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Predicting How Polyphenol Antioxidants Prevent DNA Damage by Binding to Iron

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Prevention of oxidative DNA damage due to hydroxyl radical is important for the prevention and treatment of disease. Because of their widely recognized antioxidant ability, 12 polyphenolic compounds were assayed by gel electrophoresis to directly quantify the inhibition of DNA damage by polyphenols with Fe^{2+} and H_2O_2. All of the polyphenol compounds have IC_{50} values ranging from 1−59 μM and inhibit 100% of DNA damage at 50−500 μM concentrations. Gel electrophoresis results with iron(II)EDTA and UV−vis spectroscopy experiments confirm that binding of the polyphenol to iron is essential for antioxidant activity. Furthermore, antioxidant potency of polyphenol compounds correlates to the pK_a of the first phenolic hydrogen, representing the first predictive model of antioxidant potency based on metal-binding. Understanding this iron-coordination mechanism for polyphenol antioxidant activity will aid in the design of more-potent antioxidants to treat and prevent diseases caused by oxidative stress, and help develop structure−activity relationships for these compounds.

Introduction

Reactive oxygen species, such as hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), superoxide ($O_2^{-\bullet}$), peroxynitrite ($ONOO^-$), and others, are major sources of oxidative stress in cells, damaging proteins, lipids, and DNA.[1] Oxidative DNA damage is implicated as a cause of tissue damage resulting from heart attack and stroke,[2] cardiovascular diseases including arteriosclerosis,[3] cancer,[4] aging and Alzheimer's and Parkinson's diseases.[5,6] Therefore, prevention of DNA damage has obvious and important implications for the prevention and treatment of disease.

The hydroxyl radical may come from multiple sources, such as the breakdown of peroxynitrous acid,[7] or from the metal-mediated reduction of peroxides. Hydrogen peroxide is commonly reduced in vivo by iron(II), which results in the formation of $^{\bullet}OH$ via the Fenton reaction.[8,9]

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$$

While both sources of $^{\bullet}OH$ are biologically relevant, iron-mediated oxidative DNA damage by the hydroxyl radical is the primary cause of cell death under oxidative stress conditions in both prokaryotes and eukaryotes, including humans,[10−12] and is therefore our focus. Oxidative DNA damage occurs at both the phosphate backbone (strand breakage) and nucleotide bases, and both types are widely used to determine the extent of DNA damage.[13−21] Damage to both nuclear and mitochondrial DNA occurs in cancer

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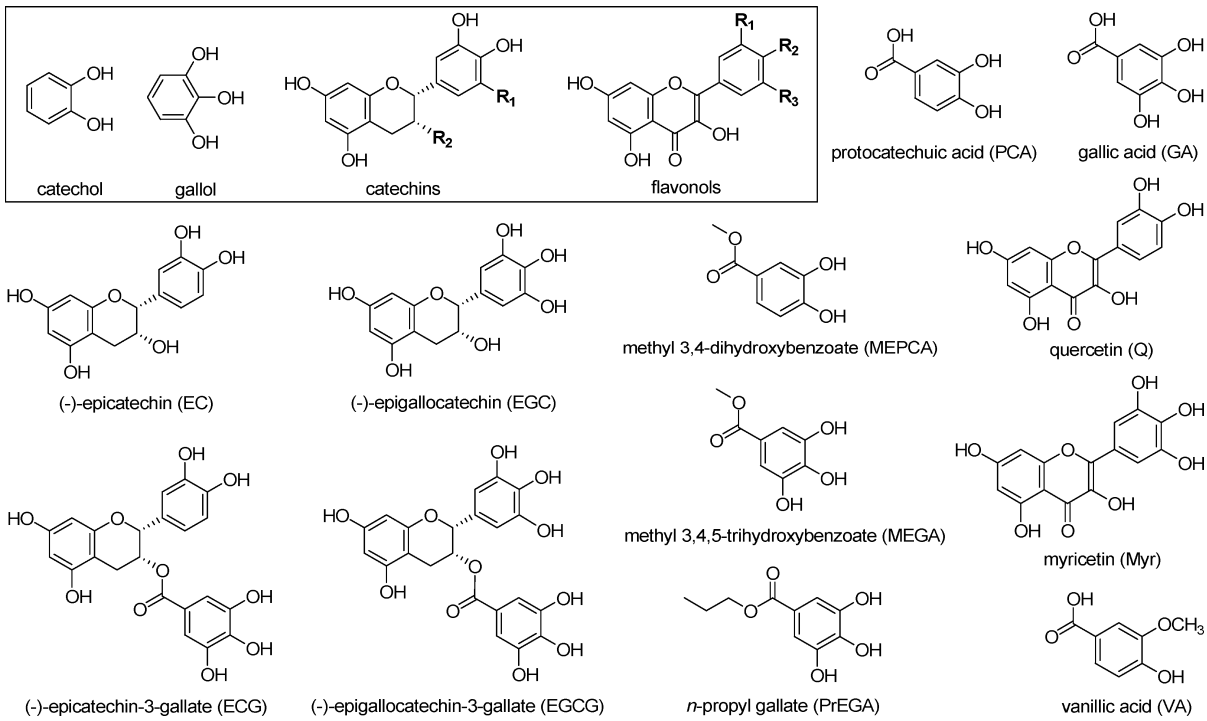

Figure 1. Inside box: structures of catechol and gallol, and general structures of catechins and flavonols. Outside box: structures of specific phenolic compounds used in this research (and their abbreviations).

and other diseases linked to iron misregulation,[22,23] and mitochondrial DNA is particularly at risk for oxidative damage due to its proximity to respiratory processes that produce H_2O_2 and other reactive oxygen species (ROS).[24–26] In fact, oxidative damage to mitochondrial DNA may be a more significant cause of cell death than nuclear DNA damage.[27,28] Oxidative damage to nuclear DNA is observed even in the presence of histone proteins, and several studies have found that the presence of histone proteins can increase metal-mediated oxidative DNA damage.[29–32]

In *E. coli*, the concentration of nonprotein bound (free) iron is 10−30 μM,[33] and it is believed to be coordinated to low-molecular-weight cellular ligands such as ascorbate.[34,35] However, if iron homeostasis is not maintained, the intracellular concentration of free iron may increase to between 80 and 320 μM,[33,36] causing a much greater susceptibility to oxidative DNA damage.[36,37] EPR studies indicate that most of this free iron in *E. coli* exists as iron(II).[33] In humans, increases in cellular free iron concentrations are associated with oxidative stress and Alzheimer's, Parkinson's, and cardiovascular diseases,[38–40] and even mildly elevated iron levels have been linked to increased cancer incidence in humans.[4] Oxidative stress also causes release of iron from proteins, resulting in increased free-iron concentrations.[36,41–43]

Polyphenol compounds, such as those found in green and black teas, fruits and vegetables, olive oil, wines, and chocolate have been shown to be excellent antioxidants, and are found in high milligram quantities per serving for these foods.[44–47] Within just 2 h after consumption of only one cup of green or black tea (350−600 mL),[48–51] catechins (Figure 1) have been found in concentrations of 0.3−1 μM in human plasma and may even approach 10 μM with higher doses.[52] Flavonols such as quercetin are reportedly less bioavailable than catechins; however, they may reach similar

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Table 1. pK_a Values for Phenolic Compounds, Wavelengths of Maximum Absorbance for Iron(III)−Polyphenol Complexes, and IC_{50} Values for DNA Damage Inhibition by Phenolic Compounds

compound	IC_{50} (μM)[a]	pK_{a1}[b]	pK_{a2}	λ_{max}(nm) of iron (III) complex	oxidation potential, Ep_a (V)[c]	reduction potential, Ep_c (V)	pK_a reference
EGCG	1.1	7.55 ± 0.03	8.74 ± 0.03	546	0.293		86
Myr	2.0	6.89 ± 0.60		443, 589[d]	0.169		87
ECG	2.3	7.6	8.8	548	0.316	0.140	86
MEGA	4.0	7.90 ± 0.23		542	0.293		87
PrEGA	5.1	7.77 ± 0.04	10.9 ± 0.1	542	0.288		88
EGC	9.8	8.51 ± 0.04	9.38 ± 0.01	561	0.255		86
Q	10.7	7.65 ± 0.07	8.77 ± 0.07	548[d]	0.250, 0.454	0.187, −0.093	89
GA	14.0	8.45 ± 0.06	11.30 ± 0.10	551	0.433		90
MEPCA	15.6	8.12 ± 0.18		561	0.380	0.294	87
PCA	34.4	8.64 ± 0.05	13.13 ± 0.05	586	0.538	0.084	90
EC	59.1	8.76 ± 0.02	9.46 ± 0.01	578	0.356	0.060	86
VA	140.0	9.39	N/A	None	0.771	−0.123	91

[a] IC_{50} is defined as the concentration at which the compound inhibits 50% of DNA damage, IC_{50} values given are the average of three separate trials, and standard deviations for the listed IC_{50} values are all ±1 μM. [b] pK_a values given are for phenolic hydrogens; pK_a values of carboxylic acid groups for GA, PCA, and VA are 4.44 ± 0.03, 4.26 ± 0.05, and 4.42, respectively. [c] Potentials are reported versus Ag/AgCl/3 M KCl. [d] Absorbance maximum not well defined (shoulder).

plasma concentrations to catechins (high nanomolar to low micromolar levels) in people with diets high in fruits and vegetables or intentionally supplemented with flavonoids.[53,54] Because polyphenols are such a large and integral part of the human diet, it is essential to understand their biological functions and modes of antioxidant activity.

In addition to their antioxidant functions, polyphenols have numerous other biological activities, such as antihistamine activity,[55] as well as anti-inflammatory,[56] vasodilatory, and cardiovascular effects.[57,58] They are also implicated in the prevention of neurodegeneration,[59,60] prevention and senescence of cancer,[61,62] and bind to proteins such as caseins,[57] inhibit enzymes such as telomerase,[63] α-amylase, pepsin, trypsin, and lipase,[64] among many others, and increase endothelial nitric oxide synthase (eNOS) activity by over 400%.[57] Polyphenols also induce apoptosis in cellular studies,[65,66] the primary reason given for their cancer-preventive properties.

Scavenging of ROS by polyphenols is the generally accepted mechanism of their antioxidant activity;[67,68] however, mechanisms involving metal binding have also been proposed and are gaining popularity. Lopes, et al. have shown by UV−vis spectroscopy that tannic acid prevents the Fenton reaction by chelating Fe^{2+}.[69] In addition, Sestili et al. have reported that binding of polyphenolic compounds to iron accounts for prevention of nuclear DNA damage in halo assays of human cancer cells exposed to peroxides, where they observed that catechol derivatives were more potent antioxidants than compounds without the catechol moiety. Antioxidant activity was therefore inferred to result from binding of iron at the catechol group, and this was measured by UV−vis spectroscopy. However, calculation of lipophilicity (C log P) values led the authors to ultimately conclude that the relative antioxidant potencies of the tested catecholate compounds were a direct function of the lipophilicity,[70] likely due to the cell membrane permeability of the compounds. Similar effects of polyphenol iron-chelating ability and lipophilicity on DNA damage in H_2O_2-treated human cancer cells were also noted by Melidou et al.[71]

Despite significant evidence to suggest that iron coordination is responsible for antioxidant activity, definitive DNA

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damage inhibition by iron coordination has been reported for only one compound, verbascoside.[72] No quantification and comparison of the ability of a range of polyphenol compounds on DNA damage prevention under Fenton reaction conditions. Cellular DNA damage assays, such as the halo and comet assays, have most commonly been used to assess the antioxidant activity of polyphenols,[73] yet whole-cell assays often involve too many variables to definitively attribute antioxidant activity to metal binding. Furthermore, the choice of compounds in the antioxidant literature has historically been somewhat scattershot or focused on only a few compounds. Although attempts have been made to extrapolate the antioxidant activities of a few compounds to encompass broad classes, these predictions routinely exclude the possibility of iron-binding as a viable antioxidant pathway. Instead, structure−activity papers focus on aspects such as quenching of 1,1-diphenyl-2-picrylhydrazyl radicals, scavenging and inhibition of $O_2^{\bullet-}$ production by xanthine/xanthine oxidase, inhibition of reactive oxygen species in bone marrow leukocytes,[74] protection of horse butyrylcholinesterase protein from oxidative damage,[75] and prevention of lipid peroxidation.[76]

Thus, a systematic approach toward experimentally identifying the useful structural components or chemical and physical properties that lead to enhanced antioxidant potency specifically via metal coordination has not yet been reported. Therefore, our goal was to quantify the potency of biologically relevant and logically chosen polyphenols by probing their ability to prevent iron-mediated DNA damage from $^{\bullet}OH$. Quantification of DNA damage inhibition enables determination of structure−activity relationships and identification of polyphenol pK_a as an important predictor of antioxidant efficacy.[77]

Materials and Methods

General. Water was purified using a Barnstead NANOpure DIamond Life Science (UV/UF) water deionization system (Barnstead International, Dubuque, IA). (−)-Epicatechin, protocatechuic acid, *n*-propyl gallate, vanillic acid, and quercetin (MP Biomedicals), myricetin (Indofine), (−)-epicatechin-3-gallate, (−)-epigallocatechin (Aldrich), (−)-epigallocatechin-3-gallate (Cayman Chemical Company), gallic acid (TCI America), methyl 3,4,5-trihydroxybenzoate, methyl 3,4-dihydroxybenzoate, $FeSO_4 \cdot 7H_2O$, $NaOH \cdot H_2O$ (99.996%), NaCl (99.999%) (Alpha Aesar), and MES buffer (99.3%) (Calbiochem), were all used as received. High-purity NaOH, NaCl, and MES were essential to avoid metal contamination. H_2O_2 (Fisher) was a 30% solution in water; absolute ethanol (Acros), TRIS base, Na_2EDTA (J.T. Baker), ethidium bromide (Lancaster), and agarose (VWR) were also used as received. UV−vis absorption spectra were measured on a Shimadzu UV-3101PC spectrophotometer (Shimadzu Corp., Kyoto, Japan).

Because of the necessity for all experiments involving DNA damage to be as free from redox-active metals as possible, all microcentrifuge tubes were washed in 1 M HCl for at least 30 min, doubly rinsed with deionized water, and dried. To avoid adding large amounts of radical scavenging solvents such as DMSO, methanol, or ethanol[78–80] that are typically used to dissolve polyphenol compounds, the addition of a small amount of NaOH (usually 20−100 μL of 1 M NaOH per 10 mL polyphenol stock solution) was sufficient to fully dissolve the polyphenol compounds in water, and upon adjustment to pH 6.0, these compounds remained in solution.

Transfection and Amplification of *E. coli*, and Purification of Plasmid DNA. Plasmid DNA pBSSK was purified from *E. coli* strain DH1 using a Qiaprep Spin miniprep kit (Qiagen, Chatsworth, CA). The plasmid DNA was dialyzed using fully hydrated Spectra/Por molecular porous membrane tubing (Spectrum Laboratories, Inc., Rancho Dominguez, CA) at 4 °C against 1 mM EDTA, 50 mM NaCl for 24 h and then against 130 mM NaCl for 24 h to remove transition metals from the DNA. For all experiments, the DNA absorbance ratios $A_{250}/A_{260} \leq 0.95$ and $A_{260}/A_{280} \geq 1.8$ were ensured for the dialyzed DNA sample.

Gel Electrophoresis Experiments. In aqueous solution, Fe^{2+} forms insoluble hydroxides at physiological pH but is soluble at pH 6.0 (Figure S3, Supporting Information),[81,82] thus all solutions were buffered to pH 6.0 with MES buffer (10 mM final concentration, final pH 6.0 measured experimentally). Furthermore, the polyphenol solutions were combined with buffer prior to the addition of $FeSO_4$ to ensure that no iron precipitation occurred (Figure S3, Supporting Information). All $FeSO_4$ solutions were freshly prepared from solid $FeSO_4 \cdot 7H_2O$ immediately prior to each experiment.

The indicated concentration of phenolic compound, $FeSO_4$ (2 μM), ethanol (100%, 10 mM), and NaCl (130 mM) at pH 6 were combined and allowed to stand at room temperature for 5 min. Ethanol was added as a small, controlled amount of radical scavenger intended to mimic intracellular organic components that may scavenge free radicals.[9] Plasmid DNA (pBSSK, 0.1 pmol in 130 mM NaCl) was then added, and the reaction mixture was allowed to stand for an additional 5 min prior to H_2O_2 (50 μM) addition. All concentrations indicated are the final concentrations in a 9 μL reaction volume. After 30 min, EDTA (1 μL, 50 μM) was added for a final volume of 10 μL. The nicked and supercoiled forms of the plasmid were separated by electrophoresis on a 1% agarose gel in TAE buffer (140 V for 30 min). The gels were stained with ethidium bromide and imaged under UV light, and the percentage of nicked and supercoiled DNA was quantified using UVIproMW (Jencons Scientific Inc., Bridgeville, PA, 2003). Ethidium stains supercoiled DNA less efficiently than nicked DNA, so supercoiled DNA band intensities were multiplied by 1.24 prior to comparison.[83,84] Intensities of the nicked and supercoiled bands were normalized for each lane so that % nicked + % supercoiled

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= 100%. For gels run using iron(II)EDTA as the iron source, a similar procedure was used, substituting iron(II)EDTA (400 μM) for the $FeSO_4$. Iron(II)EDTA stock solution was made by adding EDTA (220 μL of 0.5 M, pH 8.0) to $FeSO_4 \cdot 7H_2O$ (27.8 mg) and diluting to 10 mL in water.

Percentage of DNA Damage Inhibition Calculations. Percent DNA damage inhibition was determined using the formula 1 − [%N/%B]*100, where %N = percentage of nicked DNA in the polyphenol-containing lanes and %B = percentage of nicked DNA in the Fe^{2+}/H_2O_2 lane. All percentages were corrected for residual nicked DNA prior to calculation. Results were obtained in triplicate for all experiments, and standard deviations are represented as error bars.

IC_{50} Determination. The plots of percent inhibition of DNA damage versus log concentration of polyphenol compound (in μM) were fit to a variable slope sigmoidal dose−response curve using *SigmaPlot 2004 for Windows*, version 9.01 (Systat Software, Inc.: San Jose, CA). Errors reported for IC_{50} values in Table 1 represent standard deviations calculated from fitting three separate experiments.

pK_a vs IC_{50} graph. The IC_{50} values obtained from the gel electrophoresis experiments were plotted against literature (or calculated) pK_a values for polyphenol compounds. The best-fit exponential curve ($y = 3 \times 10^{-8}\ e^{2.39x}$; $R^2 = 0.92$) through the data points for catecholate compounds, including vanillic acid, was plotted. Quercetin was omitted because it possesses additional noncatecholate iron-binding sites.

Electrochemistry of Polyphenol Compounds. The oxidation and reduction potentials of polyphenol compounds were measured on a CH Electrochemical Analyzer (CH Instruments, Inc.: Austin, TX) in phosphate buffer (64 mM final concentration, pH 6.0) containing KNO_3 (64 mM final concentration) as a supporting electrolyte. Polyphenol solutions (375 μM final concentration) were cycled between −350 mV and 650 mV versus Ag/AgCl/3 M KCl (+210 mV vs NHE)[85] using a glassy carbon (GC) working electrode and a platinum wire counter electrode. The GC electrode was polished with alumina before each trial, and the scan rate was 100 mV/s. All solutions were prepared with ddH_2O, which had been deoxygenated with N_2 for >4 h. Prior to performing the cyclic voltammetry experiment, the sample was blanketed with flowing N_2 above the surface of the solution.

UV−vis Spectroscopy Experiments. Water and MES buffer (50 mM final concentration, pH 6.0) were first added to $FeSO_4 \cdot 7H_2O$ (145 μM final concentration), followed by addition of the polyphenol solution. Solutions of all phenolic compounds were adjusted to a final concentration of 290 μM. The absorbance was measured after 30 min to allow iron coordination. Control spectra for the polyphenol compounds and iron were obtained by replacing the iron or polyphenol compound solution, respectively, with water.

Kinetic Measurements of Iron Oxidation. Solutions of $FeSO_4$ (145 μM) and the selected polyphenol compound (435 μM) in MES buffer (50 mM, pH 6.0) were combined, and kinetics data were measured at the λ_{max} for each iron(III) complex. All pH and concentration values given are final in a 3 mL reaction volume. Kinetics data with catecholate ligands were collected over the course of 1 h, whereas those with gallate ligands were measured over 30 min. The initial rate was obtained by fitting the linear portion of the absorbance versus time graph, and the slope of this best-fit line is reported as k_{obs}. Results are the average of two trials. A graph of IC_{50} value versus k_{obs} is shown in Figure S8 (Supporting Information).

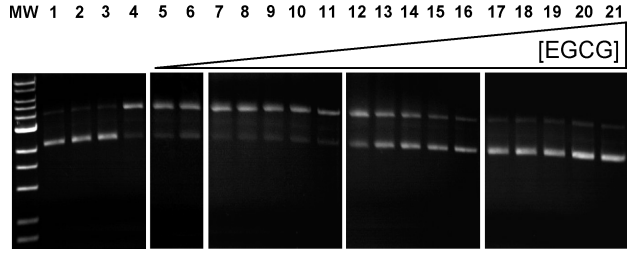

Figure 2. Gel electrophoresis image of DNA damage inhibition by (−)-epigallocatechin-3-gallate (EGCG), under Fenton reaction conditions (2 μM Fe^{2+} + 50 μM H_2O_2). Lanes: **MW** = 1 kb DNA ladder; **1** = plasmid DNA (*p*); **2** = *p* + 50 μM H_2O_2; **3** = *p* + 500 μM EGCG + 50 μM H_2O_2; **4** = *p* + 2 μM Fe^{2+} + 50 μM H_2O_2. Lanes **5**−**21**: Increasing concentration of EGCG: 0.0002, 0.0005, 0.001, 0.002, 0.02, 0.05, 0.1, 0.2, 2, 4, 10, 50, 100, 200, 300, 400, and 500 μM, respectively.

Results and Discussion

Inhibition of Fe^{2+}-Induced DNA Damage by Phenolic Compounds. The 12 compounds shown in Figure 1 were tested for their ability to inhibit DNA damage by an iron-generated hydroxyl radical using gel electrophoresis. This technique is effective for measuring DNA damage by Fe^{2+} and H_2O_2,[9] as well as for quantifying the inhibition of Cu^+/H_2O_2-mediated DNA damage by selenium compounds.[86]

Figure 2 shows the inhibition of DNA damage with increasing concentrations of EGCG (data for all other compounds are in Supporting Information). As seen in lanes 2 and 3, respectively, H_2O_2 (50 μM) alone or with polyphenol compound does not damage DNA in the absence of iron(II), although such H_2O_2 concentrations readily kill *E. coli*.[8] Hydrogen peroxide (50 μM) combined with 2 μM iron(II) results in a large amount of damaged DNA (Figure 2, lane 4). It is important to note that all $FeSO_4$ solutions were freshly prepared prior to each experiment and used immediately and that iron(II) is soluble at pH 6.0 for the duration of the experiment.[82]

Of the 12 phenolic compounds tested, EGCG is the most potent antioxidant, inhibiting 93% of the iron-mediated DNA damage by $^{\bullet}OH$ at a concentration of only 10 μM (see Supporting Information for data and calculations for all compounds). A graph of percent inhibition of DNA damage as a function of EGCG concentration (Figure 3) shows the antioxidant behavior of this polyphenol, with the solid line representing the best-fit dose−response curve for EGCG. From this fit, the concentration of EGCG necessary to inhibit 50% of DNA damage (IC_{50}) was calculated to be 1.1 μM. This IC_{50} value for ECGC is certainly biologically relevant, because plasma concentrations of EGCG typically reach ∼1

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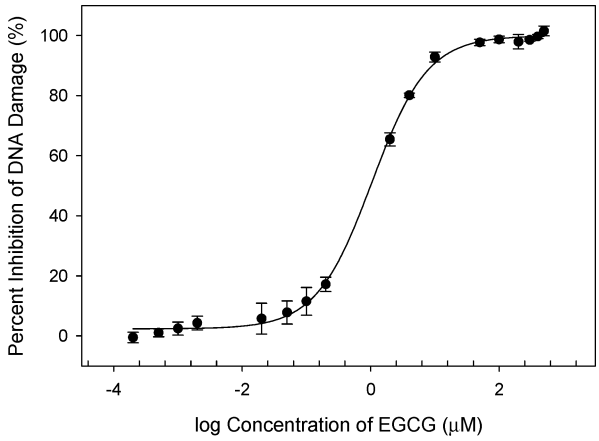

Figure 3. Percent inhibition of DNA damage graph for (−)-epigallocatechin-3-gallate (EGCG), under Fenton reaction conditions (2 μM Fe^{2+} + 50 μM H_2O_2). Error bars represent standard deviation calculated from three separate trials at each of the concentrations shown. The solid line represents the best-fit variable-slope dose−response curve calculated from the data.

μM after consuming just one cup of green tea.[52] Given the 2 μM concentration of iron in these experiments, EGCG inhibits 50% DNA damage at approximately a 0.5:1 mol ratio of EGCG to iron. Whereas this may seem to indicate a stoichiometric effect, a 1:1 mol ratio of EGCG to iron did not inhibit 100% of the DNA damage, nor did the IC_{50} values for most other polyphenols correlate to stoichiometric equivalents of polyphenol to iron. Similarly to EGCG, DNA damage inhibition studies were conducted with all of the polyphenol compounds in Figure 1 and their calculated IC_{50} values are listed in Table 1. All of the compounds tested, with the exception of VA, inhibit 100% of DNA damage at concentrations ranging from 50 to 500 μM, and have IC_{50} values ranging from 1 to 59 μM. The antioxidant behavior of VA was expected to be much lower than the other polyphenol compounds due to the methyl substituent on one phenolic hydroxyl group that hinders metal binding, and this is reflected by its high IC_{50} value of 140 μM, more than twice the IC_{50} found for the least-effective catechol compound (EC).

Elucidation of Chemical Principles Governing Antioxidant Efficacy. Gallate compounds are significantly more potent antioxidants than their catecholate analogues. ECG differs from EGCG by substitution of one gallate group for a catecholate, leading to a higher IC_{50} for ECG (2 μM). In addition, GA and its methyl ester, MEGA (IC_{50} = 14 and 4 μM, respectively), have significantly lower IC_{50} values than their corresponding catecholates PCA and MEPCA (IC_{50} = 34 and 16 μM, respectively).

Polyphenol compounds with carboxylic acid substituents are much less potent antioxidants compared to their corresponding esters, illustrated by comparing IC_{50} values of GA (14 μM) with MEGA (4 μM) and PrEGA (5 μM), which is approved by the FDA for use as a food preservative.[93] Because the carboxylic acids are deprotonated at pH 6 (pK_a ~4.4), deprotonation of the phenolic hydrogens would be correspondingly more difficult due to charge repulsion. In addition, a positively charged iron ion may also be attracted to a negatively charged carboxylic acid group as well as a phenolate group. VA is similar to PCA or GA in that it possesses a carboxylic acid but cannot bind iron as a catecholate due to the methyl substituent on one phenol. The very high IC_{50} value for VA, along with higher IC_{50} values for polyphenols with carboxylic acid groups, suggests that binding of iron to a carboxylate group does not promote antioxidant activity.

Compounds with two polyphenol substituents (EGCG and ECG) are capable of binding more than one iron ion and can therefore inhibit significantly more DNA damage than analogous compounds with only one polyphenol group (EGC and EC, with IC_{50} values of 10 and 59 μM, respectively), further suggesting that iron binding specifically at catecholate or gallate moieties is responsible for inhibition of DNA damage. The gallate Myr and its catecholate analog Q are also very potent antioxidants with IC_{50} values of 2 and 11 μM, respectively. In fact, these flavonols were significantly more potent than the other monogallate or monocatecholate compounds. Both of these compounds have a potential bidentate iron binding site near the keto group in addition to the gallate or catecholate group, and researchers have hypothesized that iron binds to Q at multiple locations on the molecule.[94,95] Because Myr is similar in structure to Q, it is also expected to be capable of multisite coordination, and the low IC_{50} value found for Myr is consistent with other compounds having multiple iron-binding sites. Notably, within error the IC_{50} value determined for Q is essentially identical to the IC_{50} value for prevention of human cell death by Q (10.7 ± 1 and 12.67 ± 0.86, respectively; Q was the only tested compound in common between the two studies) determined by Sestili et al.,[70] indicating that compounds with low micromolar activity in these gel electrophoresis studies may have similar low micromolar effects in human cells.

If iron binding to polyphenol groups of these compounds is responsible for their inhibition of DNA damage, the antioxidant potency of these compounds should be related to their pK_a values. Although pK_a values for the phenolic hydrogens of polyphenols are somewhat high (pK_{a1} for gallols is ~7.5, whereas pK_{a1} for catechols is typically ~8.5 or greater), the presence of a metal cation allows proton displacement at or below physiological pH,[96] promoting metal chelation. Polyphenol compounds with lower pK_a values should bind iron more readily under physiologically relevant conditions. Solely on the basis of pK_a values for the most acidic phenolic hydrogen (Table 1), the trend in antioxidant potencies for the polyphenolic compounds tested was hypothesized to be VA < EC < PCA < EGC < GA <

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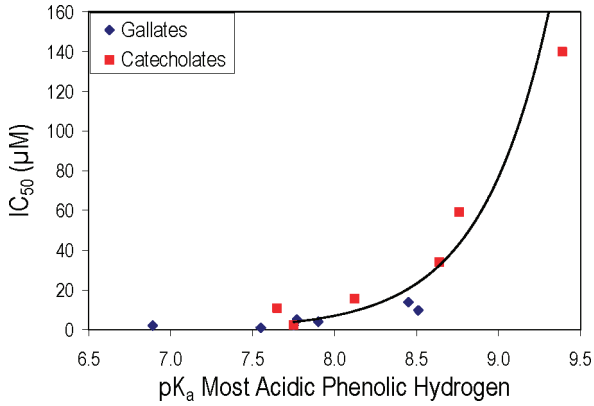

Figure 4. Graph of IC_{50} vs pK_a (first phenolic hydrogen) showing the best-fit exponential correlation to the data for catecholate polyphenols (solid line, $R^2 = 0.92$). The data point for quercetin was omitted from the catecholate data set because of its noncatechol binding site. Error bars for IC_{50} values are within the size of the data points.

MEPCA < MEGA < PrEGA < Q < ECG < EGCG < Myr. This order was generally comparable to the experimental order of VA < EC < PCA < MEPCA < GA < Q < EGC < PrEGA < MEGA < ECG < Myr < EGCG. It should be noted that the pK_a values for MEGA, MEPCA, and Myr are calculated values[97] and therefore have significant uncertainty. Regardless, pK_a was determined to be an effective predictor of relative antioxidant potency of phenolic compounds.

A graph of IC_{50} versus pK_a of the first phenolic hydrogen of the tested polyphenolic compounds is shown in Figure 4, and it is clear that gallate compounds are more potent than catecholates with similar pK_a values. In addition, the antioxidant potency of the catecholate compounds, including vanillic acid but excluding quercetin because of its multiple iron-binding sites, varies exponentially with pK_a as shown by the solid line ($y = 3 \times 10^{-8}\ e^{2.39x}$; $R^2 = 0.92$). It should be noted that a linear fit to the catecholate data points gives a similar correlation ($R^2 = 0.89$; Supporting Information, Figure S4), although an exponential relationship is expected for these data.[98,99] The correlation of IC_{50} values with the pK_a of the catecholates gives a significantly better fit than is observed when correlating either reduction or oxidation potentials of the polyphenol compounds ($R^2 = 0.79$ and 0.76, respectively; see Table 1 and Supporting Information Figures S5 and S6). This correlation is extremely important, because the polyphenol family contains thousands of compounds and a straightforward predictor of antioxidant behavior, such as pK_a, is a valuable screening tool to identify compounds with maximum antioxidant potential for further testing.

Our results represent one of the first attempts toward developing a predictive model to estimate the antioxidant activity of polyphenolic compounds via metal binding. In our studies, iron-binding is essential for the antioxidant activity of polyphenol compounds; however, the ability of these polyphenolic compounds to inhibit DNA damage in our studies does not correlate with lipophilicity as noted by Sestili et al. (Figure S7, Supporting Information).[70] This difference likely results because our electrophoresis studies directly measure antioxidant activity and their studies indirectly measure cellular uptake, highlighting the need to balance antioxidant activity and cellular uptake to maximize antioxidant activity.

Confirmation of Iron-Binding by Polyphenols. Because iron binding is required for the antioxidant activity of the polyphenolic compounds, UV−vis spectroscopy was used to examine their iron coordination behavior. Catecholate and gallate compounds bound to iron(II) quickly oxidize due to dissolved O_2 in aqueous solution to give blue-purple iron(III) complexes.[100–102] At pH 5−6, iron is typically bound by two catecholate or three gallate ligands per metal ion[103] with molar extinction coefficients on the order of 10^3,[104] consistent with our results. However, iron polyphenol complexes have been reported with mononuclear,[105–107] dinuclear,[108] or extended polymeric[96,109] structures. Regardless of stoichiometry, the observed color change is a good indicator of iron binding and oxidation by this class of compounds. All of the polyphenol antioxidants in Figure 1, with the exception of VA, changed color after the addition of iron(II).

UV−vis spectra for EGCG with and without iron(II) are shown in Figure 5 (spectra for all other compounds are in the Supporting Information). The iron polyphenol complexes displayed λ_{max} in the ranges of 542−561 nm for gallates, and 561−586 nm for catecholates, consistent with the ligand-to-metal charge transfer (LMCT) bands observed for other polyphenol complexes with iron.[69,110] The two flavonols, Q and Myr, displayed unique absorbances because these compounds are colored prior to metal binding, and they each possess a second iron binding site in addition to the catecholate or gallate moiety. Upon the addition of iron(II), Q displayed an absorbance maximum at 407 with a shoulder at 548 nm, and Myr displayed an absorbance at 443 nm with a shoulder at 589 nm. In contrast to all of the other compounds tested, the UV−vis spectrum of VA with Fe^{2+} showed no color change as evidence of iron binding or oxidation of iron(II) to iron(III).

(97) Calculated using *Advanced Chemistry Development (ACD/Labs)* Software V8.14 for Solaris.
(98) Because an IC_{50} value is equivalent to an inhibition constant (K_i, ref 98), the correlation between an IC_{50} value and a K_a value would be linear. Thus, a plot of IC_{50} versus pK_a should have an exponential correlation.
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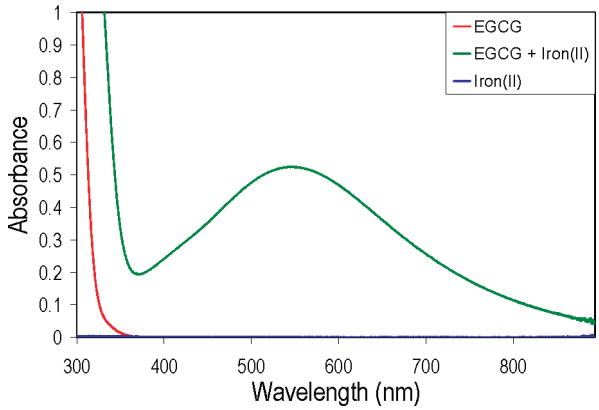

Figure 5. UV−vis spectra for 290 μM (−)-epigallocatechin-3-gallate (EGCG) and 145 μM iron(II) sulfate. All solutions were prepared in MES buffer (50 mM, pH = 6.0).

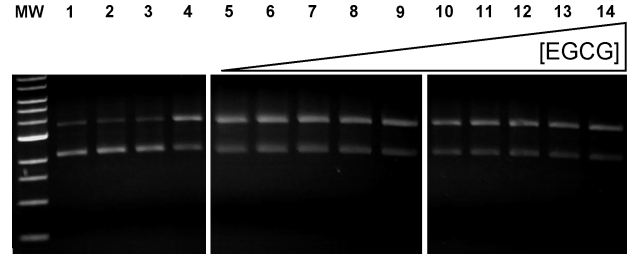

Figure 6. Gel electrophoresis image of DNA damage inhibition by (−)-epigallocatechin-3-gallate (EGCG), under 400 μM $[Fe(EDTA)]^{2-}$ + 50 μM H_2O_2 reaction conditions. Lanes: MW = 1 kb DNA ladder; 1 = plasmid DNA (p); 2 = p + 50 μM H_2O_2; 3 = p + 500 μM EGCG + 50 μM H_2O_2; 4 = p + 400 μM $[Fe(EDTA)]^{2-}$ + 50 μM H_2O_2. Lanes 5−14: Increasing concentration EGCG: 0.2, 2, 4, 10, 50, 100, 200, 300, 400, and 500 μM, respectively.

Because of the complex electrochemistry of these polyphenol compounds as well as the potential diversity of their iron complex structures, electrochemistry experiments of the polyphenol compounds in the presence of iron are extremely complex and not suitable for direct comparison. As a result, kinetics studies measuring the initial rate of iron oxidation upon polyphenol binding were also performed to determine whether iron oxidation is responsible for the observed antioxidant activity of these compounds. Although gallate compounds have significantly faster iron oxidation rates than analogous catecholate compounds (k_{obs} = 4.432 min^{-1} and 0.146 min^{-1} for MEGA and MEPCA, respectively), a graph of IC_{50} value versus k_{obs} showed only a weak correlation (R^2 = 0.78; Supporting Information, Figure S8). Thus, antioxidant activity for these polyphenol compounds correlates more strongly with iron binding, not iron oxidation. Stability constants for the iron(II) polyphenol complexes studied in this work, with the exception of quercetin,[111] have not yet been determined. The substantial effort of measuring the stability constants for this large range of compounds in conjunction with further mechanistic work and physical measurements represent the next logical chapter of this research effort.

It is well-known that catecholate ligands have very large stability constants with iron(III).[112] Thus, polyphenols in excess might be expected to compete with EDTA for iron binding (K for iron(II)EDTA ~2 × 10^{14}).[113] However, for iron(II) monocatecholate complex the K_1 of $10^{7.9}$ is relatively low,[114] and if iron(II) is chelated to EDTA prior to the introduction of 1 equiv of polyphenol, formation of the monocatecholate (or gallate) does not occur. This lack of coordination was observed when solutions containing EC, EGCG, MEPCA, or PrEGA (500 μM), and iron(II)EDTA (400 μM in MES buffer, pH 6.0) showed no blue-purple color change even after seven days, indicating that at this concentration the polyphenols do not compete with EDTA for iron.

To confirm the necessity of iron coordination for inhibition of DNA damage, gel electrophoresis experiments with iron(II)EDTA (400 μM) instead of $FeSO_4$ were performed. Because EGCG was the most potent antioxidant in the electrophoresis experiments with free Fe^{2+}, it was chosen for this experiment.

Completely coordinating the iron with EDTA, prior to addition of the polyphenol, prevented EGCG from coordinating to iron and resulted in significantly less DNA damage inhibition than observed with $FeSO_4$, as shown in Figure 6. Combining iron(II)EDTA and H_2O_2 results in significant DNA damage in the absence of EGCG (lane 4). In lanes 5−14, the concentration of EGCG is increased from 0.2 to 500 μM, and very little inhibition of DNA damage is observed, even at the highest concentrations of EGCG. With $FeSO_4$ as the iron source, a 1:1 iron to EGCG molar ratio inhibited approximately 52% of DNA damage, whereas with iron(II)EDTA, the same conditions resulted in no inhibition of DNA damage. Therefore, the results of these experiments suggest that coordination of EGCG to iron may be a biologically relevant mechanism for inhibition of DNA damage in vivo.

Because we have confirmed that iron binding is a key mechanism for DNA damage inhibition by polyphenol compounds, further understanding of how iron coordination leads to antioxidant activity is necessary. It is possible, for example, that the iron oxidation observed upon binding to polyphenol compounds may result in an iron(III) complex that cannot be reduced by cellular reductants to catalytically generate a hydroxyl radical. Therefore, determining how iron coordination controls the antioxidant potency of these compounds will be important to understanding their biological activity.

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(112) For example, bacterial siderophores may have values as large as 10^{40} to 10^{49} for chelation of iron(III).[114] The stability constant for the iron(III) triscatecholate complex is reported as ~10^{44}, whereas the stability constant for iron(III) monocatecholate (K_1) is 10^{20}.[115] The iron(III)−gallic acid complex has an overall stability constant of 10^{28}.[116]

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Conclusions

Our work to quantify the inhibition of iron-mediated DNA damage by polyphenol compounds represents the first direct comparison of antioxidant activity for a wide range of polyphenols using a biologically relevant system specific enough to validate iron binding as a viable mechanism for antioxidant activity of these compounds. Under conditions that would otherwise cause significant DNA strand breakage, the polyphenol compounds protected the DNA by binding to iron and preventing generation or release of hydroxyl radical. All of the polyphenolic compounds tested were shown to inhibit 100% of DNA damage from iron-generated $^{\bullet}$OH, with IC_{50} values between 1 and 59 μM. Half of the compounds tested had IC_{50} values below 10 μM, which is within biologically attainable concentrations for these compounds (1−10 μM).[48–52]

Correlation of antioxidant potency of DNA damage inhibition to pK_a values for the first phenolic hydrogen provide the first steps toward a predictive model for general antioxidant potency of polyphenols via metal binding. Iron-binding by these compounds resulted in antioxidant activity, and preventing their ability to bind to iron negated this activity as shown by gel electrophoresis experiments with iron(II)EDTA. Ultimately, phenolic pK_a values were found to be specific and measurable (or predictable) properties to determine which polyphenolic compounds bind iron most efficiently and therefore most potently inhibit DNA damage. Our results provide the necessary groundwork for the identification and development of more potent antioxidant compounds for the treatment and prevention of cancer and other diseases caused by oxidative DNA damage. Combining our gel electrophoresis method, which directly quantifies DNA damage inhibition, with cellular studies and *C* log *P* calculations, which describe cellular uptake, may identify the optimal balance between antioxidant activity and cellular uptake and allow the identification and/or design of superior antioxidants in the future.

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Supporting Information Available: Gel electrophoresis results for both iron(II) and iron(II)EDTA-mediated DNA damage experiments, tabulation of gel electrophoresis data, graphs of percent inhibition of DNA damage versus log concentration of phenolic compound for remaining compounds, UV−vis spectra of iron(II) at pH 6 and 7, correlations of polyphenol redox potentials to IC_{50} values, a graph of IC_{50} versus octanol/water partition coefficient (*C* log *P*) for all compounds tested, a graph of IC_{50} values versus initial rate of iron oxidation, cyclic voltammetry scans, as well as UV−vis spectra for the polyphenol compounds with and without iron(II). This material is available free of charge via the Internet at http://pubs.acs.org.

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