeG and N7-EtG seemed not to depend upon the tested doses of NDMA and/or NDEA.

3.2. Formation and persistence of 8-oxodG in liver DNA of mosquito fish treated with N-nitrosodialkylamines

The 8-oxodG concentrations in the fish liver DNA during and post-exposure to NDMA and/or NDEA were shown in Fig. 4. As compared to the stable, low values obtained for control fish (9.62 ± 1.79 μmol/mol dG during the study period), the level of 8-oxodG increased around sixfold after exposure to 10 mg/l NDMA within 1 day and decreased for another 3 days, while that in 50 mg/l NDMA group increased approximately 16-fold within 1 day and remained at about same level for 1 day and then increased for another 2 days (Fig. 4a). After cessation of exposure, this product was removed rapidly from fish liver DNA and the background level was reached at day 2 and day 4 post-exposure for 10 and 50 mg/l NDMA groups, respectively.

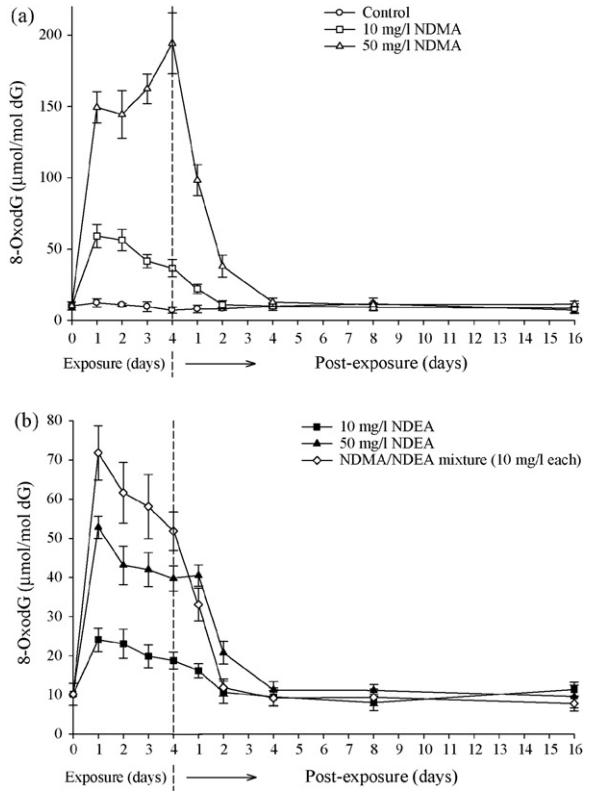

Fig. 4. Levels of 8-oxodG in fish liver DNA at various times during and post-exposure to NDMA and/or NDEA. The results are presented as mean ± S.D. (n = 3 at each time point).

Fig. 4b shows that the 8-oxodG levels increased by 2.5-, 5.5- and 7.5-fold above control within 1 day of exposure with 10 and 50 mg/l NDEA as well as NDMA/NDEA mixture (10 mg/l each), respectively, and then all decreased for another 3 days. Furthermore, it was noted that the combined treatment of NDMA and NDEA at 10 mg/l each generated comparable levels of 8-oxodG to the sum of the levels of the two individual treatments alone at 10 mg/l during exposure. After cessation of exposure, this product was removed from fish liver DNA in 2 days with its level almost reduced to the background level in 10 mg/l NDEA and NDMA/NDEA mixture (10 mg/l each) groups, whereas that in 50 mg/l NDEA group remained at similar level for 1 day post-exposure and returned to the background level in 4 days.

In this study, no reliable half-life could be given for 8-oxodG in the lower dose groups of NDMA (10 mg/l), NDEA (10 mg/l) and

Table 1
Rate constants and half-lives for elimination of N7-alkylguanines from fish liver DNA.

Exposure dose (mg/l)		N7-MeG			N7-EtG		
		Rate constant[a] (day^{-1})	R[b]	Half-life[c] (day)	Rate constants (day^{-1})	R	Half-life (day)
NDMA	10	0.0912	0.94	7.5	–[d]	–	–
	50	0.0933	0.96	7.4	–	–	–
NDEA	10	–	–	–	0.0962	0.94	7.2
	50	–	–	–	0.0883	0.95	7.8
NDMA/NDEA mixture	10 (each)	0.0978	0.99	7.1	0.1015	0.94	6.8

[a] Rate constants of N7-MeG and N7-EtG were determined by curve fitting using a regression (least squares) method, assuming that a constant fraction of these DNA adducts were eliminated from DNA during each time interval (i.e. first-order elimination kinetics). The rate constants were calculated using the data points from days 1 to 16 post-exposure.

[b] Correlation coefficients (R) indicate goodness of fit to the derived first-order eliminations.

[c] The half-lives of N7-MeG and N7-EtG in DNA were computed from the first-order rate constant. Half-life = 0.693/rate constant.

[d] Not measurable.

NDMA/NDEA mixture (10 mg/l each) since this product was excised quickly from fish liver DNA and returned to the background level within 2 days post-exposure. However, in the higher dose groups of NDMA (50 mg/l) and NDEA (50 mg/l), the half-life of 8-oxodG was roughly estimated to be 1.1 and 1.7 days, respectively, assuming the decline in concentration of this product followed a first-order relationship from days 1 to 4 post-exposure.

4. Discussion

Despite an increasing use of small fish models in cancer research, little emphasis has been placed on examining early biochemical events of carcinogenesis in small fish species. The lack of information available on the formation and persistence of alkylation and oxidative damage to DNA in liver of small fish exposed to *N*-nitrosodialkylamines promoted the present study. It has been pointed out that there are problems intrinsic in studies examining *in vivo* formation and persistence of DNA adducts following single i.p. injection, since this approach is usually ineffective at low-dose administration and the response can be missed in a short window of evaluation time due to the rapid elimination of *N*-nitrosamines [24,25]. In this study, mosquito fish were killed at various times during and post-exposure to low non-toxic levels of *N*-nitrosodialkylamines in water. The kinetics of DNA adduct formation and persistence may be more reliable and meaningful under the present experimental conditions in which fish were exposed continuously to an aqueous solution, rather than to a single pulse of carcinogen. Moreover, since there is evidence that substantial oxidation of dG occurs during the protracted treatment of DNA samples in preparation for chromatographic analysis [26], we used the protocols suggested by ESCODD [22] for liver DNA extraction and hydrolysis to minimize/eliminate the artifactual formation of 8-oxodG. The highly sensitive and specific isotope-dilution LC–MS/MS methods with on-line SPE of Chao et al. [18,19] were adapted to quantify the levels of N7-alkylguanines and 8-oxodG in small amounts of liver DNA.

Numerous studies have suggested that O^6-alkylguanines are the main premutagenic adducts generated by strongly carcinogenic alkylating agents such as NDMA and NDEA. Nevertheless, we chose to determine N7-alkylguanines since N7-alkylguanines are the major products of DNA alkylation and abundant enough to be reliably measured in liver of small fish. Our results demonstrated that N7-MeG and N7-EtG in liver DNA of fish increased with the duration and dose of *N*-nitrosodialkylamine exposure (see Figs. 1–3). The exposure–response curves for the formation of N7-alkylguanines never reached a plateau, which may be interpreted as no saturation of metabolic activation occurred at all tested doses of *N*-nitrosodialkylamines during 4 days of exposure. It was further noted that NDMA formed significantly more alkyl DNA adducts than NDEA in fish liver when compared on the basis of adducts formed per micromolar concentration during exposure (i.e. 0.182–0.253 and 0.027–0.048 μmol adducts/mol guanine formed by per micromolar concentration of NDMA and NDEA, respectively, at the end of 4 days exposure; $P < 0.001$ by Student's *t*-test). This is in agreement with previous findings showing that methylating agents are more reactive than ethylating agents [3,27]. Generally, the metabolic activation of *N*-nitrosodialkylamines to reactive intermediates by cytochrome P450 (CYP) enzymes is required to exert their genotoxicity. In rats, mice and humans, CYP2E1 and CYP2A6 have been reported to be the major enzymes for the metabolic activation of NDMA and NDEA [28–30]. Lipscomb et al. [31] found that CYP1A was readily detectable in medaka liver, while CYP2E1 was present at very low levels. However, until now, little information is available on which CYPs may be responsible for activation of *N*-nitrosodialkylamines in small fish [32].

After cessation of exposure, adduct concentrations were usually expected to be higher for all of the 0 day time points than at the 1 day points, assuming that the resulting adducts would decrease over time as repair mechanism were recruited. In this study, however, N7-alkylguanines either persisted or increased further for 1 day post-exposure, and then decreased over time but still remained significantly higher than the background levels, even at day 16 post-exposure. The metabolic activation of residual *N*-nitrosamines to form adducts may in part explain the delay seen in the formation of adducts at 1 day post-exposure [33]. In the meantime, inefficient DNA repair mechanisms in the fish (as discussed below) may also cause the N7-alkylated adduct concentrations to stabilize at elevated values.

In this study, the half-lives of N7-MeG and N7-EtG were similar (see Table 1) and estimated to be as long as 7–8 days (168–192 h), which is comparable to the 105–192 and 225 h half-lives of spontaneous depurination *in vitro* at pH 7 for N7-MeG and N7-EtG, respectively [4,34]. In contrast, N7-MeG and N7-EtG in liver DNA of rats, as reported by others [24,35] are lost faster with half-lives of approximately 45 and 35 h, respectively. It seems that fish may have a less efficient mechanism to repair N7-alkylated DNA adducts in liver (or repair processes may become saturated at the tested doses), and the loss of N7-alkylguanines could be mainly due to spontaneous depurination rather than by enzymatic DNA repair. Such a speculation could be further supported by the observation that the rates of decline in N7-alkylguanine concentrations were unrelated to the *N*-nitrosodialkylamine dose (Table 1). If DNA repair processes have a substantial impact upon the elimination of N7-alkylguanines, then half-life of N7-alkylguanines should be reduced at lower *N*-nitrosamine concentrations [34]. However, if depurination was the primary mode of N7-alkylguanines loss, then the rates of decline in N7-alkylguanines should be the same after low or high concentration *N*-nitrosamine exposure because the *in vivo* rate of loss of N7-adducted guanine should be independent of the DNA adduct load. The latter hypothesis is supported by our data. The long-term persistence of small alkyl adducts in fish liver DNA was also noted in previous work with rainbow trout [25].

Regardless of extensive studies on *N*-nitrosamines carcinogenesis and numerous investigations concerning DNA alkylation following exposure to alkylating carcinogens, surprisingly few experiments have concurrently focused upon *in vivo* oxidation of DNA by *N*-nitrosamines. The data in Fig. 4 demonstrates that both NDMA and NDEA significantly induced the formation of 8-oxodG in fish liver within 1 day, supporting the hypothesis of the production of ROS during metabolic activation [6,9]. Although antioxidative enzymes may contribute to protect DNA from oxidative damage, the excessive production of ROS by *N*-nitrosamines outstripped antioxidant defense of fish liver and resulted in oxidatively damaged DNA. In addition, it has been previously shown that both NDMA and NDEA caused inhibitory effects on the hepatic antioxidative enzymes [36,37].

After 1 day of exposure, except for the dose of 50 mg/l NDMA, 8-oxodG decreased for another 3 days of exposure at all doses tested (Fig. 4). This suggests that the excess formation of oxidatively damaged DNA, 8-oxodG, could enhance its repair activity in fish liver, but there is a time lag between the increase in 8-oxodG production and the subsequent increase in repair enzyme activity [38]. This delayed increase in repair enzyme activity may contribute to the fall in 8-oxodG levels with the time of exposure. However, at the high dose of 50 mg/l NDMA, the increase of 8-oxodG with exposure time may be due to the fact that antioxidant defense and/or oxidatively damaged DNA repair mechanisms in liver of fish, somewhat effective at other exposure levels, are saturated. Similar to those found in alkyl DNA adducts, NDMA formed more 8-oxodG than NDEA when compared on the basis of adducts formed per

micromolar concentration (i.e. 0.206–0.363 and 0.087–0.141 μmol 8-oxodG/mol dG formed by per micromolar concentration of NDMA and NDEA, respectively, at 1 day of exposure; $P < 0.01$ by Student's *t*-test). It is possible that NDMA can be more easily bioactivated by fish liver than NDEA to form a reactive alkylating agent with the concomitant formation of oxygen radicals.

Our results showed that the disappearance of 8-oxodG from liver DNA of fish was relatively rapid when compared to N7-alkylguanines (i.e. $t_{1/2} < 2$ days for 8-oxodG compared with $t_{1/2} \sim 7$–8 days for N7-alkylguanines). The levels of 8-oxodG returned to background levels within 4 days (Fig. 4), which provided the clear evidence that DNA-repair enzymes specific for oxidative damages did actually work in fish liver. In other animal models, Nakae et al. [8] similarly reported that 8-oxodG was persistent for at least 3 days in rat liver after NDEA administration at single doses. However, in contrast, the 8-oxodG was repaired much more rapidly in livers of rats and mouse after γ-irradiation, in which 8-oxodG levels were shown to return to background levels within 1 h [39,40]. One explanation for the longer persistence of *N*-nitrosamine-induced 8-oxodG is that it takes time to metabolize/eliminate the chemical agents in fish after cessation of exposure. The steady-state level of 8-oxodG could be the result of a dynamic balance between the rate of DNA repair and the continued oxidative damage to DNA induced by the residual *N*-nitrosamine present in the liver. Nevertheless, NDMA and NDEA have been suggested to be metabolized within 24 h [36,41], which was also supported by our findings in fish that N7-alkylguanines increased for only 1 day post-exposure and then decreased (Figs. 1–3). Therefore, the persistence of 8-oxodG for several days in this study may also show that the repair system may be exhausted and or disturbed in the fish liver by the exposure of *N*-nitrosamines [8].

Very recently, NDMA and NDEA are discovered as disinfection byproducts of drinking water and wastewater [42–44]. Our results showed N7-alkylguanines and 8-oxodG in fish DNA could be used as biomarkers of exposure to aquatic *N*-nitrosamines and their early biologic effects. However, alkyl adducts are specific markers for alkylating agent exposure, whereas 8-oxodG is nonspecific and can result from many (nonspecific and/or unknown) sources of oxidative stress, as diverse as UV irradiation, inflammation, oxidized foods, etc. Meanwhile, it should be noted that regardless of their specificity, alkyl adducts are better than 8-oxodG as bioindicators of fish exposure to *N*-nitrosamines, since the kinetics of 8-oxodG formation may not only depend on the rate of ROS formation from *N*-nitrosamines, but may also be highly affected by antioxidant defense and oxidatively damaged DNA repair *in vivo*. Rodríguez-Ariza et al. [45] similarly suggested that the 8-oxodG level in fish DNA seems a valuable biomarker of environmental pollution in aquatic environments, although its sensitivity is still somewhat lower than that of other well-established biomarkers of oxidative stress, such as changes in antioxidative enzymes, alteration of glutathione redox status or lipid peroxidation.

In summary, the present study represents the first time the formation and persistence of alkylated and oxidized guanines in DNA of small fish have been quantitatively determined using LC–MS/MS. The NDMA- and/or NDEA-dependent formation of N7-alkylguanines and 8-oxodG indicated that fish liver can metabolize *N*-nitrosodialkylamines to form reactive alkylating agents with the concomitant formation of ROS. Our results support the hypothesis that in addition to alkylation, *N*-nitrosodialkylamines-induced oxidative damage to DNA may strengthen the role of ROS in the carcinogencity of these compounds. This work may also contribute to a better understanding of the cellular repair of alkylation and oxidative damage to DNA in small fish species. However, some limitations of this study should be mentioned for consideration in future research. One is that in most cases the maximum of 8-oxodG was observed around 24 h after the beginning of exposure to *N*-nitrosodialkylamines. It would be of interest to make additional measurements of 8-oxodG at shorter exposure times in order to provide more information on the rise of the oxidized nucleoside and the shape of the formation curve. Subsequently, more evidence in terms of antioxidant defense measurement, other markers of oxidative stress, metabolism or repair activity would be helpful to substantiate our present findings on roles of *N*-nitrosodialkylamine-induced oxidative stress.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

This study was supported by a grant from the National Science Council, Taiwan (Grant NSC 95-2314-B-040-037-MY2). The authors thank Mr. Chun-Yen Chien for his help in sample preparation.

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