Quantifying Reactive Oxidant-Induced DNA Damage Through OPdA Adducts

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KEYWORDS. DNA, adducts, OPdA, carcinogenesis

BRIEF. An investigation of reactive oxygen species and their mechanisms of inducing reactive oxygen species.

ABSTRACT. Reactive oxygen species (ROS) are ubiquitous in our everyday lives as they range from environmental carcinogens to endogenous sources like inflammation. ROS can form reactive aldehydes when reacting with lipids, forming malondialdehyde (MDA), and DNA, forming base propenal. When such aldehydes react with 2'-deoxyadenosine (dA) and 2'deoxyguanosine (dG), mutagenic DNA adducts like M_1dG and OPdA can form. Since the mechanism of and sources for the formation of DNA adduct OPdA have been less studied, we have investigated OPdA levels induced by MDA and peroxynitrite both in vitro and in vivo. To perform these studies, MDA and peroxynitrite were synthesized and employed to treat purified calf thymus DNA in vitro and in colon carcinoma (RKO) cellular systems, with adduct prevalence assessed using liquid chromatography tandem mass spectrometry (LC-MS-MS) and high performance liquid chromatography (HPLC) assays. We hypothesized similar induction as with M_1dG and, consistent with this hypothesis, we detected a dose-dependent increase of OPdA in DNA treated with MDA and peroxynitrite. MDA gave rise to M_1dG with greater efficiency than peroxynitrite, 7 adducts/10^5 bases. High induction of OPdA from MDA than peroxynitrite in vivo suggests low reactive oxidants derived from oxidative DNA damage may contribute to cellular mutagenic burden, leading to questions about OPdA's functionality as carcinogenic biomarkers.

INTRODUCTION.

DNA is a store of genetic information, highlighting the importance of maintaining its integrity. Its chemical stability becomes of vital importance when assessing DNA damage, a term describing both mutations within the genetic code and genomic instability, or breaks and abnormal changes in DNA base pair structure. Such damage can lead to cell instability, and is linked to carcinogenic and aging pathways [1].

Reactive oxygen species (ROS) are highly reactive intracellular mutagens characterized by unpaired electrons (free radicals). Sources of ROS range from exogenous origins including chemotherapeutics to endogenous sources like respiration and macrophage activity and can damage DNA. When ROS are formed at levels that overwhelm cellular antioxidant pathways, the resulting oxidative imbalance can produce reactive byproducts. Lipids and DNA are ROS targets, producing reactive aldehydes such as malondialdehyde (MDA) and base propenal, respectively. These molecules can react with nucleic acids including 2'-deoxyadedenosine (dA) and 2'-deoxyguanosine (dG) leading to the formation of mutagenic adducts such as N^6-(3-oxo-1-propenyl)-deoxyadenosine (OPdA) and 3-(2-deoxy-b-D-erythro-pentafuranosyl)pyrimido[1,2-a]purin-10(3H)-one deoxyguanosine (M_1dG) (Figure 1). M_1dG is mutagen formed in high concentration in mammalian cancer cells, causing frameshift mutations. OPdA can cause mutagenic lesions to occur at the dA nucleoside however its function is not fully known [2].

According to Epe *et al.* changes in oxidative DNA modifications can create shifts in mutation rate, a critical step in carcinogenesis [3]. Moreover, elevated levels of oxidative DNA lesions (8-OH-dG) have been noted in various tumors, strongly implicating such damage in the cancer etiology. Thus, it appears that the DNA damage is predominantly linked with carcinogenic initiation processes. Mechanisms of DNA damage repair, such as the nucleotide excision repair pathways, and preventative antioxidant pathways, develop a balance in free radical induced DNA mutations. Yet, since ~40% of cancers have ROS induced genetic damage it is more than a correlation between sustained DNA damage and carcinogenesis [4]. However, the question still remains as to the mechanisms by which DNA adduct induction causes adducts of interest to a biologically significant level, and in understanding the pathway of subsequent substitution mutation induction [5].

Zhou *et al.* showed MDA primarily induced the Michael adduct, M_1dG [1]. In fact, Shacter *et al.* reported that mutagenic M_1dG adducts have been detected at levels of ~5,000 adducts/cell in human liver [4]. What has been less studied, however, is the formation of DNA Michael adduct OPdA and the subsequent genotoxicity [6]. To better understand mechanisms of formation, we looked at induction of OPdA from MDA and peroxynitrite (Figure 1) in a cellular system. We proposed to demonstrate a stronger connection in the intermediary formation of OPdA from MDA and peroxynitrite in order to make a more viable correlation in OPdA induction by similar ROS as induce M_1dG.

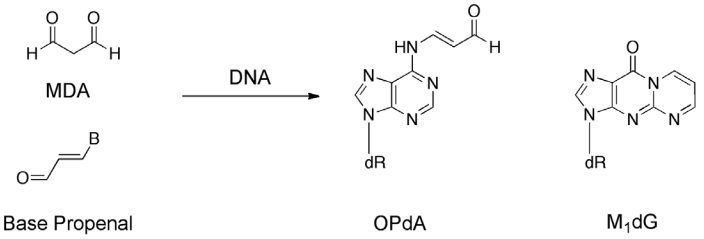

Figure 1. OPdA and M_1dG general chemical induction mechanisms from reactive aldehydes base propenal and malondialdehyde (MDA). Reactive oxygen species, like peroxynitrite or hydrogen peroxide, form reactive aldehydes when reacting with lipids to form malondialdehyde (MDA), and DNA to form base propenals.

MATERIALS AND METHODS.

Synthesis and preparation of MDA and $ONOO^-$.

In order to induce the anion form of the MDA enolate, 4.7g/L of 300 mL TMOP (tetramethoxypropanal) was mixed with 0.01 M $NaOH_{(aq.)}$. MDA enolate (UV absorbance at 267nm of deprotonated enolate form with $\varepsilon 3.18*10^4 M^{-1}cm^{-1}$) is chemically sustainable within 5 weeks of synthesis when stored at -5°C. Peroxynitrite solution was prepared at 20% yield rate as shown by Beckman *et al* with equal volumes of ice-cold solution of 50mM $NaOH_{(aq.)}$, 50mM $H2O2_{(aq.)}$. A solution of 1M $HCl_{(aq.)}$ was rapidly thrown into mixture of nitrite and hydrogen peroxide solution followed immediately by 1.5 M NaOH (half-life of peroxynitrous acid, protonated form of peroxynitrite, is 1 second) [7]. Peroxynitrite solution, beer-lampert extinction coefficient $\varepsilon = 1670\ M^{-1}cm^{-1}$) and RNase A at 1 mg/mL were added following RKO cell digestion to break cell membrane and ribose-based sugars. Peroxynitrite was then titrated to a physiological pH 7 from pH 13 by 1:1 MOPS, pH 6 buffer.

Treatment of Calf Thymus DNA with MDA and $ONOO^-$.

Freshly prepared MDA (25 mM final concentration) was added to a solution of calf thymus DNA (0.5 mg) in sodium phosphate buffer (50 mM, pH 6.5, 1 mL) and incubated at 37 °C for 8 hours. DNA was recovered by EtOH precipitation, and the supernatant was centrifuged at 13000 x*g* for 20 min at RT. The supernatant was collected and recentrifuged for 10 min at 20,000xg to obtain a crude cell pellet, which was stored at -80°C following resuspension in HPLC water. DNA was then treated and dialyzed according to Zhou *et al* by suspending

DNA in 10mg/mL BSA, 1:5 5x digest buffer (85 mM sodium succinate, 40 mM calcium chloride, pH 6), 0.008 units/μL snake venom phosphodiesterase Sigma P3243-1VL), and 2,000 gel units/ μL Micrococcal nuclease (NEB, M0247S), and 5 units/ μL Antarctic phosphatase suspended in water medium and incubated for 3 hours at 37 °C. Following addition of Alkaline phosphatase and AP Buffer (1:5 AP: Buffer) and overnight incubation at 37 °C, DNA was spin filtered through a 10 kDa spin filter and dried under N_2 gas before MS analysis.

RKO Cell Cultures and Treatments with MDA and ONOO⁻.

RKO cells were cultured in 10% DMEM, 1% FBS, 1% MEM vitamin, 10% Hepes, and 1% anti-antibiotic solution and treated with 28 mM MDA enolate solution resuspended in 0.01M $NaOH_{(aq.)}$ and 28 mM $ONOO^-$. Following 10 minutes trypsinization (180 units/mg protein) and mechanical cell scraping, RKO cells suspended in trypsinized media were collected and centrifuged at 2000x*g* for 10 minutes. RKO cell pellets were suspended in 1 vol DNA extraction buffer (100mM NaCl, 10 mM MOPS pH 8, 25 mM EDTA pH 8, 0.5% SDS, and 0.1 mg/mL proteinase K) adding RNase A to 1 mg/mL concentration, and incubated at 50 °C for 18 h, creating clear tissue samples. Following incubation, DNA was isolated with 1 vol phenol/chloroform/isoamyl-alchohol mixture at 13000x*g* for 10 min. DNA purification followed Zhou *et al* with modifications (1/10 vol of supernatant sodium acetate, pH 5.5 and 2 vol of 100% EtOH). Stringy DNA precipitate was recovered at 13000x*g* centrifugation for 10 min at 70% EtOH and resuspended and dissolved in HPLC water (65°C). Purified DNA samples were analyzed using UV spectrophotometry with Abs_{260}:Abs_{280} ratio to determine DNA concentration.

HPLC/MS-MS quantitative analysis of adduct per nucleotide.

The liquid chromatograph (Agilent 1100; Agilent Technologies, Inc., Palo Alto, CA) was coupled to a mass spectrometer with a turbo electrospray ion source (4000 Qtrap; Applied Biosystems, Foster City, CA) and was used in positive ionization mode. Mobile phase A (150 mM Ammonium Acetate, pH unadjusted) and B (MeOH + 15% A) were placed with a progressive gradient of 90% B at 15 minutes within a 15 x 0.46 cm 4u Fusion Synergi, OOF-4424 column. Flow rate was placed as 1mL/min with UV spectrophotometric detection at 260 nm . Samples of MDA and $ONOO^-$ treatments of calf thymus DNA and RKO cells were run with respect to progressive concentrations (0.1 mM, 0.5 mM, 1 mM, and 5 mM) of DNA standards with fixed ratio of dA, dG, dC, and dT injected at 10 μL each. The resulting peak area ratios were plotted against progressive concentrations of DNA standards to obtain absolute concentrations of nucleosides and DNA adduct ratios (OPdA and M_1dG), and hence abundance measured in adducts per 10^6 nucleotides.

RKO cell viability assay with $ONOO^-$ and MDA enolate.

Cells were plated at 5,000 cells/well in 96 well plate and allowed to adhere overnight. Cells were treated with increasing concentrations of MDA and $ONOO^-$ (0 mM to 5,000 uM MDA and 0 mM to 2,000 uM, respectively), cells were incubated for 2 hours and treated with viability assay as according to Lichtenfels *et al* [8]. The pH of all treatments before addition of Calcein AM were decreased to obtain physiologically stable compounds of peroxynitrite and MDA at pH 7 with $NaOH_{(aq.)}$ but addition of $NaOH_{(aq.)}$ was controlled in the process). Modifications of Calcien AM treatments included rinsing cells with 500 mL DPBS (+ calcium and magnesium) with 100 μL/well of 2 μM Calcein AM dye in PBS and incubating cells with Calcein AM for 30 minutes at 25°C. Readings of resultant fluorescence (excitation = 494 nm/emission = 517 nm) were quantified by Spectramax plate reader following removal of DPBS solutions.

RESULTS.

Correlation of OPdA formation with oxidant induced generation of MDA and peroxynitrite in vitro.

The first objective was defining oxidation chemistry in purified DNA for formation of OPdA adducts and correlating the significance of these findings with the formation of M_1dG. MDA enolate was synthesized to form solid 50g MDA from 1,1,4,4 tetramethoxypropane (TMOP), and following 1N HCl, 1M NaOH, and activated carbon filtration, was dissolved in sodium chloride (50 mM) to obtain sodium enolate form and checked by UV spectrometry at 502 nm (Figure S1). Peroxynitrite treatments were at 14 mM concentration (confirmed by UV spectrogram). After treatment with MDA and peroxynitrite from calf thymus DNA samples were conducted for two hours in incubation of 30°C with analytes (OPdA) detected using LC-MS/MS with positive ESI (electrospray ionization). Once analytes were extracted from samples and quantified using serial dilutions of internal standards (^{15},N, ^{13},C-M_1dG) prior to digestion, resulting concentrations of OPdA adduct were measured at retention time of 5.29 minutes. The results show that *in vitro* treatments of MDA (on calf thymus DNA medium) produced observable peak at 5.29 minutes, signifying a direct correlation. OPdA induced by MDA was observed to be induced at quantifiable levels with a retention time of 5.29 minutes. While the sheer detection of OPdA by mass spectrometry is qualitative, the results confirm published studies of OPdA induction by means of MDA [1].

However OPdA was not detected in statistically significant similar concentrations of peroxynitrite using the LC-MS/MS method. It must be noted that although a peak was observed with the selected reaction monitoring (SRM) transition m/z of 306 to 188 for peroxynitrite treatments, the retention time varied enough from the internal standard peak that it was concluded this peak did not correspond to that of OPdA, whereas M_1dG peaks were shown to be significant. One possible explanation for the lack of detection of OPdA in $ONOO^-$ treatments may be their proposed reaction mechanism (the creation of base propenal as an oxidative agent as an adduct) and, with two steps in reaction, the low final concentration levels of OPdA.

OPdA adducts per million nucleotide increased in a dose dependent manner with MDA concentrations *in vitro* while peroxynitrite produced no quantifiable adducts. Both adducts per nucleotide (NT) and adducts per nucleoside dA or dG increase with concentration in an quadratic manner, indicating possible adduct formation as a quadratic relationship. That is, reaction rate, given by dOPdA/dMDA = 0.176x + 1.12, suggests a dose dependency between OPdA formation and concentration MDA *in vitro*, implicating minimal local distortion of the double helix, and therefore less efficient reparation process than that of M_1dG.

Exposure to MDA induced detectable levels of OPdA and M_1dG in calf thymus DNA, which is consistent with the hypothesized reaction mechanism (Figure 1). Moreover, in accordance with the hypothesis, MDA produced DNA Michael adducts as a probable result of post-synthetic modification strategy in site specific synthesis of oligonucleotides containing MDA adducts of dG and dA.

Viability of MDA and $ONOO^-$ in RKO cellular systems

MDA and peroxynitrite dose-response curves were constructed from Calcein AM cell viability assays. Final fluorescence (excitation 494, emission 517nm) was graphed to control of 0 M (Figure 2) obtaining inhibitory concentration 50% (IC_{50}), reducing confounding factors by controlling apoptotically induced oxidative stress.

Attempts to create an inhibitory concentration from a concentration at higher levels than used in cell treatments were unsuccessful in that the alive to death ratio of each fell below the detection limit of the Calcein AM assay. This indicates that RKO cellular systems hadn't reduced in their relative reactivity with reactive aldehydes (MDA) and ROS (peroxynitrite).

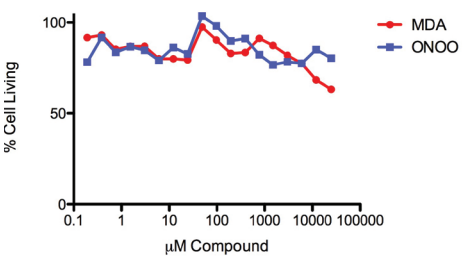

Figure 2. Final fluorescence (excitation 494 emission 517nm) was graphed to control, obtaining inhibitory concentration 50% (IC_{50}). IC_{50} of MDA and $ONOO^-$ in RKO cells was extrapolated above 20mM (highest concentration), implicating insufficient cell treatment to show IC curve characteristic.

MDA treatments on RKO cellular systems.

MDA treatments in cellular systems show no significant increase in OPdA (RT = 5.3) compared to control (low adduct formation), implicating cell-mediated inhibition of MDA and base propenal reaction pathways. That is, at a 20 mM extracellular concentration (one that far exceeds the average 5 μM MDA concentration in humans) in RKO cellular systems, created an existent, while not substantial, OPdA peak. Hence, there exists no traceable dose-dependency of OPdA induced from MDA and base propenal.

OPdA adducts per million nucleotide increased in a dose dependent manner with MDA *in vitro* while peroxynitrite had no quantifiable adducts (Figure 3). Both adducts per nucleotide (NT) and adducts per nucleoside dA or dG increased with concentration as quadratic relationship. That is, reaction rate, and hence dose dependent potency, increases with dose increments. Hence, extrapolating the causal relationship between MDA and OPdA *in vitro* induction, the question of OPdA should have been adequately higher *in vivo*, indicating the minimizing of said OPdA induction through intracellular nucleotide excision repair.

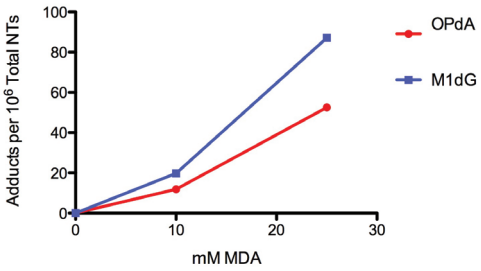

Figure 3. OPdA adducts increasing in a dose-dependent manner as hypothesized, implicating a quadratic relationship.

DISCUSSION.

There is strong evidence for endogenous DNA adducts, such as M_1dG and OPdA, as biologically potent in genetic instability and base substitution mutations induced through reactive oxidants. The goal of the present study was to address this problem for OPdA. The chemical species known to form OPdA *in vitro,* namely MDA and base propenals (a byproduct of peroxynitrite extracellular presence), arises from different sources. Lipids represent a major target for free radicals and peroxidation of PUFA generates a host of reactive electrophiles, many of which have been implicated in the formation of DNA adducts. In a similar manner, MDA is ubiquitously present in cells and tissues and has been demonstrated to react with dG to form M_1dG (a representative DNA adduct) *in vitro*, hence the proposal that it is involved in OPdA formation *in vivo*. We hypothesized that due to the reactivity of MDA as an electrophile in the formation of M_1dG and the high reactivity of base propenals than MDA toward dG (greater than 100 fold [Zhou, 2005])and their proximity to guanine bases in DNA, that MDA and peroxynitrite can mediate the reaction of carbon dioxide and biocarbonate with DNA single-strand breaks to form base propenal [1]. Yermilov *et al.* suggested that MDA and peroxynitrite might be a significant source of OPdA *in vitro* and hence MDA induction of OPdA *in vivo* [9]. The results of the present studies support this conclusion.

As a reactive aldehyde, malondialdehyde (MDA) was shown to be relatively more reactive to DNA base nucleoside 2'deoxyadenosine (dA) to form OPdA adducts both *in vitro* and in cellular systems than peroxynitrite. Such reactivity difference may be associated with differences in proposed reaction mechanisms for peroxynitrite and MDA, as peroxynitrite induces base propenal formation to later induce OPdA covalent adducts. Moreover, Calcien AM based cell viability assays show little to no cell killing within concentrations of MDA and peroxynitrite used. Peroxynitrite showed small, non-quantifiable induction of OPdA, even at higher concentrations. However, the question here is of whether *in vitro* OPdA induction of up to 50 mM $ONOO^-$ is biologically significant.

While MDA induced OPdA in a dose-dependent manner *in vitro,* in cellular systems MDA could not produce similar patterns of mutagenicity. Apoptically induced oxidative stress and mutagenicity cannot be indicated as IC_{50} of MDA in RKO cells was shown to be at a magnitude higher concentration than that treated. Hence, there was no apoptotically induced confounding factor in understanding the causal relationship between MDA and OPdA *in vivo* formation.

It is important to point out that our results do not rule out MDA as a source of OPdA in human cells. Indeed, we have observed that exposure of RKO cell lines to 10 mM MDA (37 °C, 48 hr) caused a presence of the OPdA adduct level to 2 lesions per 100 nt, which is similar to that of M_1dG level (1.5 per 10^7 nt) observed by Marnett *et al.* in studies of S. typhimurium exposed to 10 mM MDA [10]. However, given the small increases in peroxynitrite occurring with non-physiological MDA concentrations, we argue that MDA arising from oxidative DNA damage significantly contributes to the cellular burden of OPdA in a dose-dependent manner *in vitro*. In conclusion, the results from studies in purified DNA and in RKO cellular model suggest MDA, not peroxynitrite, is the major source of M_1dG in biological systems. Furthermore, intracellular concentrations of MDA, given *in vitro*, exhibited a dose-dependent trend for the induction of OPdA from MDA in treated calf thymus DNA.

CONCLUSION AND FUTURE DIRECTION.

As endogenous DNA adducts are known to cause base substitution mutations, elucidating the induction mechanism for these adducts in a biological context can lead to understanding relative potency, endogenous mutation rates, and other risk factors in the reactive aldehydes such as MDA. Previous studies indicate that patients of chronic inflammatory diseases like hyperthyroidism and coronary artery disease, on average, showed an increase in MDA concentrations (>10 fold) than control patients 3.20 mM MDA [11]. Since such data insinuates the presence of conditions of chronic inflammation with a correlation to MDA increase, questions arise about the increased carcinogenic risk associated with reactive aldehydes in a biological system.

This understanding of OPdA's involvement in carcinogenesis can be applied as a potential biomarker for carcinogenesis due to indication of two major hallmarks of cancer: oxidative stress and mutagenecity. Moreover, indicators can be marked by epidemiological studies assessing adducts per 10^6 nucleotides observed in a variety of cancer patients, helping indicate environmental factors in genetic integrity.

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SUPPORTING INFORMATION.

Figure S1. MDA synthesis from TMOP

REFERENCES.

1. Zhou, X. T. *J Biol Chem*, 50 (2005).
2. Hao W., I. D. *Chem Res Tox*, 1467 (2008).
3. Epe, B. *J Biol Chem*, 467 (2002).
4. Shacter E., W. S. *Oncology*, 217 (2002).
5. Marnett, L. J. *Toxicology*, 219 (2002).
6. Marnett, L. J. *J Clin Invest*, 583 (2003).
7. Beckman JS, C. J. *Methods in Enzymol*, 229 (1994).
8. Lichtenfels, R. B. *J Immunol Methods*, 227 (1994).
9. Yermilov V, Y. Y. *FEBS Letters*, 67-70 (1999).
10. Sevilla, C. L. *Chem Res Tox*, 172 (1995).
11. Dumitriu L, B. R. *Endocrinologie*, 35 (1988).

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