Reactive Oxygen Species, Isotope Effect, Essential Nutrients, and Enhanced Longevity

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ABSTRACT

A method is proposed that has the potential to lessen detrimental damages caused by reactive oxygen species (ROS) to proteins, nucleic acids, lipids, and other components in living cells. Typically, ROS oxidize substrates by a mechanism involving hydrogen abstraction in a rate-limiting step. The sites within these (bio)molecules susceptible to oxidation by ROS can thus be “protected” using heavier isotopes such as $^2$H (D, deuterium) and $^{13}$C (carbon-13). Ingestion of isotopically reinforced building blocks such as amino acids, lipids and components of nucleic acids and their subsequent incorporation into macromolecules would make these more stable to ROS courtesy of an isotope effect. The implications may include enhanced longevity and increased resistance to cancer and age-related diseases.

INTRODUCTION

The suggested $^1$ and currently accepted $^2$ theory of aging blames the accumulation of irreversible alterations in cell machinery and reduced efficiency of metabolic processes on the detrimental effects of free radicals and other reactive species that damage DNA, proteins, lipids, and other cell components. The correlation between aging, age-associated diseases, and the presence of oxidative stress and oxidative damage is extremely good. $^3$

In lipids, reactive oxygen species (ROS) usually oxidize 1,4-diene-methylene (3) carbons. $^4$ Protein oxidation by ROS, which leads to carbonylation or aromatic hydroxylation of some amino acids (AA), is irreparable by proteases after a certain threshold number of AA residues have been oxidized. $^5$ From a quantitative standpoint, nucleic acids (NA) may be the least significant target for ROS compared to other macromolecules. But NAs deserve special attention because of genetic/cancer and age-related consequences. $^6$

In this paper, a method is proposed that can reduce the negative effect of ROS on cellular macromolecules, potentially increasing the lifespan. The applicability of this method to major classes of macromolecules is discussed.

REACTIVE OXYGEN (AND OTHER) SPECIES

ROS is a collective term used to describe oxygen radicals ([O$_2$]$^-$) and its conjugate acid hydroperoxyl [HO$_2$$^+$], hydroxyl [HO$^-$], peroxy [ROO$^-$], alkoxyl [RO$^-$]), and certain other nonradicals that are either potential oxidizing agents or are easily converted into radicals, such as ozone (O$_3$), peroxynitrite (ONOO$^-$) singlet oxygen ($^1$O$_2$), and hydrogen...
peroxide (H₂O₂).⁷ Reactive nitrogen species (RNS) encompass nitrogen dioxide radical (NO₂⁻), peroxynitrite, nitrous acid (HNO₂), and related species. Reactive chlorine species (RCS) include moieties such as hypochlorous acid (HOCl).

These species, normally present in cells, are generated by mitochondria (respiratory aerobic metabolism). They have been implicated as a common feature that connects aging of organisms and age-related diseases. Other enzymatic sources for oxygen radicals are xanthine oxidase, located in the peroxisomal matrix or membranes, and reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, located at the plasma membrane. In addition, there are chemical, photochemical, and radiochemical sources of radicals. ROS also participate in signaling between mitochondria and nucleus.⁸

**ISOTOPE EFFECT**

Isotopes are forms of the same chemical element differing in atomic mass because of different number of neutrons in their nuclei.

The vibration frequency of the chemical bond between two atoms depends on their masses. For atoms with the same electron configuration (same chemical element) but different masses (different isotopes) this vibration parameter will be different, affecting the bond cleavage rate: heavier isotopes form stronger bonds. This is known as the primary kinetic isotope effect, which is commonly used when elucidating mechanisms and rate-determining stages of chemical and biochemical reactions. The rate of reaction involving C-H bond cleavage is typically 5 to 10 (sometimes up to 20) times faster than the corresponding C-D bond cleavage, because of the twofold difference in the masses of H and D. The difference in reaction rates is even higher for tritium (³H or T) because it is three times heavier than hydrogen but is unstable. The ¹²C atom of the C-H bond can also be substituted for a heavier ¹³C isotope, but the bond cleavage rate decrease will be smaller⁹ because ¹³C is only 8% heavier than ¹²C, similar to ¹⁵N and ¹⁸O isotopes. ROS-mediated oxidation reactions can be a good illustration of the isotope effect, since the hydrogen abstraction by an oxidizer is usually a rate limiting step of the process. Secondary isotopic effect and tunneling are related isotope based phenomena that also affect the rate of the bond cleavage.

**THE PROPOSAL: USE OF ISOTOPES TO RESIST OXIDATION AND ENHANCE LONGEVITY**

Many cellular compounds that undergo irreversible chemical transformations such as oxidation or nitration, associated with the onset of senescence or age-related diseases, belong to the group of essential¹⁰ or conditionally essential components, i.e., have to be supplied with the diet. Because these compounds are oxidatively damaged at specific sites, it may be possible to use the primary and secondary kinetic isotope effect (KIE) to make these sites more resistant to the detrimental oxidative damage; substitutions at sensitive sites for heavier stable isotopes would protect against ROS, without compromising the chemical identity of the compounds. Dietary supply of these modified AA, lipid and NA components would then provide an organism with a set of building blocks more stable to oxidation.

This can be achieved by substituting hydrogen atoms subjected to abstraction during oxidation/oxidative substitution at the most reactive carbon sites, or the sites known to undergo the ROS/RNS inflicted damage as illustrated in Figures 1–3, 5, 6, 8 with deuteriums. Substituting carbons as well could provide further protection (Figs. 4, 7–9).

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¹⁰Essential and conditionally essential nutrients—Essential food components cannot be synthesised de novo by an organism and need to be supplied with the diet. Conditionally essential compounds need to be supplied with diet under specific circumstances. For humans, 10 AAs: phenylalanine, valine, tryptophan, threonine, isoleucine, methionine, histidine, leucine, lysine, and arginine (up to the age of 5) are essential¹⁰ (mnemonic rule, “Pvt Tim Hall”). Purine and pyrimidine nucleosides can be regarded as conditionally essential.¹¹ Essential fatty acids (PUFA) are ω-3 and ω-6 while monounsaturated oleic acid (ω-9) is generally nonessential.¹² The AAs oxidized by ROS/RNS/RCS most are essential, with Arg and Lys bearing the major brunt.⁵¹⁵
Protection of proteins against ROS

Oxidized protein loses its catalytic or structural activity, but proteases are unable to disintegrate heavily carbonylated strands. The damaged species accumulate and aggregate clogging up cellular passages. This gradually wears down cellular mechanisms, ultimately causing cellular death. Apart from aging, many diseases such as Alzheimer’s, Parkinson’s, cataracts, arthritis, diabetes, etc., are associated with protein carbonylation. Physiologic levels of protein carbonyls are around 1 nmol/mg protein; pathologic levels go to 8 nmol/mg and above.

The oxidative damages inflicted by ROS on some essential AAs are shown in Figure 1. Hydrogen abstraction from the carbon that undergoes oxidation is a rate limiting step. Oxidation of Arg and Lys by ROS proceeds through sequential replacement of ω-hydrogens with hydroxyls (Like Arg, Pro is oxidized to glutamic semialdehyde). Thr is oxidized into oxo-acid. Other essential AAs undergoing ROS-driven oxidation include Leu (to 5-hydroxyleucine), Val (to 3-hydroxyvaline) and Ile (several products). Aromatic AAs undergo ROS-driven hydroxylation, which may lead to ring opening.

RNS also oxidize essential AAs (Fig. 2). Nitration of tyrosine (not shown) proceeds at the same positions as hydroxylation of phenylalanine (Fig. 1).

Yet another detrimental process occurring with proteins is a ROS-driven peptide bond cleavage, which is preceded by oxygen free radical-mediated protein oxidation (Fig. 3).

A hydrogen atom is abstracted from a Cα atom of the polypeptide chain, which then

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**FIG. 1.** Examples of reactive oxygen species (ROS)-damaged amino acids (AAs). Hydrogens shown undergo rate limiting abstraction. Side chains are subjected to the same transformations when these AAs are parts of polypeptides/proteins.
leads to formation of alkoxyl radical. This can lead either to hydroxyl protein derivative, or to peptide bond cleavage by diamide or α-amidation pathway.15

AAs containing ²H and ¹³C instead of ¹H and ¹²C should be more resistant to this oxidative damage. Only the oxidation sensitive hydrogens should be substituted with deuteriums, to minimize the risk of other metabolic processes being compromised when these compounds are used in other metabolic/catabolic pathways; this relates to NAs and lipids as well. For example, only ω-carbon atoms (Fig. 1) of Lys and Arg should be protected (Fig. 4). The AAs shown would be also more stable towards RNS (Trp and Phe, see Fig. 2).

Synthesis of deuterated analogues of AAs should not pose any serious technical problem. For instance, the protected form of Lys, 2,6-diaminohexanoic acid-6,6-D₂, can be prepared from a precursor nitrile by hydrogenolysis in D₂. Deuterated analogue of Arg, 2-amino-5-guanidinopentanoic acid-5,5-D₂, can be synthesized from a corresponding nitrile. Ornithine-D₂, obtained by hydrogenolysis in a way similar to that described for Lys, can then be converted into a protected Arg using standard protocols¹⁷,¹⁸ (Fig. 4). The ²H,¹³C-substituted compounds will be more expensive to prepare, but will further boost the stability toward the oxidative damage. For instance, the AAs shown on Figure 4 (bottom line) will be.
more stable against damages shown in Figure 1 and 2 as well as confer protection from the oxidative peptide chain cleavage shown on Figure 3.

**Protection of nucleic acids against ROS**

Oxidative damage to DNA occurs at a high rate under normal metabolic conditions. DNA in each cell of our body suffers approximately $10^4$ “oxidative hits” per day, leading to the formation of many different oxidative DNA lesions, some of which cause mutations. There are many NA repair enzymes that remove these lesions, but their efficiency is not 100% so oxidative NA damages and mutations accumulate. Another major consequence of a ROS/RCS/RNS attack on NA is depurination/depyrimidination (formation of abasic sites). Repair of these proceeds through either a base excision repair (BER) pathway or a nucleotide excision repair (NER) pathway.

An example particularly important for the mitochondrial functioning is 8-oxo-dG. Organisms can counter DNA damage through pathways that recognize and repair a variety of mutagenic and lethal base modifications, such as 8-oxo-dG that can mispair with A leading to G to T transversions widely seen in mutated oncogenes and tumor suppressor genes. DNA lesions that escape repair give rise to mutations when cells divide. Endogenous DNA damage is substantial: approximately $10^6$ oxidative lesions are present per rat cell. An important factor in the mutagenic effect of an exogenous agent, whether it is genotoxic or nongenotoxic, is the increment it causes over the background cell division rate (mitogenesis) in the stem cell. Increasing their division rate increases mutation and cancer. DNA damage and the mu-
tation rate is higher in mtDNA (1/130000 in nuclear DNA, versus 1/8000 in mtDNA), resulting from oxygen metabolism, inefficient DNA repair and absence of histones in mitochondria. Oxidative lesions in mtDNA accumulate with age in many human tissues. The amount of 8-oxo-dG in mtDNA in human diaphragm muscle is reported in an 85-year-old individual to be c. 0.5% of the dG residues in mtDNA. Comparisons with mtDNA from younger individuals show a 25-fold increase with age. A high level of 8-oxo-dG (0.87% of dG residues) is observed in mtDNA from the brain of one individual 90 years of age. Recent controversy regarding the measurements of the 8-oxo-dG (values higher than actual because of oxidation during sample preparation) does not lessen the importance of this modification but calls for development of more reliable analytical methods, some of which, for example urinary 8-OH-dG/8-oxo-dG/8-oxoguanine (Fig. 5), are used as biomarkers of in vivo oxidative DNA damage. Other modifications of purines by ROS include 8-oxo-dA and 2-OH-dA. An RNS peroxynitrite (ONOO\(^-\)), released during chronic inflammation, yields 8-nitro-dG, a possible contributor to the mutagenicity and carcinogenicity of inflammation. Peroxynitrite is produced in vivo by reaction of nitric oxide (NO) with \(\mathrm{O}_2^-\) and is involved in inflammation, neurodegeneration, and sclerosis. Its exogenous source is cigarette smoke. Under physiological conditions, the major reactive form of peroxynitrite (pK_\(a\) 7) is peroxynitrous acid (HOONO), which decomposes yielding products with the reactivity of ONO\(^-\) and HO\(^-\). ONO\(_2^--\) is more reactive toward 8-oxo-dG than to unmodified DNA bases, so with increase in the levels of 8-oxo-dG, they can compete with unmodified bases present at much higher concentration for reaction with ONO\(_2^-\).

Pyrimidines are also prone to oxidation (Fig. 6). Major oxidation products of 2'-deoxyctydine (dC) are 5-hydroxy-2'-deoxycytidine (5-OH-dC), 5-hydroxy-2'-deoxyuridine (5-OH-dU), and 5,6-dihydroxy-2'-deoxyuridine (dUg). The levels increase when DNA is exposed to ionizing radiation, \(\mathrm{H}_2\mathrm{O}_2\), UV light and other oxidizers, suggesting that 5-OH-dC, 5-OH-dU, and dUg are major oxidative DNA damage products, and that there is the link between DNA damage induced by oxidative metabolism and spontaneous mutagenesis leading
to cancer and aging. The levels of these from rat tissues range from less than 0.5 fmol/µg of DNA for 5-OH-dU to c10 fmol/µg of DNA for 5-OH-dC and dUg in liver and kidney and 22 fmol/µg of DNA for 5-OH-dC in brain. The corresponding levels of 8-oxo-dG are somewhat lower than the levels of 5-OH-dC. 5-OH-dU and dU glycol are more mutagenic than 8-oxo-dG. Thymine glycol (dUglycol with a methyl group in position 5) repair system is missing in embryonic stem cells bearing the breast cancer susceptibility gene BRCA1.

There is a ninefold elevation in the levels of 8-oxo-dG, 8-oxo-dA, and formamidopyrimidine in tumour tissues compared to surrounding normal tissues. Elevated levels of oxidized bases are also associated with inflammatory diseases hepatitis and cirrhosis.

An RCS hypochlorous acid (HOCl), generated from H2O2 and chlorine by myeloperoxidase in activated neutrophils during inflammation, reacts with dC, dU, and DNA to give 5-CIUra. In the carrageenan-induced inflammation model in rats, chlorinated nucleosides were significantly increased, compared to controls, in the exudate fluid isolated from the inflammation site.

Like AAs, NA components can also be isotopically protected (Fig. 7) against oxidative damages shown on Figures 5 and 6. ROS/RNS/RCS vary in reactivity towards NAs (e.g., O2 selectively attacks guanine whereas a more reactive HO- attacks all four DNA bases) and lead to formation of different products. But all these oxidative reactions have either a proton abstraction or a double bond (C=C, N=C) addition as a rate-limiting step.

Similar approaches can be used to reinforce other sites within nucleosides/nucleotides.

Curiously, there are no literature reports on deuterium KIE of aromatic AAs and NAs components’ oxidation by ROS. Different oxidation mechanisms are possible, and KIE for some model systems has been reported to vary from inverse to none to positive. These and other examples are not directly comparable to the AAs and NAs systems described, so only the experiment will tell. The KIE will also depend on the nature of ROS. 13C will confer a positive KIE, albeit a smaller one, to aromatic systems (Dr. P. Fitzpatrick, personal communication).
such as the glycosidic bond, that are vulnerable to damage by reactive species. Again, deuterated compounds would be easier to synthesise, but $^2$H, $^{13}$C derivatives would be even more resistant to oxidation.

**Oxidation of lipids/PUFAs**

Essential fatty acids (PUFA) undergo oxidative damage primarily by the nonpolar $\text{HO}_2^\cdot$, in the chain-propagating fashion. Superoxide radicals react several orders of magnitude faster with the lipid hydroperoxides than with the corresponding fatty acids. Moreover, there is some indication that hydroperoxyl radicals $\text{HO}_2^\cdot$ are formed inside the mitochondrial membrane (Dr. A. de Grey, personal communication) or at its very surface. Methylene groups of 1,4-diene systems are substantially less stable to ROS than allylic methylenes (Fig. 8): oleic acid (a nonessential fatty acid with one double bond) is stable in the above conditions. This ROS-driven radical chain reaction, that takes place in mitochondrial lipid bilayers, affects the integrity of the membranes and damages other macromolecules such as proteins.

Low-density lipoprotein (LDL) is prone to similar type of oxidation, which is important in development of atherosclerosis.

Fatty acids can be protected against ROS-mediated damage by reinforcing the methylene groups of 1,4-butadienyl (bis-allyl) fragments (Fig. 8). Not all PUFAs might be amenable to such derivatisation with isotopes though. An important class of biologic mediators—prostanoids (the class includes inflammatory mediators prostaglandins, thromboxanes, lipoxins, resolvins, isofurans, isoprostanes, leukotrienes, etc.) is synthesised mostly from arachidonic acid. As the first step, the COX enzymes abstract hydrogen from the methylene group of the 1,4-diene system followed by a conversion into a hydroperoxy derivative in a process similar to oxidation by ROS. Isotopic substitutions might therefore compromise these pathways.

Once again, deuterium-substituted compounds would be easier to make, whereas $^{13}$C, $^2$H-derivatives would be more difficult to synthesise but slightly more stable to oxidation than the deuterium-only ones.

**Can this work?**

An indication of a viability of this approach comes from experiments on ethanol oxidation by alcohol dehydrogenase (ADH). Ethanol is oxidized to acetaldehyde, which involves hydrogen abstraction in a rate-limiting step. Ethanol was compared against its deuterated and tritiated analogues. The KIE upon $V/K$ for (1-R)[$^1$H$_2$]- and (1-R)[$^3$H$_2$]-ethanol oxidation by liver ADH to acetaldehyde, measured at pH 6, has been found to be 3 ($D(V/K)$) and 6.5 ($T(V/K)$).

In vivo experiments in perfused rat liver gave the mean value of $D(V/K)$ of 2.89.

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**FIG. 7.** A, G, and T bases protected at oxidation-prone sites with D (top row) or with both D and $^{13}$C (bottom row) for a larger isotope effect. $^{13}$C is marked with an asterisk. $R = \text{H}$; (deoxy)ribose; (deoxy)ribosylphosphate; oligonucleotide; polynucleotide.
Lower than expected rates confirm the discrete role of the non-ADH systems as alternative pathways. In all cases the oxidation of deuterated ethanol was substantially slowed down.

The magnitude of the isotope effect (both primary and secondary) for suggested protected essential nutrients can only be obtained experimentally and will vary depending on the conditions, the nature of the radical, and the compound. Nonenzymatic examples of DNA cleavage by the hydroxyl radical37 and of a polystyrene cleavage38 (polymer aging/degradation is akin to oxidation shown in Figures 1–3, 5, 6, and 8 as it is also driven by ROS) point to a KIE similar in magnitude to ADH ($k_{H}/k_{D} \approx 2$). A somewhat larger effect should be expected for a less reactive HO$_2$.

Because the compounds worst affected by the oxidative damage often belong to the group of essential or conditionally essential, the delivery of their isotopically protected forms should not pose a problem. To minimize the risk of compromising the important metabolic/catabolic pathways, only the oxidation-vulnerable sites within the compounds should be protected with isotopes. Protected components may be administered as supplements through a digestive system to achieve a desired effect of slowing down detrimental changes associated with aging process and various diseases. Nonessential compounds delivered in this way might be diluted by the de novo synthesis, reducing the effect. Ways other than through the digestive tract, for instance an intravenous delivery, can also be envisaged.

Looking further ahead, biotechnological/nutraceutical processes can be envisaged whereby, for example, essential AAs-deficient yeast/algae/bacteria/etc., can be cultivated on appropriate isotopically protected media/sub-

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**FIG. 8.** Top, oxidation of PUFA by reactive oxygen species (ROS). The oxidation takes place at the 1,4-butadienyl methylene groups indicated by arrows. Same sites are affected when these PUFAs form lipid membranes. Linoleic and linolenic acids protected at oxidation-prone sites with D (middle row) or with both D and $^{13}$C (bottom row) for a larger isotope effect. $^{13}$C is marked with an asterisk.
strates and then the biomass obtained fed to fish or livestock, which can then be introduced into human food chain in the normal manner.

Medical and other applications

There are other potential applications for the strategy described. Metastasis is a multistep process responsible for most cancer-related deaths, and it can be influenced by both the immediate microenvironment (cell–cell or cell–matrix interactions) and the extended tumour microenvironment. Hypoxia (low oxygen) is clinically associated with metastasis and poor patient outcome. Recent studies have shown the expression of lysyl oxidase (LOX) to be elevated in hypoxic human tumor cells. Because LOX oxidizes the same position of Lys as ROS (see Fig. 1), it would be interesting to see how these cells would be affected by the protected version of Lys (Fig. 4), compared to specific LOX inhibitors.

Another interesting possibility would be to control mutagenic properties of NA bases by shifting their tautomeric equilibriums. Some mutations arise because nucleic acid bases in different tautomeric forms such as 4-oxo-T and 4-hydroxy-T pair differently. The equilibrium of this reaction might be shifted more towards the former by placing heavier versions of oxygen and carbon (18O and 13C) at position 4 of a pyrimidine ring. Similarly, C with a 13C-15NH2 fragment at position 4 may have a reduced propensity for hydrolysis yielding U.

FIG. 9. dC and 5-Me-dC protected with D and 13C. 13C is marked with an asterisk. Bottom row: dC would be more difficult to methylate, while 5-(deuteromethyl)-dC would be more difficult to de-methylate.

FIG. 10. Interaction between oxidants and cellular (macro)molecules.
Yet another application could be to provide personnel regularly exposed to some level of radiation (nuclear installations workers, cosmonauts) with diets containing isotope protected nucleic acid components, as the latter are usually the first target for the radiation-induced radical attack, leading to mutations and cancer.

CONCLUSIONS

Proposed here is a class of compounds that when ingested result in the formation of bodily constituents (e.g., proteins, NAs, fats, etc., catabolizable by both P450 and non-P450 pathways) that are functionally equivalent to normal bodily constituents but have a greater resistance to degradative/detrimental processes mediated by ROS and RNS.

For the three molecules involved in a process of oxidative damage—an oxidizer, its substrate and an antioxidant—the oxidizer and antioxidant were the subject of many studies aimed at neutralizing the pool of oxidants and boosting the pool of antioxidants to minimize the damage. So far there were no attempts to protect the target of this oxidative process itself (gray area on Figure 10), macromolecules and other cellular components damaged by ROS.

Many biochemical pathways have evolved to repair damaged cellular components. But eventually, dark forces of ROS prevail and aging and disease take over and kill the organism. The whole process may be considered as a chemical reaction with equilibrium shifted toward the undesired end. With isotopes, a tantalizing possibility—that of slightly helping out the “loosing” repair pathways, shifting the equilibrium just enough to make them overcome the killer ones—might now become a reality. Aging, cancer,46 and other diseases were never tackled from this direction before.

Sets of compounds reinforced with different isotopes should be synthesized and tested in living systems. Apart from D and 13C, other isotopes such as 15N and 18O should be investigated. A softer version of protected AAs, NAs, and fatty acids can also be tested that would only contain 13C substitutions, reducing the isotope effect but also minimizing the undesired slowing effect on other metabolic pathways.

The price of isotopes should not be a deterrent and will fall as the economy of scale will take over: the natural abundance of isotopes (H, 99.985% and D, 0.015%; 12C, 98.89%, and 13C, 1.11%) suggests that manufacturing schemes substantially cheaper than currently adopted ones47 can be developed.

Once again, there are numerous issues to be addressed: Maillard reaction (AGEing products),48 tolerance to isotopes, the loading phase, chemical issues, and many other but only the experiment will tell. So what are we waiting for?

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