JOURNAL OF NATURAL PRODUCTS

Article

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Flavonoids, Flavonoid Metabolites, and Phenolic Acids Inhibit Oxidative Stress in the Neuronal Cell Line HT-22 Monitored by ECIS and MTT Assay: A Comparative Study

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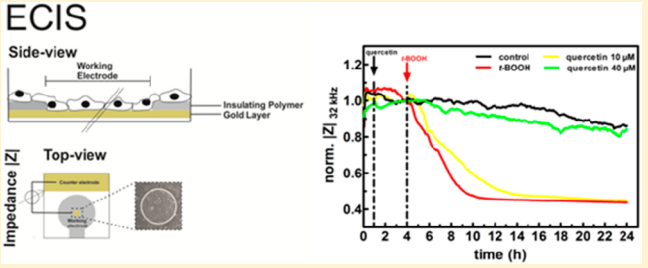
Supporting Information

ABSTRACT: A real-time and label-free in vitro assay based on electric cell-substrate impedance sensing (ECIS) was established, validated, and compared to an end-point MTT assay within an experimental trial addressing the cytoprotective effects of 19 different flavonoids, flavonoid metabolites, and phenolic acids and their methyl esters on the HT-22 neuronal cell line, after induction of oxidative stress with *tert*-butyl hydroperoxide. Among the flavonoids under study, only those with a catechol unit and an additional 4-keto group provided cytoprotection. The presence of a 2,3-double bond was not a structural prerequisite for a neuroprotective effect. In the case of the phenolics, catechol substitution was the only structural requirement for activity. The flavonoids and other phenolics with a ferulic acid substitution or a single hydroxy group showed no activity. Electrochemical characterization of all compounds via square-wave voltammetry provided a rather specific correlation between cytoprotective activity and redox potential for the active flavonoids, but not for the active phenolics with a low molecular weight. Moreover this study was used to compare label-free ECIS recordings with results of the established MTT assay. Whereas the former provides time-resolved and thus entirely unbiased information on changes of cell morphology that are unequivocally associated with cell death, the latter requires predefined exposure times and a strict causality between metabolic activity and cell death. However, MTT assays are based on standard lab equipment and provide a more economic way to higher throughput.



Imbued by the dramatically increasing incidence of Alzheimer's disease (AD) worldwide, the search for compounds with neuroprotective activity has been enhanced in the last years. As AD is correlated with neuronal damage in different brain areas and is still incurable, protection of neuronal cells by inhibition or retardation of the destruction process is a promising therapeutic concept.[1]

Limited defense strategies against oxidative stress play an important role in neuronal damage and the generation of AD.[2] In vitro assays to evaluate the neuroprotective effects of anti-AD drug candidates are therefore often based on studying the impairment of neuronal cell lines after exposure to different stress factors such as radicals, glutamate, and amyloid-β peptide.[3−5] The viability of the damaged cells is frequently quantified by well-established assays based on tetrazolium dyes (MTT, XTT, or WST-1) or lactate dehydrogenase release (LDH) and compared with an untreated control.[5−8] The decrease of cell viability caused by the toxic agents is reversed by the test compounds, resulting in an increase in cell viability after co-incubation of the noxious agent and the test compound.

Over the last twenty years, results from several in vitro and in vivo studies indicated that plant phenols are an important class of defense antioxidants, showing cytoprotective effects for different cell types.[9,10] Cumulative evidence has shown that phenols can act as radical scavengers, antioxidants, and metal-chelating compounds.[11,12] Furthermore, their cytoprotective effects may be mediated via direct interference with signaling cascades, the enhanced expression of enzymes with antioxidant activity including catalase and glutathione reductase, or other cellular defense strategies such as the GSH/GSSG ratio.[13] Phenols are widespread in fruits and vegetables and include mainly flavonoids, phenolic acids, and tannins. Whereas various in vitro and in vivo studies document protective effects of different flavonoids on neuronal cells,[5,14,15] data for the neuroprotective activity of procyanidins, flavonoid metabolites such as phase-II conjugates or C-ring cleavage products, and

Special Issue: Special Issue in Honor of Otto Sticher

Received: June 27, 2013

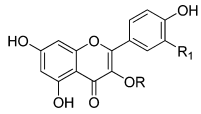
dx.doi.org/10.1021/np400518k | *J. Nat. Prod.* XXXX, XXX, XXX−XXX

phenolic acids and their esters are scarce. Furthermore, comprehensive investigations addressing structural requirements of flavonoids and related phenolic compounds to become neuroprotective are available only for individual stress factors such as glutamate.[4,5]

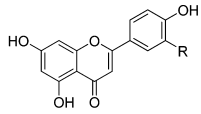
Monitoring neuroprotection assays in real time using label-free readout technologies offers many methodological advantages over traditional end-point assays, since the former provide continuous information over the entire length of the experiment. Moreover, the independence of labels allows for cellular or molecular follow-up experiments with the same cell population. Among the different types of label-free approaches, impedance analysis is the most well developed. Impedance-based cellular assays were described initially by Giaever and Keese[16,17] and are now referred to as electric cell-substrate impedance sensing (ECIS). The technique is based on growing the cells of interest on a pair of gold-film electrodes (the working and the counter electrode), which are deposited on the bottom of a cell culture dish.[16−18] The cell culture medium serves as an electrolyte and provides the electrical connection between the electrodes. During measurement, the impedance of the cell-covered electrodes is recorded continuously at a predefined set of AC frequencies. The cell bodies, surrounded by their dielectric plasma membranes, behave like insulating particles, forcing the current to flow through the small electrolyte-filled channels beneath and between the cells at most frequencies. This restriction of current flow within these channels creates extra impedance that is dependent on the three-dimensional shape of the cells on the electrode surface. If the cells change their morphology, this alters the geometry of the channels and consequently impedance readings. Thus, ECIS reports on cell shape changes when the cells are exposed to biological, chemical, or physical stimuli with a resolution that is well below optical microscopy.[17,19] As adherent cells tend to swell and eventually lose the insulating properties of their plasma membranes during necrosis, whereas they shrink and round up during apoptosis, both forms of cell death are sensitively monitored by ECIS recordings. Membrane rupture during necrosis allows the current to flow rather unimpeded through the cells, whereas apoptotic cell shrinkage provides open spaces between cells. In either case, a severe decrease of impedance down to values of a cell-free electrode is observed.[52]

In this study, ECIS was applied to monitor continuously the neuronal cell line HT-22 upon exposure to flavonoids, flavonoid metabolites, and phenolic acids and their methyl esters (**1**−**19**) after induction of oxidative stress with *tert*-butyl hydroperoxide (*t*-BOOH). The organic hydroperoxide, *t*-BOOH, is able to induce oxidative stress in cells via the generation of alkoxyl and peroxyl radicals that accelerate lipid peroxidation.[20,21] This is associated with several intracellular stress factors such as increased ROS, decreased GSH levels, and DNA strand breaks, as observed for C6 glial cells.[22] The ECIS assay was run for 24 h, and a traditional MTT assay was performed for all compounds at the end of the exposure time.

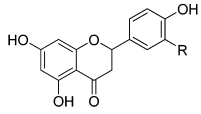
Besides their biological activity, all test compounds (**1**−**19**) were studied with respect to their electrochemical redox behavior using square-wave voltammetry (SWV). SWV is a pulsed voltammetric measuring technique that allows for a highly sensitive quantification of redox-active flavonoids and the determination of their individual oxidation potentials.[23−25] So far, several correlations have been established in the literature for flavonoids between their redox potentials and different biological activities, such as antioxidative processes and mitochondrial enzyme inhibition,[26] the inhibition of lipid peroxidation in rat liver microsomes,[27] and a series of spectrometric cell-free antioxidant capacity assays[28] such as the DPPH and TEAC assays, using cyclic voltammetry for the electrochemical measurements. In contrast to cyclic voltammetry and differential pulse voltammetry (DPV), which have also been reported in the literature, SWV allows very short analysis times and is often more sensitive (e.g., in the case of fast electron transfer rates) than DPV. In addition, undesired adsorption effects of analyte molecules at the electrode surface can be diminished.[23] Comparative SWV measurements of the phenolic compounds studied in the present investigation were carried out by the use of screen-printed carbon electrodes. It has been reported that flavonoids with a low positive oxidation potential possess a high radical-scavenging activity, i.e., representing higher receptiveness to electrochemical oxidation.[27,29] It was the aim of the present work to identify putative correlations between the oxidation potentials obtained in SWV experiments and the antioxidative, neuroprotective effects on HT-22 cells for compounds **1**−**19**.



1 R = H; R_1 = OH
2 R = H, R_1 = H
3 R = H, R_1 = OCH_3
4 R = glucuronyl, R_1 = OH



5 R = H
6 R = OH



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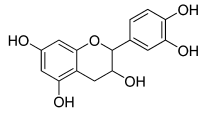
8 R = OH



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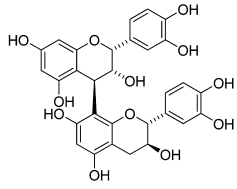

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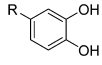
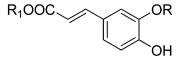
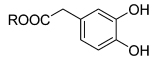


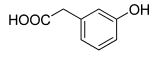
11 R = H
12 R = CH_3



13 R = R_1 = H
14 R = H, R_1 = CH_3
15 R = CH_3, R_1 = H
16 R = R_1 = CH_3



17 R = H
18 R = CH_3



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RESULTS AND DISCUSSION

Addition of *t*-BOOH is a well accepted in vitro model to mimic oxidative stress in neuronal cells.[22,38,39] In recent studies, several different neuronal cell lines, for example, PC-12 (rat pheo-chromocytoma),[38] C6 (rat glial),[22] and HT-22 (mouse hippocampal) neuronal cells,[39] have been exposed to individual concentrations of *t*-BOOH. Thus, the optimal *t*-BOOH concentration was initially determined to induce oxidative stress in HT-22 cells by monitoring the dose-dependent impairment of cell viability via the MTT assay and time-resolved ECIS recordings (Figure 1). Concordantly, both assays showed only a partial reduction of HT-22 viability at

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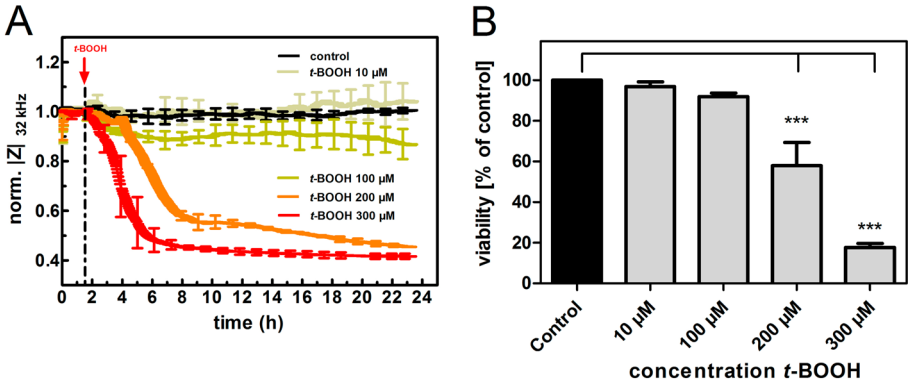

Figure 1. Dose-dependent neurotoxicity of *t*-BOOH on HT-22 cells monitored by (A) time-resolved ECIS readings and (B) MTT end-point assays after 24 h of incubation. (A) The red arrow indicates addition of *t*-BOOH at different concentrations. The impedance magnitudes |*Z*| of three independent ECIS experiments have been averaged and were normalized to the last value of |*Z*| recorded before the addition of *t*-BOOH. (B) Cells were incubated with *t*-BOOH for 24 h on confluent cells, and the viability was quantified by a modified MTT assay. Results of cell viability are expressed as percentage to untreated control cells. Results of three independent experiments were averaged and are presented as means ± SD. Data were subjected to one-way ANOVA followed by Dunnett's multiple comparison post-test using GraphPad Prism 5 software (level of significance, $***p < 0.001$).

concentrations of 10 or 100 μM *t*-BOOH. Incubation with 200 and 300 μM *t*-BOOH led to a strong reduction of cell viability, as observed in both the MTT assay and the ECIS readings. The latter *t*-BOOH concentration of 300 μM was chosen as the standard concentration in all protection assays conducted, since it showed an earlier and stronger effect in the ECIS measurements and more pronounced significant toxic effect in the MTT assay at 300 μM compared to 200 μM (Figure 1).

The flavonol quercetin (**1**) is one of the best investigated cytoprotective flavonoids. It has proven antioxidative and radical-scavenging activity in various tetrazolium and LDH release-based assays.[5,22,40] Therefore, it was chosen as a positive control for the cytoprotection assays used herein. In accordance with literature studies, it showed a significant and concentration-dependent neuroprotective effect at a concentration range between 10 and 50 μM (Figure 2).

To exclude false negative results due to any inherent cytotoxicity of the putative protective substances, all test compounds were first investigated for their influence on HT-22 viability at a fixed concentration of 40 μM, which was selected as a standard test concentration for the subsequent neuroprotection assay (Figure 2S of the Supporting Information).

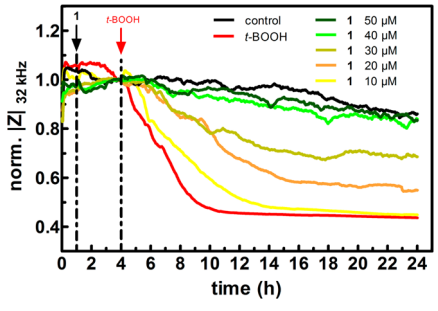

Figure 2. Dose-dependent neuroprotective effect of quercetin (**1**) against *t*-BOOH-induced oxidative stress on HT-22 cells using time-resolved ECIS readings. The black arrow indicates the addition of **1** at different concentrations for a preincubation period of 3 h. The red arrow indicates addition of *t*-BOOH at a final concentration 300 μM. The impedance magnitude |*Z*| at any time of the measurement was normalized to the last value of |*Z*| recorded before the addition of *t*-BOOH.

The protective effect of **1** against oxidative stress in HT-22 cells was saturated at 40 μM, as revealed by ECIS readings (Figure 2). Some of the "protective" test compounds did indeed exhibit cytotoxic effects at concentrations above 40 μM (data not shown) but not at 40 μM. Except for **11**, none of the phenolics significantly influenced the cell viability of HT-22 cells at 40 μM.

Neuroprotection by the flavonols **1**–**3**, the flavones **5** and **6**, and the flavanones **7** and **8** were evaluated at a standard concentration of 40 μM by the ECIS method. As a result, the presence of a 3′,4′-dihydroxy substituent (catechol substructure) in the B-ring seems to be an essential requirement for a strong neuroprotective effect by flavonoids in HT-22 cells after exposure to *t*-BOOH (Figures 3 and 4). Neither B-ring monosubstitution with a OH-4′ group nor B-ring disubstitution with 3′-methoxy- and 4′-hydroxy substituents provided any neuroprotection. The inability of **3** to exert a neuroprotective effect is noteworthy, as phenolic derivatives with a feruloyl substructure are also often strong radical scavengers/antioxidants and only slightly less effective in comparison to the corresponding derivatives with a catechol moiety.[41] Consequently, protection against oxidative stress cannot be explained entirely by radical scavenging or the antioxidant effect of flavonoids with 3′,4′-dihydroxy substitution. This is supported by the fact that two compounds with a catechol moiety, namely, **9** and **10**, also did not show any effect in the neuroprotection assay (Figures 3 and 4, data for **10** in Figure 3 not shown). Both compounds are also reported as strong radical scavengers and cytoprotective phenolics.[42,43] Thus, according to the ECIS-based protection assays conducted, it is apparent that an additional oxo group at C-4 is a further prerequisite for the neuroprotective activity of flavonoids in *t*-BOOH-treated HT-22 cells in vitro.

Phenolic derivatives with a low molecular weight, which are derived biosynthetically from the shikimate pathway, are widespread in the plant kingdom. Several studies have reported on the biological effects of caffeic acid (**13**) and ferulic acid (**15**) and their ester derivatives.[44,45] In the neuroprotection assay with *t*-BOOH, neither **13** nor **15** showed any in vitro effect at 40 μM (Figure 4, data for **13** and **15** in Figure 3 not shown). In contrast, the corresponding methyl ester of caffeic acid (**14**) proved to be as active as the flavonoids **1**, **6**, and **8**,

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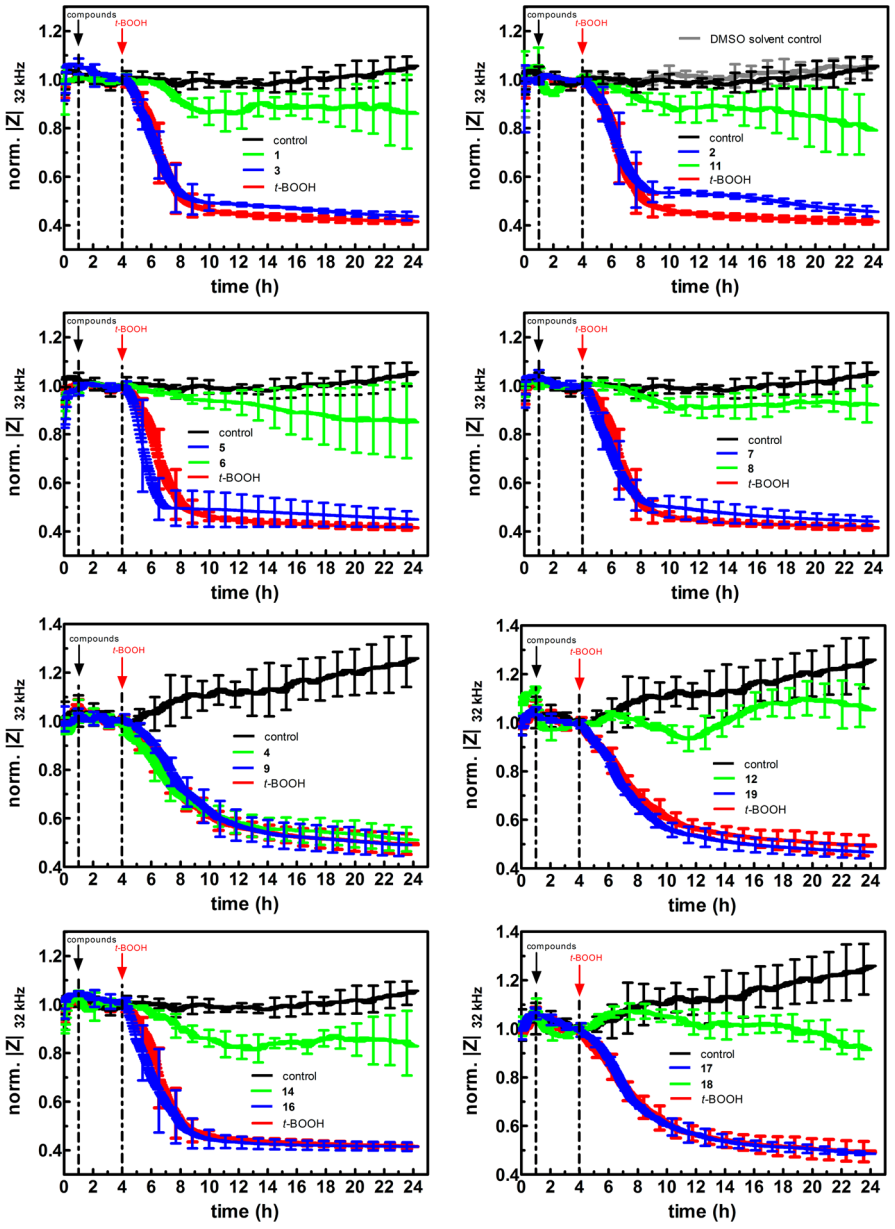

Figure 3. Neuroprotection of different flavonoids, flavonoid metabolites, or related phenolics against *t*-BOOH-induced oxidative stress on HT-22 cells using time-resolved ECIS readings. The black arrow indicates the addition of each test compound at the final concentration 40 μM for a preincubation period of 3 h. The red arrow indicates addition of *t*-BOOH at the final concentration 300 μM. Results are presented as means ± SD of three independent experiments. The impedance magnitude |*Z*| at any time of the measurement was normalized to the last value of |*Z*| recorded before the addition of *t*-BOOH. In each graph, curves of two test compounds were compared to an untreated or *t*-BOOH-treated control.

whereas the methyl ester of ferulic acid (**16**) was also inactive, in accordance with the negative result obtained for **3**. The dissociation of **13** and **15** to the corresponding carboxylates presumably prevents the free acids from permeating through the cell membrane. Thus, the observed inactivity of these molecules is a further hint that the neuroprotective effect requires an intracellular mechanism and is not the result of direct scavenging reactions on the cell surface with the alkoxyl and peroxyl radicals generated by the decay of *t*-BOOH. This assumption was supported by the results obtained for **17**, an important C-ring cleavage product of the in vivo metabolism of **1**, its glycosides, and several other flavonols and flavones.[46] The free acid is reported to be a strong radical scavenger in PMNs stimulated with formyl-methionyl-leucyl-phenylalanine (FMLP) or opsonized zymosan,[47] but showed no neuroprotective effect. As expected, esterification to **18** restored the neuroprotective activity (Figures 3 and 4). Furthermore, **12**, which is also a common C-ring cleavage product of flavonoids in vivo,[46] and **11**, a metabolic product of salicortin,[48] were active (Figures 3 and 4).

A very interesting investigation on the cytoprotective effect of flavonoids toward neurons was performed on HT-22 cells after exposure to glutamate,[5] which is a method of activating stress cascades in neurons. As these cells lack ionotropic glutamate receptors, addition of extracellular glutamate in high concentrations inhibits cystine (as oxidized form of cysteine) transport via the cystine/glutamate antiporter.[49] This neuronal oxidative stress induced toxicity (referred to as oxytosis) leads to intracellular cysteine and therefore glutathione depletion,

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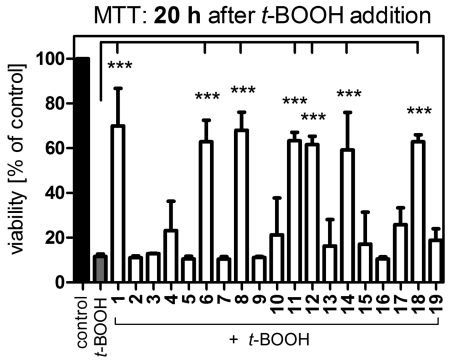

Figure 4. Depicted are the results of the MTT end-point assay. A colorimetric MTT test was additionally performed in the ECIS array directly after the ECIS measurement (Figure 3) was terminated (which was 20 h after the addition of *t*-BOOH). Results of cell viability are expressed as percentages to untreated control cells. The results of three independent experiments have been averaged and are presented as means ± SD. Data were subjected to one-way ANOVA followed by Dunnett's multiple comparison post-test using GraphPad Prism 5 software (level of significance, ***$p < 0.001$).

which induces intracellular ROS accumulation and cell injuries.[50]

Ishige et al. used a set of test compounds based on flavonoids with a lower number of hydroxy groups in comparison to the present investigation, but flavonoids **1**, **2**, **5**−**7**, and **9** as well as **13** and **14** were also tested.[5] The conformity of the present results was very high, as **1**, **6**, and **14** were active, whereas **5**, **7**, **9**, and **13** were not. Molecular investigations have shown the influence of different flavonoids on diverse molecular pathways. Nevertheless, one of the most interesting points is the diverging neuroprotective behavior of **2** in different types of stress-related experiments. Whereas **2** is not active in the *t*-BOOH work (Figures 3 and 4), the glutamate-based assay revealed significant activity.[5] Therefore, the glutamate assay was used according to standard procedures[4,34,37] to study cytoprotective potential toward HT-22 neurons against glutamate-induced oxidative stress, but also potential self-toxic effects of **2** were studied at exactly the same conditions and the same seeding density applied for the cytoprotection assay. This was found to be essential when studying self-toxic effects of compounds since cytotoxic effects were found to be different according to the cell density (the glutamate assay was performed with unconfluent cells,[4,34,37] while the modified MTT assays under ECIS conditions were performed on confluent cells for direct comparability with ECIS experiments). The glutamate assay reproduced the neuroprotective effect of **2** at 5, 10, and 25 μM in concordance with Ishige et al.[5] (Figure 5B), revealing that **2** targets a special molecular pathway of the glutamate stress response, but not the pathways downstream of *t*-BOOH. However, **2** by itself significantly reduced the cells' viability at 25 and 50 μM (Figure 5A), which may explain the decrease of its protective effect from 10 to 25 μM in the glutamate assay.

Ishige et al. considered an unsaturated C-ring as a structural requirement for the neuroprotective activity of flavonoids in the glutamate toxicity model.[5] Nevertheless, they did not include in their investigation the flavanone **8**, which was found to be protective in the *t*-BOOH assay (Figures 3 and 4). Compound **8** was tested in the glutamate toxicity model (Figure 5B) and showed a significant protective activity as quantified by MTT and ECIS, indicating that an unsaturated flavonoid C-ring is not a structural prerequisite for neuroprotective activity. However, similar to **2**, compound **8** showed cytotoxicity at increasing concentrations on unconfluent cells under the glutamate assay conditions (Figure 5A), demonstrating a concentration-dependent loss of its protective effects at increasing concentrations (Figure 5B).

Besides C-ring cleavage, deglycosylation, absorption, and phase-II metabolism of intact flavonoids are key steps in the in vivo flavonoid metabolism,[51] leading to flavonoid glucuronides and sulfates. The quercetin phase-II derivative quercetin 3-*O*-β-glucuronide (**4**) showed no activity in the neuroprotection assay, despite its catechol moiety, either with MTT or with the ECIS readout. To evaluate whether dissociation of the glucuronic acid unit is the reason for a reduced permeation through the plasma membrane, the effect of the methyl ester of **4** was also tested (data not shown). The methyl ester of **4** did not show any neuroprotective activity, as the molecule is probably unable to penetrate the membrane, too.

All results obtained from experiments that were monitored by continuous ECIS readings were confirmed by an MTT test

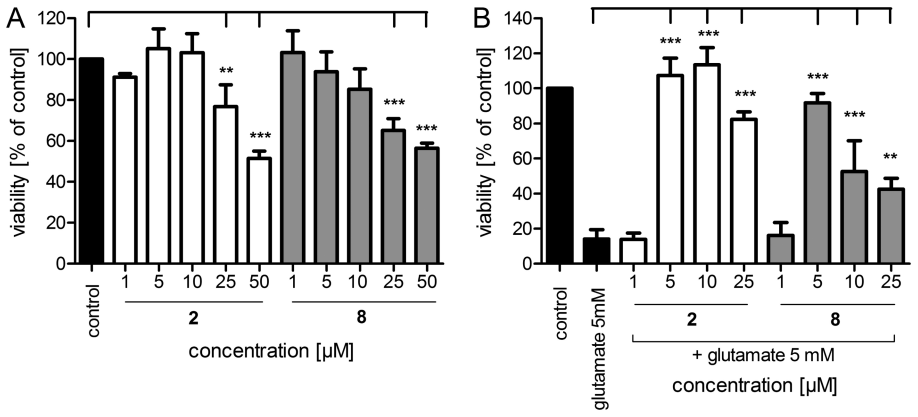

Figure 5. Dose-dependent self-toxicity (A) and dose-dependent neuroprotection against glutamate-induced oxidative stress (B) of kaempferol (**2**) and eriodictyol (**8**) on HT-22 cells using a modified MTT test on unconfluent cells according to standard protocols as described in the Experimental Section. Briefly, HT-22 cells were incubated for 24 h either in the absence (A) or presence (B) of glutamate with compounds **2** and **8** at the indicated concentrations. Results of cell viability were expressed as percentages to untreated control cells and presented as means ± SD of three independent experiments each performed in quadruplicate. Data were subjected to one-way ANOVA followed by Dunnett's multiple comparison post-test using GraphPad Prism 5 software (level of significance, **$p < 0.01$, ***$p < 0.001$).

dx.doi.org/10.1021/np400518k | *J. Nat. Prod.* XXXX, XXX, XXX−XXX

performed directly (after ECIS data acquisition was terminated) in the wells of the electrode array (Figure 4), showing that the impedance measurements match the results of the tetrazolium-based method. An additional comparison study between the time-resolved ECIS measurement curves (Figure 3) and the MTT test results (Figure 4) can be found in Figure 3S of the Supporting Information. Briefly, viability of HT-22 cells was studied for compounds **1**−**19** under the exact experimental ECIS conditions, but with MTT reaction 4.5 h (Figure 3S A) and 9 h (Figure 3S B) after exposure to *t*-BOOH. After 4.5 h only three (namely **1**, **12**, and **18**) of the seven active compounds of Figures 3 and 4 were able to reduce *t*-BOOH-induced toxicity in this MTT setup, and also the toxicity of *t*-BOOH is relatively low, at 70% (Figure 3S A). After 9 h the results match the data of Figure 4, indicating that at least 9 h is required for *t*-BOOH incubation to detect neuroprotective properties sufficiently with MTT quantification (Figure 3S B of the Supporting Information).

Summing up the results from a structural point of view, flavonoids showed a neuroprotective activity when *t*-BOOH is used, provided they possess a catechol substructure and a 4-keto group (**1**, **6**, and **8**), whereas a 2,3-double bond is not necessary. In the case of low molecular weight phenolics, the only prerequisite for neuroprotective activity is a catechol substitution (**11**, **12**, **14**, and **18**). The flavonoids and other phenolics with a ferulic acid substitution or with only one hydroxy group are not protective against *t*-BOOH. This is a striking difference in comparison with a glutamate model in which kaempferol (**2**) is active according to the literature and to our results. All phenolic compounds with a free and dissociable carboxylic acid group did not obey the general trend independent from the substitution pattern, as they showed in general no activity, maybe due to insufficient permeation across the plasma membrane into the cells.

The oxidation propensity of flavonoids has been considered an important parameter describing their antioxidative potential; therefore, it has often been used in studies that evaluate the antioxidant activity.[26−29] Thus, SWV was used to measure the oxidation potential of all compounds under study (**1**−**19**) to identify putative relationships between oxidation potential and an ability to serve as an antioxidant in neuroprotection assays.

SWV abbreviates a pulsed voltammetric measurement technique that enables a highly sensitive quantification of redox-active flavonoids and the determination of their corresponding oxidation potential.[24] In terms of electrode setup and square-wave voltammetric experimental conditions, there is no uniform protocol to study the compounds of interest. Only a few flavonoids were characterized in this manner in previous reports.[23−25] In the present study, all substances were investigated by applying the same electrochemical protocol to enable a comparative evaluation. Additionally, some phenolic acid derivatives and metabolites have been investigated in this study that have not been evaluated with square-wave voltammetry previously. A potential range of 0.2−1.5 V was chosen to explore the putative radical-scavenging activities of all compounds under physiological pH conditions. The peaks of interest are the first peaks of the measurements occurring at negative or low positive potentials that indicated the susceptibility of a molecule to be oxidized by free radicals. Compounds showing a similar electrochemical behavior were grouped with respect to their pharmacological activity (Table 1).

Table 1. Peak Potentials Determined by Square-Wave Voltammetry[a]

compound	peak 1 (mV)	peak 2 (mV)	peak 3 (mV)	peak 4 (mV)
quercetin (**1**)	+40 (p)	+189 (w)	+361 (w)	+919 (m)
kaempferol (**2**)	− 33 (m)	+611 (w)		
isorhamnetin (**3**)	−109 (p)	+391 (w)		
quercetin 3-*O*-glucuronide (**4**)	+105 (p)	+804 (m)		
apigenin (**5**)	+298 (w)	+540 (m)		
luteolin (**6**)	+64 (vp)	+835 (m)		
naringenin (**7**)	+391 (w)	+677 (m)		
eriodictyol (**8**)	+82 (p)	+851 (p)		
catechin (**9**)	+205 (m)	+506 (m)		
procyanidin B1 (**10**)	+242 (m)	+490 (m)		
catechol (**11**)	+213 (m)			
4-methylcatechol (**12**)	+163 (m)			
caffeic acid (**13**)	+161 (m)			
caffeic acid methyl ester (**14**)	+94 (vp)			
ferulic acid (**15**)	+205 (m)	+702 (w)		
ferulic acid methyl ester (**16**)	+141 (p)			
3,4-dihydroxyphenylacetic acid (**17**)	+276 (m)			
3,4-dihydroxyphenylacetic acid methyl ester (**18**)	+197 (m)	+591 (w)		
3-hydroxyphenylacetic acid (**19**)	+413 (m)			

[a]The small letters in brackets specify the peak height in the square-wave voltammograms at the indicated peak potentials as very pronounced (vp), pronounced (p), moderate (m), or weak (w). Peak heights of very pronounced peaks are around 300 μA, of pronounced peaks in the range 150−200 μA, of moderate peaks in the range 50−130 μA, and of weak peaks below 50 μA.

The first observation that could be made is that all active flavonoids with a catechol moiety and a 4-oxo-group produce very pronounced and sharp peaks (with a peak width of approximately 200 mV) at low positive potentials: 40 mV for **1**, 64 mV for **6**, and 82 mV for **8**. This was true also for caffeic acid methyl ester (**14**), which gave rise to a similar peak at 94 mV. The molecule has a catechol moiety connected to an oxo group as a part of an ester functionality. All of these three flavonoids but not **14** exhibited a second oxidation reaction in a very narrow range between 835 (**6**) and 919 mV (**1**). Quercetin 3-*O*-*β*-glucuronide (**4**) showed pronounced electrochemical activity at 105 mV, and another oxidation reaction occurred at 804 mV. Two very weak oxidation peaks observed for **1** at 190 and 360 mV were not present for **4**. Thus, no substantial impact due to the glucuronide substituent could be observed with respect to the redox properties and antioxidant activity of **1**. Thus, its low activity in the cell-based protection assays used is most likely due to reduced membrane permeability.

dx.doi.org/10.1021/np400518k | *J. Nat. Prod.* XXXX, XXX, XXX−XXX

Isorhamnetin (**3**) and kaempferol (**2**) showed moderate oxidation peaks at very low potential ranges (−109 and −33 mV), but exhibited no activity in the *t*-BOOH-based neuroprotection assay. Moreover, both molecules were characterized by a second oxidation reaction, which appeared as a weak peak for **3** at 391 mV and for **2** at 611 mV, respectively.

Catechol (**11**) and 4-methylcatechol (**12**) were also active in the neuroprotection assay but showed different electrochemical characteristics in the SWV experiments conducted in comparison to **1**, **6**, and **8**. They showed moderate oxidation peaks at relatively low potentials of 163 (**12**) and 213 mV (**11**). No further oxidation reactions were detected for this pair of compounds. A similar characteristic was observed for the inactive compounds caffeic acid (**13**) and 3,4-dihydroxyphenylacetic acid (**17**). Esterification led to a shift of ca. −65 to −80 mV from the free phenolic acids to the ester as observed for the pairs **13**/**14**, **15**/**16**, and **17**/**18**.

The electrochemical profile of the active compound **18** showed no characteristic pattern. This compound behaved in a similar manner to the inactive **9** and **10** and belongs to a group for which two oxidation reactions were detected. The first was observed as a moderate oxidation reaction at 200 and 240 mV for **9** and **10** and at 197 mV for **18**. The second oxidation reaction was observed at around 591 mV. The peak for **9** and **10** was again moderate, but for **18** less intensive than its first peak.

It can be concluded from these physicochemical results that biological activity is not associated directly with one specific electrochemical profile. Nevertheless, for one group of flavonoids (**1**, **6**, and **8**) with a characteristic electrochemical pattern, neuroprotective activity was indicated. It would be of interest to evaluate whether structurally different compounds with the same electrochemical profile as observed for these flavonoids are also active in in vitro neuroprotective assays.

EXPERIMENTAL SECTION

Chemicals and Reagents. Quercetin 3-*O*-*β*-glucuronide (**4**) and procyanidin B1 (**10**) were kind gifts from Prof. Dr. I. Merfort (Universität Freiburg, Germany) and Prof. Dr. A. Nahrstedt (Universität Münster, Germany), respectively. Kaempferol (**2**), luteolin (**6**), and racemic eriodictyol (**8**) were purchased from Extrasynthese (Genay Cedex, France, purity each with ≥99.9% determined by HPLC). Isorhamnetin (**3**, purity 99.9%, determined by HPLC), apigenin (**5**, purity 99.9%, determined by HPLC), racemic naringenin (**7**, purity ≥95%, determined by HPLC), caffeic acid (**13**, purity ≥95%, determined by HPLC), and ferulic acid (**15**, purity ≥95%, determined by HPLC) were obtained from Roth (Karlsruhe, Germany). 3-Hydroxyphenylacetic acid (**19**), monosodium-L-glutamate, and glutaraldehyde were purchased from Merck (Darmstadt, Germany; purity of each ≥99% determined by HPLC). Caffeic acid methyl ester (**14**, 99.9% HPLC) and ferulic acid methyl ester (**16**, purity 99%, determined by GC) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). 3,4-Dihydroxyphenylacetic acid (**17**, purity ≥98% determined by HPLC) was obtained from Lancaster Synthesis (Frankfurt am Main, Germany). Quercetin (**1**, purity ≥98% determined by HPLC), catechol (**11**, purity ≥99%, determined by HPLC), 4-methylcatechol (**12**, purity ≥95%, determined by HPLC), and (±)-catechin (**9**, purity ≥96%, determined by HPLC) as well as all other chemicals used were purchased from Sigma (Steinheim, Germany). 3,4-Dihydroxyphenylacetic acid methyl ester (**18**) was synthesized by dropwise addition of $SOCl_2$ to **17** in methanol at 0 °C (purity ≥98%, determined by HPLC).[30] NMR spectroscopic and mass spectrometric data were in agreement with those reported in the literature for this compound.[30]

Cell Culture. All experiments in this study were performed using the HT-22 neuronal cell line,[31,32] which was originally derived from murine hippocampal tissue.[33] HT-22 cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Karlsruhe, Germany) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS, Biochrom, Berlin, Germany).[34] Cells were kept under standard cell culture conditions (37 °C, 5% CO_2) in a humidified incubator. Cells were subcultured three times a week.

Impedimetric Monitoring Using ECIS. Time-resolved impedimetric monitoring of HT-22 cells was performed using the ECIS 1600R instrument purchased from Applied BioPhysics Inc. (Troy, NY, USA). Disposable electrode arrays of type 8w1e (Applied BioPhysics) were used in all experiments. They consisted of eight-well cell culture dishes with gold electrodes deposited on the bottom of the wells. Each well contained a small working electrode (area 5×10^{-4} cm^2) and a larger counter electrode (area 0.15 cm^2). Due to this difference in surface area of the electrodes, the total impedance of the system was dominated by the impedance of the small working electrode.[19] Thus, the ECIS readout mirrored the averaged response of approximately 100 cells fitting on the surface of the small electrode. Data were recorded at 23 selected frequencies between 25 and 10^5 Hz (MFT mode), but only the impedance at 32 kHz was used for analysis. Previous studies have shown that impedance readings at 32 kHz are the most robust indicator for cytotoxicity.[35] The complete ECIS device is depicted in Figure 1S (Supporting Information).

Prior to cell seeding, the array was precoated with a layer of cross-linked gelatin to provide better attachment conditions to the HT-22 cells, as these cells easily detach on regular cell culture surfaces when grown to confluence. Therefore, the wells were incubated with 200 μL of a 0.5% (w/v) aqueous gelatin solution for 1 h at room temperature. After aspiration, 200 μL of 2% (v/v) glutaraldehyde solution was added and the incubation was continued for another 15 min. The wells were then thoroughly washed 10 times with sterile Millipore water to remove any cytotoxic glutaraldehyde. Cells were then seeded in a density of 6×10^5 cells per well and grown to confluency over 2−3 days.

The eight-well electrode array was placed in a humidified cell culture incubator at 37 °C with 5% CO_2 and connected to the electronic devices located outside the incubator. The commercial ECIS software was used for data acquisition, storage, and analysis. Prior to treatment, cells were allowed to equilibrate for 1 h to provide stable baseline data. Subsequently, either medium or medium containing the test compounds was added to the wells and allowed to preincubate for 3 h. Then *t*-BOOH was added to the wells in 300 μM concentration, and impedance data were recorded for a further 20 h. The impedance magnitude |*Z*| at any time of the measurement was normalized to the last value of |*Z*| recorded before the addition of *t*-BOOH.

All compounds tested were dissolved in DMSO and diluted with fresh medium, with the DMSO concentration always below 0.1% (v/v). The aqueous *t*-BOOH solution was directly diluted with fresh medium.

MTT Cell Viability Assay. This modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma, Steinheim, Germany) assay[36] was applied to study *t*-BOOH-induced dose-dependent neurotoxic effects. Furthermore, the phenolic compounds were prior tested with this MTT assay for their cytotoxicity against HT-22 cells to exclude any cytotoxic response to the test compounds during the ECIS measurements. For comparability to ECIS measurements, the MTT assay was performed on a confluent layer of HT-22 cells. Therefore, the seeding density was atypically high when compared to standard protocols, and the MTT reagent incubation time was shortened. Briefly, a 96-well plate was precoated with 50 μL of a 0.5% (m/v) aqueous gelatin solution for 1 h at room temperature. After removal, cells were seeded in 96-well plates at a density of 5×10^4 cells per well and cultured to confluence over 24 h. Subsequently, cells were incubated for another 24 h with either medium or the test compounds. The MTT solution (4 mg/mL in PBS) was diluted 1:10 with medium, and the mixture was added to the wells after removal of the previous medium. After 1 h of incubation, the supernatant was removed and 100 μL of lysis buffer (10% SDS, pH 4.1) was added to the wells. Absorbance was determined at 560 nm on the next day with a multiwell plate photometer (Spectra Fluor Plus, Crailsheim,

dx.doi.org/10.1021/np400518k | J. Nat. Prod. XXXX, XXX, XXX−XXX

Germany). The results of cell viability studies were expressed as a percentage to untreated control cells.

Dose-Dependent Neurotoxicity of *t*-BOOH. To identify a suitable concentration of *t*-BOOH capable of inducing a pronounced cytotoxic response of HT-22 cells, 10, 100, 200, and 300 μM *t*-BOOH were evaluated in both the ECIS- and MTT-based assays with the conditions described above.

Dose-Dependent Neuroprotective Effect of Quercetin (1) in ECIS. To establish a suitable positive control, the dose-dependent effect of quercetin was evaluated at concentrations of 10, 20, 30, 40, and 50 μM in the ECIS assay as described above.

Evaluation of Neuroprotection from ECIS Readings. All test compounds were evaluated at a 40 μM final concentration in the ECIS assay as described above.

End-Point MTT Assay after ECIS Readings. Directly after ECIS data acquisition was terminated at the end of the *t*-BOOH exposure time, the viability of cells in the wells of the electrode array was determined additionally using an MTT assay.[36] The MTT solution (4 mg/mL in PBS) was diluted 1:10 with medium, and the mixture was added to the wells after removal of previous medium. After 1 h of incubation, the supernatant was removed and 400 μL of lysis buffer (10% SDS, pH 4.1) was added to the wells. Absorbance was determined at 560 nm on the next day using a multiwell plate photometer (Spectra Fluor Plus) after transferring the test solutions into a fresh 96-well plate. The results of cell viability were expressed as percentage to untreated control cells.

Neuroprotection against Glutamate-Induced Toxicity. This test was performed as reported before.[34,37] Briefly, HT-22 cells were seeded in 96-well plates at a density of 5×10^3 per well and cultured for 24 h. Subsequently, cells were incubated for another 24 h with either medium or test compounds with potential cytoprotective activity in either the absence (to test for toxic effects) or the presence (to test for the protective potential against glutamate-induced oxidative stress) of 5 mM glutamate. MTT solution (4 mg/mL in PBS) was diluted 1:10 with medium, and the mixture was added to the wells after removal of the previous medium. The plates were then incubated for another 3 h. Then, the supernatant was removed, and 100 μL of lysis buffer (10% SDS, pH 4.1) was added to the wells. Absorbance at 560 nm was determined on the next day using a multiwell plate photometer (Spectra Fluor Plus). The results of these cell viability assays were expressed as percentages relative to untreated control cells. All compounds were dissolved in DMSO and diluted with fresh medium, with the DMSO concentration always below 0.1% (v/v).

Square-Wave Voltammetry. All voltammetric measurements were performed using a μAutolab III potentiostat (Metrohm, Switzerland), in combination with GPES software (version 4.9). The screen-printed electrodes used for SWV were obtained from Dropsens (Llanera, Spain) and consisted of a 4 mm diameter carbon-based working electrode. The working electrode was surrounded by an auxiliary electrode from the same material, and all potentials were determined relative to a silver pseudoreference electrode. A 50 μL aliquot of sample solution was sufficient to cover the three-electrode arrangement. Square-wave voltammetry was carried out applying a pulse amplitude of 50 mV, a step potential of 1.95 mV, and a frequency of 180 Hz, according to Adam et al.[24] Scanning the potential window from −0.2 to 1.5 V was performed at a scan rate of 350 mV/s. The phenolic test compounds were dissolved in DMSO at a concentration of 50 mM. The stock solution was further diluted to a concentration of 1 mM in a mixture of 75 mM phosphate buffer (pH 7.4)/DMSO (90:10, v/v) to prevent the phenolics from precipitating. Additionally, higher background conductivity for SWV experiments was achieved by using an aqueous buffer system. For data evaluation a SWV scan from the blank buffer/DMSO mixture was recorded and subtracted from SWV scans recorded in the presence of phenolic compounds.

Statistics. Data of all MTT tests were expressed as means ± SD of at least three independent experiments. Data were subjected to one-way ANOVA followed by Dunnett's multiple comparison post-test using GraphPad Prism 5 software (levels of significance $*p < 0.05$; $**p < 0.01$; $***p < 0.001$).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

A schematic overview of the experimental setup to perform ECIS measurements with 8W1E arrays and further MTT assay results in addition and for comparison to the ECIS experiments are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Prof. Dr. I. Merfort (Universität Freiburg, Germany) and Prof. Dr. A. Nahrstedt (Universität Münster, Germany) are gratefully acknowledged for providing quercetin 3-*O*-β-glucuronide and procyanidin B1, respectively. Special thanks are due to Dr. B. Kraus (Universität Regensburg, Germany) for introducing B.K. to cell culture work and for fruitful discussions. Appreciation is expressed to Dr. X. Chen (Universität Würzburg, Germany) for the synthesis of 3,4-dihydroxyphenylacetic acid methyl ester. Sincere gratitude is extended to Professor S. Elz (Universität Regensburg, Germany) for financial support.

■ DEDICATION

Dedicated to Prof. Dr. Otto Sticher of ETH-Zurich, Zurich, Switzerland, for his pioneering work in pharmacognosy and phytochemistry.

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