Incorporation of extracellular 8-oxodG into DNA and RNA requires purine nucleoside phosphorylase in MCF-7 cells

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ABSTRACT

7,8-Dihydro-8-oxo-2′-deoxyguanosine (8-oxodG) is a well-known marker of oxidative stress. We report a mechanistic analysis of several pathways by which 8-oxodG is converted to nucleotide triphosphates and incorporated into both DNA and RNA. Exposure of MCF-7 cells to [^{14}C]8-oxodG combined with specific inhibitors of several nucleotide salvage enzymes followed with accelerator mass spectrometry provided precise quantitation of the resulting radiocarbon-labeled species. Concentrations of exogenously dosed nucleobase in RNA reached one per 10^6 nucleotides, 5–6-fold higher than the maximum observed in DNA. Radiocarbon incorporation into DNA and RNA was abrogated by Immucillin H, an inhibitor of human purine nucleoside phosphorylase (PNP). Inhibition of ribonucleotide reductase (RR) decreased the radiocarbon content of the DNA, but not in RNA, indicating an important role for RR in the formation of 8-oxodG-derived deoxyribonucleotides. Inhibition of deoxycytidine kinase had little effect on radiocarbon incorporation in DNA, which is in contrast to the known ability of mammalian cells to phosphorylate dG. Our data indicate that PNP and RR enable nucleotide salvage of 8-oxodG in MCF-7 cells, a previously unrecognized mechanism that may contribute to mutagenesis and carcinogenesis.

INTRODUCTION

Reactive oxygen species (ROS) are important metabolic by-products that cause cellular damage, particularly to proteins, lipids and nucleic acids (1,2). ROS are produced through normal cellular metabolism in most cell types, primarily through oxidative phosphorylation. Incomplete transfer of electrons to O_2 during oxidative phosphorylation yields products such as hydrogen peroxide, hydroxyl radicals and singlet oxygen species, which jointly comprise ROS (3). The oxidative modifications caused by ROS lead to cell dysfunction and possibly cell death (4) and are implicated in aging, cancer and other diseases (5,6).

Much has been published as to how ROS chemically modify DNA and the nature of these modifications. It has been well characterized that when nucleotides are exposed to ROS, a variety of altered bases are formed (7,8). Amongst the four normal nucleobases, guanine (Gua) is the most susceptible to oxidation due to its low oxidation potential (9–11). The most abundant oxidized nucleobase found in DNA is 7,8-dihydro-8-oxoguanine (8-oxoGua, Figure 1A) (12). When present in DNA, 8-oxoGua can pair with both cytosine and adenine leading to G→T transversion mutations during replication and DNA repair. Likewise, 8-oxodGTP can mispair with adenine nucleotides to cause A→C transversion point mutations.

The formation of 8-oxoGua in DNA has been shown to occur via two pathways: (i) through direct oxidation of Gua in DNA or (ii) indirectly via oxidation of dGTP in the nucleotide pool to 8-oxodGTP, followed by incorporation of 8-oxodGTP into the DNA by DNA polymerase(s) (1,13). A third pathway, metabolism of the 2′-deoxynucleoside 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxodG) to 8-oxodGTP has only recently been reported (14,15), but with incomplete mechanistic detail. The nucleotide pool containing 8-oxodG may be modulated by extracellular sources. It is known that the repair products from other cells, cell turnover and the diet of the individual contribute to the 8-oxodG load as well.

A

Gua   **dG**   **8-oxoGua**   **8-oxodG**   **8-oxoG**
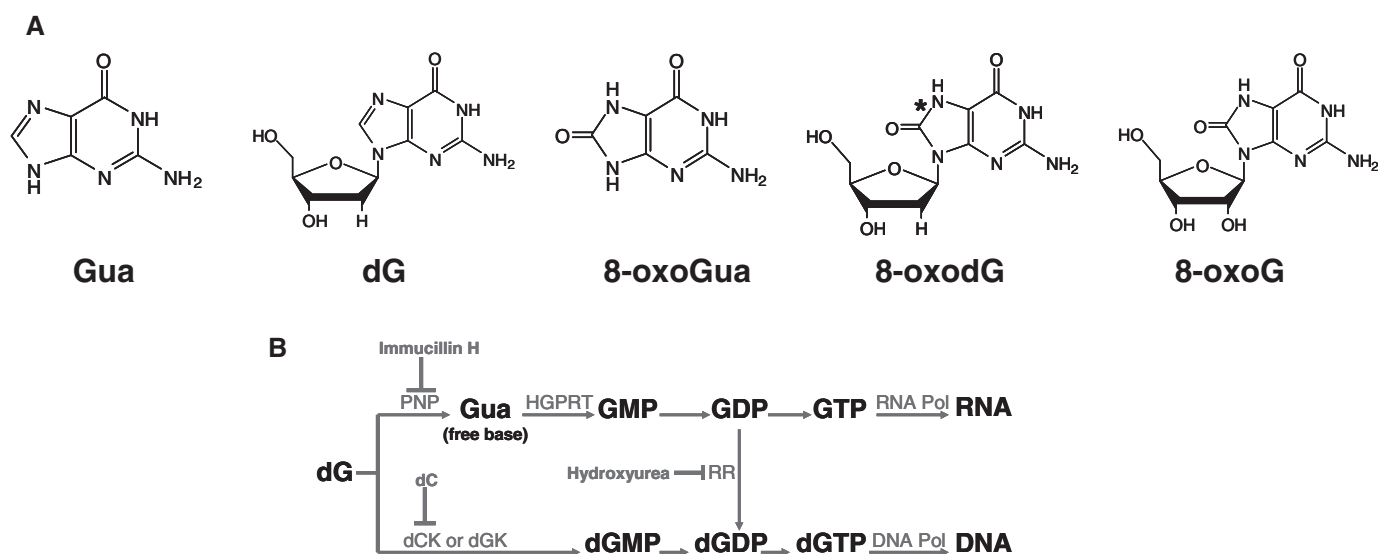
B

Figure 1. (**A**) Structures and abbreviations of guanine (Gua), deoxyguanosine (dG), 8-oxoguanine (8-oxoGua), 8-oxodeoxyguanosine (8-oxodG) and 8-oxoguanosine (8-oxoG), respectively. The asterisk on 8-oxodG indicates the ^{14}C label. (**B**) Nucleotide salvage pathways for dG and inhibitors that can be used to elucidate which are the predominant metabolic pathways. In the cytoplasm, dG undergoes phosphorolysis to the free nucleobase Gua and 2′-deoxyribose-1′-phosphate by human purine nucleoside phosphorylase (PNP, top left). The resulting free base is then phosphoribosylated by hypoxanthine-guanine phosphoribosyltransferase (HGPRT). Two additional phosphorylation steps result in formation of GTP, which serves as a substrate for RNA polymerase-dependent incorporation into RNA. Alternatively, deoxycytidine kinase (dCK, bottom left) in the nucleus or deoxyguanosine kinase (dGK) in mitochondria use dG as a substrate to form dGMP. This in turn is phosphorylated twice to form dGTP, a substrate for DNA polymerase-dependent incorporation into DNA. These two major salvage pathways are connected by ribonucleotide diphosphate reductase (RR), which forms dGDP from GDP. Upon subsequent phosphorylation, the resulting dGTP serves as a nucleotide for DNA synthesis. IH, dC and HU stand for Immucillin H, deoxycytidine and hydroxyurea, respectively.

Extracellular 8-oxodG has been identified in human plasma (16,17) and cerebrospinal fluid (18).

8-OxoGua is premutagenic and the cell employs a number of repair pathways to limit the presence of this species in DNA. Cells primarily use base excision repair (BER) to remove oxidized purines (19). In humans, the hOGG1 glycosylase removes 8-oxoGua from DNA when incorporated opposite cytidine (20,21). The *Escherichia coli* (*E. coli*) MutY homolog, MYH, removes the misincorporated adenine when opposite 8-oxoGua in DNA (19,22). In the nucleotide pool, the *E. coli* MutT homolog, hMTH1, hydrolyzes 8-oxodGTP to 8-oxodGMP to prevent 8-oxoGua incorporation into DNA (23,24). More recently, evidence has emerged that mismatch repair is synergistic with both hMTH1 and hOGG1 in lowering mutation frequencies due to 8-oxodG-derived species (22,24,25).

In contrast to 8-oxoGua in DNA, little information is available on the mutagenic potential and cellular responses to the 8-oxodG. Based upon known nucleotide salvage pathways (26,27), we hypothesized that 8-oxodG (Figure 1A) can be metabolized in two different ways. Direct mono-, di- and triphosphorylation of the nucleoside would result in formation of 8-oxodGTP, a substrate for DNA replication. Alternatively, processing of 8-oxoGua (free nucleobase) can form the nucleotide triphosphates 8-oxoGTP and 8-oxodGTP for both RNA and DNA synthesis after removal of the 2′-deoxyribose moiety. Both pathways reflect the known metabolism of the canonical 2′-deoxynucleoside dG (28) (Figure 1B).

Briefly, as shown in Figure 1B, in the cytoplasm of human cells, dG undergoes phosphorolysis to the free nucleobase Gua and 2′-deoxyribose-1′-phosphate by purine nucleoside phosphorylase (PNP). The resulting free base is then phosphoribosylated by hypoxanthine–guanine phosphoribosyltransferase (HGPRT) (16). Two additional phosphorylation steps result in the formation of GTP, which serves as a substrate for RNA polymerase-dependent incorporation into RNA. Alternatively, deoxycytidine kinase (dCK) in the cytoplasm, or deoxyguanosine kinase (dGK) in mitochondria, phosphorylates dG to form dGMP. The monophosphate is subsequently phosphorylated to form dGTP, a substrate for DNA polymerase-dependent incorporation into DNA. These two major salvage pathways are connected by ribonucleotide diphosphate reductase (RR), which forms dGDP from GDP by replacement of the 2′-OH with a hydrogen atom. Upon subsequent phosphorylation, the resulting dGTP serves as a nucleotide for DNA synthesis.

In the present article, we report evidence that 8-oxodG is a substrate for nucleotide salvage. We investigated whether cells dosed with extracellular [14C]8-oxodG are able to employ either the dCK- or the PNP-dependent pathway (via RR) by quantitation of the radiocarbon on the nucleobase portion of the molecule present in the nucleotide pool and nucleic acids of MCF-7 cells under a variety of conditions. Using accelerator mass spectrometry (AMS), we measured the radiocarbon content in DNA and RNA of cells dosed with [14C]8-oxodG in concert with inhibitors of several nucleotide salvage

enzymes. AMS is a well-established technology for radio-carbon dating and is now in development for tracing ^{14}C-labeled compounds in biological systems, affording sub-attomole ^{14}C per milligram of total carbon sensitivity with a few percent precision (15,29,30). This sensitivity allowed us to observe 8-oxodG metabolism in cells without using large concentrations of 8-oxodG that may have rendered artifactual data due to pathway saturation. Our combined results support the conclusion that the PNP-dependent mechanism is the major pathway by which 8-oxodG is incorporated both into DNA and RNA of MCF-7 breast cancer cells.

MATERIALS AND METHODS

Cell culture and incubation with [^{14}C]8-oxodG

MCF-7 human breast cancer cells originally obtained from ATCC were maintained in DMEM-F12 (Invitrogen/Gibco, Carlsbad, CA, USA) media supplemented with 10% fetal bovine serum (FBS) (Invitrogen/Gibco) and 10 μg/ml insulin (Invitrogen/Gibco). A total of 5×10^5 cells were plated and grown for 24 h in a 10-cm dish before dosing. Cells were incubated with radiocarbon-labeled 8-oxodG for time periods indicated in figure legends, prior to harvest (10.8 pmol per plate with a specific activity of 12.5 mCi/mmol, measured as 300 d.p.m./ml by liquid scintillation counter). [^{14}C]8-OxodG synthesis was performed as described (14).

DNA and RNA extraction from MCF-7 cells

At harvest, the media was removed and the cells were washed with phosphate-buffered saline (PBS, Invitrogen/Gibco), removed from the plate with trypsin (Invitrogen/Gibco) and washed by centrifugation in PBS. DNA and RNA were harvested using Trizol (Invitrogen, Carlsbad, CA, USA). To remove RNA contamination, the DNA was treated with RNase and purified using a DNeasy reverse-phase cartridge (Qiagen, Valencia, CA, USA). Likewise, for RNA samples, contaminating DNA was removed using an RNeasy kit from Qiagen and the on-column DNase kit was employed. The [^{14}C]8-oxodG was ~99% chemically and radiochemically pure; the DNA isolation method was previously reported to not cause spurious oxidation of [^{14}C]8-oxoG in DNA (15). In the previously reported work, the DNA was digested to deoxyribonucleosides and there were negligible ribonucleotides as assayed by HPLC. Furthermore, exposure of the purified DNA to the DNA glycosylase, Fpg, which excises 8-oxodG from duplex DNA, resulted in loss of >95% of the radiocarbon from the DNA, indicating little or no contamination from [^{14}C]RNA. AMS samples were prepared in triplicate from purified DNA or RNA (1 μg) after addition of carrier carbon in the form of 1 μl of tributyrin (ICN Chemical Co.). AMS samples were analyzed as described (15).

Inhibitor studies

Cells were dosed for inhibitor studies in combination with [^{14}C]8-oxodG as described earlier, except with a pre-treatment with the inhibitor 6 h prior to [^{14}C]8-oxodG dosing (30 h total inhibition). Inhibitor doses were chosen such that the maximum dose displayed no effect on cell proliferation and cell death compared to a no-dose control. Immucillin H was prepared as described (31,32) and was used in concentrations increasing 10-fold from 10 nM to 10 μM. Deoxycytidine (dC, Sigma Chemical Co.) was used at 20 μM, 100 μM and 1 mM concentrations, and when dosed in combination with [^{14}C]8-oxodG and 100 nM Immucillin H, 100 μM dC was used. Hydroxyurea (HU, Sigma Chemical Co.) was used at 100 μM, 250 μM and 500 μM.

HPLC separation following cell lysis

After 2 days of incubation with [^{14}C]8-oxodG, the media was removed and the cells were washed three times with PBS. Cells were removed from the plate with trypsin, washed by centrifugation in PBS and lyzed with 1 ml of Lysis Solution from the DNA Extractor WB Kit (Wako Pure Chemical Industries, Ltd.). The resulting lysate was filtered using a Millipore Ultrafree-MC centrifugal filter. The lysate (100 μl) was analyzed by reverse-phase (RP) and anion-exchange (AE) HPLC–AMS using published protocols (15,33–35). Radiocarbon content and elution times were calibrated with authentic mono-, di- and triphosphorylated 2′-deoxyribosyl and ribosyl derivatives of 8-oxoGua. The standards were purchased from Trilink BioTechnologies Inc. (San Diego, CA, USA), except for 8-oxodGMP, which was synthesized according to the literature (15,36).

Proliferation assay and cell death assay

Cells were plated and incubated for 24 h before treatment. Samples were dosed in triplicate for a total of 30 h with each specific inhibitor. For cell death, cells were plated in 60-cm plates and harvested by trypsination. At least 200 cells per plate were counted using the trypan blue exclusion assay. The mean for the replicates is represented and standard error is shown.

Cell proliferation was measured by reduction of the yellow tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO, USA) by metabolically active cells, following quantitation of the resulting intracellular purple formazan. Cells were plated into a 96-well plate at ~1000 cells/well. After 24 h incubation, cells were dosed with indicated concentrations of Immucillin H, dC and HU. After additional 24-h incubation, 100 μl of 12.1 mM each compound was added per well and incubated for 3 h at 37°C. The MTT was removed and 100 μl of 100% DMSO was added to each well and the plate was read at 550 nm.

Statistical analysis

For each experiment, the calculated mean of a representative example is shown. Each point was conducted in triplicate and standard error is indicated. For the Student's *t*-test, Microsoft Excel was used to calculate one tail and two tail probabilities and the results were considered significant if $p < 0.05$.

RESULTS

Incorporation of radiocarbon from [^{14}C]8-oxodG into DNA and RNA

MCF-7 cells grown in the presence of radiolabeled 8-oxodG for up to 5 days were harvested and analyzed by AMS for radiocarbon content in DNA and RNA (Figure 2A). The maximal incorporation of 8-oxoGua into DNA after 24 h was 3.4 ± 0.2 fmol per microgram of DNA compared to 13.0 ± 0.2 fmol per microgram of RNA. After washing the cells and replenishing with radiocarbon-free media, the radiocarbon content in DNA decreased by roughly half, each day during a 3-day time course. This reduction is likely due to DNA repair and dilution of signal due to cell division, approximating the doubling time of MCF-7 cells common in our laboratory. After 3 days of growth, the average daily reduction was only 17% probably due to the slowed growth as the cells reached confluence. Average radiocarbon content in RNA decreased by 35% each day.

Cell lysates from MCF-7 cells grown in the presence of radiolabeled 8-oxodG for 2 days were syringe-filtered in preparation for HPLC–AMS. The samples were injected onto a reverse phase (RP) HPLC system in order to characterize the formation of 8-oxodG metabolic products in the nucleotide pool (34,35). Fractions collected at 1-min intervals were analyzed by AMS for radiocarbon content. The elution times of fractions containing elevated radiocarbon concentrations were compared to those of authentic standards of mono-, di- and triphosphorylated 2′-deoxyribosyl and ribosyl derivatives of 8-oxoGua, for which the retention times were 9.1, 6.1, 4.7, 3.3, 2.9 and 2.8 min, respectively. The fractions containing elevated radiocarbon levels corresponded to retention times consistent with these authentic standards (Figure 2B). Importantly, we also found elevated radiocarbon levels that co-eluted with unmodified 8-oxodG (retention time of 22.6 min).

In addition, the samples were also injected onto an AE HPLC column in order to alternatively confirm the formation of 8-oxodG metabolism products in the nucleotide pool (Figure 2C). Fractions collected at 1-min intervals were analyzed by AMS for radiocarbon content, as described earlier. The elution times of fractions containing elevated radiocarbon concentrations were comparable to those of authentic standards of 8-oxodG, mono-, di- and triphosphorylated 2′-deoxyribosyl and ribosyl 8-oxoGua, of which the retention times are 4.5, 7.4, 9.7, 12.4, 7.7, 10.6 and 13.1 min, respectively. The fraction co-eluting with 8-oxodG shown in panel 2B has relatively low radiocarbon content, whereas the content of radiocarbon in the component co-eluting with 8-oxodG shown in panel 2C dominates the content of radioactivity. A significant amount of radiocarbon was found for fractions eluting between 8-oxoGMP and 8-oxodGTP. This raises the possibility of the presence of significant amount of unknown radiocarbon-containing components present in the cell lysate. These differences may, in part, be due to specific characteristics of the HPLC method, such as elution of radiocarbon with the solvent front, as is likely in panel 2C. Also, we did not use a standard of
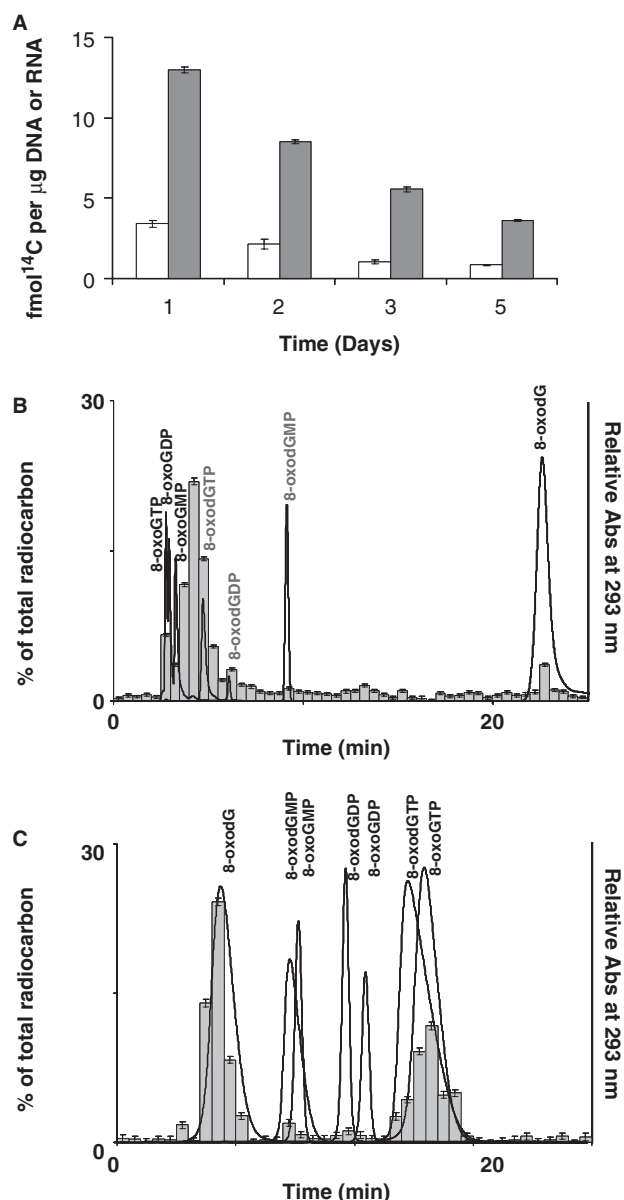

Figure 2. 8-OxodG incorporates into DNA and RNA. (**A**) AMS data from a 5-day time course after exposure to [^{14}C]8-oxodG in MCF-7 cells. (DNA in white, RNA in gray) (**B**) Reverse-phase HPLC–AMS and (**C**) anion-exchange HPLC–AMS data from cell lysates after a 2-day exposure to [^{14}C]8-oxodG in MCF-7 cells. HPLC trace represented on right axis, AMS data represented as a bar graph for each HPLC fraction, with axis on the left.

8-oxoGua, since there is an issue of poor solubility in the buffers used. Since AMS–HPLC relies on coelution with standards, the method is limited in capability for producing new molecular information. However, it is clear that 8-oxodG is metabolized in these cells.

To test the PNP involvement in 8-oxodG metabolism directly, we used Immucillin H, a PNP-specific inhibitor, in cell culture experiments. Immucillin H is a transition-state analog inhibitor that is currently in Phase II trials as an anti-leukemia agent (32,37). Cells were pre-treated with Immucillin H for 6 h and then dosed with [^{14}C]8-oxodG.
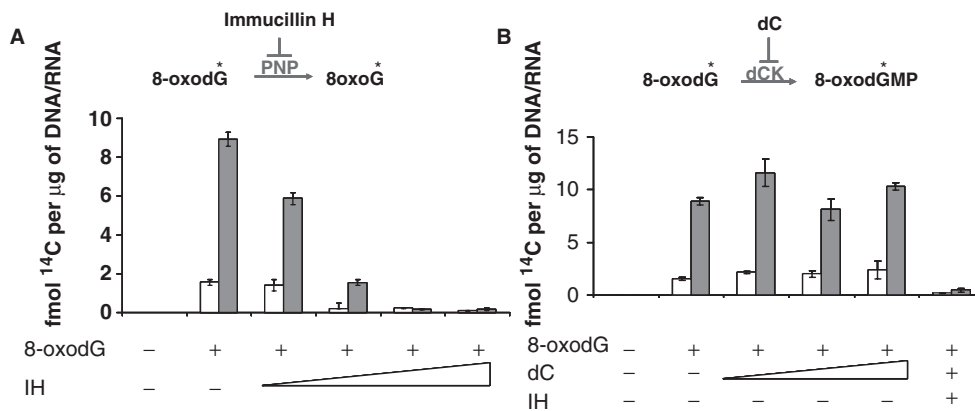
Figure 3. 8-OxodG is a substrate for PNP, but not dCK. MCF-7 cells were dosed with [^{14}C]8-oxodG in combination with increasing concentrations of (**A**) Immucillin H (IH) or (**B**) dC where indicated. RNA (gray) and DNA (white) were harvested and purified from the cells 24 h after dosing. One microgram of DNA or RNA was analyzed by AMS.

After 24 h, the ^{14}C content in both DNA and RNA was measured by AMS in triplicate samples. We found 1.5 ($\pm$0.2) fmol ^{14}C per microgram of DNA and 8.9 ($\pm$0.4) fmol ^{14}C per microgram of RNA when the cells were dosed with [^{14}C]8-oxodG in the absence of inhibitor (Figure 3A). At 10 nM Immucillin H, there was no significant inhibition (Student's *T*-test $p < 0.05$) of [^{14}C]8-oxodG incorporation in DNA and a relatively small, but significant inhibition of incorporation into RNA (34%). In contrast, 100 nM Immucillin H resulted in nearly complete inhibition of incorporation into both DNA and RNA (86% and 74% average reduction, respectively). At this dose, the reduction of 8-oxodG incorporation into DNA was not statistically significant when compared to micromolar concentrations of Immucillin H. The 1 μM dose, but not the 10 μM dose, resulted in a significant reduction of incorporation into RNA compared to the 100 nM concentration, although overall the magnitude of the change at any dose was small. Combined, these data suggest that PNP is a primary nucleotide salvage pathway mediator of 8-oxodG metabolism.

We also examined the contribution of direct phosphorylation of 8-oxodG via the dCK pathway. dC is a known inhibitor of dCK and operates through competition between dCTP and dC to act as a substrate. Cells were pre-treated with increasing concentrations of dC for 6 h prior to the addition of [^{14}C]8-oxodG (Figure 3B). Incorporation of ^{14}C 24 h after 8-oxodG dosing was examined in both DNA and RNA using AMS. We observed no significant reduction of radiocarbon incorporation in both DNA and RNA compared to untreated cells regardless of the concentration of dC used. These data support that dCK does not contribute to 8-oxodG incorporation from the nucleotide pool into nucleic acids.

Since there was a residual amount of incorporation observed even in the presence of high concentrations of Immucillin H and dC, it was possible that the inhibition of PNP caused shunting of purine salvage through the dCK pathway. If this is true, then inhibition of PNP and dCK should result in a complete depletion of incorporation of ^{14}C into DNA or RNA. When we performed this experiment at the highest concentrations of Immucillin H and dC used for the previous experiments there was no further reduction in the ^{14}C incorporation (Figure 3B, far right). This result suggests either incomplete inhibition of the enzymes or the use of another pathway that makes a minor contribution to 8-oxodG metabolism and incorporation into DNA and RNA compared to the PNP-mediated nucleotide salvage.

Assuming that PNP is the primary participant in the nucleotide salvage of 8-oxodG, the incorporation of [^{14}C]8-oxodG into DNA would depend on the activity of RR to form the 8-oxodGTP derivative required by DNA polymerase. In this case, inhibition of RR should result in ^{14}C incorporation into RNA but not into DNA. To test this possibility, we used HU to inhibit RR. HU is a radical scavenger that inactivates RR by directly reducing the tyrosyl radical of an RR subunit during catalysis. Unlike many other RR inhibitors, it does not require activation by dCK (38,39).

We incubated MCF-7 cells with HU for 12 h and then added [^{14}C]8-oxodG for 24 h before harvesting the DNA and the RNA (Figure 4). ^{14}C incorporation into DNA was reduced by an average of 21% and 42% at 250 and 500 μM, respectively. This inhibition was statistically significant compared to the untreated control ($p < 0.05$), whereas no change was observed in the incorporation of [^{14}C]8-oxodG into RNA. These observations are consistent with 8-oxoGua forming from 8-oxodG via PNP-mediated *N*-glycosidic bond cleavage, with subsequent phosphoribosylation and conversion to 8-oxoGTP prior to incorporation into RNA. Our data indicate that the primary mechanism of 8-oxodG metabolism is through the PNP and RR enzymes, which supports our observation that [^{14}C]8-oxodG can be incorporated into both RNA and DNA.

An alternative possibility is that the reduced incorporation observed was due to the toxicity of the inhibitors, or modulation of proliferation rates. Immucillin H is potentially toxic because dG is shunted through dCK possibly causing accumulation of enough dGTP to block DNA synthesis (32).
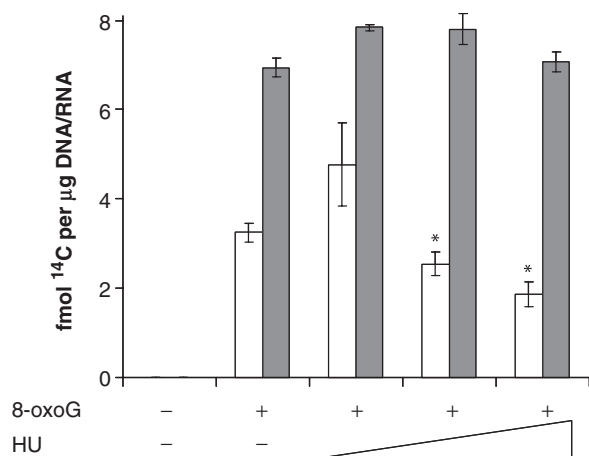
Figure 4. 8-OxoGDP is a substrate for RR. MCF-7 cells were exposed to increasing concentrations of hydroxyurea (HU, 100, 250 and 500 μM), an RR inhibitor, and [^{14}C]8-oxodG where indicated. DNA (white) and RNA (gray) were harvested and purified from the cells 24 h after dosing and analyzed by AMS. The asterisk (*) indicates values that are significantly different from the untreated control ($p \leqslant 0.05$).
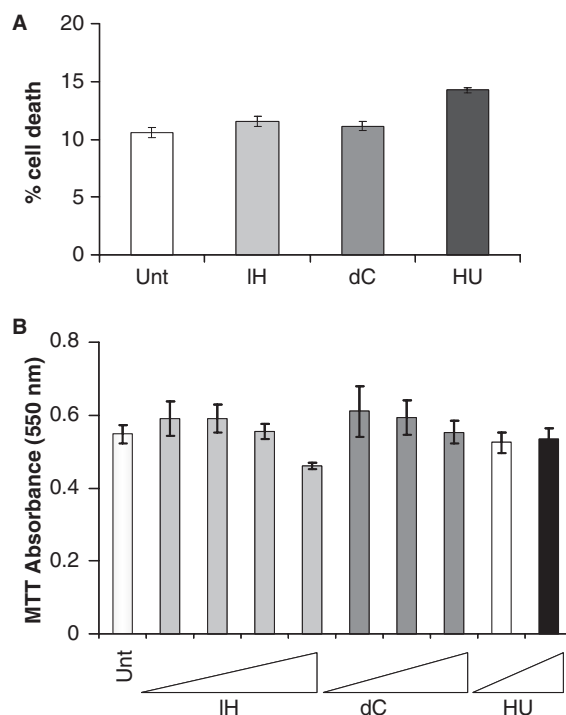

Figure 5. Immucillin H (IH), deoxycytidine (dC) and hydroxyurea (HU) do not inhibit proliferation and are not cytotoxic in MCF-7 cells at the concentrations used. (**A**) Trypan Blue Exclusion Assay for cell death. (**B**) MTT proliferation assay.

To address these concerns, we tested the toxicity of the inhibitors for 30 h (6 h of pre-treatment plus 24 h of 8-oxodG exposure) at the highest concentrations used in the 8-oxodG metabolism studies. Cells were dosed, harvested and assessed for cell death using the trypan blue exclusion assay, where dead cells are counted by virtue of their ability to absorb the dye. We found that the treatments were not substantially toxic compared to the untreated control (Figure 5A).

Additionally, we examined the effects of these inhibitors on cell proliferation (Figure 5B) using the MTT assay, where only living cells are capable of carrying out the enzymatic reaction necessary for the colorimetric change. There was no difference in proliferation in the dosed cells compared to controls at all inhibitor concentrations. Therefore, we could conclude that the reduced incorporation of [^{14}C]8-oxodG in the presence of Immucillin H or HU was due to enzyme-specific inhibition, and not due to toxicity or growth arrest artifacts.

DISCUSSION

Given the high levels of 8-oxodG found excreted from cancer patients (40), and its prominent accumulation as an oxidized nucleobase, 8-oxodG has been regarded as a biomarker of disease. There are many reports examining the role that the nucleotide pool plays in contributing to the mutagenic consequences of 8-oxodG (7,8), but measurement of the effects of oxidative stress in a cellular environment is often hampered by poor measurement sensitivity and precision. Our present data make use of the highly sensitive AMS technology to follow radiocarbon-labeled 8-oxodG as it is metabolized by human breast cancer cells. Experiments from multiple laboratories conducted in parallel by ESCODD reported that 8-oxodG levels are within 0.3 and 4.2 per 10^6 guanines in human lymphocyte DNA (41). Our results fall within

these levels. Furthermore, Park and coworkers (16) found concentrations of 69 ± 15 fmol/ml of 8-oxodG in human plasma. Our supplemental concentration of 108 fmol/ml of 8-oxodG is roughly equivalent to this, thus making our dosage physiologically relevant to the study.

There are conflicting reports as to the ability of different cell types to incorporate exogenous 8-oxodG into nucleic acids and by which mechanisms. For example, there is evidence that neither dCK nor dGK is able to metabolize 8-oxodG (42,43). There is also evidence that [^{14}C]8-oxodG is not a substrate for the PNP enzyme, nor for DNA polymerases (42). Importantly, however, these published data were gathered using purified enzymes and cell extracts that may lack cofactors or cellular integrity required for full utilization of the proposed pathways for 8-oxodG metabolism. In contrast, our data were obtained in intact cells containing all known or unknown cofactors required for nucleotide salvage cells, using physiologically relevant concentrations of 8-oxodG. Our results are facilitated by the use of AMS. AMS is specific only to the labeled compound in any biological or chemical medium without prior speciation or introduction of molecular modifications or internal standards.

The observed incorporation of 8-oxodG into DNA in MCF-7 cells seems to contrast with Kim and Chung (43) who found incorporation of [^{3}H]dG but not [^{3}H]8-oxodG in similar experiments using scintillation counting. However, the mechanistic analysis presented herein provides an explanation, since the tritium label from Kim *et al.* was located on the 2′-deoxyribose ring.
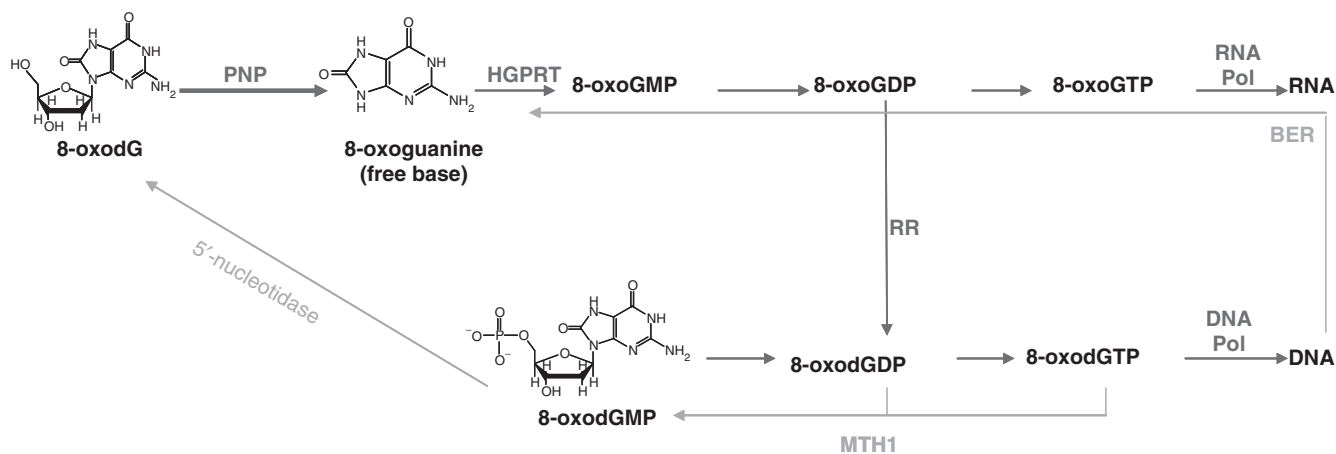
Figure 6. Proposed mechanism of 8-oxodG metabolism in MCF-7 cells. 8-OxodG undergoes phosphorolysis to the free nucleobase 8-oxoGua and 2′-deoxyribose-1′-phosphate by human purine nucleoside phosphorylase (PNP). The resulting free base is then phosphoribosylated by a hypoxanthine-guanine phosphoribosyltransferase (HGPRT). Two additional phosphorylation steps result in formation of 8-oxoGTP, which serves as a substrate for RNA polymerase-dependent incorporation into RNA. Ribonucleotide diphosphate reductase (RR), forms 8-oxodGDP from 8-oxoGDP. Upon subsequent phosphorylation, the resulting 8-oxodGTP serves as a nucleotide for DNA synthesis. Prevention of the incorporation of 8-oxoGua into DNA or RNA derives from the activity of MTH1 and 5′-nucleotidase. MTH1 is a pyrophosphatase capable of cleaving the pyrophosphate from either 8-oxodGTP or 8-oxoGTP, thus preventing the accumulation of a potentially mutagenic species. 5′-Nucleotidase removes the phosphate from 8-oxodGMP to form the 8-oxodG species, which can be excreted in urine *in vivo*.

PNP phosphorylates the ring as it removes the purine nucleobase, which would preclude incorporation of the tritium label into DNA and RNA. In our present article, 8-oxodG was labeled with ^{14}C on the nucleobase, allowing tracking of its metabolism in the absence of the sugar ring, which allowed elucidation of the contribution of the PNP-mediated salvage pathway. The reported elevated incorporation of [^{3}H]dG into DNA compared to [^{3}H]8-oxodG supports more facile direct phosphorylation of dG, compared to 8-oxodG, which is independent of PNP.

Our experiments clearly demonstrate that 8-oxodG from the nucleotide pool is incorporated both into the DNA and the RNA (Figure 2). ^{14}C content in RNA was measured to be 5–6 times higher than in DNA, which may be explained by the constitutive transcription of RNA or the larger pool size of ribonucleotides (42). The radiocarbon concentrations that we observed for this work are higher than previously reported (15,44), possibly due to optimized media composition with respect to supplemental insulin. While there could be trace amounts of RNA in the harvested DNA, our samples were digested with a protocol that was optimized against residual RNA (15). We attribute these higher levels of incorporation to the higher proliferation rate of MCF-7 cells in the presence of supplemental insulin, which resulted from optimization of the cell culture conditions. The levels of 8-oxodG reported here are approximately 1 molecule per 10^6 nucleotides, which is equivalent to the background level of 8-oxodG in many cell types (45). Our results remain consistent with the levels of 8-oxodG in the cells as predicted by ESCODD by labs using HPLC with electrochemical detection (46).

The RP HPLC–AMS data support that 8-oxodG in the nucleotide pool is metabolized to the corresponding ribosyl and 2′-deoxyribosyl triphosphorylated species that serve as substrates for both RNA and DNA polymerases (Figure 2B), which is consistent with the same experiment performed with AE HPLC–AMS (Figure 2C). Since these experiments simply show co-elution of 8-oxodG metabolites with authentic standards, these data alone do not prove the conversion of 8-oxodG to triphosphorylated 2′-deoxyribosyl and ribosyl derivatives of 8-oxoGua. It would be desirable to have better separation of the HPLC peaks in order to allow quantitation of [^{14}C]8-oxodG metabolites, particularly with exposure of cells to Immucillin H. The data support that the radiolabeled compound is metabolized to several species whose retention times are consistent with nucleosides and nucleotides.

The HPLC–AMS data combined with the observation that [^{14}C]8-oxodG incorporates into both DNA and RNA strongly support the interpretation that, free 8-oxodG is converted to triphosphorylated derivatives prior to incorporation into DNA and RNA. The RP- and AE-HPLC–AMS data are consistent with metabolism of 8-oxodG to several phosphorylated species that are expected to be existent based upon the PNP- and RR-mediated nucleotide salvage of dG.

Our results further demonstrate that the PNP enzyme is important to the incorporation of 8-oxoG into RNA and that RR is used to form the deoxyribonucleotides necessary for incorporation into DNA (Figure 6). At this point, the perturbation of other pathways relevant to nucleotide salvage by Immucillin H cannot be ruled out, but there are currently no reports of other enzymes for which Immucillin H is a substrate. However, Immucillin H is now in phase IIb clinical trials and has had extensive testing in cells, animals and humans. To date, no additional effect other than PNP inhibition has been attributed to the inhibitor. For example, administration

of Immucillin-H causes a change in blood patterns in humans that are the same as those found in humans with a genetic deletion in PNP (47).

Each inhibitor experiment was carried out at least three times with triplicate plates of cells for each inhibitor concentration sampled. AMS measurements were taken repeatedly on each sample until a threshold number of counts were recorded. The variation in the data is certainly due to differences in the cell culture experiments. We do not understand the small but statistically significant increase in the DNA radiocarbon from the $100 \mu M$ dose of HU shown in Figure 4. The minimal role for dCK in 8-oxodG salvage is consistent with previous publications that argue against a step-wise phosphorylation mechanism for 8-oxodG incorporation (42,43). Given that the inhibition of dCK and PNP concurrently did not reduce incorporation to zero, an alternative pathway may function in a minimal capacity.

This work re-identifies 8-oxoGua as an important and potentially mutagenic substrate for incorporation into DNA. Our results ultimately imply that 8-oxoGua generated by the BER process may be cycled back for incorporation into DNA. Furthermore, since 8-oxodG is first converted to 8-oxoGua, extracellular 8-oxodG likely contributes more to mutagenesis than previously recognized. Given the metabolism of 8-oxoGua to a substrate that can be incorporated into DNA, the role of the Nudix-hydrolases becomes much more central to prevent mutations. In light of these new data, the contribution of the nucleotide pool with respect to oxidation of DNA-incorporated modified guanines becomes significant and needs to be assessed further (48). Future work will examine the cell-specific nature of these results, since there are several reports that support the possibility that 8-oxoG levels DNA may be enhanced in cancer cells (49).

Conflict of interest statement. None declared.

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