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Metal-Mediated DNA Damage and Cell Death: Mechanisms, Detection Methods, and Cellular Consequences

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Abstract

The redox activity of metal ions can lead to the formation of highly reactive species that damage DNA, producing different oxidation products and types of damage depending upon the redox potentials of the DNA bases, formation of intermediate adducts, and identity of the reactive species. Other factors are also important in determining the degree of metal-mediated DNA damage, such as localization and redox chemistry of the metal ions or complexes and lifetimes of the reactive oxygen species generated. This review examines the types of DNA damage mediated by first-row transition metals under oxidative stress conditions, with emphasis on work published in the past ten years. Similarities and differences between DNA damage mechanisms of the first-row transition metals *in vitro* and in *E. coli* and human cells are compared and their relationship to disease development are discussed. Methods to detect this metal-mediated DNA damage, including backbone breakage, base oxidation, inter- and intra-strand crosslinking, and DNA-protein crosslinking are also reviewed, as well as detection methods for reactive oxygen species generated by these metal ions. Understanding the conditions that cause metal-mediated DNA damage and metal generation of reactive oxygen species *in vitro* and in cells is required to develop effective drugs to prevent and treat chronic disease.

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Metal imbalance and DNA damage: Cellular oxidative stress and health consequences

Transition metal ions have long been implicated in the incidence of human disease, often resulting from mis-regulation of metal ion levels under non-homeostatic conditions. The main problem with these imbalances are the deleterious reactions in which metal ions can participate, such as generation of reactive oxygen species (ROS), substitution of non-natural metal ions into enzymes (e.g. $Co^{2/3+}$ replacement of $Fe^{2/3+}$ by in Fe-S clusters),[1] disruption of metabolic and antioxidant pathways (e.g. depletion of ascorbate by Co^{2+} and Ni^{2+} inhibition of cellular ascorbic acid uptake),[2] and inhibition of DNA repair enzymes.[3,4] The most common metal-generated ROS are hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$), and singlet oxygen ($^1O_2$). These four species are generated by different processes that are often interrelated; for example, metal-containing superoxide dismutase (SOD) enzymes catalyze the conversion of $O_2^{\cdot -}$ to H_2O_2. DNA damage caused by metal-generated ROS yields various products (strand breaks, base oxidation or loss, and DNA-protein crosslinks),[5-8] but ROS can also cause lipid peroxidation[7-9] and protein oxidation[7,9] leading to abnormal cellular functioning. Uncontrolled ROS generation can lead to conditions such as male infertility,[10] prostate cancer,[11] and aging.[12-14] Other diseases are related to specific metal ions, including cardiovascular[15,16]and respiratory[17] diseases, cancer,[9,18-21] neurodegenerative disorders such as autism, Alzheimer's, Parkinson's, and Huntington's diseases,[4,22-25,26,27] diabetes,[5,28] and inflammatory responses (Table 1).[4]

Iron is one of the most studied DNA-damaging metals, and iron-mediated DNA damage is an underlying cause of multiple diseases. In Huntington's disease, a correlation is observed between higher-than-normal iron concentrations,[24,25] increases in lipid, DNA, and protein oxidation products, and a reduction of cellular antioxidant capacity; higher levels of DNA oxidation products correlate with more aggressive symptoms.[23] Disruption of metal homeostasis

leading to high iron levels are also related to prostate[18] and other cancers,[29,30,31] Alzheimer's and Parkinson's diseases,[32,33] type 2 diabetes,[34] and vascular disorders such as atherosclerosis.[16] Similarly, high copper levels are related to cancer[29,30,31] as well as Alzheimer's and Parkinson's diseases.[32,33]

Table 1. Common types of DNA damage and human diseases related to metal ion imbalance or exposure.

Metal Ion	Type of DNA Damage	Reactive Oxygen Species or Oxidant	Diseases Related to Metal Imbalance or Exposure
Sc^{3+}	Undetermined	$^1O_2$[35]	--
Ti^{4+} (TiO_2)	Single- and double-strand breaks, base oxidation[36]	$^1O_2$, and $O_2^{\cdot -}$[37,38]	--
VO_2^+, VO^{2+}	Single- and double-strand breaks, base oxidation[39]	$^1O_2$, $^{\cdot}OH$,[40] and oxovanadium species	--
Cr^{3+}, Cr^{4+}, Cr^{5+}, Cr^{6+}	DNA-protein crosslinking,[41] base oxidation, DNA-Cr^{3+} adducts[42]	Cr^{4+},[43] ascorbyl radical,[43] $^{\cdot}OH$,[44] $O_2^{\cdot -}$[44]	Diabetes,[28] cardiovascular diseases,[45] lung cancer,[46,47] inflammatory responses[4]
Mn^{2+}, Mn^{4+}	Single strand breaks and thymine oxidation[48]	None directly detected[49,50]	Adverse neurological effects[27,49] Inflammatory responses[4] respiratory diseases[51]
Fe^{2+}	Single-strand breaks,[52] base oxidation[53]	$^{\cdot}OH$, ferryl ($[Fe{=}O]^{2+}$)[52] species	Parkinson's and Alzheimer's diseases,[33] atherosclerosis,[16] type 2 diabetes,[34] prostate tumors,[18] Huntington's disease[24,25]
Co^{2+}	Backbone cleavage, adenine and cytosine cleavage[54]	$^{\cdot}OH$, $^1O_2$[55]	Autism,[26] hypoxic response,[56] liver and kidney toxicity[57]
Ni^{2+}	Backbone cleavage, guanine oxidation[58]	$^1O_2$,[59] hydrolytic cleavage[60]	Lung cancer,[17] prostate tumors,[18] inflammatory responses[4]
Cu^{+}	Bases and sugar oxidation[61]and backbone cleavage[62]	$^{\cdot}OH$, $^1O_2$,[63] $O_2^{\cdot -}$, CuOOH	Alzheimer's disease,[33,64] prostate tumors,[18] inflammatory responses[4]
Zn^{2+}	Backbone cleavage[65]	Hydrolytic cleavage[65]	Alzheimer's disease,[33] prostate tumors[18]

Acute administration (intraperitoneal injection) of hexamine cobalt(III) chloride in mice (20 mg/kg for 3 days) causes severe kidney toxicity (lipid peroxidation, and oxidative damage, as well as reductions in glutathione (GSH) levels, superoxide dismutase (SOD) activity, and catalase activity) in a dose-dependent manner, in addition to liver and spleen toxicity.[57] Upon environmental exposure, cobalt typically accumulates in the lungs, and lung epithelial cells

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(H460) exposed to cobalt (300 μM for 24 h) show an increase in ROS generation (as measured by 2,7'-dichloroflorescein diacetate fluorescence) that results in poly-ADP ribose polymerase (PARP) cleavage and double-strand DNA breaks.[58] Apoptosis of H460 cells is also observed after treatment with Co^{2+} (300 μM), Ni^{2+} (200 μM), or both metal ions simultaneously.[58] Cobalt released from arthroplasty prostheses (presumably Co^{2+}) was reported to bind and modify serum albumin (presumably at the *N*-terminal binding site), causing more free Co^{2+} in the blood available for transport into neurons via the divalent metal transport protein (DMT1).[66] Higher concentrations of manganese, chromium, nickel, and copper are present in the frontal cortex, hippocampus, and olfactory bulb of children and young adults exposed to the polluted air in Mexico City compared to residents of two low-pollution cities (Tlaxcala and Veracruz). These metal increases correlate with an increase in inflammatory response, as measured by COX2 and IL1β expression, and a reduction of OGG1 levels (an enzyme that recognizes and removes oxidized guanine), but they report no increase in DNA damage.[4]

Manganese can be neurotoxic if present in excess,[49] and chromium has been heavily implicated in the formation of DNA adducts and generation of damaging ROS in cells, with high concentrations of chromium measured in cancer patients.[29-31,67,68] The diagram in Figure 1 highlights the types of DNA damage associated with each metal and compares the number of publications in the past ten years that discuss DNA damage for each first-row transition metal.

Metals in cells: Metalloproteins, cofactors, and labile ions

Measuring biological concentrations of metal ions in both bacterial and mammalian cells is an active research area. Table 2 lists average concentrations of the first-row transition metal ions found in various human biological matrices. Unsurprisingly, the number of reports of

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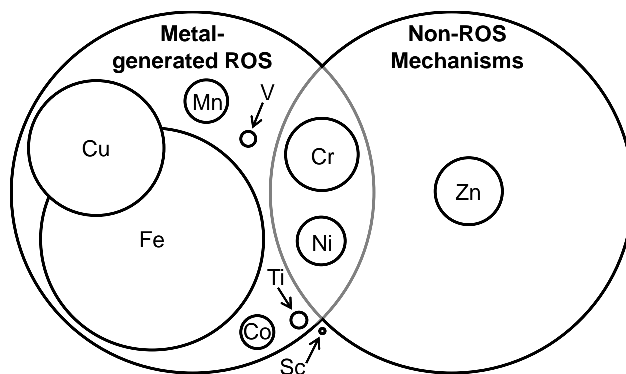

Figure 1. Visual representation of articles published on DNA damage by first-row transition metals. Large circles correspond to the primary mechanism(s) by which the metals damage DNA, and the size of the smaller circles for each metal corresponds to the number of publications in the past ten years (2003-2014).[69] For reference, the circle labeled Sc corresponds to ~4 publications, Ti to ~164, and Fe to ~2300.

particular metal concentrations are directly proportional to their biological significance, and metal ion distribution can vary widely between tissues and within cellular compartments (Table 2). Scandium, titanium, and vanadium are present only in trace amounts with no known biological functions, and are therefore not well studied. Rikhanov *et al.*[70] used inductively coupled plasma mass spectrometry (ICP-MS) and instrumental neutron activation analysis (INAA) to determine scandium concentrations ranging from 0.0001 mg/kg to 0.1 mg/kg in human tissue samples. They also found vanadium in significantly larger quantities, ranging from 0.05 mg/kg to 10 mg/kg.[70] Additional studies quantified vanadium at 0.21 to 0.25 μM in human blood serum (Table 2).[71] Although not a vital element in humans, vanadium is an important cofactor in several enzymes such as haloperoxidases from *Ascophyllum nodosum* (algae) and *Corallina officinalis* (seaweed) and nitrogenases from bacteria in the *Azotobacter* genus.[72,73]

Titanium is of more interest in biological systems due to leaching from medical implants, although it is commonly believed to pass through the body safely, causing minimal DNA damage.[74] Blood titanium levels in healthy humans are less than 0.01 μM. However, in people with titanium implants, these levels can reach 0.1 μM.[75] Many studies related to titanium-mediated DNA damage involve titanium dioxide nanoparticles[37,38] instead of titanium ions and

Table 2. Average concentrations of first-row transition metals in various human biological matrices.

Metal	Methods[a]	Matrix	Concentration (μM)	References
Scandium	ICP	Serum	0.54	76
Titanium	ICP	Serum	0.01-3.13	75,76,77
Vanadium	ICP	Serum	0.21-0.25	76,77
Chromium	ICP	Serum	0.0023-4.8	76,78,79
Manganese	ICP	Serum	0.0083-0.042	80
	ICP	Cerebrospinal fluid	0.0083	76
Iron	ICP	Serum	20-30	81
	INAA	Cerebrospinal fluid	1-5	81
	PIXE	Brain tissue	4	82
	Mössbauer spectroscopy	Mitochondria	210	83
	FAAS	Liver tissue	6315	84
	FAAS	Bone marrow	3581	85
Cobalt	SF-ICP-MS	Serum	0.0031-0.025	86
Nickel	ICP	Serum	0.004 – 0.80	77
Copper	ICP	Serum	10-25	87
	ICP	Brain tissue	102	88
	FAAS	Liver tissue	85.5	84
Zinc	ICP	Serum	15.75-19.27	77
	FAAS	Liver tissue	903	84
	FAAS	Kidney tissue	533	89

[a]ICP = inductively coupled plasma measurements; INAA = instrumental neutron activation analysis; PIXE = particle-induced X-ray emission; SF-ICP-MS = sector field inductively coupled plasma mass spectrometry; FAAS = flame atomic absorption spectroscopy. Metal concentrations in tissue were calculated from wet weight percentages using the average density of human brain (1040 g/L[90]), liver (1106.5 g/L), and kidney (1106.5 g/L[91]) tissue.

are thus outside the scope of this review. Currently, no studies exist focusing on the ability of titanium ions alone to damage DNA.

Chromium is also a required trace element and has attracted considerably more interest than scandium, vanadium, and titanium due to its role in glucose metabolism (as Cr^{3+}) and its well-known genotoxic and carcinogenic effects (as Cr^{6+}).[67] It has also been well-studied with respect to diabetes mellitus, but its status as an essential trace element has been recently called into question.[92,93] Cr^{3+} is present in human serum from 6.0 nM to 0.5 μM,[77] and more recent studies have determined typical chromium levels to be less than 1 μg/L in blood and urine.[79] Cr^{3+} is not membrane permeable, and thus transferrin and chromodulin are implicated in its biological

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transport and cellular uptake.[94] Cr^{6+}, however, exists as chromate (CrO_4^{2-}) and enters cells via sulfate channels due its structural similarities to sulfate.[42] Once inside the cell, Cr^{6+} can be reduced by ascorbic acid or other cellular reductants, producing damaging chromium metabolites.[95] The extent of this damage and the formation of Cr-DNA adducts are discussed in the "In vitro *DNA damage: Metals, mechanisms, and products*" section of this review.

Manganese has also attracted considerable interest due to its neurotoxicity,[96,97] as well as its functions in calcium absorption, metabolism, bone formation, blood sugar regulation, and its essential role in enzymes such as arginase and superoxide dismutase.[98] Manganese levels in human serum typically range from 0.003 to 0.068 μM,[99] and most cellular manganese is found as Mn^{2+} in the mitochondria of brain and liver cells. Although biological manganese can exist in multiple oxidation states, there is a distinct lack of information related to the possible DNA damage caused by manganese-mediated ROS formation. In fact, most studies involving this metal focus on manganese antioxidant activity due to its presence in superoxide dismutase (SOD). However, Mn was recently implicated as a cause of ROS-mediated DNA damage and has also been thought to have a critical role in neurodegeneration.[48,100]

Iron is an essential nutrient required for many life processes, as well as a major generator of DNA-damaging ROS, and has attracted considerable research interest over many years. In *E. coli*, non-protein-bound Fe^{2+} concentrations are around 10 μM, but reach levels of 80-320 μM under oxidative stress conditions.[101-103] In human cells, the first ever "ironome" was recently reported by Jhurry and coworkers;[83] iron concentrations in Jurkat (human T lymphocyte) cells were established for labile, protein-bound, and mitochondrial iron pools. Iron in these cells range from 30 μM of labile Fe^{2+} in the cytosol to 210 μM for mitochondrial iron. These authors also distinguished concentrations of natural Fe^{3+} nanoparticles and non-heme-bound Fe^{2+}.[83] In normal

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human serum, iron concentrations typically range from 20 to 30 μM but reach concentrations as high as 0.5 to 1 mM in the brain.[81,104] The high levels of iron in stressed *E. coli* and in the mitochondria of human cells highlight iron's critical relationship to oxidative DNA damage. Mitochondria, in particular, are well-established sites of oxidative DNA damage due to their high iron levels, and mitochondrial DNA damage has emerged as its own focus in this field.[105]

Cobalt is another trace metal ion required for life and is most well-known for its presence in cobalamin, or vitamin B_{12}.[106] Cobalt concentrations in human serum range from 0.18 to 1.62 ng/mL (0.0031-0.025 μM).[86] It is suggested that Co^{2+} ions participate in hydroxyl radical generation, Ca^{2+} and Fe^{2+} antagonism, and upregulation of many hypoxia-inducible genes following hypoxia-inducible transcription factor (HIF-1) activation.[107] It has been suggested that iron-like generation of hydroxyl radical by Co^{2+} leads to similar DNA damage as observed for Fe^{2+},[107,108] but the relatively high redox potential of the Co^{2+}/Co^{3+} couple (1.92 V) compared to Fe^{2+}/Fe^{3+} (0.77 V) may preclude cobalt redox cycling in biological systems.[109,110] Studies with cobalt often show damaged proteins or DNA, but few experiments directly examine cobalt-generated ROS. Co^{2+} has also been implicated in the displacement of other, redox-active divalent metal ions (such as iron) from metalloproteins, resulting in indirect DNA damage.[2]

Nickel's biological role is more extensive among plant and microbial systems than in mammals.[111] Despite this, nickel deficiency is linked to adverse effects in rats, such as inhibited iron uptake.[112] Nickel is present in human serum at concentrations ranging from 0.004 to 0.8 μM,[77] concentrations higher than other trace metal ions such as cobalt, manganese, or vanadium. Thus, nickel has been more strongly correlated with DNA damage and carcinogenesis than most other metal ions discussed in this review.[113,114]

Copper, like iron, is essential to life in a variety of roles and has been thoroughly studied.

Serum concentrations of copper range from 10 to 25 μM but reach concentrations as high as 0.1 mM in the human,[87,88] where it is required for several metabolic processes and signaling mechanisms during neural activity.[115] It is also a cofactor in many other oxygen-related proteins such as cytochrome c oxidase, copper superoxide dismutase, and ceruloplasmin.[81] However, copper is much more tightly controlled by chaperone and other proteins than iron due to its smaller window of redox activity (0.16 V compared to iron's 0.77 V).[116] This redox activity can lead to undesired reactivity and DNA-damaging ROS generation.[117]

Although zinc is technically classified as a post-transition element, it is included in this review due to its similarities with copper and its undeniable biological significance. In *E. coli*, zinc concentrations are between 0.1 to 0.5 mM; whereas in humans, concentrations range between 1030 to 1260 ng/mL (16-19 μM) in human serum and 500 μM in the brain.[118] Zinc is not redox active, but has structural functions in protein folding and also acts as Lewis acid catalyst in enzymes.[119,120] Labile zinc is involved in cellular signaling, similar to Ca^{2+}, as a secondary messenger in the brain.[121] Zinc deficiency has been linked to oxidative stress, but it does not directly generate ROS—its contribution to DNA damage is discussed in the "In vitro *DNA damage: Metals, mechanisms, and products*" section.[122]

Detection methods for reactive oxygen species generation

Reactive oxygen species (ROS) are the price that life pays for requiring oxygen to survive,[123] and they have been a major focus of biological research for decades. ROS are more concentrated in the mitochondria of cells,[124-126] which is especially troubling since mitochondrial DNA damage is often more persistent than nuclear DNA damage.[127] Superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\cdot}OH$) are all present in the mitochondria at different stages of

the respiratory cycle, and act as signals and messengers in low concentrations for several receptor-mediated pathways.[125,128,129] In excess, they cause cellular damage and oxidative stress.[130,131] Enzymes such as catalase and superoxide dismutase decompose H_2O_2 and $O_2^{\bullet -}$, respectively, and are often expressed in higher concentrations in response to oxidative stress.[132,133] Glutathione peroxidase (GPx) enzymes also decompose hydrogen peroxide and perform other antioxidant functions.[134] The four most common metal-generated ROS (Figure 2) are discussed in this section, as are selected methods to detect these ROS that have been developed or modified in the past ten years.

Any disturbance that shifts biological homeostasis can cause ROS overproduction and damage to cellular organelles and critical biomolecules. ROS can be generated by the one-

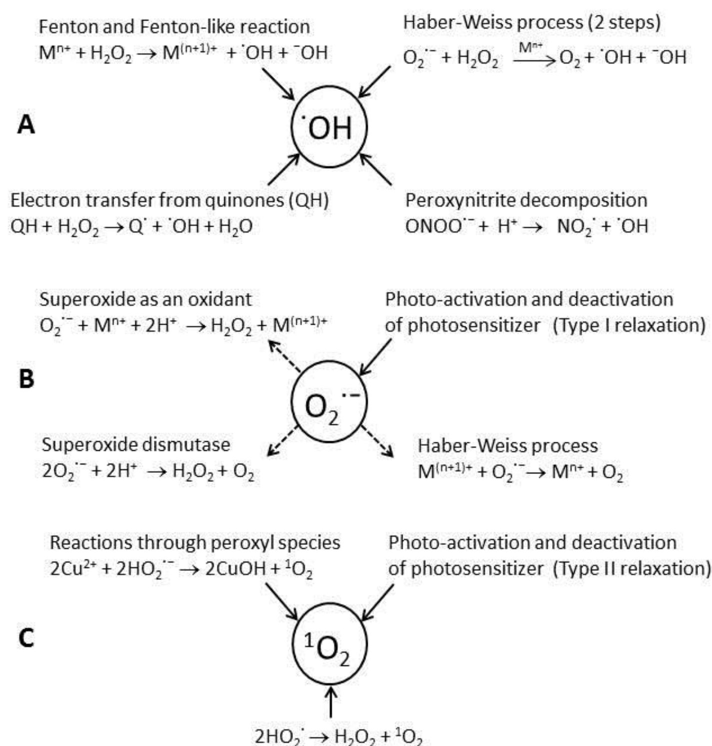

Figure 2. Reactions involved in generation and consumption of the most common reactive oxygen species (ROS) linked to DNA damage: A) hydroxyl radical, B) superoxide, and C) singlet oxygen. Solid and dotted arrows indicate generation or consumption of each reactive oxygen species, respectively.

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electron oxidation of protein or non-protein bound metal ions (VO^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, and Cu^{+}), and transfer of the electron to oxygen-containing substrates (Haber-Weiss, Fenton, and Fenton-like reactions, etc.; Figure 2).[135,136] The oxidized metal ion can then be reduced by cellular reducing agents (e.g. NAD(P)H, FAD(P)H, and ascorbic acid, etc.), redox cycling the metal ion and catalytically producing $^{\cdot}OH$ (Figure 2). ROS also can be generated by the excitation of organic photosensitizers,[137,138] nanoparticles,[139] and metal complexes.[13] The relaxation of an excited photosensitizer occurs in two ways: the excitation energy is used to transfer an electron from the photosensitizer to a nearby molecule to generate radical species ($O_2^{\cdot-}$, $^{\cdot}OH$), or the energy is transferred directly to a second molecule, changing it from a triplet to a singlet state ($^{1}O_2$).[140] All these ROS generation mechanisms involve metal ions or complexes and can lead to DNA damage and disease (Figure 1 and Table 1). This section provides a brief description of some of the more easily performed and highly sensitive methods for ROS detection and is not intended to be a comprehensive examination of this field. Detailed reviews about many different ROS species, their physical and chemical properties, their metabolism, and associated detection methods have been previously published.[124,141-145]

Hydrogen peroxide. Although hydrogen peroxide (H_2O_2) is a common ROS with an estimated generation rate in rat liver of 0.82 μM s^{-1} and a steady-state concentration of approximately 10 nM,[135] hydrogen peroxide alone cannot damage DNA. The one-electron reduction of H_2O_2 produces $^{\cdot}OH$, the typical damaging agent.[146] Since hydrogen peroxide disrupts Fe-S clusters[147,148] and disables Fe^{2+} ions in protein prosthetic groups,[149] it can lead to cellular toxicity. The Fe^{3+}-containing enzyme catalase decomposes H_2O_2 to H_2O and O_2, maintaining H_2O_2 homeostasis and preventing oxidative damage.[150] In 2010, Rhee *et al.*[143] reviewed spectroscopic methods for H_2O_2 detection, including sensitive methods such as

detecting the fluorescence emission of resorufin (587 nm) produced from the reaction of H_2O_2 with Amplex Red® (Figure 3), or the less sensitive measurement of ferrithiocyanate absorbance after oxidation of Fe^{2+} ions by H_2O_2. Resorufin formation results when H_2O_2 is homolitically cleaved by horseradish peroxidase (HRP), and the resulting $^{\cdot}OH$ radicals oxidize Amplex Red®. A similar method is also used to detect H_2O_2 electrochemically by square wave voltammetry (SWV).[151] This electrochemical method has several advantages, including a low detection limit (8 pM with soluble HRP or 20 nM with immobilized HRP), a high signal-to-noise ratio, and the ability to detect H_2O_2 in biological samples without interference from ascorbic or uric acids.[151]

Spin traps such as 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), and 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) react with specific reactive oxygen species to give characteristic signals in electron paramagnetic resonance (EPR) spectroscopy. For example, the $O_2^{\cdot-}$ adduct of DMPO gives rise to a 1:1:1:1 quartet

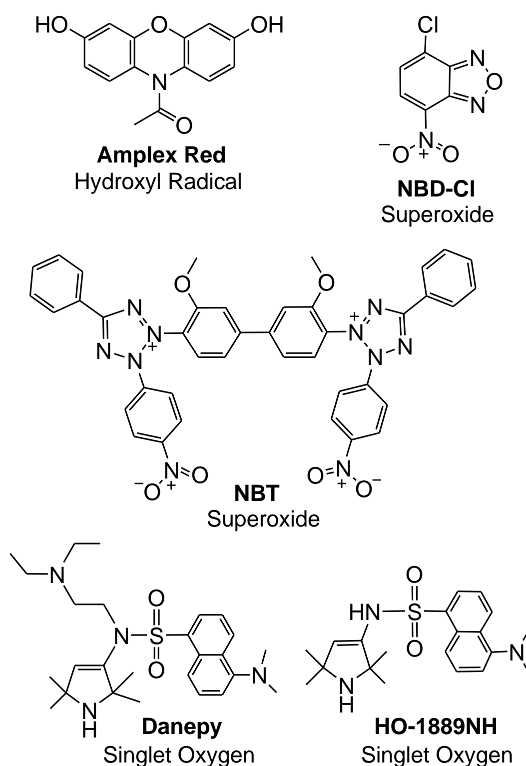

Figure 3. Structures of probes used specifically to detect $^1O_2$, $^{\cdot}OH$, and $O_2^{\cdot-}$.

resonance,[145] and the $^{\cdot}OH$ adduct of DMPO generates a quartet signal with greater intensities for the two central resonances compared to the two outer resonances.[145] These types of experiments are used widely to detect ROS and correlate their formation with DNA damage.[152-154]

Hydroxyl radical. This radical ($^{\cdot}OH$, Figure 2A) is the most common and damaging ROS, causing DNA base oxidation and single-strand breaks.[136,155] Hydroxyl radical is generated by several metal ions (VO^{2+}, Fe^{2+}, Ni^{2+}, and Cu^{+}),[135,156] and the resulting oxidized metal ions are reduced by cellular reductants, making hydroxyl radical generation catalytic.[62,157] Hydroxyl radical has an extremely short lifetime (about a nanosecond), and the longest distance that $^{\cdot}OH$ travels is approximately 12 bases of stretched DNA (about 8.6 nm).[158] Production of $^{\cdot}OH$ in rat liver is calculated to be 4 nM s^{-1}, based in a Fenton reaction constant of 6.8×10^{4} M^{-1} s^{-1} and a concentration of labile iron of 5 μM, suggesting that iron overload may result in higher $^{\cdot}OH$ generation rates.[135] Peroxynitrite decomposition also produces $^{\cdot}OH$ without the need for metal ions, and several reviews analyze the reaction conditions[159,160] and the consequences[161,162] of this source of $^{\cdot}OH$. Hydroxyl radical is also generated in the Haber-Weiss process from $O_2^{\cdot -}$, H_2O_2, and a metal ion such as Fe^{2+},[7,136] and by one-electron quinone oxidation.[157]

The SWV electrochemical detection method for H_2O_2 reported by Lyon and Stevenson[151] can be used to directly detect $^{\cdot}OH$, since one molecule of Amplex Red® consumes two $^{\cdot}OH$ radicals. EPR spectroscopy using spin traps (DMPO, TEMPO, etc.) has also been used to detect $^{\cdot}OH$,[145] and despite its low signal/noise ratios near detection limits, this technique helped confirm $^{\cdot}OH$ generation in Co- and Ni-peptide-mediated DNA damage[54,163] and from irradiation of anthraquinones in the presence of trace iron levels.[138]

Superoxide. Superoxide reactions, such as those that occur in superoxide dismutase (SOD) enzymes containing Mn, Fe, Cu and Zn, as well as its generation and functions *in vivo* have been extensively reviewed.[136,164-166] Under physiological conditions, the electrochemical potential of dioxygen reduction to superoxide (Figure 2B) is 0.12 V, similar to the reduction potential of ascorbyl radical to ascorbate anion (0.10 V),[135] making it unlikely that biological $O_2^{\cdot-}$ regenerates by direct reaction of dioxygen with ascorbic acid.

Superoxide detection by the absorbance of the 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) -$O_2^{\cdot-}$ adduct (470 nm, $\varepsilon = 4000\ M^{-1}\ cm^{-1}$; Figure 3) is effective for concentrations as low as 12 μM of $O_2^{\cdot-}$, comparable to detection methods using cytochrome *c*.[167] Nitroblue tetrazolium (NBT, a yellow, cell-permeable dye; Figure 3) is also used to detect $O_2^{\cdot-}$ in phagocytic cells. In this method, NBT is reduced by $O_2^{\cdot-}$, yielding insoluble formazan that is dissolved in dimethylsulfoxide/KOH to measure the absorbance at 620 nm.[168] Although Choi *et al.*[168] reported no detection limit for this method, they reported no interference from NO after phagocytic cell stimulation with phorbol 12-myristate 13-acetate.

Konovalova *et al*.[38] detected $O_2^{\cdot-}$ by EPR measurements using α-phenyl-*N-tert*-butylnitrone (PBN) as spin trap. The $O_2^{\cdot-}$ adduct of PBN exhibits a triplet resonance by EPR spectroscopy with hyperfine coupling constants of (a_N=14.2 G and a_H= 2.8 G). They also confirmed $O_2^{\cdot-}$ formation by observing the typical EPR resonance (quartet of similar intensity) of the $O_2^{\cdot-}$-DMPO adduct.[38] Similar experiments to detect superoxide using DMPO, PBN, and α-(4-pyridyl-1oxide)-*N-tert*-butylnitrone (POBN) spin traps were performed by Brezová *et al*.[37]

Singlet oxygen. Several reviews analyze the physical properties, generation, deactivation, and applications of singlet oxygen ($^1O_2$),[142,169,170] a ROS that oxidizes DNA bases similarly to $^{\cdot}OH$.[55] $^1O_2$ is also popularly believed to be a signaling molecule in plants.[171] Upon irradiation,

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

TiO_2 nanoparticles[37,38] as well as some metal complexes (e.g. cobalt-terperydine based complexes[55]) generate $^1O_2$. This ROS also can be generated by the dimerization of $^{\cdot}OOH$[37] or reactions of Cu-OOH[61] (Figure 2C). The lifetime of $^1O_2$ in water is about 3 μs,[172] increasing to 10.2 μs in methanol[173] and to 35 μs in rat hippocampal neurons.[172]

Niedre *et al.* measured the luminescence of $^1O_2$ in the infrared region (1270 nm) to detect and quantify its concentration in water, plant cells, and whole leaves, showing the advantage of this method for intracellular detection of $^1O_2$.[173] The sensitivity of this technique is not specifically reported, but based upon the quantum yield for $^1O_2$ generation, it can be estimated around 1 μM—a good sensitivity considering the lifetime of the species. Another detection method for $^1O_2$ is based on the reduction in the fluorescence (330 nm) of Danepy or an analog molecule (HO-1889NH; Figure 3).[174] This method detects $^1O_2$ without interference from $^{\cdot}OH$, at roughly the same concentrations reported for the Danepy luminescence. The disadvantage of this technique is that the dye fluorescence is quenched by radiation in the 400 -700 nm range and, for HO-1889NH, by $O_2^{\cdot -}$.[174] Thus, this method of $^1O_2$ detection is not useful when its generating photosensitizer is excited in the visible range, but it is useful for $^1O_2$ detection in plant tissues. The use of Danepy or HO-1889NH are more direct methods than the traditional method of $^1O_2$ detection by absorbance decrease of *N,N*-dimethyl-*p*-nitrosoaniline (RNO) caused by reaction of the $^1O_2$-imidazole adduct with RNO.[175]

EPR spectroscopy also helped infer $^1O_2$ generation after irradiation of TiO_2 nanoparticles using 2,2,6,6-tetramethyl-4-piperidone(4-oxo-TMP) as a spin trap.[38] The $^1O_2$-4-oxo-TMP adduct exhibits a triplet resonance in the EPR spectrum, a drawback since this spin trap is not specific for $^1O_2$, but detection can be confirmed by addition of $O_2^{\cdot -}$ scavengers such as SOD.[38]

Metal-mediated DNA damage can be enhanced by species that cycle the generated ROS[62,157] or by forming other oxidants. For example, chelation of Fe^{2+} by ATP increases the kinetics of $^{\cdot}OH$ radical production, but also reduces $^{\cdot}OH$ yield, likely due to stabilization of Fe^{3+} by ATP binding that prevents re-reduction to Fe^{2+}.[176] In addition, the strong oxidant peroxymonocarbonate (HCO_4^-) is formed from H_2O_2 and dissolved CO_2 (Reaction 1), and either HCO_4^- or $CO_3^{\cdot -}$ (Reaction 2) is responsible for DNA lesions and an increase in mutation frequency in tetracycline-resistant *E. coli*.[177] HCO_4^- can be regenerated by $CO_3^{\cdot -}$ reaction with $^{\cdot}OH$ (Reaction 3).[177]

$$CO_2 + H_2O_2 \rightarrow HCO_4^- + H^+ \qquad [1]$$

$$HCO_3^- + {}^{\cdot}OH \rightarrow CO_3^{\cdot -} + H_2O \qquad [2]$$

$$CO_3^{\cdot -} + {}^{\cdot}OH \rightarrow HCO_4^- \qquad [3]$$

In addition to ROS, reactive nitrogen species (RNS) are also cell signaling metabolites and respiration byproducts.[162,178] Although many RNS do not require the metal ions for their formation, they also damage DNA.[161] In RAW 264.7 macrophages, Lim *et al.*[171] estimate the concentration of peroxynitrite ($ONOO^-$) in the nanomolar range, nitrogen dioxide ($NO_2^{\cdot}$) in the picomolar range, and dinitrogen trioxide (N_2O_3) in the femtomolar range based upon theoretical calculations with a starting NO concentration of 1 μM. Koppenol and coworkers[159,160] report that HOONO decomposition occurs mostly (95%) via a heterolytic pathway, producing $ONOO^-$ and H^+, but also that evidence supports homolytic cleavage to NO_2 and $^{\cdot}OH$ and recombination to produce HNO_3. When peroxynitrite reacts with CO_2, $CO_3^{\cdot -}$ and NO_2 are produced, resulting in DNA base nitration and DNA inter- and intra-strand crosslinks.[179] Several reviews discuss the *in vivo* generation and fate of $ONOO^-$ as well as $ONOO^-$ detection methods.[144,162,180,181] Another less common ROS is HOCl, generated by myeloperoxidase[182,183] present in neutrophils, a known

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precursor of ROS such as $^1O_2$, $^{\cdot}OH$, and O_3.[183] Many different ROS and RNS generate both metal-mediated and non-metal mediated DNA damage, and developing methods to selectively detect these highly reactive species at very low biological concentrations continues to be a challenge.

Types of DNA damage and their detection methods

In some cases, it is easier to detect the products of ROS-DNA interactions than the ROS themselves, due to their short lifetimes and the difficulty of analyzing ROS *in vivo* or in complex biological matrices. Several methods are used to determine DNA damage; some of these methods do not identify the specific type of damage (base oxidation, backbone cleavage, inter- or intra-crosslinks, or a combination of these), such as the comet assay, polymerase chain reaction assays, electron micrography,[184] and gel electrophoresis. Three reviews written by Dorfman *et al.,*[185] Collins,[186] and Dahlmann *et al.*[187] explain in great detail many of these DNA damage detection methods. Detection methods that also identify the type of DNA damage often include combinations of two or more techniques, such as coupling liquid chromatography and mass spectrometry (LC-MS). Other techniques to detect types of DNA damage include electrochemistry, biosensor techniques, and double mass spectrometry (MS/MS) measurements.

DNA backbone damage. Since damaged DNA must be distinguished from undamaged DNA, recognizing undamaged DNA is as important as detecting DNA damage. Methods to determine undamaged double stranded DNA (dsDNA), such as minor groove binding by polyamides conjugated to fluorescent dyes, formation of DNA triplexes with oligonucleotides, and interactions with DNA-specific binding proteins are reviewed by Ghosh *et al.*[188] Several methods have been developed to detect DNA damage, for example, Liang *et al.*[189] used the

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strong dsDNA intercalator $Ru(bpy)_2(dppz)^{2+}$ (bpy = 2,2'bypirimidine; dppz = dipyrido[3,2-a:2'3'-c]phenazine) as a damage indicator, a complex that does not bind DNA with single-strand breaks as strongly as intact dsDNA. Current passing through the intercalated DNA was measured, and a reduction in this current was observed due to less efficient intercalation when the DNA was damaged (using 1 mM Fe^{2+} and H_2O_2 generated *in situ* from glucose oxidation by glucose oxidase). This technique has the advantage of being relatively cheap compared to other methods and can be used to detect DNA damage during a photochemical reaction since current is the detected signal.

Another method for real-time detection of DNA strand breaks has been demonstrated by Rawle *et al*.[190] In this case, DNA is deposited on a polyethyleneimine surface adsorbed to a silica-coated quartz crystal disk, and after DNA damage caused by Cu^{2+} and quercetin, the resulting single-strand DNA (ssDNA) hybridizes with complimentary strands provided in solution, increasing the mass of the sample on the quartz crystal disk. This mass increase measurably lowers the frequency of the disk vibration.[190,191] Real-time DNA damage detection is achieved, non-invasively with small amounts of DNA, but the instrumentation is expensive and this is not a sequence-specific detection method when the complimentary hybridization DNA is composed of several sequences.

Electrospray ionization ultra-performance liquid chromatography tandem mass spectrometry (ESI-UPLC-MS/MS) was used to detect formation of C4-AP abasic sites in a 15-mer oligonucleotide after treatment with Fe^{2+} and bleomycin (10 mM each).[192] The C4-AP abasic sites (Figure 4) were detected without interference from other DNA damage byproducts because the C4-AP sites were treated with methoxyamine to increase the sample mass of the oligonucleotide, a compound that reacts with aldehydes and ketones but not 2'-

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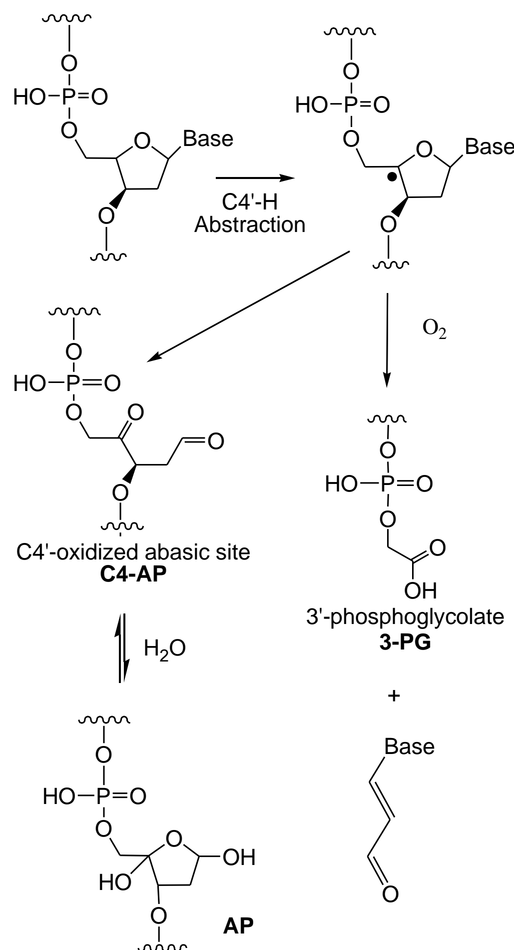

Figure 4. Products formed from H atom abstraction at the C4' position of deoxyribose in DNA.

deoxyribonolactone or 3'-phosphoglycolate (3-PG). This method can be used to detect DNA backbone damage at specific sites in oligonucleotides, does not require hydrolysis of the DNA into individual nucleosides, and can detect a variety damaged products, but larger DNA samples would significantly complicate analysis.[192]

DNA base damage. Of the four common DNA bases, guanine oxidation products, such as 8-deoxyguanine (8-oxodG; Figure 5), are the most studied because guanine has the lowest reduction potential.[193] Vadhanam *et al.*[194] detected 8-oxodG and other non-identified oxidation products by ^{32}P-postlabeling/thin-layer chromatography. 8-OHdG was also measured in the urine of female toll station workers exposed to vehicle exhaust using an enzyme-linked immunoassay

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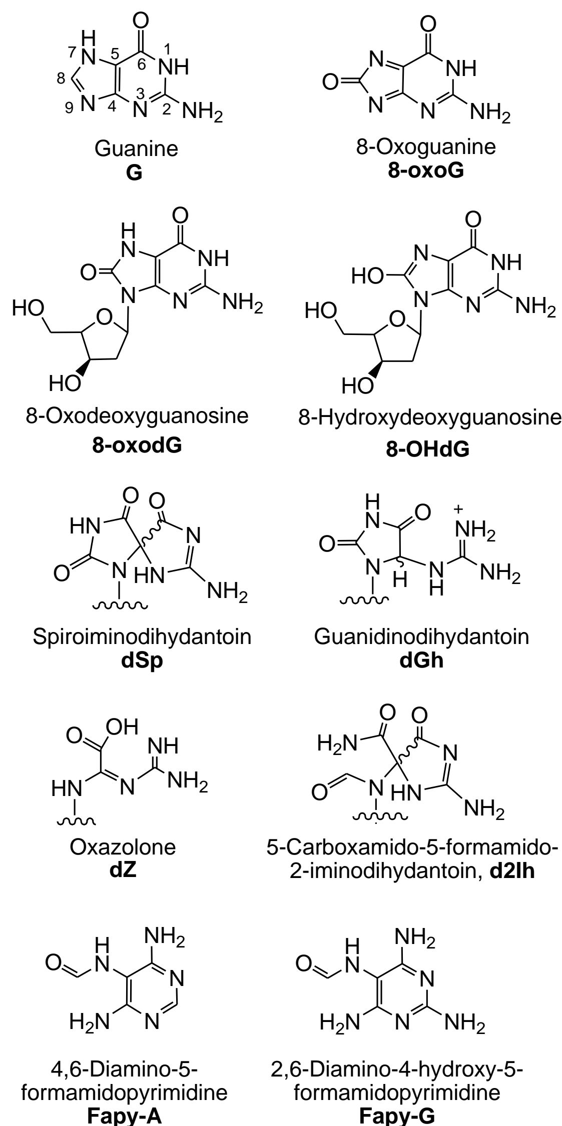

Figure 5. Structures of guanine, guanine oxidation products, and the two formamidopyrimidine (Fapy) oxidation products that Fpg enzyme recognizes in the DNA damage repair process.

method (ELISA) with monoclonal antibodies,[195] and these workers had higher levels of 8-OHdG than female office workers.[195] Fleming *et al.*[61] used high-pressure liquid chromatography (HPLC) with a Hypercarb column and mass spectrometry (MS) to separate and identify nucleosides of 8-oxoguanine (8-oxodG), spyroiminodihydantoin (dSp), guanidinodihydantoin (dGh), oxazolone (dZ), and the major product 5-carboxamido-5-formamido-2-iminohydantoin (d2Ih; Figure 5), formed from Cu^{2+}/H_2O_2/ascorbic acid or *N*-acetyl-cysteine (NAC) oxidation of

guanine in single- and double-stranded oligonucleotides. This work showed that 8-oxodG is not the final product of guanosine oxidation, but it is an intermediate for further oxidized products. HPLC-MS methods are very common for this type of DNA damage analysis, although the instrumentation is expensive and the experiment destroys the sample.

Kelly *et al.*[196] separated bases, nucleosides, and their oxidation products using HPLC with an Phenomenex Onyx monolith RP-18 column in shorter times (4 min) with product peak resolution improved or equal to separations using a standard reversed-phase column (40 min). Reducing experiment time without resolution loss is a great advantage because DNA oxidation products may undergo further oxidation during long analysis times.[61] Using this HPLC method, 8-oxoguanine and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-OHdG, Figure 5) were detected in the nanomolar range (50 nM), a significant improvement since DNA oxidation products are typically quantified in the micromolar range.[196] To achieve greater biological relevance, this method must be optimized to separate and observe additional oxidation products.

Oxoguanine glycosylase (OGG1) is a DNA repair enzyme that recognizes and excises 8-oxoguanine,[197,198] and this protein was recently used in a luminescent sensor to detect DNA oxidation.[199] The sensor consists of a 5-methylcytosine binding domain (MBD1) protein, which detects a methylated cytosine in the 23-mer dsDNA, attached to half of a split luciferase enzyme; OGG1 is attached to the other half of the split luciferase. When guanine is oxidized to 8-oxoG, both MBD1 and OGG1 bind the oligonucleotide, bringing together the two halves of luciferase and causing luminescence. Replacement of OGG1 by DNA-damage-binding protein 2 (DDB2) allowed this sensor to detect UV-induced lesions such as cyclobutane pyrimidine dimers and 6-4 pyrimidine-pyrimidone photoproducts.[200-202] This promising method can be applied in samples as low as 50 ng of DNA and can detect as low as ~200 fmol 8-oxoG under optimized oxidation

conditions (30-60 μM $CuCl_2$ and 1 mM H_2O_2).[199] Such a low detection limit is of great importance for identifying DNA damage since LC-MS typically detects damage only down to ~30 pM. In the future, this luciferase split-assembly biosensor method may also be used recognize other DNA lesions by changing the DNA damage recognition enzyme. For instance, using 8-oxoguanine glycosylase (Fpg) or adenine glycosylase (MutY) may allow detection of 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG, Figure 5)[203] or mispaired adenine with 8-oxoG,[204,205] respectively.

Kuznetsov et al.[206] examined damaged DNA interactions with the *E. coli* repair enzyme Fpg by pulse electron double resonance (PELDOR) spectroscopy. This enzyme bends the DNA duplex to strengthen binding interactions and recognition of not only 8-oxodG and 8-OHdG, but also formamidopyrimidine derivatives of adenine and guanine (Fapy-A and FapyG; Figure 5).[206] Analysis of the gas-phase interactions of MutY with several adenine analogs established that this enzyme recognized the adenine 1, 3, and 7 nitrogen atoms (Figure 5; the numbering is the same for guanine and adenine).[207] These experiments illustrate the high selectivity of Fpg and MutY for specific DNA base lesions that might make them useful in luminescent biosensensors.

Although much work has focused on guanine oxidation detection, advances have also been made in detecting pyrimidine oxidation. Liquid chromatography separations by Samson-Thibault *et al.*[208] efficiently separate intact purine and pyrimidine nucleosides from oxidized nucleosides, including cytosine oxidation products (Figure 6), using coupled octadodecyl silica gel and graphite columns.[208] Although this method is more involved than traditional separations of oxidized nucleoside products by HPLC, it represents a significant advance in identifying cytosine oxidation products.

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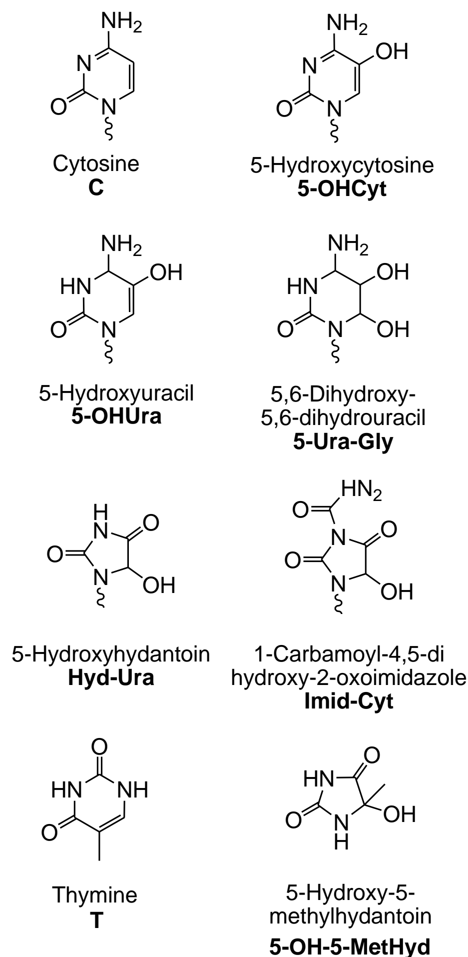

Figure 6. Selected cytosine and thymine oxidation products generated by Fenton reaction conditions or γ-irradiation.

Although methods are now available to separate and to detect many different types of DNA damage, most can only detect one or a few specific types of damage. Thus, it is important to develop methods to identify many different types of DNA damage in the same analysis. Although much work has focused on detection of oxidized guanine and adenine products, methods are also needed to improve detection of cytosine and thymine oxidation products. Many of these methods (such as mass spectrometry and derivatization) destroy the samples during the analysis, but some (such as luciferase biosensor assays) do not. In addition, some methods now can detect DNA lesions at the nanogram level. For analysis of minor oxidation

products, methods that amplify the signal are critical, and faster analyses of small sample volumes or samples with large DNA molecules (> 100 base pairs) are also areas with significant potential in this field.

In vitro DNA damage: Metals, mechanisms, and products

Scandium, titanium, and vanadium. No investigations focusing on scandium-mediated DNA damage have been reported, although a scandium-hypocrellin A complex was recently reported to generate potentially-DNA-damaging $^1O_2$ and $O_2^{\cdot -}$.[35] The vast majority of titanium-related work focuses on DNA damage by TiO_2 nanoparticles,[209,210] a topic outside the scope of this review. Titanocene (Cp_2Ti; Cp = cyclopentadienyl) forms Cp_2Ti-DNA adducts,[211] and its dichloride analog (Cp_2TiCl_2) have been successfully tested in phase I and II clinical trials as antitumor agents.[212,213] Abeysinghe and Harding[214] summarized in their review the metabolic route by which cancer cells uptake Cp_2TiCl_2, but they report only strong interactions of Cp_2TiCl_2 with DNA but not the mechanism of damage. Strong interactions with DNA are also reported by Gonzalez-Pantoja *et al.*[215] in a series of bimetallic titanocenes with Cp rings derivatized by organometallic chains containing Au, Pd, or Pt, and it was reported that the Au-titanocene derivatives stabilize DNA, but Pd- and Pt-titanocene derivatives destabilize DNA as measured by calf-thymus DNA melting point experiments. This destabilization led to the cytotoxic effects observed in human cervical carcinoma (HeLa) and prostate cancer (DU-145) cell lines.

Vanadium's status as an essential trace element is still the subject of debate, but some vanadium complexes have anticancer properties and other potential biological applications.[216] Vanadium compounds have been used for over a century to treat diabetic patients, with vanadyl complexes of malonate ($VO(mal)_2$), tartrate ($VO(tar)_2$), and oxalate ($VO(ox)_2$) being particularly

effective.[217] Vanadium compounds stimulate hexose transport, uptake, and metabolism,[218] and Na_3VO_4 (0.15 - 15 mM) prevents DNA alkylation by $(C_2H_5O)_2SO_2$ (1.5 mM) in a concentration-dependent manner after treatment of plasmid DNA.[219] This is attributed to formation of anionic oxospecies (e.g. $V_5O_{14}^{3-}$) that inactivate the alkylating agent by converting it into the corresponding alcohol.

Despite the ability of vanadium complexes to prevent DNA alkylation, Stemmler and Burrows[39] reported DNA strand scission by vanadyl sulfate ($VOSO_4$, 30 μM) in the presence of $KHSO_5$. Since $VOSO_4$ treatment caused guanine-specific oxidation, the authors ruled out $^{\cdot}OH$ formation under these conditions, instead hypothesizing that vanadyl ion (VO^{2+}) binds the N7 of guanine or the phosphodiester backbone and that the oxidizing agent is $SO_5^{\cdot-}$ or a metal-bound sulfate radical (e.g. $[V^VO(SO_4)]^{2+}$). DNA cleavage increases with increasing $VOSO_4$ and $KHSO_5$ concentrations as well as longer incubation of VO^{2+} with DNA, prior addition of $KHSO_5$, and increased reaction times.[39]

High concentrations of vanadate (VO_2^+, 0.5-10 μM) increase DNA damage in human fibroblasts. Single strand breaks (SSB) were detected in samples treated with VO_2^+, and double strand breaks (DSB) were observed in samples treated with VO_2^+ and UV irradiation,[220] but mechanistic detail for these DNA damage reactions is lacking. Ivancsits *et al.*[220] reported that this DNA damage does not correlate with increased 8-OHdG formation, but speculated that VO_2^+ could unwind DNA strands during the repair process. In contrast, other researchers provided evidence for $^{\cdot}OH$ as the damaging species, but excluded formation of singlet oxygen ($^1O_2$).[221-223] Sam *et al.*[221] reported $^1O_2$ and $^{\cdot}OH$ generation by bis(peroxo) vanadium(V) phenanthroline caused non-specific DNA damage (in thymine 20-mers and AG 20-mers) and 60 times greater strand scission in irradiated samples versus non-irradiated samples. The proposed mechanism

suggests formation of a hydroperoxylradical species by cleavage of a V-O_{peroxo} bond that reacts with mono(peroxo)vanadium to produce H_2O_2, $^3O_2$, and VO^{2+}. VO^{2+} then reduces H_2O_2 in a Fenton-like reaction, forming damaging $^{\cdot}OH$.[221] Thus, evidence for the oxidizing species for vanadium-mediated DNA damage is ambiguous, and only a few oxidized DNA products have been identified from these studies.

Sasmal *et al.*[40,222,223] investigated DNA damage by vanadium (IV) complexes with dipyrido[3,2-d:2',3'-f]quinoxaline (dpq), dipyrido[3,2-a:2',3'-c]phenazine (dppz), and 1,10 phenanthroline (phen) ligands as well as preferential binding of these complexes to poly(dA)·poly(dT) compared to poly(dG)·poly(dC) or calf thymus DNA that occurs due to minor groove binding (dpq complexes), major groove binding (dppz complexes) and partial or non-classical intercalation into DNA.[40,222,223] Some vanadium complexes, such as vanadyl bis-(benzimidazolylmethyl)amine phenathroline and vanadyl *N*-salicyledene-L-arginine phenathroline, enhance DNA damage in the presence of H_2O_2 upon UV irradiation by forming $^1O_2$ and $^{\cdot}OH$, but only $^{\cdot}OH$ is formed upon near-infrared irradiation.[222,223] Considering the therapeutic potential of vanadium complexes for diabetes or cancer treatment, additional research to understand vanadium-mediated DNA damage mechanisms is warranted.

Chromium and manganese. Toxicity of Cr^{6+} is related to the most common form of this ion, chromate (CrO_4^{2-}),[42,95] an ion transported into cells by phosphate (PO_4^{3-}) or sulfate (SO_4^{2-}) uptake channels. Holland and Avery[224] briefly reviewed the consequences of cellular Cr^{6+} incorporation: after Cr^{6+} transport, Cr^{6+} is reduced to Cr^{3+}, and the toxic effects (DNA-protein crosslinks, base oxidation, strand breaks) are due primarily to Cr^{3+}. It is also is postulated that the Cr(V)-NADPH complex and H_2O_2 can also generate damaging $^{\cdot}OH$,[225] and that $Cr^2O_7^{2-}$ may also generate $O_2^{\cdot-}$ in cells.[44] In addition, Macfie *et al.*[41] reported that bovine serum albumin

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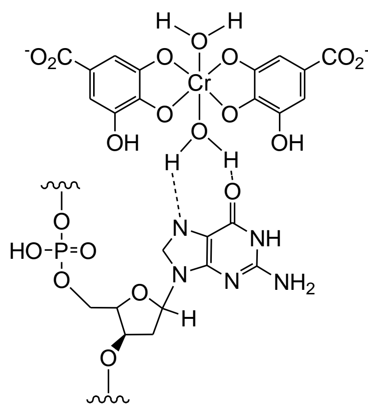
3 (BSA, 60 μg) and calf thymus DNA (5 μg) crosslink after treatment with $K_2Cr_2O_4$, ascorbic acid, cysteine, and glutathione. This DNA-protein crosslinking is pH dependent, and the rate-limiting step is the formation of Cr-DNA adducts. Crosslinking occurs with Cr^{3+}-bound DNA, but not if BSA is pretreated with Cr^{3+} and then combined with DNA. Chelation of Cr^{3+} using EDTA or phosphate prevents this damage.

DNA base oxidation is primarily focused on 8-oxoguanine (8-oxoG) as the major product, since guanine has the lowest reduction potential (1.28 V) of the four DNA bases.[193,226] However, 8-oxo-guanine's reduction potential is lower than guanine's,[227] so it is readily oxidized to spiroiminodihydantoin (Sp; Figure 5). After treatment of a 22-mer DNA oligonucleotide with Cr^{6+} (3.1 – 50 μM) and ascorbate (31 – 500 μM) for 1 h, formation of Sp occurs in a dose-responsive manner and is at least 20 times greater than that of 8-oxoG.[43] These oxidation products are not exclusive to Cr^{3+}-mediated damage; other ions such as Fe^{2+},[228] Ni^{2+},[229] and Cu^{+}[61] also generate these oxidized guanine products. Slade *et al.*[43] postulate that DNA treatment with Cr^{6+} and ascorbic acid forms Cr^{4+} and dehydroascorbate as intermediates. The Cr^{4+} is then further reduced by ascorbic acid, resulting in Cr^{3+} and ascorbyl radical. Since H_2O_2 was not added in this experiment and no oxygen radical species were detected,[43] the authors suggest direct metal oxidation of DNA.

Another type of interaction between Cr^{3+} and the N7 of guanine is reported by Arakawa and Tang[230] upon treatment of plasmid DNA with ethyl gallate and gallic acid Cr^{3+} complexes. After DNA treatment with both complexes (0-5 μM), they postulate formation of Cr^{3+}-guanine-phosphate-DNA or Cr^{3+}-(guanine)$_2$-DNA adducts (Figure 7), but not Cr^{3+}-phosphate-DNA adducts,[230] based on studies using the UvrABC scission enzyme that shows great sequence specificity for Cr^{3+}-modified DNA adducts.[231] Due to its many accessible oxidation states,

chromium causes DNA damage in a variety of ways, leading to the high toxicity of this element.

Manganese is present in superoxide dismutase (SOD), and most of the ROS-related manganese studies concern its role in this enzyme.[164,232] Mn^{2+} itself associates with DNA,



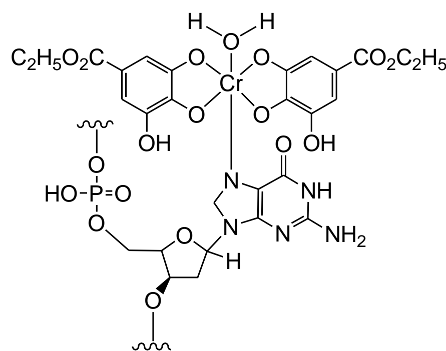


Figure 7. Proposed structures for Cr^{3+}-guanine binding after treatment of plasmid DNA with Cr^{3+} complexes of gallic acid (GA) and ethyl gallate (EGA).[230] Reprinted from Arakawa, H.; Tang, M. –S. *Chem. Res. Toxicol*. **2008**, *21*, 1284-1289. Copyright 2014 American Chemical Society.

binding to multi-G sequences with a markedly increased preference for GGG (where binding occurs at the central G) over GG (where binding occurs at the 5' G) sequences.[233] This binding preference correlates with electron density in the highest occupied molecular orbitals (HOMO) for these sequences.[233] Several authors have reviewed the genotoxic effects of manganese and its relationship with diseases such as cancer and Parkinson's disease,[27,234,235] and recent studies examine the cytotoxic effects of manganese and, in some cases, show complementary DNA

damage. Cellular manganese-mediated DNA damage has been investigated in greater detail and is discussed in the "*Beyond* in vitro *metal-mediated DNA damage: Metal toxicity and DNA damage in cells*" section.

Iron, cobalt, and nickel. Iron-mediated DNA damage has been studied for over thirty years and is relatively well understood compared to other DNA-damaging metal ions.[236-240] DNA damage by Fe^{2+} results from its ability to participate in one-electron reduction of hydrogen peroxide to generate $^{\cdot}OH$.[241,242] Highly reactive hydroxyl radical is the main ROS species formed (Figure 2),[241] although some researchers postulate a ferryl species ($[Fe{=}O]^{2+}$) as the active DNA-oxidizing species, causing damage through a mechanism similar to that for the hydrogen abstraction of oxygenases.[52] In addition, Rachmilovich-Calis *et al.*[243] present kinetic and mechanistic evidence for the formation of another intermediate species, $[Fe(^{-}O_2H)]^{2+}$, from the reaction of Fe^{2+} and H_2O_2.[243] The most commonly studied types of iron-mediated DNA damage are single strand breaks (SSB) and base oxidations. For example, Barbouti *et al.*[244] reported SSB by intracellular iron in human T lymphocyte (Jurkat) cells upon challenge with H_2O_2 generated *in vivo* (1 – 2 μM) by glucose oxidase addition. Similarly, Nieto-Juarez *et al.*[245] reported inactivation of MS2 coliphage by $^{\cdot}OH$ radical upon treatment with Fe^{2+} (1 – 10 μM) and H_2O_2. oxoguanine. Frelon *et al.*[228] reported formation of 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua, Figure 5C) and other further oxidized guanine products by $^{\cdot}OH$ generated in the Fenton reaction (1 – 100 μM Fe^{2+} and 1 – 200 μM H_2O_2), γ-irradiation, or both. Under these conditions, FapyGua formed three times more frequently than 8-oxoG in calf thymus DNA, and SSB by H atom abstraction from deoxyribose produced pyimidinopurine malonaldehyde-2'-guanosine adducts in higher yields than the oxidized bases.[228]

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Fe^{2+} binding to 16-mer oligonucleotides was examined using NMR spectroscopy, determining that Fe^{2+} ions localize sequence-specifically on the N7 of guanine in RTGR[246] and RGGG[247,248] sequences (R = adenine or guanine) with dissociation constants of 0.9 and 2.0 mM, respectively.[246,248] Similar to Cr^{3+}, Mn^{2+}, and Co^{2+}, Fe^{2+} binds preferentially to guanine N7 rather than the phosphate oxygen atoms, likely due to the electron density at these G-rich sequences[233] and the greater stabilization of Fe^{2+} by borderline nitrogen donors than the hard oxygen atoms of the phosphate backbone. Performing localization experiments with longer DNA sequences, such as human telomeric sequences, and competition studies with DNA containing several potential iron-binding sequences would determine Fe^{2+}-DNA sequence-binding preferences so that the disease-causing effects of iron-mediated DNA damage can be better understood.

Iron-mediated DNA damage is observed even in the absence of H_2O_2 when reducing agents such as ascorbic acid and quinones are present, and iron can react with antioxidants to cause DNA damage under specific conditions.[53,157] For example, Li *et al*.[157] detected formation of ˙OH via the Haber-Weiss and Fenton reactions (Figure 2) as well as ascorbyl and semiquinone radicals by EPR spectroscopy, likely due to Fe_2O_3 or Fe_3O_4 on the quartz surface of the sample holder. When reduced by ascorbic acid or anthraquinone in solution, trace amounts of Fe^{2+} could be released to form the observed radical species. Consistent with detection of ˙OH by Li *et al.*, Furukawa *et al.*[53] observed 8-oxoG formation in calf thymus DNA without H_2O_2 addition after treatment with $[Fe(EDTA)]^{2-}$ (20 μM) and various catechins (catechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate, 1 to 20 μM). These results indicate the necessity of using strictly metal-free conditions when determining DNA damage under oxidative conditions, since even trace amounts of redox-active metal ions can generate damaging ROS.

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In the past ten years, Co^{2+}-mediated DNA damage has been the focus of only a few studies. Similarly to Fe^{2+}, Co^{2+} preferentially binds RTGR sequences,[246] and NMR experiments indicate that Co^{2+} localizes preferentially on the 5' G of GG sequences and the middle G of GGG[233] due to the HOMO electron density at these positions.[249,250] Baldwin *et al.*[3] used gel electrophoresis to determine that human (but not yeast) topoisomerase IIα cleaves supercoiled plasmid DNA upon treatment with $CoCl_2$ (1 or 5 mM), relative to treatment with other divalent cations (Ca, Mn, Cd, Ba, Sr, Cu, or Zn) and that this cleavage was 6-13 times higher than cleavage observed in presence of Mg^{2+} (the natural divalent cation typically used by this enzyme). The authors attribute these SSB to substitution of Co^{2+} for Mg^{2+} in topoisomerase IIα, causing it to cleave DNA at sites other than the scission sites of the Mg^{2+}-containing enzyme.

In addition the peptide complex, Co^{2+}-GGH (50 μM) with $Na_2S_2O_5$ (as a S(IV) source) caused about 90% of plasmid DNA strand breaks at low S(IV) concentrations (1-80 μM) in a rapid reaction that is dependent upon S(IV), oxygen, and Co^{2+}-GGH ratios.[163] Based upon EPR data and literature reports, Alipázaga and collaborators hypothesize that this DNA damage is caused by $SO_4^{\cdot-}$ radical formed after several redox steps, similar to the mechanism proposed for Cu^{2+} and Ni^{2+}- peptide-mediated DNA damage (*vide infra*).[54,163,251-253]

Binding studies of diimine-cobalt(II) complexes (10 μM) with 1,10-phenanthroline (phen), 5,6-dimethyl-1,10-phananthroline (dmp), and dipyrido [3,2-d:2',3'-f]-quinoxalin (dpq) ligands showed calf-thymus DNA damage by $^{\cdot}OH$ in the presence of H_2O_2 (200 μM) and revealed that small changes in the ligand significantly affect DNA binding modes, causing greater DNA damage when the cobalt complex binds in the major groove than when binding occurs in the minor groove.[254] A cobalt-containing brominated porphyrin complex, $(Br_8TMPyP)Co$, binds DNA more strongly than the non-brominated porphyrin complex,

(TMPyP)Co and causes more DNA damage.[255] Both complexes interact with DNA by external binding rather than intercalation and show preferential cleavage into DNA fragments at adenine-thymine (A-T) base pairs instead of G-C base pairs. This preference was determined electrochemically by recording the voltammograms of DNA samples and observing an increase in the oxidative cleavages potential after addition of adenine or thymine to the DNA samples.[255] However, the authors do not provide further information about the mechanism for this damage.

Roy *et al.*[55] treated plasmid DNA with H_2O_2 and Co^{2+} complexes with 4'-phenyl-, 4'-anthracenyl, and 4'-(1-pyrenil)-2,2':6',2''-terpyridines (0.5 – 500 μM) under UV irradiation, similar to experiments with vanadium complexes reported by Sasmal *et al.*[40,222,223] Singlet oxygen and $^{\cdot}OH$ were detected upon UV-A irradiation of these complexes, but only $^{\cdot}OH$ formation was observed when these studies were repeated in ambient light.[55] Because the DNA damage activity of cobalt has been examined with both chelated cobalt complexes and cobalt ions with labile aqua ligands, different DNA binding sites, cleavage mechanisms, and generated ROS are observed.[55,163,254] Additional studies are necessary to elucidate specific ligand effects and DNA damaging mechanisms for many of these complexes.

Ni^{2+} (10 μM) in the presence of diethylenetriaminepentaacetic acid (DTPA, 2.5 μM) and thiols, such as dithithreitol (DTT), 1,4-dithio-L-threitol, dithioerythritol, mercaptopyruvate, and dihydrolipoic acid (0.2 – 5 μM), caused 8-oxoG formation without backbone damage.[59] Catalase (Figure 2) inhibited this damage, but superoxide dismutase (SOD) enhanced guanine oxidation, indicating that H_2O_2 and $O_2^{\cdot-}$ are involved in ROS generation. The authors state that the ROS responsible for this damage is likely $^{\cdot}OH$ but do not examine how these ROS are formed.[59] Similar to Fe^{2+} and Co^{2+}, a preference for Ni^{2+} DNA damage at GGG sequences is observed, and this specificity is speculated to be due to a lowering of the HOMO energy of 5'G bases when

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3 they are stacked.[59] Oikawa *et al.*[59] discarded $^{1}O_2$ generation as the cause of damage, since autoradiograms obtained after DNA treatment with a $^{1}O_2$ generator and by Ni^{2+} and DTT were not sufficiently similar.[59]

A Ni^{2+} tripeptide complex, Ni-GGH, also causes DNA strand breaks and guanine oxidation in presence of sulfur species,[54,251,252] and the resulting DNA damage occurs faster upon Ni^{2+}-peptide treatment than with Cu^{2+}-peptide ($[Cu\text{-}G_4]^{2+}$) treatment under the same conditions.[251-253] Co^{2+}-, Ni^{2+}-, and Cu^{2+}-peptide mediated DNA damage in the presence of S(IV) is postulated to occur in the same way, forming DNA-damaging sulfate radical.[54,163,251,252,253]

An especially interesting aspect of nickel-mediated DNA damage was reported by Kelly *et al.*[229] who observed oscillatory concentrations of 8-oxoG when calf thymus DNA was treated with $NiSO_4$ (1.5 mM) and H_2O_2. These oscillatory concentrations were the result of further guanine oxidation to oxoguanidinohydatoin (oxGH) and guanidinohydantoin (GH) and spyroiminodihydantoin (Sp; Figure 5) through a proposed 8-oxoG^{+} intermediate where two 8-oxoG^{+} regenerate one guanine and one molecule of the further-oxidized base.[229] Nickel complexes (100 μM) with ATP, histidine, or both are reported to cleave plasmid DNA in the presence of H_2O_2. In these studies, UV spectroscopy indicated Ni^{2+} oxidation to Ni^{3+} concomitant with guanine oxidation, and this time-dependent oxidation is not associated with $^{\cdot}OH$ or $O_2^{\cdot-}$ generation.[256]

Tan *et al.*[60] also reported that an intercalated nickel-quercetin complex ($Ni(Que)_2(H_2O)_2$, 50 to 400 μM) caused single and double strand breaks in plasmid DNA in a pseudo-first-order reaction without involvement of $^{\cdot}OH$ or H_2O_2. The cleavage decreases as ionic strength increases, suggesting that electrostatic interactions may contribute to the damage. A hydrolytic mechanism has been proposed to explain this damage, involving coordination of Ni^{2+} by an

oxygen of the phosphate backbone followed by nucleophilic attack on the phosphorus of that phosphate by a water molecule (either coordinated to Ni^{2+} or hydrogen bound to a hydroxyl group of quercetin). The resulting pentacoordinate phosphorous intermediate then eliminates one P-O bond, nicking the DNA strand at this position.[60]

Treatment of plasmid or calf-thymus DNA with nickel-2,12-dimethyl-3,7,11,17-tetraazabicyclo-[11.3.1]-heptadeca-1(17),2,11,13,15-penta-ene ($NiCR^{2+}$) or the oxidized version of this ligand ($Ni(CR-2H)^{2+}$, 600 μM) in the presence of oxone show very different results. Whereas the reduced complex ($NiCR^{2+}$) does not oxidize DNA, the oxidized complex causes single-strand breaks and complete hydrolysis in presence of a molar excess of [$Ni(CR-2H)^{2+}$] without an oxidizing agent.[257] $NiCR^{2+}$ damages DNA only in the presence of an oxidizing agent (oxone). This widely differing DNA damaging activity for these two similar complexes is attributed to the additional double bond in the oxidized complex that permits two-electron reduction and confers planarity and better minor groove binding; unfortunately, further evidence for this premise and for the mechanism is not reported.[257] However, planarity and DNA minor groove binding in nickel complexes does not guarantee DNA damage. For example, the nickel-porphyrin complex (Ni-TMPyP) also interacts with the minor groove of a 19-mer double-stranded DNA oligonucleotide and inhibits DNA damage by a Mn-porphyrin complex (Mn-TPMyP) and $KHSO_5$ (10 μM).[258] The strong minor groove binding of Ni-TMPyP prevents Mn-TPMyP from reaching the minor groove and reacting with $KHSO_5$ to cause DNA damage.[258]

Coordination geometry and ligand substitution effects have been analyzed for several additional nickel complexes and compared to their DNA-damaging ability.[259-261] The distorted square planar geometry of nickel-ferrocenyl-hydrazone complexes promotes DNA groove binding, allowing more efficient plasmid DNA cleavage than for non-planar copper-ferrocenyl-

hydrazone complexes.[259] This higher affinity for DNA groove binding results in greater DNA damage in the presence of H_2O_2, but also increases scavenging of damaging $^{\cdot}OH$ and $NO^{\cdot}$ radicals. No mechanistic reasons are provided for this dual behavior, but it may occur by the same phenomena of blocking the minor groove and preventing attack by these reactive species. The variety of geometries that Ni^{2+} complexes adopt provide a range of DNA-damaging (or -preventing) properties difficult to find for any other first-row metal ion.

Copper and zinc. Copper DNA damage and toxicity has been widely studied in the past decade,[117] primarily due to the neurological disorders associated with copper mis-regulation.[22] Copper homeostasis and transport is much more tightly regulated in cells than that of iron, reflecting copper's greater ability to catalyze the formation of damaging ROS.[262,263] Analysis of reaction kinetics for Cu^{2+}, ascorbic acid, H_2O_2, and DNA indicates that the rate limiting step for strand breaks in human genomic DNA is the reaction of a Cu^{+}-DNA complex with H_2O_2 to oxidize the neighboring DNA base where Cu^{+} is bound.[264] The calculated rate constant for the Fenton-like reaction between Cu^{+} and H_2O_2 ranges from 1×10^{-5} s^{-1} to 4.1×10^{3} M^{-1} s^{-1} depending the data-fitting model used (pseudo-first-order or first-order reaction), a difference of 8 orders of magnitude![265,266] Despite this divergence, there is consensus that the rate constant for metal-mediated hydroxyl radical generation is higher for Cu^{+} than with Fe^{2+}.[238,240,267,268]

Interactions of Cu^{2+} with guanine were computationally modeled, and the four-coordinate complex of Cu^{2+} bound to guanine N7 (Figure 5) and three water molecules or chelated by guanine (N7 and O6) and two water molecules are the most stable complexes.[269] Metal-guanine N7 interactions have been observed for many metal ions, and this interaction may be responsible for the majority of DNA damage. In addition, Cu^{2+} in the presence of catechins damages DNA in a proposed one-electron transfer from the catechin to the Cu^{2+}, followed by reduction of O_2 to

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$O_2^{\cdot -}$. The generated $O_2^{\cdot -}$ disproportionates to H_2O_2, which then oxidizes another Cu^+ ion to generate $^{\cdot}OH$ radical.[53] DNA-damaging sulfate radical ($SO_4^{\cdot -}$) has a very high potential for reduction to SO_4^{2-} (E > 2.43 V vs. NHE) and is generated in a complex series of reactions by a copper-tetraglycine complex (0.1 mM $[CuGGGG]^{2+}$ with 0.1 μM Ni^{2+}) complex in the presence of sodium bisulfite ($Na_2S_2O_5$).[253] This mechanism for generation of DNA strand breaks is similar to that reported for Co^{2+} and Ni^{2+}-peptide complexes.[54,163,251-253]

Despite the rarity of Cu^{3+} complexes, this species is reported as an intermediate in single- and double-stranded oligonucleotide damage upon treatment with Cu^{2+} (10.0 μM), H_2O_2 and ascorbic acid or NAC, resulting in several guanine and deoxyribose oxidation products.[61] The yields of C5 (d2Ih and dZ; Figure 5) were approximately twice that of either C8 (8-oxodG, dSp, and dGh) or deoxyribose oxidation products when samples were treated with ascorbic acid, whereas NAC treatment produced the three types of oxidation products in almost the same yields. Because of the variety of observed products, Fleming *et al.*[61] proposed a mechanism for $^1O_2$ formation from a copper-peroxide dimer that hydrolyzes under acidic conditions to $^1O_2$ and two Cu^{3+}-OH ions, and this mechanism is supported by experiments carried out in D_2O, where the yield out of C8 oxidation products is higher than in H_2O,[61] consistent with the longer lifetime of $^1O_2$ in D_2O.[170,173]

Several Cu^{2+}-mediated DNA damage studies include catechol- or quinone-derived compounds to generate the observed damage, highlighting the pro-oxidant tendencies of these compounds in the presence of copper. In these systems, DNA damage is caused by ROS generated by reducing Cu^{2+} to Cu^+ or by direct reduction of O_2 to various ROS, including CuOOH, $^1O_2$, H_2O_2, and $^{\cdot}OH$.[62,270-274] Wang *et al.*[62] treated viral DNA with Cu^{2+} (10 μM) and epigallocatechin gallate (EGCG, 1 -50 μM) and observed DNA cleavage in a dose dependent

manner with respect to EGCG concentration. The authors proposed $^1O_2$ formation from reaction of Cu^+ and H_2O_2, likely through the formation of a CuOOH species.[62] Another copper redox cycling mechanism involving Cu^{2+}, an aromatic compound, and a reducing agent was elucidated by Murata and Kawanishi[275] who treated DNA with Cu^{2+} (20 μM), NADH, and the hydroxyl derivative of 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhPIP(NHOH), 0.2 – 5.0 μM), a compound that does not have catechol or quinone groups in the aromatic ring. At low concentrations, 8-OHdG formed specifically at the 5' position of GG and GGG sequences, but at higher concentrations, this site specificity is lost, likely due to proton transfer among complimentary bases.[275] In their mechanism, Murata and Kawanishi[275] proposed generation of $O_2^{\cdot -}$ and a short-lived DNA-Cu(I)-OOH complex able to cause the DNA damage.

A reactive DNA-Cu(I)-OOH complex was also proposed by Tan *et al.*[276] upon treatment of plasmid DNA with a Cu^{2+}-quercetin complex ($Cu(Que)_2(H_2O)_2$, 10 – 400 μM) for 1 h and analysis by gel electrophoresis and UV absorption spectroscopy. In this case, the proposed hexacoordinate Cu^{2+} is bound to two bidentate quercetin molecules and two water molecules, based upon elemental analysis and infra-red spectroscopy results, but no structural data are presented to support this coordination geometry. Although similar quercetin complexes are reported for Ni^{2+} and Zn^{2+}, these complexes are thought to damage DNA via hydrolysis.[60] In contrast, the proposed DNA damage mechanism for this Cu^{2+} complex involves an oxidative pathway with a CuOOH[276] as an intermediate, as well as $^{\cdot}OH$, $O_2^{\cdot -}$, and H_2O_2 formation.

In summary, Cu^+ can participate in one-electron transfer reactions to form not only $^{\cdot}OH$, but also $^1O_2$, $O_2^{\cdot -}$, and H_2O_2 (Figure 2). Although the typical mechanism for $^1O_2$ formation requires UV irradiation, in the presence of Cu^{2+}, $^1O_2$ also can be generated without irradiation. The wide variety of ROS that copper produces, in combination with the fact that Cu^+ reacts with

H_2O_2 significantly faster than Fe^{2+} to produce $^{\cdot}OH$[268] explains why copper is one of the most damaging metals under non-homeostatic conditions.

Although Zn^{2+} is a redox inactive metal ion, some reports indicate that it also causes DNA damage. Chuang *et al.*[63] report an increase in ΦX174 phage DNA damage by epigallocatechin gallate (EGCG, 50-200 μM) and Zn^{2+} (50 μM) compared to damage observed with EGCG alone (50-200 μM). The most surprising part of this result is not DNA damage enhancement by Zn^{2+}, but that the proposed mechanism to explain the damage involves H_2O_2 generation (as a product of the reaction of EGCG and Zn^{2+}) and subsequent generation of $^{\cdot}OH$ by a Fenton-like reaction without any supporting evidence. Augustyniak *et al*.[277] also reported an increase in DNA damage in brain ganglia cells from diapausing grasshoppers (*Chortippus brunneus*) collected from polluted and unpolluted areas of southern Poland and supplemented with zinc salts. Although zinc concentration does not correlate with observed DNA damage, they mention the possibility of $^{\cdot}OH$ and $O_2^{\cdot -}$ formation by Zn^{2+}.[277] These unsupported proposals are of significant concern, since Zn^{2+} does not participate in one-electron redox reactions under biological conditions. In addition, Augustyniak *et al.* do not consider the fact that the increase of Zn^{2+} in grasshoppers' brains was also accompanied by an increase in Cu^{2+}, a more likely culprit for the observed increases in DNA damage. Although Zn^{2+} is redox inert, zinc may also cause DNA damage through a hydrolytic mechanism, as suggested by Tan *et al.*,[60] who observed DNA damage by a Zn^{2+}-quercetin complex (50-400 μM) similar to the Cu^{2+}- and Ni^{2+}-quercetin complexes already discussed. To support this zinc-mediated hydrolytic mechanism, they observed DNA re-ligation by T4 ligase.[60]

A great deal of work has been conducted to better understand the mechanisms and properties that govern metal-mediated ROS formation and DNA damage. Nevertheless, many

questions remain to be answered, especially for the lesser-studied metals such as Sc, Ti, Mn, Co, and Zn. Additional studies are also needed to determine the specific conditions that promote (or prevent) metal-mediated DNA damage; in some cases the initial ROS generated are precursors of the actual damaging agents, and many of the mechanisms and intermediates for this damage are unknown. Clearly, DNA damage by metals is a complex process that greatly depends on the metal and other reactions conditions, and understanding the many mechanistic aspects of this damage is key to preventing diseases caused by metal ions.

Beyond *in vitro* metal-mediated DNA damage: Metal toxicity and DNA damage in cells

The potential mechanisms by which metal ions damage cellular genetic material are quite varied and are influenced by a huge variety of factors. Although many studies in this field are limited to reporting experimental or observational data, this section focuses primarily on recent mechanistic studies of metal-mediated DNA damage in bacterial and human cells. Whereas other reviews in this field have typically focused attention on only one or a few metal ions to include a greater number of related studies,[7,67,117,278,279] this review compares mechanisms of metal-mediated DNA damage and cell death for the first-row transition metals.

Scandium, vanadium, and titanium. Metal-mediated DNA damage is often studied in simpler bacterial systems as well as in mammalian systems to grasp the key mechanistic features involved, as well as to correlate *in vitro* and *in vivo* studies for the purposes of discovering therapeutic targets for DNA damage prevention. However, bacterial studies involving the trace metal ions scandium, vanadium, and titanium are scarce. Anticancer properties of complexes containing these ions have been documented,[280] but investigations into the cellular mechanisms of Sc, V, or Ti-induced DNA damage are nonexistent. Work in this area has been performed

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using mammalian cells, so perhaps similar studies may be conducted with bacterial systems in the future.

No representative body of work for non-nanoparticle Sc- or Ti-induced cellular DNA damage exists. Vanadium ions, however, have been directly linked to DNA damage in several different types of mammalian cells. Human lymphocytic, HeLa, and peripheral blood cells exhibit single-strand breaks and sister chromatid exchanges upon treatment with VO^{2+} and V^{4+} respectively.[281,282] Vanadate and oligovanadate (10 μM) induce necrosis in neonatal rat cardiomyocytes by caspase 3 activation after 24 h treatment.[70] These investigations used comet assays and single cell gel electrophoresis (SCGE) assays to determine DNA damage as described by Singh *et al*.[283] However, these techniques merely assess the extent of DNA damage and do not elucidate specific types of DNA damage or damage mechanisms. Recently, investigations by Hosseini *et al*.[284] showed that V^{5+} damages rat liver cells by mediating ROS production in mitochondria. Isolated mitochondria showed elevated oxidative stress responses after vanadium supplementation ($NaVO_3$) in concentrations as low as 100 μM, leading to apoptosis signaling, but it is unlikely that biological systems accumulate such high concentrations of vanadium (Table 2). These data are still critical, due to studies of vanadium complexes as insulin mimetics.[285]

Chromium and manganese. Recent bacterial studies with chromium have primarily focused on using *E. coli* to bioremediate water sources contaminated with toxic Cr^{6+}. Studies in mammalian systems have been primarily concerned with exploring the extent of DNA damage in cells treated with Cr^{6+} and Cr^{3+}, or more broadly, chromium-induced carcinogenesis in human cells and mice.[286] El-Yamani *et al*.[287] treated human lymphoblastoid cells with Cr^{3+} (as $CrCl_3$, 0.2-1.0 mM) and Cr^{6+} (as Na_2CrO_4, 0.2-1.0 mM) and observed increasing dose-dependent DNA

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3 damage and cytotoxicity using the comet assay. Thompson and coworkers[288] observed a similar
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6 dose-dependent DNA damage response in an intestinal cell line (Caco-2) when treated with Cr^{6+}
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8 in the form of $Na_2Cr_2O_7$ (0.1-100 μM).
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11 Other studies have focused on the necessity of ascorbate for chromium-mediated DNA
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13 damage, since Cr^{6+} must be intracellularly reduced to form Cr-DNA adducts or for Cr^{3+} to
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15 generate hydroxyl radical. Reynolds and coworkers[289] found that Cr^{6+} (as K_2CrO_4, 2 to 10 μM)
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18 did not induce biologically significant DNA damage in human lung epithelial (H640) cells
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20 without added ascorbate. Often, cellular studies do not supplement ascorbate in typical media,
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23 yet it is often present in biological systems. Upon ascorbate (1 mM) addition, the observed DNA
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25 damage was mitigated, suggesting that ascorbate is an important part of Cr^{6+} cellular metabolism
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27 and should be included in any study involving Cr-mediated DNA damage. The authors
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30 acknowledge the difficulty of detecting every type of DNA damage inflicted by Cr; thus,
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32 complete mechanisms of Cr genotoxicity are still unclear. Focused cellular studies utilizing
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35 specific DNA damage assays that differentiate base lesions from Cr-DNA adducts would shed
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37 considerable light on Cr-mediated DNA damage.
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39 In general, studies of manganese-mediated DNA damage mirror those with chromium.
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42 Recent investigations regarding DNA damage by manganese in bacterial systems are non-
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44 existent, but a host of studies examine Mn's ability to damage DNA in human cells. Interest has
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47 been chiefly directed toward neuronal cells, since manganese has an established role in
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49 neurodegeneration.[49] Stephenson and coworkers[48] found that Mn^{2+} (as $MnCl_2$, 2 to 625 μM)
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51 induces thymine base lesions in human neuroblastoma cells (SH-5YSY) and that this damage is
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54 inhibited by antioxidants such as *N*-acetylcysteine and glutathione. GC/MS methods identified 5-
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56 OH-5MetHyd (Figure 6) as the major DNA oxidation product with minor amounts of and 8-
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OHG and FapyG (Figure 5).[48] In contrast, Bornhorst *et al.*[49] did not observe significant strand breaks in human astrocytes (CCF-STTG1) after incubation with Mn^{2+} ($MnCl_2$, 1, 250, and 500 µM) for 2, 24, or 48 h. When cells were pre-incubated with H_2O_2 (250 µM) before Mn^{2+} addition, they reported a decrease in the poly(ADP-ribosyl)ation DNA repair process. In an separate experiment, Bornhorst *et al.*[49] also observed DNA strand breaks only after addition of H_2O_2 (100 µM) at the highest concentrations of Mn^{2+} (250 and 500 µM).[49]

In another example of the diverse results for Mn^{2+}-mediated damage, Mn^{2+} ($MnCl_2$, 200 and 800 µM) promoted oxidative stress and cell death in murine neuroblastoma (Neuro-2a, CCL-131) cells by disrupting membrane-bound ATPases, and this damage was prevented by the flavonoid silymarin (10 and 50 µM).[290] Sava *et al.*[291] reported Mn^{2+} ($MnCl_2$, ~1.8 µM) increased lipid peroxidation but not DNA damage as measured by 8-oxoG formation, but addition of melanin and Mn^{2+} promoted DNA damage. From these results, manganese's role in DNA damage likely involves direct or indirect ROS production in neuronal mitochondria.[100]

Manganese species show dual activity, with some of the species (Mn-porphryrins) helping to detoxify ROS,[292] whereas others (Mn^{3+}-salen complexes) cause nuclear DNA fragmentation.[293] For example, the Mn-porphyrin complexes (10-30 µM) present in mouse embryonic cells were shown to reduce mitochondrial levels of H_2O_2 and inhibit DNA-damage-signaled apoptosis. Ansari *et al.* showed that Mn-salen complexes were selective at inducing apoptosis in breast and colon cancer (MCF7, MCF10, and CCL228) cells at physiologically relevant concentrations (~15 µM). Because manganese-mediated DNA damage depends highly on experimental conditions, more focused studies are required to determine the types of damage, the ROS generated, and the specific conditions required for DNA damage by this metal.

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Iron, cobalt, and nickel. Iron remains the most well-studied element of the first row transition metals in terms of its DNA-damaging effects, and iron essentiality, regulation, and toxicity in bacterial and mammalian systems have been extensively reviewed recently.[110,294-297] Because of its well-known biological functions, detection of cellular iron-mediated DNA damage is a popular research area compared to that of other metal ions. In seminal work, Linn *et al.*[298-300] used gel electrophoresis to quantify the extent of iron-mediated DNA damage *in vitro* and in *E. coli* and established that this iron-mediated damage is the primary cause of cell death in *E. coli* under oxidative stress conditions.

Other studies with iron have utilized techniques ranging from the Ames test to sister chromatid exchange assays and base oxidation detection in a variety of mammalian cells (primary rat hepatocytes, Jurkat C6-1, HepG2 hepatocytes) to determine the extent of iron-mediated DNA damage and resultant cell death.[301,302] Detection of mitochondrial DNA base oxidation from Fe^{2+} ($FeSO_4$, 1.5 to 300 μM) and H_2O_2 (0.01 μM to 100 mM) in porcine thyroid tissue has also been performed using HPLC.[303] In all of these cellular studies, iron consistently generates harmful ROS and DNA damage in a dose-dependent manner. Unsurprisingly, recent work in human and other mammalian systems far outstrips bacterial studies.

Research efforts directed towards understanding cobalt-mediated DNA damage in cells are relatively sparse if nanoparticle studies are excluded. $CoCl_2$ is a cellular hypoxia mimetic[304] and can induce genetic damage in yeast (0.75 mM $CoCl_2$).[305] However, the comet assay used to determine DNA damage in this study, does not reveal the nature of DNA damage and only a single $CoCl_2$ concentration was used. In contrast, Wang *et al.*[56] reported mitochondrial, but not nuclear DNA damage upon exposure to $CoCl_2$ (100 and 200 μM) in rat neuronal cells mimicking hypoxia conditions. Tan and coworkers[306] reported that $CoCl_2$ (300 μM) generated intracellular

ROS and induced morphological changes in human endothelial cells using Western blots and UV-vis assays with formazan. Also using Western blots, Patel *et al.*[58] determined that Co^{2+} alone and in combination with Ni^{2+} ($CoCl_2$ and $NiCl_2$, 50 to 300 μM) caused significant double-strand breaks in human lung epithelial DNA. The exact nature of the relationships among cobalt's inducement of hypoxia, ROS generation, and inhibition of DNA repair remain to be elucidated.

Both homeostasis of nickel in bacterial and mammalian cells[307] and toxicity of nickel in microorganisms[308] have been reviewed recently. Nickel is well established as a potent carcinogen and ROS generator and can induce DSB in human cells.[309] Chronic exposure to relatively high Ni^{2+} concentrations ($NiCl_2$, 10 mM) induces ROS generation and causes complete DNA fragmentation and cell death in human leukemia cells (HL-60). Damage is increased upon H_2O_2 addition but can be decreased by addition of ascorbic acid or *N*-acetyl-cysteine (NAC) reducing agents.[310] Additionally, Xu *et al.*[311] used the polymerase chain reaction (PCR) and fluorescence spectroscopy to detect mitochondrial DNA damage caused by nickel ($NiCl_2$, 125 to 500 μM) in murine neuroblastoma cell lines. Besides these specific studies, most of the literature relating nickel and DNA damage involves detecting the presence of DNA damage alone (often in mammalian cancer cell lines) and does not focus upon the mechanisms by which nickel damages DNA.[278,312]

Copper and zinc. Copper and its relationship to DNA damage in humans has been recently well-reviewed by Linder.[313] Copper homeostasis and transport is much more tightly regulated in cells than that of iron, reflecting its greater ability to catalyze formation of damaging ROS.[262,263] As for the other transition metals, techniques employed to analyze copper-generated DNA damage range from agarose gel electrophoresis to the comet assay. These methods are well established with copper, and it is more extensively implicated in ROS generation and subsequent

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DNA damage than many of the earlier transition metals.[117,314]

Bacterial studies involving copper are few, but interesting developments regarding copper toxicity in *E. coli* have been recently published. Macomber *et al.*[315] used quantitative PCR to demonstrate that copper toxicity in *E. coli* is not likely due to oxidative DNA damage in the nucleus, since fewer DNA lesions were detected in mutants with excess copper. Using mutant *E. coli* strains that do not efficiently export copper (GR17, *recA copA cueO cusCFBA*) in addition to copper supplementation ($CuSO_4$, 2.0 mM), the authors showed that excess copper inhibits growth, but not exclusively through oxidative DNA damage. In human cells, copper has repeatedly been shown to cause many types of DNA damage as Cu^{+}/Cu^{2+} in the presence of cellular reductants such as NADH and ascorbic acid.[117] Also, tumors from various cancers have anomalously high copper levels compared to normal tissues.[316,317] Thus, several organic ligands that enhance copper's DNA damaging abilities in cells have been investigated in cancer research (phenanthrolines, bipyridines, and thiosemicarbazones).[318,319] Recently, copper's genotoxic ability (as copper salts) has been called into question by Valko *et al.*,[8] but abundant evidence exists showing copper's role in the production of harmful ROS and the subsequent DNA damage.[117,278,315,320] Although much *in vitro* work has been performed, much remains unknown about the exact mechanisms by which cellular copper damages DNA.

Most studies involving zinc and DNA damage focus on zinc protection against cytotoxicity *in vivo*. In fact, zinc deficiency, rather than excess, is linked to oxidative stress.[122,321] A few studies, as discussed above in the "In vitro *DNA damage: Metals, mechanisms, and products*" section, have suggested that Zn participates in ROS generation to damage DNA, such as the study by Augustyniak *et al.*[277] in grasshoppers. Zinc is not redox active and is unlikely to generate damaging ROS through one-electron transfer in a reducing cellular environment.[122] A

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few studies have demonstrated that zinc overload (50-100 μM $ZnCl_2$) can damage neuronal cells (primary rat and C8-D1A astrocytes), but the mechanism remains unclear.[322-325] It is suspected that a combination of excess zinc and hypoxia conditions lead to hypoxia-inducible factor (HIF-1) overexpression, resulting in neuronal cell death.[323]

The most striking challenges facing cellular DNA damage studies involve the difficulties associated with the complicated biological matrix in which assessment of damage is attempted. Most of the studies examining transition-metal-mediated DNA damage supplement their chosen ion or complex (often at concentrations much higher than would be biologically relevant) and then quantify the resultant cellular DNA damage. However, metal overload conditions can induce a variety of different cellular responses that are distinct from metal ion effects. In *E. coli*, for example, high iron levels cause induction of the Fur-box, a series of genes responsible for reducing iron uptake. The Fur-box *E. coli* is thought to contain over 90 genes, many related to oxidative stress responses.[326] Copper ions are thought to generate hydroxyl radical that targets iron-sulfur clusters instead of DNA in *E. coli*, suggesting that DNA damage must occur via different mechanisms.[327]

Studies also have demonstrated that metal ions inhibit other processes vital to DNA upkeep, such as DNA repair mechanisms. A review by Hartwig *et al.*[279] catalogs several investigations in which metals such as Ni, Cu, and Co were shown to inhibit different DNA repair processes. If these metals both directly damage DNA and inhibit its repair, a cellular understanding of DNA damage mechanisms becomes significantly more complex. Thus, simply measuring the extent of DNA damage after metal ion exposure may not accurately reflect direct DNA-damaging effects of the metal ion itself, and additional effort should be invested to ascertain cellular mechanisms for the observed damage. Future cellular studies must more

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specifically target suspected pathways (e.g., ROS production, inhibition of DNA repair, sites and types of DNA damage) to fully understand metal-mediated DNA damage.

Since the specific details of metal-mediated DNA damage in cells are not entirely understood, a great deal of attention has been focused on metal chelation therapy. It has long been known that metal chelators such as *o*-phenanthroline can block iron-mediated DNA damage in *E. coli* and mammalian cells,[300,328] and iron chelation has been recently reviewed as a strategy for cancer treatment.[329] Copper-chelating drugs intended for treatment of Wilson's disease, such as tetrathiomolybdate, *D*-penicillamine, and trientine, also inhibit tumor formation and have experienced reasonable success in Phase I and II clinical trials.[330-332] The majority of anti-cancer studies focus on metal chelation therapy for cancer treatment rather than prevention.[333] Iron chelators such as methylphenidate have been shown to ameliorate symptoms of Parkinson's disease in several clinical studies.[334] A more thorough understanding of metal-mediated DNA damage mechanisms will enable development of more effective chelating drugs to treat and prevent diseases caused by metal-mediated oxidative stress and DNA damage.

Conclusions, Challenges, and Outlook

In the past decade, great advances have been made in elucidating metal-mediated DNA damage mechanisms and how this DNA damage contributes to cell death and disease development. More sensitive techniques to detect DNA damage and identify distinct DNA oxidation products as well as the generated reactive oxygen species are now available, but these areas require significantly more research to develop a comprehensive understanding of how metals damage DNA. Mechanistic aspects of metal-generated DNA damage such as kinetic studies, stability of intermediaries such as the postulated CuOOH implicated in $^1O_2$ formation,

and metal-dependent differences in cellular damage mechanisms are still largely unexplored. Where possible, DNA binding and localization of metal ions or metal complexes have been discussed in this review, and this is a critical area for future study. Much is also unknown about metal-mediated DNA damage *in vitro* and in cells, such as the causes of metal imbalance, how this mis-regulation directly leads to disease development, and whether metal chelating drugs can successfully prevent these diseases.

Analytical methods to detect and characterize DNA damage have improved over the past decade: base oxidation products can be detected in nanomolar concentrations and DNA damage locations can be determined in real-time. Improvements in these techniques are still needed to detect specific sites of damage in larger DNA samples, damage-induced changes in DNA conformation, and less common DNA lesions. Determining affinity constants of metal ions and their complexes with DNA also are required to determine the relationships between metal binding, ROS generation, types of DNA damage, and cellular effects of this damage. Most published studies examine DNA damage caused by only one type of metal ion, and analyses describing treatments with more than one metal ion are scarce. In the cellular environment, many metal ions can be present simultaneously, so it is necessary to study the rates of damage under these more complex conditions. Although studies to determine mechanisms, products, and effects of metal-mediated DNA damage are often challenging, developing a comprehensive picture of these processes is critical to understanding and preventing this significant source of cellular damage and disease.

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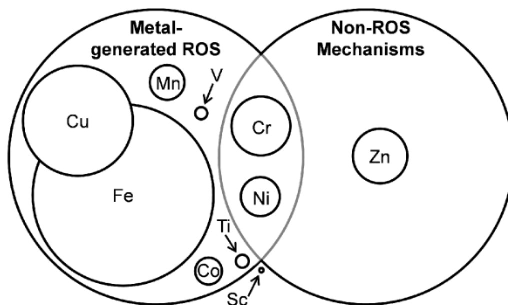
Table of Contents Abstract



Metal ions cause various types of DNA damage by multiple mechanisms, and this damage is a primary cause of cell death and disease.