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Antioxidant Effects of Isoflavonoids and Lignans, and Protection Against DNA Oxidation

ANGELA HARPER[a], DAVID J. KERR[b], ANDREAS GESCHER[c] and J. KEVIN CHIPMAN[a,*]

[a]*School of Biochemistry,* [b]*Cancer Research Campaign Institute of Cancer Studies, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK;* [c]*Medical Research Council Toxicology Unit, University of Leicester, Leicester, LE1 9HN, UK*

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The antioxidant capability of a series of isoflavonoid and lignan compounds in both cellular and cell-free systems has been investigated, and related to structure. Nordihydroguaiaretic acid exhibited a potent antioxidant activity in both HepG2 and MDA-MB-468 cells (IC_{50} 5.3 and 1.1 μM respectively), as determined by inhibition of 2′,7′-dichlorofluorescin oxidation via *t*-BOOH, although no inhibition was observed with other compounds tested in this system. All compounds inhibited the formation of 8-oxodeoxyguanosine in DNA exposed to hydroxyl radicals via gamma irradiation or the Fenton reaction. Whilst almost complete inhibition of gamma irradiation-induced damage was achieved (IC_{50} ranged from 0.2 to 0.8 μM), inhibition was less pronounced with the Fenton system. The ability of all compounds to interact with DNA (as well as with reactive oxygen and iron) was also demonstrated by scanning UV spectroscopy, suggesting that the compounds may inhibit DNA oxidation at least in part by binding to DNA. Hydroxyl radical-scavenging, iron-chelating and DNA-binding activity of these compounds supports their proposed role as natural cancer-protective agents.

Keywords: Reactive oxygen species, isoflavonoids, lignans, free radicals, 8-oxodeoxyguanosine

Abbreviations: NDGA, nordihydroguaiaretic acid; HPLC-EC, high-performance liquid chromatography with electrochemical detection; ROS, reactive oxygen species; 8-oxodG, 8-oxodeoxyguanosine; dG, deoxyguanosine; *t*-BOOH, *tert*-butyl hydroperoxide; DCFH-DA, 2′,7′-dichlorofluorescin-diacetate; DCFH, 2′7′-dichlorofluorescin; DCF, 2′,7′-dichlorofluorescein; DMF, *N,N*-dimethyl-formamide;

INTRODUCTION

It is thought that reactive oxygen species (ROS) play a fundamental role in numerous pathological events, such as cellular aging, inflammation and cancer development.[1] ROS generated during normal cell metabolism are usually inactivated by host antioxidant defence mechanisms, however oxidative stress can occur when these mechanisms become compromised. This can lead to an excessive accumulation of oxygen metabolites, such as oxygen radicals (superoxide, hydroxyl), singlet oxygen and hydrogen peroxide, which vary in their capacity to damage cell molecules.

*Corresponding author. Tel.: +44(0)121 414 5422. Fax: +44(0)121 414 3982. E-mail: J.K.Chipman@bham.ac.uk.

In particular, hydroxyl radicals produced *in vivo* by superoxide-driven Fenton-type reactions[2] are highly reactive and can cause severe damage to DNA and cellular proteins and lipids.[3] These events may contribute to the initiation and promotion of cancer.

Epidemiological evidence suggests that the incidence of breast and prostate cancer may be lowered by a diet high in soybean and wholegrain products, likely due to the protective effects of isoflavonoids and lignans.[4–8] These occur in plants as glycosides and are converted by intestinal bacteria to biologically active compounds, which have similar diphenolic structures and are weakly oestrogenic. Phytoestrogens have demonstrated a wide range of biological activities which support their proposed role as natural cancer-protective agents (reviewed in[5,8–11]). In particular, their antioxidant effects have been widely documented (see below).

The ability of the isoflavone genistein to enhance the activities of antioxidant enzymes and to inhibit expression of the proto-oncogene c-*fos* in mouse skin has been reported.[12,13] Certain flavonoids and lignans have also been shown to scavenge superoxide and hydroxyl radicals.[14–20] Hydroxyl radicals in turn can initiate peroxidation of membrane lipids, causing continuous production of peroxyl and alkoxyl radicals,[21] and it has been reported that phytoestrogens can also scavenge these intermediate radicals and thus protect against lipid peroxidation.[22–25] On the other hand, the chelation of iron ions by certain phenolic compounds, which would depress the superoxide-induced Fenton reaction, has also been demonstrated.[16,26] Hence the antioxidant effect of flavonoids could occur via concomitant activities of iron chelation and free radical scavenging.

In contrast, certain flavonoids and other phenolic compounds have also been shown to exhibit pro-oxidant effects under certain *in vitro* conditions.[15,17,23,27] Hence these agents cannot always be classified solely as antioxidants.

This study examines the antioxidant capability of the isoflavonoid compounds genistein and equol (a metabolite of the isoflavone daidzein[28]), and the lignans enterolactone, enterodiol and nordihydroguaiaretic acid (NDGA); for structures see Figure 1. Previous studies with genistein by Wei *et al.*[12,29,30] demonstrated its ability to inhibit tumour promoter-induced hydrogen peroxide production by cultured cells and mouse skin, and to scavenge both superoxide anion and hydrogen peroxide extracellularly. Furthermore, Ruiz-Larrea *et al.*[31] demonstrated that the ability of genistein to scavenge a radical species in the aqueous phase and to protect against LDL oxidation was greater than that of several other related structures. Here we present work in which the relative antioxidant capability of the five compounds within cultured cells has been assessed using both human-derived HepG2 cells (which retain many features of normal hepatocytes[32]) and MDA-MB-468 cells (a breast cancer cell line). To our knowledge the relative intracellular antioxidant capacity of these substances has not previously been examined.

One of the major DNA lesions caused by ROS is the altered nucleoside 8-oxodeoxyguanosine. (8-oxodG;[33]). 8-oxodG may be produced via a variety of processes, such as the Fenton reaction, UV light and ionizing radiation.[34] We have therefore examined the effect of these isoflavonoids and lignans on the formation of 8-oxodG in calf thymus DNA exposed to hydroxyl radicals formed via gamma irradiation or the Fenton reaction.[35] This approach was used to help distinguish between effects mediated by the ability of compounds to scavenge hydroxyl radicals, chelate iron or to bind to DNA. Overall, these studies provide further insight into the relative antioxidant status of these structurally-related phenolic compounds.

MATERIALS AND METHODS

Chemicals and Reagents

Genistein (4′,5,7-trihydroxyisoflavone) and equol (4′,7-dihydroxyisoflavan) were purchased from Apin Chemicals Ltd (Abingdon, UK) and were

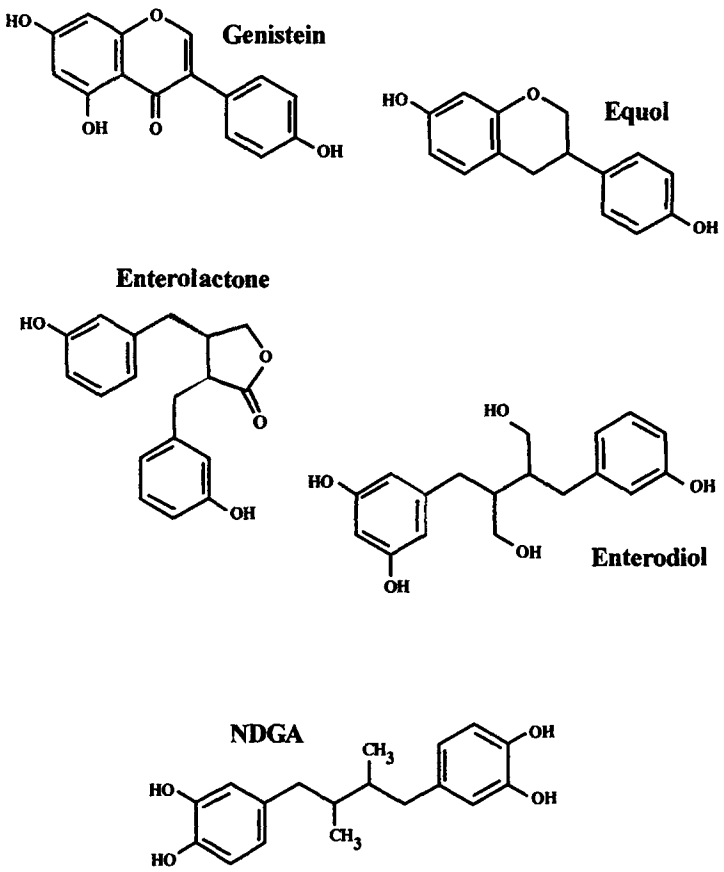

FIGURE 1 Structures of the isoflavonoids genistein and equol, and the lignans enterolactone, enterodiol and NDGA.

more than 98% pure. NDGA was purchased from Fluka Chemie AG (Switzerland). Enterolactone (*trans*-2,3-bis[(3-hydroxyphenyl)methyl]-butyrolactone) and enterodiol (2,3-bis[(3-hydroxyphenyl)methyl]-butane-1,4-diol) were obtained from Dr. K. Wähälä (Chemistry Dept., University of Helsinki, Finland) and were more than 99% pure. 2′,7′-Dichlorofluorescin-diacetate (DCFH-DA) was purchased from Cambridge Bioscience (Cambridge, UK). $FeSO_4$, EDTA, sodium acetate and sodium citrate were from BDH Chemicals Ltd (Poole, UK). 8-oxodG was synthesized by the Udenfriend system[36] and its structure confirmed by mass spectroscopy.[37] Calf thymus DNA, H_2O_2 (30%), *tert*-butyl hydroperoxide (*t*-BOOH (70%)), *N*,*N*-dimethyl-formamide (DMF), nuclease P1, alkaline phosphatase and all other general laboratory chemicals were purchased from Sigma Chemical Co. (Poole, UK).

Cell Culture

HepG2 cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK) and routinely cultured in Williams E medium (Gibco, Paisley, UK) supplemented with 10% (v/v) foetal calf serum, 0.25 mM L-cysteine,[38] 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 µg/ml). MDA-MB-468 cells were obtained from Prof. J. Carmichael (University of Nottingham, UK) and grown in RPMI 1640 medium (Gibco) with the above supplements, except L-cysteine. Stock cultures were maintained

in 25 or 80 cm^2 flasks (Gibco) at 37°C, 5% CO_2/air. All media supplements were from Sigma.

Intracellular Detection of ROS Using DCFH-DA and *t*-BOOH

The assay for detection of ROS inside HepG2 or MDA-MB-468 cells was based on a fluorimetric method by Rosenkranz *et al.*,[39] using DCFH-DA. This non-polar compound diffuses into cells where it is hydrolyzed by intracellular esterases to the non-fluorescent polar derivative 2′,7′-dichlorofluorescin (DCFH), which is trapped inside the cells. In the presence of ROS, DCFH is oxidized to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF). In these studies, *t*-BOOH was used as a source of ROS (see Discussion).

Cells were cultured in 96-well plates (Canberra Packard, UK) at a density of 8×10^5 cells/well. When confluent, after 24 h (HepG2) or 48 h (MDA-MB-468), monolayers were rinsed with 200 µl phosphate buffered saline (PBS; pH 7.4, 37°C) and 200 µl serum-free medium was added. This consisted of Eagle's Minimal Essential Medium with Earle's salts (Gibco), supplemented with 1% (v/v) non-essential amino acids (Gibco), 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 µg/ml). DCFH-DA (2 µl of 3 mg/ml solution in methanol) was added to wells and the microplate was incubated at 37°C, 5% CO_2/air for 30 min. Monolayers were then treated with different concentrations of test compounds in ethanol (2 µl volumes), and 1 mM *t*-BOOH. Ethanol vehicle (2 µl) was also added to control wells. The plate was incubated as described above, for 1 h.

For detection of intracellular fluorescence, medium was aspirated from wells and monolayers were rinsed with 200 µl ice-cold PBS. Triton X-100 (200 µl; 0.5%) was added and after 20 min shaking at room temperature to release cellular contents, fluorescence was measured using a microplate spectrofluorimeter (Perkin-Elmer LB50) at excitation and emission wavelengths of 485 (bandwidth 5.0 nm) and 535 nm (bandwidth 20.0 nm) respectively.

Treatment of DNA with $FeSO_4$ plus H_2O_2 (Fenton Reaction System) or *t*-BOOH

Calf thymus DNA was dissolved in 'ultra high quality' water (reverse osmosis membrane filtered; Elga Ltd, High Wycombe, UK) at a concentration of 0.5 mg/ml. DNA (250 µl) was incubated in PBS (pH 7.4) with 250 µM $FeSO_4$, 100 µM H_2O_2 or 10 µM *t*-BOOH and different concentrations of test compound at 37°C for 15 min (1 ml total volume). Stock compounds (100 mM) were dissolved in DMF and diluted in either PBS (genistein) or water (other compounds). The DMF vehicle had no significant effect ($P > 0.05$) at the concentrations used in experiments, and for this reason was used in preference to ethanol or other solvents. The reaction was terminated by addition of 100 µl sodium acetate (0.3 M) and 4 ml ethanol (−20°C). The samples were kept overnight at −20°C to precipitate the DNA, which was then pelleted by centrifugation (1000 g for 10 min) and redissolved in 100 µl sodium citrate (5 mM)/sodium chloride (20 mM) buffer, pH 6.5. Samples were heated (95°C, 6 min) to denature the DNA, and cooled on ice prior to digestion of the DNA.

Exposure of DNA to γ-irradiation

Solutions containing DNA and different concentrations of test compound in PBS, as described above, were irradiated in flat-bottomed glass vials using a cobalt-60 gamma ray source at a dose rate of 2.34 Gy/min for 9 min. Samples were kept on ice both prior to and after irradiation. DNA was then precipitated and prepared as described above.

Measurement of 8-oxodG and Deoxyguanosine (dG)

Amounts of 8-oxodG and dG in the DNA were determined by HPLC, based on a method by

Floyd *et al.*,[40] with some modifications. Samples in sodium citrate/sodium chloride buffer, as described above, were incubated with 95 µl sodium acetate (20 mM, pH 4.8) buffer containing 0.1 mM $ZnCl_2$, and 2.5 U nuclease P1 at 45°C for 30 min. Samples were then incubated with 90 µl Tris-HCl (50 mM, pH 7.4) and 1.5 U alkaline phosphatase at 37°C for 1 h. DNA hydrolysates were centrifuged (2000*g*, 10 min) and supernatants kept at −70°C prior to analysis by HPLC, using a Pye Unicam LC-XPD HPLC pump. The column was a reverse-phase Technopak 10 C_{18} (HPLC Technology, Cheshire, UK). The mobile phase consisted of sodium acetate (25 mM), citric acid (12.5 mM), sodium hydroxide (30 mM) and acetic acid (10 mM) in 5% methanol, pH 5.1, with EDTA (2 mg/l) added. The flow rate was 1 ml/min. A Pye Unicam LC-UV detector with UV absorbance of 254 nm enabled quantitation of dG. 8-OxodG was quantitated by electrochemical activity using a BAS Model LC-4B amperometric detector (Biotech, Luton, UK). The detector used a glassy carbon electrode at a potential of +0.6 V, measured against an Ag/AgCl/3 M NaCl reference electrode. The amounts of dG and 8-oxodG in samples were calculated from their peak areas, relative to the corresponding co-injected standards.

Reaction of Hydroxyl Radicals, Iron Ions and DNA with Isoflavonoids and Lignans

Test compound solutions (100 mM in DMF) were diluted in PBS (pH 7.4) before treatments. Compounds (25 µM) were exposed to hydroxyl radicals by γ-irradiation at a dose of 20 Gy, as described above. Interaction of iron ions or DNA with compounds was examined by addition of 250 µM $FeSO_4$ or 250 µl DNA (0.5 mg/ml) to 100 µM compound in PBS, and incubation at 37°C for 15 min. After treatments, optical spectra were recorded using a dual beam Uvicon spectrophotometer (Kontron Instruments).

Statistical Analysis

Data are expressed as means ± SD for triplicate samples. Statistical significance was analysed by one-way ANOVA, and the *t* test was used to make comparisons between individual treatments.

IC_{50} Determinations

IC_{50} values for compounds were approximated from curves plotted using Grafit (Erithacus), with data for at least 6 concentrations.

RESULTS

Optimal Conditions for the Determination of DCF Fluorescence by ROS in Cultured Cells

In preliminary studies we found *t*-BOOH to be more effective than H_2O_2 at oxidizing DCFH in this cellular system, hence experiments of a factorial design were done to determine the appropriate incubation time and concentrations of DCFH-DA and *t*-BOOH for detection of optimal fluorescence (data not shown). Incubation of cells for 1 h enabled detection of fluorescence values which increased with both *t*-BOOH and DCFH-DA concentration. With 1 mM *t*-BOOH, the fluorescence produced from DCFH-DA (30 µg/ml) was doubled. No significant loss in cell viability was detected using these conditions which were employed in all experiments with cultured cells. We also demonstrated that DCFH was not oxidized by *t*-BOOH in the absence of cells (data not shown).

Effect of Isoflavonoid and Lignan Compounds on Oxidation of DCFH by *t*-BOOH in HepG2 and MDA-MB-468 Cells

The compounds genistein, equol, enterolactone and enterodiol (0.25, 2.5 and 25 µM) had no inhibitory effects on the oxidation of DCFH by *t*-BOOH in either HepG2 (not shown) or

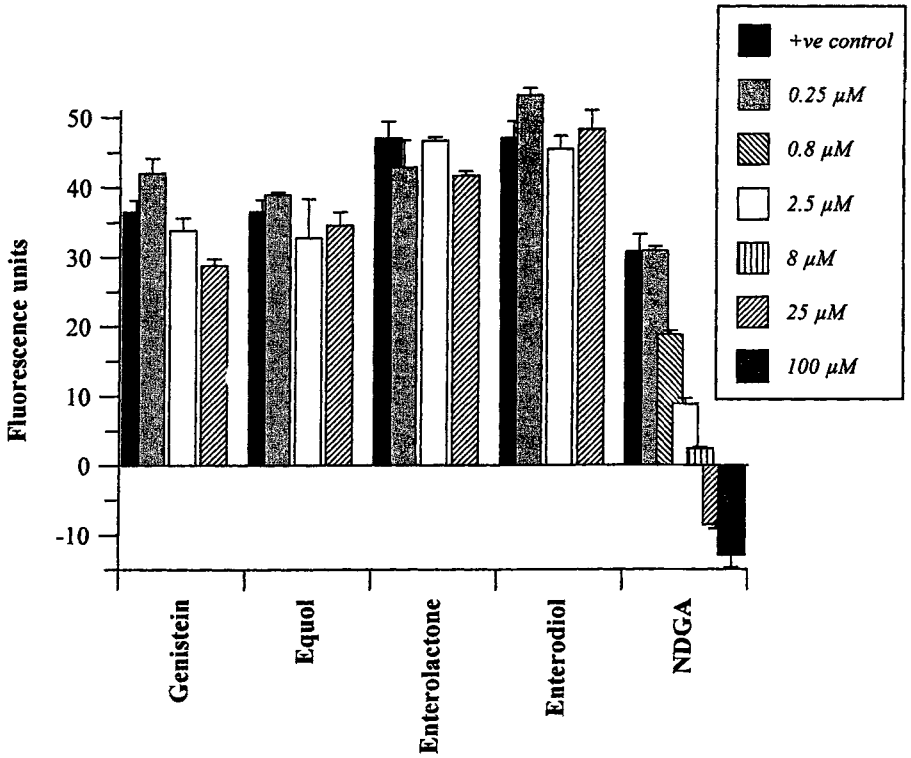

FIGURE 2 Effect of isoflavonoids and lignans on *t*-BOOH-induced oxidation of DCFH in MDA-MB-468 cells. The value for DCFH oxidation by *t*-BOOH alone (positive control) is shown in each case and the background fluorescence of DCFH alone has been subtracted from data points. Hence NDGA lowered the background oxidation of DCFH. The results are means ± SD of an experiment done in triplicate, representative of two.

MDA-MB-468 cells (Figure 2). In contrast, NDGA (0.8–100 μM) was a very potent inhibitor of DCFH oxidation in MDA-MB-468 cells ($P < 0.01$), acting in a concentration-dependent manner. Similar results were obtained in HepG2 cells (not shown). IC_{50} values for NDGA were approximately 5.3 μM (HepG2) and 1.1 μM (MDA-MB-468). In these latter cells a significant reduction in the background level of DCF fluorescence was also observed with 25 and 100 μM NDGA ($P < 0.05$).

Inhibition of 8-oxodG Formation in DNA Exposed to Fenton Reaction Products or γ-Irradiation

Exposure of calf thymus DNA to $FeSO_4$ with 100 μM H_2O_2 or to γ-irradiation led to a significant increase in formation of 8-oxodG, expressed as the percentage ratio of 8-oxodG to dG (Table I). 8-oxodG was increased 28-fold (Fenton system) or 26-fold (γ-irradiation) over background levels. For the purpose of comparison, results with isoflavonoids and lignans are normalized as the percentage of oxidative DNA damage caused by treatments (i.e. percentage of positive control). All compounds tested inhibited the formation of 8-oxodG via both the Fenton reaction system and γ-irradiation, in a concentration-dependent manner, whereas the vehicle DMF had no effect. Representative data are shown for genistein in Figure 3; similar curves were obtained for all other compounds. However, the inhibitory effect of all compounds on γ-irradiation-induced DNA damage was much more pronounced, with almost complete inhibition in each case (Table II). In the Fenton reaction system, none of the

TABLE I Formation of 8-oxodG in calf thymus DNA treated with H_2O_2/Fe^{2+} or γ-irradiation

Treatment	% Ratio 8-oxodG/dG[a]	
	Control	Treated
H_2O_2/Fe^{2+}	0.0094 ± 0.003	0.2611 ± 0.03
γ-irradiation	0.0152 ± 0.003	0.3953 ± 0.08

[a]Calf thymus DNA was either incubated with H_2O_2 and $FeSO_4$ for 15 min at 37°C, or exposed to γ-irradiation at a dose of 20 Gy. Data represent means ± SD of 5 to 6 experiments with 3 samples in each. The amounts of 8-oxodG and dG were determined by HPLC with electrochemical (8-oxodG) and UV (dG) detection.

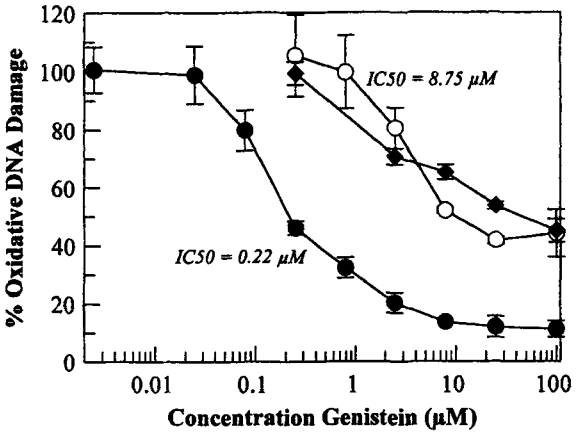

FIGURE 3 Protective effect of genistein on 8-oxodG formation via the Fenton reaction and γ-irradiation. Results represent the inhibitory effect of genistein on the level of 8-oxodG relative to dG produced in DNA treated with H_2O_2/Fe^{2+} (○), γ-irradiation (●) or *t*-BOOH/Fe^{2+} (◆). Values for DNA alone have been subtracted. Values for treated DNA (positive control), as the % ratio of 8-oxodG to dG, were as follows: 0.281 ± 0.02 (○), 0.500 ± 0.09 (●), 0.142 ± 0.01 (◆). Data are means ± SD for triplicate samples.

compounds achieved complete inhibition. The maximal inhibition values achieved by NDGA and genistein were 65% and 58% respectively, compared with 96% and 89% for γ-irradiation-induced DNA damage. The IC_{50} values for inhibition of 8-oxodG formation via the Fenton reaction or γ-irradiation are also shown in Table II, except for enterolactone and enterodiol, neither of which reached 50% inhibition in the Fenton system. These values account for at least a 20-fold difference in potency of the compounds between the two systems. The results overall suggest that the rank order of effectiveness at inhibiting 8-oxodG formation is NDGA = genistein > equol > enterolactone = enterodiol.

Whilst all compounds inhibited the formation of 8-oxodG via treatment with $FeSO_4/H_2O_2$ and γ-irradiation, only NDGA was previously shown to inhibit the oxidation of DCFH by *t*-BOOH in cultured cells (see above). To determine if this may have been due to different radicals produced in the two systems, we investigated the effect of genistein on formation of 8-oxodG via $FeSO_4$ and *t*-BOOH. Exposure of calf thymus DNA to $FeSO_4$ and 10 μM *t*-BOOH led to a significant increase in formation of 8-oxodG, to 25 times above the background level. Moreover, genistein inhibited this oxidative DNA damage maximally by 55%, similar to the percentage achieved with $FeSO_4/H_2O_2$ (Table II) and the effect was concentration-dependent (Figure 3). The IC_{50} value obtained was about 30 μM.

Interaction of Phytoestrogens with Hydroxyl Radicals, Iron Ions and DNA

When solutions of each isoflavone and lignan were exposed to hydroxyl radicals generated by γ-irradiation, the intensity of each resulting absorption spectrum was decreased (data not shown). The absorption spectrum for genistein alone had a maximum at 263 nm (Figure 4(a), curve 1). When genistein was incubated with $FeSO_4$, the intensity of this peak was increased. More importantly, changes in the differential spectra for the genistein–iron ion complex recorded against the $FeSO_4$ solution (curve 3) or against the genistein solution (curve 4) were observed, notably in the UV region. Analogous spectra were obtained for all other test compounds (not shown).

In the presence of DNA, similar changes were observed with differential spectra (Figure 4(b)). In particular, the spectrum for the genistein–DNA complex recorded against the DNA solution (curve 3) showed a decrease in intensity at 205 nm, which was the mirror image of a

TABLE II Inhibition by isoflavonoid and lignan compounds of 8-oxodG formation via the Fenton reaction or γ-irradiation

Compound	8-oxodG via Fenton reaction		8-oxodG via γ-irradiation	
	Maximal inhibition (%)[a]	IC_{50} (μM)[b]	Maximal inhibition (%)	IC_{50} (μM)
Genistein	58 ± 1.9	8.75	89 ± 23	0.22
Equol	55 ± 6.2	50	90 ± 14	0.30
NDGA	65 ± 6.2	5.5	96 ± 22	0.27
Enterolactone	45 ± 7.7	—	87 ± 11	0.80
Enterodiol	37 ± 1.5	—	87 ± 8	0.77

[a]Data represent the percent maximal inhibition of 8-oxodG formation achieved by incubating compounds with calf thymus DNA during exposure to H_2O_2/Fe^{2+} or γ-irradiation. Values are means ± SD for triplicate determinations.
[b]Based on values for at least 6 concentrations. Where no value is shown, 50% inhibition was not obtained.

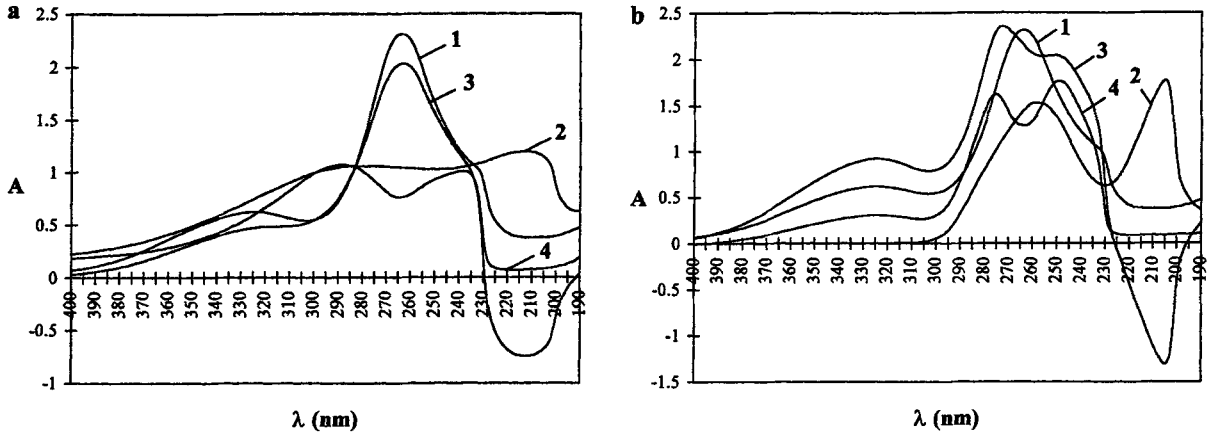

FIGURE 4 Absorption spectra for genistein (100 μM) complexed with ferrous ion (a) or DNA (b). (a) 1 = Absorption spectrum of genistein in PBS, pH 7.4; 2 = spectrum of ferrous ion as 250 μM $FeSO_4$ solution; 3 = differential spectrum of ferrous ion–genistein complex against the $FeSO_4$ solution; 4 = differential spectrum of ferrous ion–genistein complex against the genistein solution. (b) 1 = Absorption spectrum of 100 μM genistein; 2 = spectrum of DNA; 3 = differential spectrum of DNA–genistein complex against the DNA solution; 4 = differential spectrum of DNA–genistein complex against the genistein solution.

maximum observed with DNA alone (curve 2). This peak was also absent from the spectrum of the genistein–DNA complex recorded against genistein (curve 4). Analogous spectra were again observed with all other compounds (data not shown). The compound-iron/DNA complexes were relatively stable, with little change in the spectra observed after 48 h.

DISCUSSION

Several studies have demonstrated both the antioxidant and pro-oxidant effects of different plant phenolic compounds, depending on the experimental system used.[15,17,23,27,31] This study has used both cellular and cell-free systems to investigate the relative antioxidant capabilities of a series of isoflavonoids and lignans, potential cancer-protective agents. Potential determinants of the protective effects, in tems of radical-scavenging, iron-chelating or DNA-binding ability, have also been analysed.

In both HepG2 and MDA-MB-468 cells only NDGA exhibited a potent antioxidant capability against *t*-BOOH, and no effect was observed with either genistein, equol, enterolactone or enterodiol. This contrasted with the ability of all five

compounds to protect against DNA oxidation by hydroxyl radicals and with genistein's reported ability to scavenge radical species in cell-free systems.[29–31] In our cellular system, ROS derived from *t*-BOOH were used to oxidize DCFH inside cells, a reaction which is markedly increased by the presence of peroxidase or intracellular iron.[41] It has been reported that *t*-BOOH can react with ferrous ions to produce hydroxyl and *tert*-butyl alkoxyl (t-BO$^{\bullet}$) radicals in a Fenton-type reaction.[42,43] In addition, both these radicals are able to induce peroxidation of membrane lipids and promote production of other reactive species, such as *tert*-butyl peroxyl (*t*-BOO$^{\bullet}$) radicals.[44,45] Hence a variety of ROS that could potentially oxidize DCFH may be present in the system we used; other studies have also shown that several species can oxidize DCFH.[46,47] Antioxidant effects of genistein, equol, enterolactone and enterodiol against particular individual radical(s) may therefore have been masked and not detected.

It is also possible that these compounds were sequestered inside the cells at sites separate from that of DCFH oxidation. DCFH is a polar compound, whereas the flavonoids and lignans are highly lipid soluble.[48] Since the reaction of hydroxyl radicals, in particular, occurs very close to their production site,[2] DCFH may only detect ROS in the soluble fraction of the cell. The compounds might still be capable of antioxidant effects in, for instance, cell plasma membranes, which have been suggested to be the main site of *t*-BOOH-induced damage.[42,49] In contrast, the highly water soluble antioxidant ascorbate (100 μM) did inhibit DCFH oxidation in both HepG2 and MDA-MB-468 cells (not shown). Moreover, we have also shown that genistein was able to protect against oxidative DNA damage induced by *t*-BOOH in a cell free system. The fact that NDGA was a potent antioxidant in both cell types, yet exhibited effects very similar to those of genistein in all other systems examined, might be explained by a different cellular distribution of this compound.

The intracellular protective effects described above for NDGA could be attributed to radical-scavenging and/or iron-chelating ability. Indeed, UV scanning spectroscopy has suggested an ability of all five compounds tested to react with both hydroxyl radicals and iron, hence both these effects could contribute to the overall effect on free radical processes. It is not known if iron complexes formed can prevent the formation of ROS. All compounds inhibited the formation of 8-oxodG in DNA by hydroxyl radicals formed via Fenton chemistry or γ-irradiation, but whereas almost complete inhibition was achieved with γ-irradiation, inhibition was less pronounced with the Fenton system. No further protection was obtained by decreasing the concentrations of either hydrogen peroxide or iron (not shown), therefore these were not in excess. Iron chelation may have contributed to protection in the Fenton system whereas ROS scavenging may have protected against damage produced by both the Fenton system and irradiation. In addition, the ability of all compounds tested to interact with DNA was also demonstrated by UV absorption spectra. It therefore seems likely that protection against oxidative DNA damage may also have occurred, at least in part, by compounds binding to sites in the DNA. This hypothesis was recently proposed for genistein by Wei *et al.*,[30] supported by the fact that both genistein and its derivative biochanin A were able to inhibit UV light-induced DNA oxidation, whereas only genistein significantly scavenged hydrogen peroxide. It might also provide another explanation for genistein's ability to inhibit DNA oxidation via *t*-BOOH, despite its lack of effect on *t*-BOOH-induced oxidation of DCFH inside cells. The inability of compounds to completely inhibit 8-oxodG formation in the Fenton system could be explained by the presence of iron ions closely bound to DNA,[1] forming ROS inaccessible to the compounds. Moreover, if the theory of Wei *et al.*,[30] is correct in that DNA binding of flavonoids is important for protection, then the presence of iron may limit such binding.

In contrast to the observed antioxidant capabilities of the test phytoestrogens, genistein, equol and NDGA also exhibited some pro-oxidant effects in a cell-free system in the absence of the reductant ascorbate (data not shown), as found with other similar studies.[15,17,23,27] The test system involved oxidation of DCFH by horse radish peroxidase, in the presence and absence of hydrogen peroxide. Whilst there are several possible explanations for these findings, the potential pro-oxidant effects of these compounds should not be disregarded. It is important to consider conditions that are likely to occur *in vivo*. Iron is not freely available to drive Fenton-type reactions, since it is largely chelated to organic acids such as citrate and adenine nucleotides.[15] Moreover, the presence of natural reducing agents would tend to make the redox-cycling activity of compounds less likely. Under these conditions, it is probable that isoflavonoids and lignans have a greater potential to act as antioxidants.

In summary, all isoflavonoids and lignans tested exhibited a marked ability to protect against oxidation by ROS in at least one system examined. This protection appears to be mediated by abilities to scavenge hydroxyl radicals, chelate iron and bind to DNA, showing potential for use in the treatment of free radical pathologies. NDGA was the most potent inhibitory agent in all systems tested; genistein was similar in action except no antioxidant effects towards *t*-BOOH were observed in cultured cells. Enterolactone and enterodiol were the least effective agents. The data further demonstrate the importance of the 5,7-dihydroxy structure (in the A ring) combined with the 2,3-double bond in conjugation with a 4-oxo function (C ring). These parameters have already been recognized as being important in the relative antioxidant capacity of genistein.[18,31,50,51] Effective free-radical scavenging may also be enhanced by the dual hydroxyl groups substituted in the aromatic B-ring of NDGA which may also facilitate iron binding. The relative importance of each potential mode of action of the compounds may vary depending on the experimental system used, but it is necessary to establish precisely how these compounds act *in vivo*. Why did only NDGA exhibit antioxidant effects inside cells, for instance? More information is needed on the cellular distribution and possible metabolism of the compounds *in vivo*.[29] It is also clearly necessary to make use of systems which remove the ambiguity as to which radical species are formed or detected. Nevertheless, involvement of a variety of mechanisms of protection against oxidation and the relative potency of a series of isoflavones and lignans have been demonstrated.

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