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Potent Antioxidant Dendrimers Lacking Pro-oxidant Activity

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Abstract

It is well known that antioxidants have protective effects against oxidative stress. Unfortunately, in the presence of transition metals, antioxidants including polyphenols with potent antioxidant activities may also exhibit pro-oxidant effects, which may irreversibly damage DNA. Therefore, antioxidants with strong free radical scavenging abilities and devoid of pro-oxidant effects would be of immense biological importance. We report two antioxidant dendrimers with a surface rich in multiple phenolic hydroxyl groups, benzylic hydrogens and electron donating ring substituents that contribute to their potent free radical quenching property. In order to minimize their pro-oxidant effects, the dendrimers were designed with a metal chelating tris(2-aminoethyl)amine (TREN) core. The dendritic antioxidants were prepared by attachment of six syringaldehyde or vanillin molecules to TREN by reductive amination. They exhibited potent radical scavenging properties: 5 times stronger than quercetin and 15 times more potent than Trolox according to the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. The antioxidant dendrimers also protected low-density lipoprotein, lysozyme and DNA against 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced free radical damage. More importantly, unlike quercetin and Trolox, the two TREN antioxidant dendrimers did not damage DNA via their pro-oxidant effects when incubated with physiological amounts of copper ions. The dendrimers also showed no cytotoxicity towards Chinese hamster ovary cells.

Keywords

Polyphenolic dendrimer; antioxidant; pro-oxidant; lipoprotein; lysozyme; DNA

Introduction

Oxygen- and nitrogen-based free radicals are produced as a result of metabolism and the immune response. At low levels, radicals have important physiological roles, such as cell signal transduction resulting in gene expression and modulation of cell growth characteristics. However, excessive amounts of radicals can be harmful to biomolecules including DNA, proteins, and lipid. Persistent overproduction may result in a condition of oxidative stress to cells, leading to the pathogenesis of many ailments, such as inflammation, cancer, asthma, cardiovascular and neurological diseases, and in the etiology or progression

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of age-related disorders. Among the myriad of antioxidants, polyphenols have been reported to possess numerous health benefits. Anti-cancer [1], anti-lipoperoxidation [2], anti-ischemic [3], anti-allergic and anti-inflammatory [4] properties have been reported for these compounds. Quercetin, a popular polyphenolic flavonoid has been clinically shown to be a chemo-preventive agent [5]. Unfortunately, antioxidants including polyphenols with potent antioxidant activity also exhibit carcinogenic and mutagenic effects [6]. Rats fed with excessive amounts of quercetin developed renal tubule adenomas, adenocarcinomas, intestinal and bladder cancer [7]. Although seldom seen clinically, there is substantial evidence associating high concentrations of flavonoids and other popular antioxidants, such as vitamin C and E and carotenoids, with mutagenic and DNA damaging ability [8]. It is well known that chelation of iron by ascorbate can lead to the formation of hydroxyl and superoxide radicals. Some of the damaging effects on DNA by antioxidants are associated with their pro-oxidant activity in the presence of transition metals like copper and iron [9]. Other potential mechanisms of the genotoxic effects of antioxidants have been proposed [8]. Identification of natural or synthetic potent antioxidants that have little or no pro-oxidant effects would clearly be beneficial for biological applications.

A vast number of antioxidants have been synthesized in attempts to develop more potent and bioavailable compounds. Strong antioxidants share certain common structural features. They often possess multiple phenolic hydroxyl groups (e.g. flavonoids) or extensively conjugated π system (e.g. carotenoids). In the case of phenolic antioxidants, stronger antioxidant capacity is observed with increasing number of phenolic hydroxyl groups [10]. The high antioxidant efficiency of polyphenols stems from their ability to form stable phenoxyl radicals that can resonate with the benzene rings after donation of H atoms to free radicals. If the ring has an extended conjugation *ortho* or *para* to the hydroxyl group, the resonance stabilization of the antioxidant radical increases, resulting in enhanced antioxidation capacity [11]. Substitution of the phenolic ring also plays an important role in antioxidant potential. For example, an electron-donating group *ortho* or *para* to hydroxyl increases the hydrogen donating capability by stabilizing phenoxyl radical via electron donation, increasing antioxidant efficiency [12]. Commonly found electron-donating groups in potent antioxidants include substituents that do not form intramolecular hydrogen bonds with the phenolic hydroxyl groups (e.g. methyl groups in α-tocopherol) or that can form nonlinear intramolecular hydrogen bonds with phenolic hydrogen at the *ortho* position (such as methoxy) [13]. Benzylic hydrogens have also been reported to be beneficial toward enhancing antioxidant potential [14]. These hydrogen atoms are chemically labile and analogous to phenolic hydrogens in terms of their ability to stabilize the resulting radical by resonance delocalization with the benzene ring.

The dendritic architecture allows us to incorporate multi-functionality in a molecule. Dendrimers are “soft” nanomaterials whose size can be systematically increased to provide precise structures (generations). We recently reported the synthesis and antioxidant properties of three generation 1 (G1) dendritic polyphenols consisting of syringaldehyde, vanillin and 5-iodovanillin emanating from a 4-aminomethylbenzylamine core [15]. Among these three dendritic antioxidants, quercetin and a vitamin E analog (Trolox), the syringaldehyde-based antioxidant dendrimer showed the strongest antioxidant activity (measured by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical assay), and was the most effective in protecting LDL, linoleic acid and DNA from free radical attack. Interestingly, when the pro-oxidant effects of the G1 antioxidants on copper-induced DNA oxidation was compared with quercetin and Trolox, they were found to be less harmful than the latter two antioxidants. These promising results led us to prepare similar G1 antioxidants which possess interior amines that have potential metal chelation property surrounded by a peripheral layer of phenol rings which are efficient radical scavenging groups. An antioxidant dendrimer of this design should show beneficial antioxidant potential with

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reduced undesirable pro-oxidant activity towards DNA. Structures of the two newly synthesized syringaldehyde and vanillin-based dendrimers with a tris(2-aminoethyl)amine (TREN) core (**1** and **2**), a previously reported syringaldehyde-based dendrimer with a 4-aminomethylbenzylamine core (**3**), and a naturally occurring polyphenol (quercetin) are shown in Figure 1.

Materials and Methods

Syringaldehyde, vanillin, quercetin, TREN (97%), sodium triacetoxyborohydride ($Na(OAc)_3BH$), tetra-butylammonium fluoride (n-Bu_4NF, 75 wt% solution in water), tert-butyldimethylsilyl chloride (TBDMS-Cl, 50% in toluene), DPPH, Fat Red 7B, phosphate-buffered saline (PBS), potassium persulfate, glacial acetic acid, sodium acetate and methanol were purchased from Sigma Aldrich and were used without further purification. Lysozyme (egg white) was purchased from Worthington Biochemical Corporation. 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was obtained from Cayman Chemical (Ann Arbor, MI, USA). Human low-density lipoprotein (LDL) was obtained from Kalen Biomedical (Montgomery Village, MD, USA). The lipoprotein solution (protein = 5 mg/mL) contained 154 mM NaCl and 0.34 mM EDTA.

^{1}H-NMR spectra were recorded with a Varian Mercury-300 spectrometer operating at 300 MHz with tetramethylsilane ($Si(CH_3)_4$) as an internal standard. ^{13}C-NMR spectra were recorded using a Varian Mercury-300 spectrometer operating at 75 MHz with $Si(CH_3)_4$ as an internal standard. The mass spectra were obtained on Bruker Autoflex MALDI-TOF using 2,5-dihydroxybenzoic acid as a matrix. All spectrophotometric data were obtained using Perkin Elmer UV/Vis spectrometer (Lambda 20) and Molecular Devices Corp. Spectra Max (M2^e).

General procedures for the protection of phenolic hydroxyl groups with TBDMS-Cl

Phenolic hydroxyl groups were protected with TBDMS as previously described [15].

TBDMS-protected syringaldehyde

Yield: 98%; R_f value: 0.75 (hexane-ethyl acetate = 1:1); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 0.15 (s, 6H, $Si(CH_3)_2C(CH_3)_3$), 1.00 (s, 9H, $Si(CH_3)_2C(CH_3)_3$), 3.86 (s, 6H, 2 × OCH_3), 7.09 (s, 2H, C_2-*H* and C_6-*H*), 9.82 (s, 1H, C*H*O); ^{13}C-NMR (75 MHz, $CDCl_3$) δ −4.3 ($Si(CH_3)_2C(CH_3)_3$), 19.0 ($Si(CH_3)_2C(CH_3)_3$), 25.9 ($Si(CH_3)_2C(CH_3)_3$), 56.0 (OCH_3), 106.9 (C_2 and C_6), 129.5(C_1), 140.8(C_4), 152.2 (C_3 and C_5), 191.3 (*C*HO); MS: m/z 297 [$M+H^+$].

General procedures for the formation of dendrimers 1 and 2

The dendrimers **1** and **2** were synthesized using the previously reported methods [15].

Dendrimer 1

Yield: 63%; R_f: 0.33 (acetone); ^{1}H-NMR(300 MHz, CD_3COCD_3) δ 2.49 (s, 6H, 3 × core N-CH_2-CH_2-N-), 2.63 (s, 6H, 3 × core N-CH_2-CH_2-N-), 3.41 (s, 12H, 6 × Ph-CH_2-N-), 3.77 (s, 36H, 12 × OCH_3), 5.4 (s, 6H, 6 × O*H*), 6.65 (s, 12H, 6 × Ph C_2-*H* and C_6-*H*); ^{13}C-NMR (75 MHz, CD_3COCD_3) 50.9 (3 × core N-CH_2-CH_2-N-), 52.7 (3 × core N-CH_2-CH_2-N-), 56.4 (12 × OCH_3), 59.0 (6 × Ph-CH_2-N-), 106.5 (6 × Ph C_2 and C_6-H), 130.5 (6 × Ph C_1-CH_2-), 135.4 (6 × Ph C_4-OH), 148.4 (6 × Ph C_3 and C_5-OCH_3); MS: m/z 1143.5 [$M+H^+$].

Dendrimer 2

Yield: 51%; R_f = 0.21 (acetone); ^{1}H-NMR (300 MHz, CD_3COCD_3) δ 2.46 (t, J = 5.7 Hz, 6H, 3 × core N-CH_2-CH_2-N-), 2.57 (t, J = 5.4 Hz, 6H, 3 × core N-CH_2-CH_2-N-), 3.42 (s,

12H, 6 × Ph-CH_2-N-), 3.79 (s, 18H, 6 × OCH_3), 6.75 (s, 12H, 6 × Ph C_2-*H* and C_6-*H*), 6.95 (s, 6H, 6 × Ph C_5-*H*); ^{13}C-NMR (75 MHz, CD_3COCD_3) δ 51.3 (3 × core N-*C*H_2-CH_2-N-), 53.1 (3 × core N-CH_2-*C*H_2-N-), 56.1 (6 × O*C*H_3), 58.8 (6 × Ph-*C*H_2-N-), 112.9 (6 × Ph C_2-H), 115.3 (6 × Ph C_5-H), 122.1 (6 × Ph C_6-H), 131.6 (6 × Ph C_1-CH_2), 146.3 (6 × Ph C_4-OH), 148.1 (6 × Ph C_3-OCH_3); MS: m/z 963.2 $[M+H]^+$.

DPPH assay

The reduction of DPPH radical was determined for dendrimers, quercetin, syringaldehyde, and vanillin as previously reported [16]. The DPPH reagent and antioxidants were dissolved in methanol. Antioxidant sample (10 μL) was added to 1.0 mL of reagent and the absorbance was monitored at 515 nm until a plateau was reached. All samples were run in triplicates at room temperature. The within-run coefficient of variation of the % inhibition values was less than 6%.

LDL oxidation-electrophoresis

Low-density lipoprotein was incubated with 20 mM AAPH in PBS and increasing concentration of antioxidant (0–37 μM, made in methanol) at 37 °C for 21 h. The mixture was then subjected to electrophoresis on 1% agarose gels (Helena Labs, Beaumont, TX) using the Ciba Corning Clinical Electrophoresis System. The gels were stained with Fat Red 7B.

Enzyme inactivation

Lysozyme (1.8 mg/mL) was incubated with 20 mM AAPH in PBS and 80 μM antioxidant (made in methanol) at 37 °C for 24 h. Appropriate control with solvent (methanol) instead of antioxidant was also run. After incubation, the sample was diluted with PBS and assayed for lysozyme activity. Lysozyme assays were carried out at room temperature (25 °C) by monitoring the loss of apparent absorbance at 450 nm, which results from the addition of lysozyme to a suspension of lyophilized Micrococcus lysodeikticus in 67 mM phosphate buffer (pH 6.2). Coefficient of variation for within-run % enzyme activity was $< 8\%$.

Antioxidant effects on DNA

DNA electrophoresis was performed on pBR 322 after 4 h incubation at 37 °C in the presence of 10 mM AAPH in PBS and various concentrations (0–45 μM) of antioxidants (made in methanol).

Pro-oxidant effects on DNA

DNA (pBR 322) was incubated with 10 μM $CuCl_2$ in the presence of 0–45 μM antioxidants (made in methanol) at 37 °C for 1 h. DNA damage was monitored by agarose electrophoresis.

Cell culture

Cytotoxic effects of dendrimer **1** and **2** were assessed using 3-(4,5-di-methylthizol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Chinese hamster ovary (CHO-K1) cells were cultured in F-12K medium supplemented with 10% fetal bovine serum. The cells (3×10^5/mL) were seeded in 100 μL volumes in 96 well culture plates and incubated with the solvent control (DMSO, 0.3%), dendrimer **1** or **2** at 3.1, 6.2, 12.5, 25, or 50 μM for 1, 3, or 5 days. Two hours prior to experimental termination, 20 μL of a 5 mg/mL MTT solution in 0.01M PBS was added to each well. Plates were centrifuged at 450 × g for 10 min and supernatant was removed. The resultant formazan crystals were dissolved in 100 μL DMSO and mixed. Absorbance was measured using a Biolog microplate reader (Biotek Instruments) at dual wavelengths, 590 and 650 nm. Percent control response was calculated as follows:

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(absorbance of treatment/absorbance of control) × 100. All experiments were performed in triplicate and repeated. The data were analyzed using Systat 12 for windows. Multiple groups were compared using a one-way analysis of variance (ANOVA) and a Tukey test for mean separation. A p value <0.05 was considered statistically significant.

Results and Discussion

Since the dendrimer has interior and exterior layers, which can be appropriately manipulated to produce a nanomolecule with various desirable properties, the dendritic architecture is ideal for the design of a nanoantioxidant that is able to chelate metal ions as well as scavenge free radicals. Dendrimers such as poly(amidoamine) (PAMAM) and poly (propylene imine) (PPI) are known to encapsulate metal ions such as Cu^{2+} and Fe^{3+}. If the exterior layer of the dendrimer does not bind metal ions, then most of the bound metal ions are found in the interior of the dendrimer attached to its tertiary amines [17]. In the case of our antioxidants **1–3**, such a binding can occur with the tertiary amines located in the core since TREN is well known for its metal ligating abilities and compounds derived from TREN have been reported to bind cobalt, nickel, copper, lanthanides and actinides [18]. Unlike quercetin with a catechol group that is known to bind metal ions such as Cu^{2+} [19], the surface groups of **1** and **2** lacking the catechol moiety is not expected to bind metal ions favorably. In addition to the metal chelating capability of the TREN core in compounds **1–2**, six phenol rings, either derived from syringaldehyde or vanillin, on the exterior of the antioxidant dendrimers form a potent radical scavenging layer. The presence of multiple phenolic hydroxyl groups, benzylic hydrogens, and electron donating substituents (on the benzene rings) should contribute favorably towards the radical quenching property of these antioxidants.

Syntheses of compounds **1** and **2** were initiated by protecting building blocks (syringaldehyde and vanillin) with TBDMS-Cl (Scheme 1). The protected building blocks were then attached to the amino groups of TREN via their aldehyde groups. The three resulting unstable imine bonds were subsequently reduced with $Na(OAc)_3BH$ to form secondary amines which further react with additional aldehyde groups, eventually forming tertiary amines after reduction by $Na(OAc)_3BH$. Unlike other amine alkylation using alkyl halide (which usually produce a mixture of species ranging from primary amine to quaternary ammonium), the reaction between amine and aldehyde can only form a tertiary amine as an end product. Hence, it is an attractive method to form branches in a controlled manner, producing a well-defined dendritic structure. Although $Na(OAc)_3BH$ has been reported to be an efficient reductant for imine and iminium bonds [20], we found that its selective reduction of these groups over aldehyde was poor. The arylaldehydes reduced to their corresponding alcohols fairly rapidly when all reagents were mixed together in one pot. To avoid this problem, three equivalents of TBDMS-protected building block was first reacted with one equivalent of TREN core and the reaction was allowed sufficient time (> 5 h) before addition of $Na(OAc)_3BH$. This was to ensure that the aldehyde reacted with three amino groups first with limited exposure to the reducing agent. After $Na(OAc)_3BH$ addition, the reaction was run for at least 24 h before another three equivalents of building block was added. Based on our experience, $Na(OAc)_3BH$ worked more efficiently in 1,2-dichloroethane than other solvents like CH_2Cl_2. Protection of syringaldehyde and vanillin with TBDMS-Cl enhanced percent yield by increasing solubility of the dendrimers **1** and **2** in 1,2-dichloroethane. Both TBDMS-dendrimer **1** and **2** have much better solubility than the corresponding dendrimers in the solvent. Although the water by-product cleaved some of the TBDMS protecting groups, the target compounds with varying number of TBDMS groups remained soluble during the reaction. Prior to column purification, the protecting groups were cleaved with *n*-tetrabutyl ammonium fluoride in ethanol, affording the antioxidant dendrimers **1** and **2**.

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Polyacrylamide gel electrophoresis (PAGE) is a useful tool for characterization of dendrimers [21]. Like proteins, these low-polydispersity nanoparticles show bands in polyacrylamide gels. The antioxidant dendrimers **1–3** were run under acidic conditions on a 15% PAGE gel and stained with Coomassie Blue (Figure 2). Each lane contained 10 nmol of antioxidant. All antioxidants produced well-stained bands. For comparison, a generation 4 PAMAM dendrimer with amine surface is shown in lane 1. Dendrimer **2** (lane 2) showed the highest mobility towards the cathode followed by **3** (lane 3) and **1** (lane 4). In addition to the major band, dendrimer **1** also showed a broad minor band with lower mobility while **3** displayed a less intense band with higher mobility. These minor bands probably represent defective target compounds (e.g., species with missing arms).

The free-radical scavenging ability of the antioxidants was evaluated by the DPPH assay [16]. The two TREN-based dendrimers, **1** and **2**, showed similar potency (IC_{50} mean of three experiments = 2 μM; SD = 1 μM at 120 min). Within experimental error, these values were also similar to the previously reported syringaldehyde-based 4-aminomethylbenzylamine core dendrimer **3** (IC_{50} mean = 4 μM; SD = 0.9 μM at 120 min). Under identical experimental conditions, average IC_{50} value for quercetin was 10 μM (SD = 0.2 μM) at 120 min, Trolox was 30 μM (SD = 0.2 μM) at 120 min while the starting materials used for syntheses of the antioxidants displayed negligible DPPH activity (IC_{50} values of syringaldehyde and vanillin were >100 μM).

The ability of the antioxidant dendrimers to protect lipids, protein, and DNA from free radical damage was evaluated. For LDL, AAPH was used instead of Cu^{2+} to induce free radical damage since the latter may cause pro-oxidant interference [22,23]. Oxidation of human LDL was used as a model for evaluating the ability of the G1 dendrimers as chain-breaking antioxidants for lipid. LDL oxidation leads to changes in both the lipid and the protein moieties of the lipoprotein particle. Lipid peroxidation in LDL has been evaluated by a variety of methods. Due to the complexity of events that occur in lipoprotein oxidation, the electrophoretic migration pattern of oxidized LDL is one of the best methods to evaluate the extent of lipoprotein oxidation [24]. LDL was incubated with 20 mM AAPH and increasing concentrations of the G1 dendrimers at 37 °C for 21 h. The agarose gel obtained with dendrimer **1** is shown in Figure 3.

Under these conditions, oxidized LDL (lane 2, Figure 3A), unlike native lipoprotein (lane 1), showed a smear, characteristic of heterogeneous species formed as a result of lipoprotein oxidation. The lipoprotein was fully protected against free radical damage at 18.5 μM and 37 μM of dendrimer **1** (lanes 3 and 4, Figure 3A). Lower concentrations of dendrimer **1** (lanes 5–8, Figure 3A) were ineffective. Under similar conditions, dendrimers **2** and **3** were slightly less effective. They showed a sharp LDL band at 37 μM but not at 18.5 μM (data not shown). In comparison, quercetin, Trolox and the starting materials were ineffective at all antioxidant concentrations (1–37 μM) (lanes 3–8, Figure 3B).

Protective effect of the dendritic antioxidants on protein was determined using hen egg white lysozyme as a model protein. The enzyme was incubated with 20 mM AAPH and 80 μM antioxidant at 37 °C for 24 h. Both dendrimers **1** and **2** completely protected the enzyme (100% recovery), while dendrimer **3**, quercetin and Trolox gave 54%, 34% and 12% recovery, respectively. All starting materials gave <10% recovery. Enzyme recovery decreased with lower antioxidant concentrations. At 40 μM antioxidant concentration, both dendrimers **1** and **2** gave 70% recovery, while dendrimer **3**, quercetin and Trolox gave 50%, 19% and 10% recovery, respectively. Lower antioxidant amounts gave even lower recoveries. For example, dendrimer **1** gave recoveries of 40%, 16%, 13% at antioxidant concentrations of 20 μM, 10 μM, and 5 μM, respectively (control without antioxidant gave 7% activity).

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Protection of DNA from free radical damage by the antioxidant dendrimers was also evaluated. Plasmid DNA (pBR 322) samples were incubated with AAPH (final concentration, 10 mM) at 37 °C for 4 h with antioxidants (final concentrations, 0.35–45 μM) and subjected to agarose electrophoresis [25]. A gel obtained with dendrimer **1** is shown in Figure 4. The native DNA was mostly in its supercoiled (S) form (small amount of open circular, O, form was also visible, lane 1, Figure 4). In the presence of AAPH, the DNA was transformed almost entirely into its O form (lane 2, Figure 4). In the presence of 11–45 μM of dendrimer **1**, DNA was well protected; the S band intensity was similar to control containing no AAPH (lanes 3–5, Figure 4). Quercetin as well as dendrimers **2** and **3** also displayed similar gel patterns to dendrimer **1** (data not shown). Some protection was also obtained for the three dendrimers at 5 μM as shown by traces of supercoiled DNA (lane 6, Figure 4) but not with quercetin (data not shown). Negligible DNA protection was afforded by all dendrimers at < 3 μM (lanes 7–10, Figure 4). Under similar conditions, Trolox as well as the starting materials for dendrimer synthesis were ineffective at all concentrations.

All of the above antioxidant activity tests clearly indicate that the antioxidant dendrimers are effective radical scavenging agents. This may be attributed to the presence of multiple phenolic hydroxyl groups, benzylic hydrogens, and electron donating substituents in these compounds.

The pro-oxidant property of antioxidants, such as quercetin, in the presence of transition metals ions (e.g., Cu^{2+} and Fe^{3+}) has been associated with their mutagenic and carcinogenic health hazard [7,19]. This is a serious drawback for their potential clinical applications. Since potent antioxidants often possess strong pro-oxidant activity, it was important for us to examine the pro-oxidant behaviors of our dendritic antioxidants. This was performed by co-incubation of DNA (pBR 322), copper (II) ion and antioxidant. It is believed that an antioxidant such as quercetin which displays strong pro-oxidant activity binds to Cu (II)-DNA complex through its catechol and forms a DNA-Cu(II)-quercetin complex. This ultimately forms DNA-Cu(I)OOH, initiating a free radical cascade by which DNA damage occurs [26]. pBR 322 was incubated with 10 μM Cu^{2+} in the presence of antioxidants at 37 °C for 1 h. Figure 5 shows an agarose gel obtained with dendrimer **1** (A) and quercetin (B). No DNA damage was obtained at all concentrations of dendrimer **1** between 0.35–45 μM. Dendrimer **2** also gave similar gel patterns (data not shown). As previously reported, DNA damage was clearly observed with dendrimer **3**, quercetin (Fig. 5B) and Trolox at concentrations between 11–45 μM [15]. The starting materials did not show any pro-oxidant effect on DNA under these conditions. These results suggest that the lack of pro-oxidant effects displayed by the two TREN-based dendrimers may be due to chelation of copper ions by the tertiary amines in the core. At high copper concentrations (e.g. 60 μM), the O-form of DNA was also observed with the TREN dendrimers (data not shown). This suggests that the metal chelating property of the dendrimers was likely overwhelmed at higher copper concentrations resulting in pro-oxidative effects and DNA damage. However, it should be noted that physiological concentrations of copper are less than 10–20 μM in plasma and approximately 10^{-18} M for the cation in cells [27]. Therefore, chelation of even low amounts of cation can markedly affect free radical formation, especially in the chromatin region where one copper ion is present per kilobase [28].

Pro-oxidant effect of our novel antioxidant dendrimers was also evaluated with Fe^{3+}. DNA was incubated at 37 °C for 1 h in the presence of physiological concentration of Fe^{3+} (30 μM) and antioxidant **1** (45 μM) or quercetin (45 μM). Neither the dendritic antioxidant **1** nor quercetin showed any pro-oxidant behavior (similar to lane 3, Figure 5A, data not shown). We also wanted to see if there was any deleterious interaction of our dendrimers with non-transition metal ions, such as Zn^{2+} that is present in significant amount in plasma (~12 μM),

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under similar conditions. Both dendrimer **1** and quercetin at 45 μM had no effect on DNA in the presence of 12 μM Zn^{2+} (similar to lane 3, Figure 5A, data not shown).

Preliminary data on cell toxicity of dendrimers **1** and **2** were obtained with Chinese hamster ovary (CHO) cells using 3-(4,5-di-methylthizol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) [29]. Incubation of the CHO cells (3×10^5/mL) in the presence of 3.1 – 50 μM of antioxidants did not show any reduction in cell viability over 5 days (Figure 7).

Conclusion

We have successfully demonstrated the concept of designing dendrimers with free radical scavenging as well as metal chelating properties. The antioxidant dendrimers we synthesized show high radical scavenging activity and strong protective effects on biomolecules (lipid, protein and DNA). In general, potent antioxidants also possess strong pro-oxidant activity. The potent TREN-based dendritic antioxidants were however devoid of pro-oxidant activity thus providing a significant biological benefit. Their strong radical scavenging potential and lack of pro-oxidant effects as well as cell toxicity make them promising candidates as therapeutic agents for pathological conditions strongly associated with oxidative stress including asthma, atherosclerosis and neurodegenerative diseases.

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List of abbreviations

AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
$CDCl_3$	Deuterated Chloroform
CD_3COCD_3	Deuterated acetone
CHO-K1	Chinese hamster ovary cells
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPPH	1-Diphenyl-2-picrylhydrazyl
LDL	Low-density lipoprotein
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization-Time of Flight
MTT	3-(4,5-Di-methylthizol-2-yl)-2,5-diphenyltetrazolium bromide
$Na(OAc)_3BH$	Sodium triacetoxyborohydride
$n\text{-}Bu_4NF$	Tetra-butylammonium fluoride
PAGE	Polyacrylamide gel electrophoresis
PAMAM	poly(amidoamine) dendrimer
pBR 322	Plasmid DNA
PBS	Phosphate-buffered saline

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PPI	poly (propylene imine) dendrimer
$Si(CH_3)_4$	Tetramethylsilane
TBDMS-Cl	Tert-butyldimethylsilyl chloride
TREN	Tris(2-aminoethyl)amine

References

1. (a) Deschner EE, Ruperto J, Wong G, Newmark HL. Quercetin and rutin as inhibitors of azoxymethanol-induced colonic neoplasia. Carcinogenesis 1991;12:1193–1196. [PubMed: 2070483] (b) Elangovan V, Sekar N, Govindasamy S. Chemopreventive potential of dietary bioflavonoids against 20-methylcholanthrene-induced tumorigenesis. Cancer Lett 1994;87:107–113. [PubMed: 7954363] (b) Brown JP. A review of genetic effects of naturally occurring flavonoids, anthraquinones and related compounds. Mutat. Res 1980;75:243–277. [PubMed: 6770263]
2. Terao J, Piskula M, Yao Q. Protective effect of epicatechin, epicatechin gallate, and quercetin on lipid peroxidation in phospholipid bilayers. Arch. Biochem. Biophys 1994;308:278–284. [PubMed: 8311465]
3. Rump AF, Schussler M, Acar D, Cordes A, Ratke R, Theisohn M, Rosen R, Klaus W, Fricke U. Effects of different inotropes with antioxidant properties on acute regional myocardial ischemia in isolated rabbit hearts. Gen. Pharmacol 1995;26:603–611. [PubMed: 7789735]
4. (a) Gil B, Sanz MJ, Terencio MC, Ferrándiz ML, Bustos G, Payá M, Gunasegaran R, Alcaraz MJ. Effects of flavonoids on Naja naja and human recombinant synovial phospholipases A2 and inflammatory responses in mice. Life Sci 1994;54:333–338. (b) Middleton E Jr, Kandaswami C. Effects of flavonoids on immune and inflammatory cell functions. Biochem. Pharmacol 1992;43:1167–1179. [PubMed: 1562270]
5. Ferry DR, Smith A, Malkhandi J, Fyfe DW, deTakats PG, Anderson D, Baker J, Kerr DJ. Phase I clinical trial of the flavonoid quercetin: pharmacokinetics and evidence for in vivo tyrosine kinase inhibition. Clin. Cancer Res 1996;2:659–668. [PubMed: 9816216]
6. (a) Rahman A, Fazal F, Greensill J, Ainley K, Parish JH, Hadi SM. Strand scission in DNA induced by dietary flavonoids: role of Cu(I) and oxygen free radicals and biological consequences of scission. Mol. Cell Biochem 1992;111:3–9. [PubMed: 1588940] (b) Sahu SC, Washington MC. Effects of antioxidants on quercetin-induced nuclear DNA damage and lipid peroxidation. Cancer Lett 1991;60:259–264. [PubMed: 1756517]
7. Yamashita N, Tanemura H, Kawanishi S. Mechanism of oxidative DNA damage induced by quercetin in the presence of Cu(II). Mutation Res 1999;425:107–115. [PubMed: 10082921]
8. Rietjens IMCM, Boersma MG, Haan Ld, Spenkelink B, Awad HM, Cnubben NHP, van Zanden JJ, Woude Hvd, Alink GM, Koeman JH. The pro-oxidant chemistry of the natural antioxidants vitamin C, vitamin E, carotenoids and flavonoids. Environ. Toxicol. Pharmacol 2002;11:321–333.
9. (a) Rahman A, Shahabuddin HSM, Parish JH, Ainley K. Strand scission in DNA induced by quercetin and. Cu(II): role of Cu(I) and oxygen free radicals. Carcinogenesis 1989;10:1833–1839. [PubMed: 2791202] (b) Cao G, Sofic E, Prior RL. Antioxidant and prooxidant behaviour of flavonoids:structure-activity relationships. Free Radic. Biol. Med 1997;22:749–760. [PubMed: 9119242] (c) Lebeau J, Furman C, Bernier JL, Duriez P, Teissier E, Cotelle N. Antioxidant properties of di-tert-butylhydroxylated flavonoids. Free Radic. Biol. Med 2000;29:900–912. [PubMed: 11063915]
10. (a) Pirvu L, Nichita C, Giurginca M, Meghea A. Correlation structure-antioxidant activity of some polyphenols of vegetal origin. Revista de Chime 2006;57:699–705. (b) Tripoli E, Guardia ML, Giammanco S, Majo DD, Giammanco M. Citrus flavonoids: Molecular structure, biological activity and nutritional properties: A review. Food Chem 2007;104:466–479.
11. (a) Natella F, Nardini M, Felice MD, Scaccini C. Benzoic and Cinnamic Acid Derivatives as Antioxidants: Structure–Activity Relation. J. Agric. Food Chem 1999;47:1453–1459. [PubMed: 10563998] (b) Rice-Evans CA, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic. Biol. Med 1996;20:933–956. [PubMed: 8743980] (c)

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Cai YZ, Sun M, Xing J, Luo Q, Corke H. Structure–radical scavenging activity relationships of phenolic compounds from traditional Chinese medicinal plants. Life Sci 2006;78:2872–2888. [PubMed: 16325868] (d) Cuvelier ME, Richard H, Berset C. Biosci. Biotech. Biochem 1992;56:324–325.

12. (a) Cuvelier ME, Richard H, Berset C. Comparison of the antioxidative activity of some acid-phenols:structure-activity relationship. Biosci. Biotech. Biochem 1992;56:324–325. (b) Rice-Evans CA, Miller NJ, Paganga G. Antioxidant properties of phenolic compounds. Trends Plant Sci 1997;2:152–159. (c) Shahidi F, Janitha PK, Wanasundara PD. Phenolic antioxidants. Crit. Rev. Food Sci. Nutr 1992;32:67–103. [PubMed: 1290586]
13. De Heer MI, Mulder P, Korth HG, Ingold KU, Lusztyk J. Hydrogen atom abstraction kinetics from intramolecularly hydrogen bonded ubiquinol-0 and other (poly)methoxy phenols. J. Am. Chem. Soc 2000;122:2355–2360.
14. (a) Okada Y, Tanaka K, Sato E, Okajima H. Antioxidant activity of the new thiosulfinate derivative, *S*-benzylphenylmethanethiosulfinate, from *Petiveria alliacea* L. Org. Biomol. Chem 2008;6:1097–1102. [PubMed: 18327337] (b) Hu C, Yuan YV, Kitts DD. Antioxidant activities of the flaxseed lignan secoisolariciresinol diglucoside, its aglycone secoisolariciresinol and the mammalian lignans enterodiol and enterolactone in vitro. Food Chem. Toxicol 2007;5:2219–2227. [PubMed: 17624649] (c) Mendiara SN, Coronel MEJ. Evidence for the development of persistent carbon-centered radicals from a benzyl phenolic antioxidant: an electron paramagnetic resonance study. Appl. Magn. Reson 2006;30:103–120. (d) Nishiyama T, Sakita K, Fuchigami T, Fukui T. Antioxidant activities of fused heterocyclic compounds, xanthene-2,7-diols with BHT or catechol skeleton. Polym. Degrad. Stab 1998;62:529–534.
15. Lee CY, Sharma A, Cheong JE, Nelson J. Synthesis and antioxidant properties of dendritic polyphenols. Bioorg. Med. Chem. Lett 2009;19:6326–6330. [PubMed: 19822425]
16. Brand-Williams W, Cuvelier ME, Berset C. Use of free radical method to evaluate antioxidant activity. Lebensm. -Wiss. U.-Technol 1995;28:25–30.
17. (a) Balogh L, Tomalia DA. Poly(amidoamine) dendrimer-templated nanocomposites. 1. Synthesis of zerovalent copper nanoclusters. J. Am. Chem. Soc 1998;120:7355–7356. (b) Niu Y, Crooks RM. Dendrimer-encapsulated metal nanoparticles and their applications to catalysis. C.R. Chimie 2003;6:1049–1059.
18. Cregg, PJ., editor. A practical guide to supramolecular chemistry. John Wiley and Sons; 2005. p. 19-21.
19. Lebeau J, Furman C, Bernier JL, Duriez P, Teissier E, Cotelle N. Antioxidant properties of di-tert-butylhydroxylated flavonoids. Free Radic. Biol. Med 2000;29:900–912. [PubMed: 11063915]
20. Abdel-Magid AF, Carson KG, Harris BD, Maryanoff CA, Shah RD. Reductive amination of aldehydes and ketones with sodium triacetoxyborohydride. Studies on direct and indirect reductive amination procedures. J. Org. Chem 1996;61:3849–3862. [PubMed: 11667239]
21. Sharma A, Desai A, Ali R, Tomalia D. Polyacrylamide gel electrophoresis separation and detection of polyamidoamine dendrimers possessing various cores and terminal groups. J. Chromatography A 2005;1081:238–244.
22. Salah N, Miller NJ, Paganga G, Tijburg L, Bolwell GP, Rice-Evans CA. Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. Arch. Biochem. Biophys 1995;322:339–346. [PubMed: 7574706]
23. All solutions were made in ultra-pure water.
24. Foxx KK, Roberts RL, Waxdal MJ. Kalen Biomedical Technical Note 2: Effect of copper ion concentration on the oxidation of human LDL. Available: http://www.kalenbiomed.com/50tech_info.php via the INTERNET.
25. Sakihama Y, Cohen MF, Grace SC, Yamasaki H. Plant phenolic antioxidant and prooxidant activities: phenolics-induced oxidative damage mediated by metals in plants. Toxicol 2002;177:67–80.
26. Yamashita N, Tanemura H, Kawanishi S. Mechanism of oxidative DNA damage induced by quercetin in the presence of Cu(II). Mutat. Res 1999;425:107–115. [PubMed: 10082921]
27. Babaeva EE, Vorobyova UA, Zharkova MS, Cheknyov SB. *Human serum* γ-globulin binds copper cations. Bull. Exp. Biol. Med 2006;141:59–62.

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28. Drouin R, Rodriquez H, Gao SW, Gebreyes Z, O'Connor TR, Holmquist GP. Cupric ion/ascorbate/hydrogen peroxide-induced DNA damage: DNA-bound copper ion primarily induces base modifications. Free Radic. Biol. Med 1996;21:261–273. [PubMed: 8855437]

29. Pestka JJ, Uzarski RL, Islam Z. Induction of apoptosis and cytokine production in the Jurkat human T cells by deoxynivalenol: Role of mitogen-activated protein kinases and comparison to other 8-ketotrichothecenes. Toxicol 2005;206:207–219.

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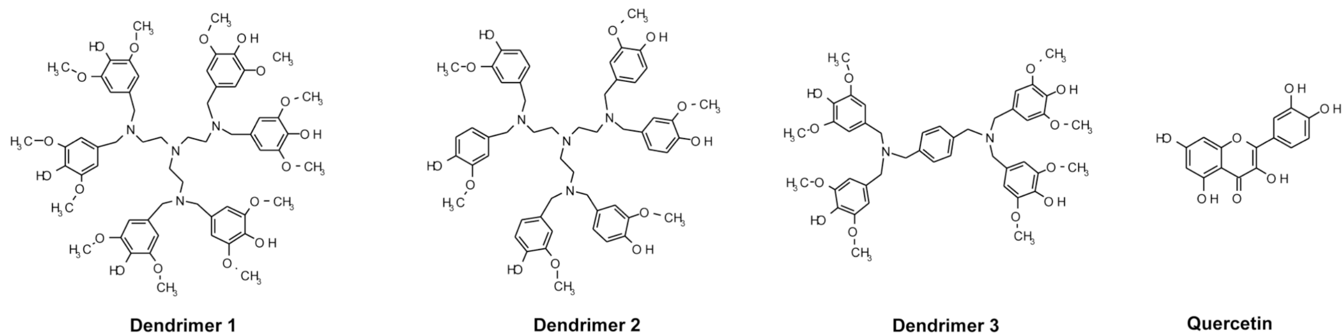

Figure 1.

Structures of dendrimers **1–3** and quercetin.

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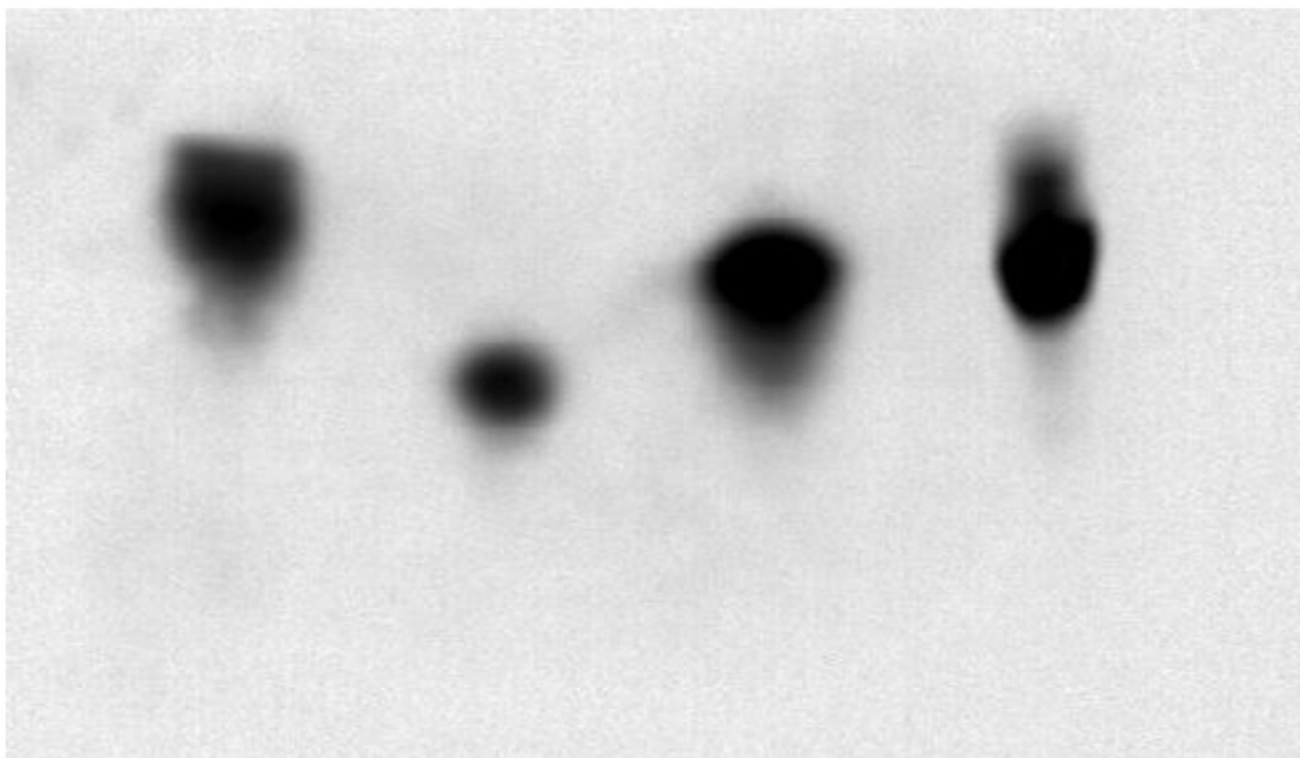

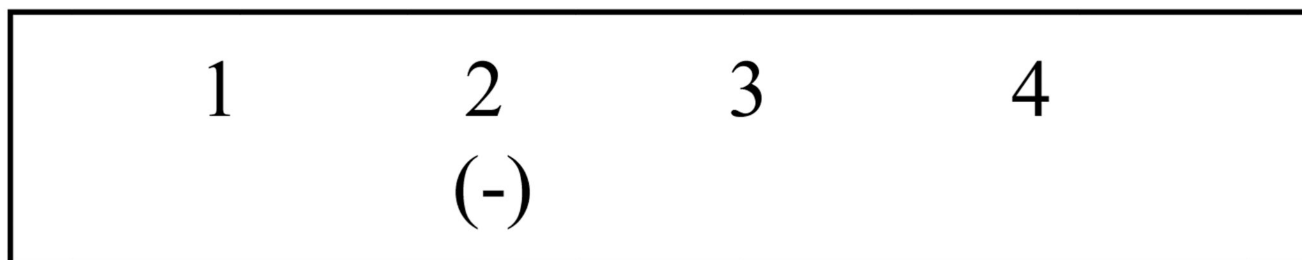

Figure 2.
Electrophoresis of antioxidant dendrimers.
Lane 1 (G4 PAMAM); lane 2 (dendrimer **2**); lane 3 (dendrimer **3**); lane 4 (dendrimer **1**).
Each lane contains 10 nmol dendrimer.

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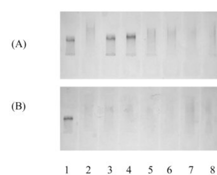

Figure 3.
Effect of antioxidants on LDL oxidation.
(A) Lane 1 (native LDL); lanes 2–8 (AAPH-oxidized LDL with 0, 37, 18.5, 9, 4.5, 2 and 1 μM dendrimer **1**, respectively). (B) Lane 1 (native LDL); lanes 2–8 (AAPH-oxidized LDL with 0, 37, 18.5, 9, 4.5, 2 and 1 μM quercetin, respectively).

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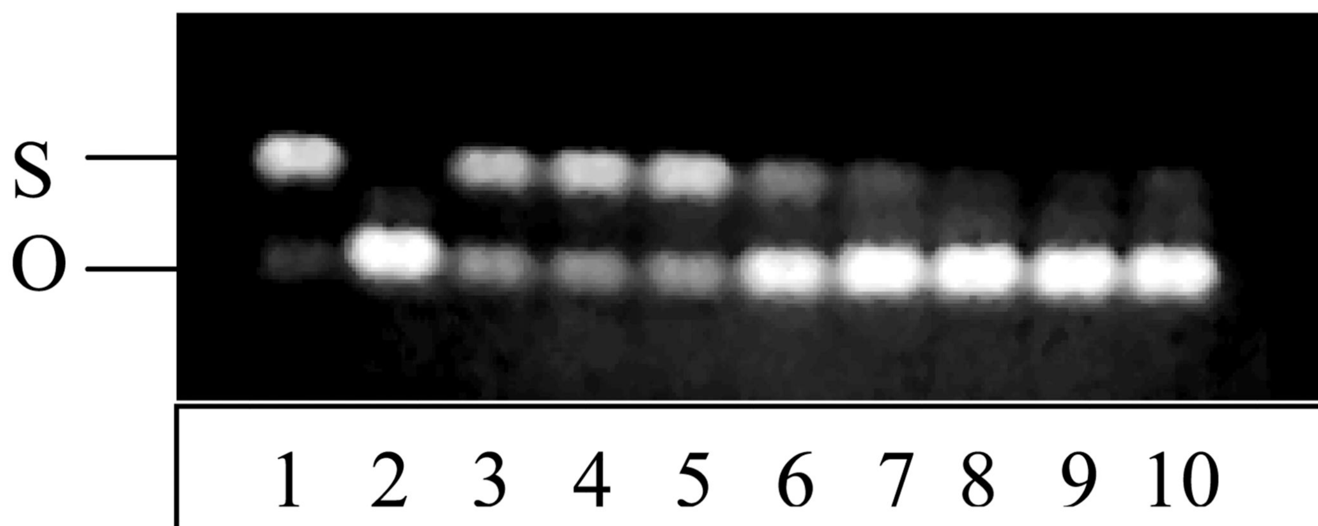

Figure 4.
Protection against AAPH-induced DNA oxidation by dendrimer **1**.
Lane 1 (native DNA); lanes 2–10 (AAPH-oxidized DNA with 0, 45, 23, 11, 5, 3, 1.5, 0.7, and 0.35 μM antioxidant, respectively).

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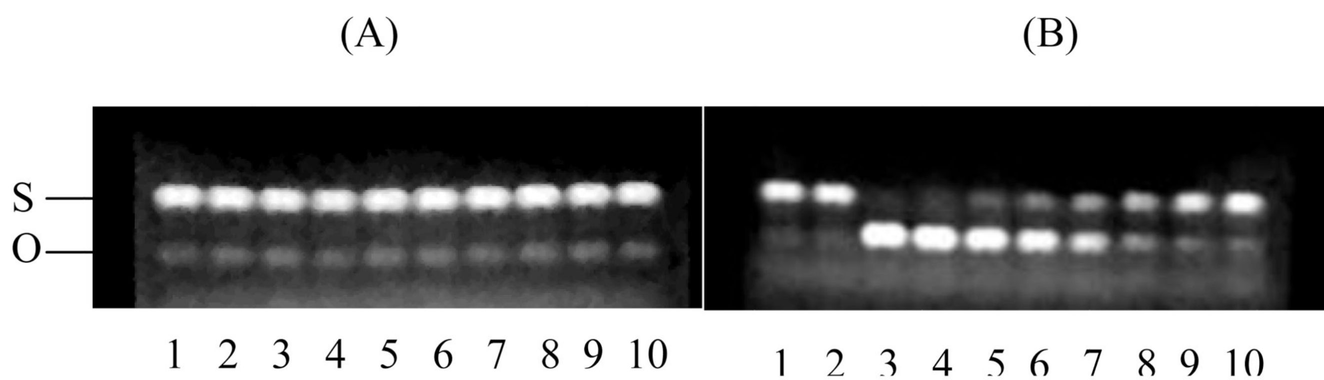

Figure 5.
Pro-oxidant effect of dendrimer **1** (A) and quercetin (B) on DNA (pBR 322). Lane 1 (native DNA); lanes 2–10 (Cu^{2+} oxidized DNA with 0, 45, 23, 11, 5, 3, 1.5, 0.7, and 0.35 μM antioxidant, respectively).

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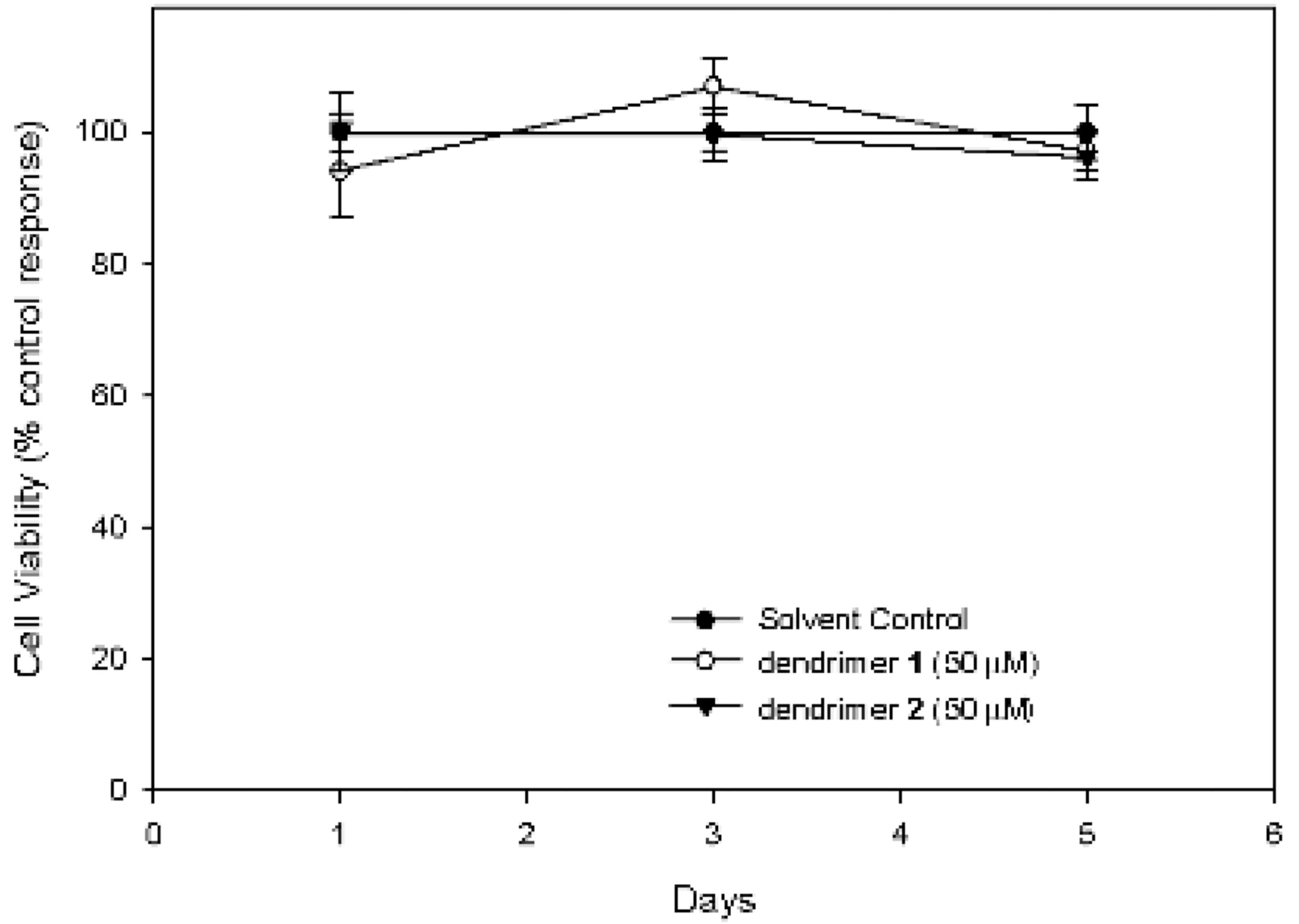

Figure 6.
Comparative effects of dendrimers **1** and **2** on CHO cell viability. CHO cells were incubated with DMSO control, dendrimer **1** or dendrimer **2** at 50 μM for 1, 3, and 5 days.

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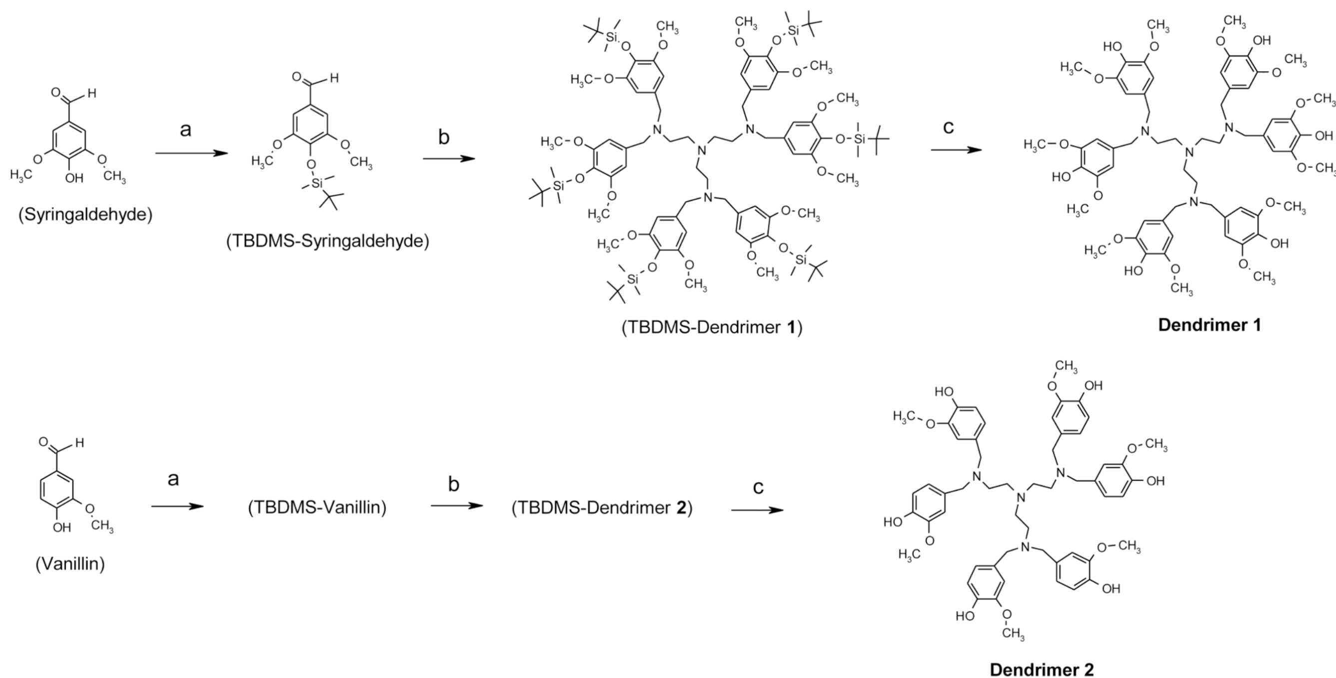

Scheme 1.

Syntheses of dendrimers **1** and **2**.

Reagents and conditions: (a) TBDMS-Cl, triethylamine, CH_2Cl_2, 0 °C (b) TREN, $Na(OAc)_3BH$, 1,2-dichloroethane (c) n-Bu_4NF, ethanol.