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Nrf2/ARE-Mediated Antioxidant Actions of Pro-Electrophilic Drugs

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Abstract

Living cells maintain a balance between oxidation and reduction, and perturbations of this redox balance are thought to contribute to various diseases. Recent attempts to regulate redox state have focused on electrophiles (EPs), which activate potent cellular defense systems against oxidative stress. One example of this approach is exemplified by carnosic acid (CA) and carnosol (CS), compounds that are found in the herb rosemary (*Rosmarinus officinalis*). Importantly, CA and CS themselves are not electrophilic, but in response to oxidation, become electrophilic, and then activate the Keap1/Nrf2/ARE (antioxidant response element) transcription pathway to synthesize endogenous anti-oxidant 'phase 2' enzymes. As a result of our efforts to develop these compounds as therapeutics for brain health, we have formulated two innovative criteria for drug development: the first concept is the use of Pro-Electrophilic Drugs (PEDs) that are innocuous in and of themselves; and the second concept involves the use of compounds that are Pathologically-Activated Therapeutics (PATs), i.e., these small molecules are chemically converted to their active form by the very oxidative stress that they are designed to then combat. The chemical basis for PED and PAT drugs is embodied in the *ortho*- and *para*-hydroquinone electrophilic cores of the molecules, which are oxidized by the Cu²⁺/Cu⁺ cycling system (or potentially by other transition metals). Importantly, this cycling pathway is under stringent regulation by the cell redox state. We propose that redox-dependent quinone-formation is the predominant mechanism for formation of PED and PAT drugs from their precursor compounds. In fact, redox-dependent generation of the active form of drug from the "pro-form" distinguishes this therapeutic approach from traditional EPs such as curcumin, and results in a decrease in clinical side effects at therapeutic concentrations, e.g., lack of reaction with other thiols such as glutathione (GSH), which can result in lowering GSH and inducing oxidative stress in normal cells. We consider this pro-drug quality of PED/PAT compounds to be a key factor for generating drugs to be used to combat neurodegenerative diseases that will be clinically tolerated. Given the contribution of oxidative stress to the pathology of multiple neurodegenerative diseases, the Keap1/Nrf2/ARE pathway represents a promising drug target for these PED/PAT agents.

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Keywords

Glutathione; Electrophile; Keap1; Pathologically-Activated Therapeutic; Electrophilic Counterattack

(1) Electrophile-cell interaction

Electrophiles (EPs), or positively charged compounds that are attracted to and react with an electron rich center, have diverse chemical structures and induce various biological actions. A single EP can have both protective and toxic effects on cells. In order to describe this complicated type of compound-cell interaction, several types of action have been characterized, including chemoprevention [1–3], stress responses [4, 5], hormesis [6, 7] and electrophilic counterattack [8–12]. These studies are generally characterized by a dose–response in which a low-dose stimulates and a high-dose inhibits activity [5–7]. This mode of action can be graphically represented by an inverted U-shaped dose-response curve, as shown in Fig. 1A. Low-dose stimulation most likely represents an adaptive biological response that is manifested as a modest increase in neuroprotection and longevity [5–11]. In contrast, high-dose stimulation results in disruption of homeostasis and serious side effects, in part because of indiscriminant reaction with thiol groups, as discussed below [12–14].

The EPs considered here have various chemical structures but share a common property – they have electron-deficient (electrophilic) carbon centers because the electron density is drawn to the carbonyl oxygen in the structure. The result is that the carbon is relatively electron deficient [9]. EPs react with nucleophiles, including protein thiols or sulfhydryl groups (–SH), as found in reduced glutathione (GSH) or guanine bases in DNA. Each of these groups has an unshared pair of electrons. Facile reaction of an electrophile with the –SH group (or, more correctly, with a thiolate anion, $-S^-$) of a cysteine residue results in alkylation [9]. Formally, this reaction may resemble that of nitrosonium ion (NO^+) as an intermediate of the reaction of the NO group and thiolate anion [15, 16]

Although reaction of most electrophiles with reduced cysteine residues, for example those of GSH, can contribute to neurotoxicity by decreasing the reductive capacity of the cell, a minor population of EPs have been reported to be neuroprotective, at least within limited concentrations [9]. Such neuroprotective EPs, although not necessarily possessing antioxidative activity themselves, have been reported to transcriptionally induce antioxidative enzymes to afford neuroprotection [8–12]. Thus, these EPs have an advantage over antioxidant molecules because their action is more sustained and amplified by transcription-mediated signaling pathways [1–4]. However, highly-reactive EPs have a tendency to react rather indiscriminately with thiol groups; since GSH is arguably the most abundant thiol in the cell, it may be depleted by EPs, paradoxically rendering otherwise normal cells more vulnerable to oxidative stress, as discussed in more detail below.

(2) Distinctive groups of EPs

We have described the fact that EPs fall into three distinctive categories in terms of their protective vs. toxic effects: EP1, EP2 and EP3, as shown in Fig. 1B [9, 12]. First, we will describe the chemical and biological properties of these three groups of EPs. The most basic distinction involves two opposing actions involving GSH depletion, which can contribute to toxicity, vs. ‘electrophilic counterattack,’ which can afford cell protection from oxidative insult. For example, the anti-cancer drug Doxorubicin (DOX, trade name Adriamycin®) is a good example of the first category, EP1, which induces toxicity [9]. DOX predominantly causes cell death by depletion of GSH in cells, while barely activating an ‘electrophilic counterattack’ that affords protection. DOX is therefore useful as a chemotherapeutic for the

treatment of solid tumors. This quinone-containing compound is well known for producing oxidative stress not only *in vitro* but also *in vivo* [17, 18]. DOX significantly decreases GSH levels and alters the GSH/GSSG ratio in mouse brain [18]. GSH levels in the murine brain vary from 1–3 mM, depending on the region (cortex>cerebellum>hippocampus) and cell type (astrocytes>neurons) [19, 20]. Intraperitoneal administration of DOX decreases GSH levels by up to 20% [18], suggesting that side effects in the brain of patients treated with DOX as chemotherapy against solid tumors may be due, at least in part, to a decrease in GSH levels in neural cells.

On the other hand, we and others have reported that neurite outgrowth-promoting prostaglandin (NEPP) 11, manifests protective effects, at least within a limited concentration range [8]. NEPP11 is representative of the EP2 category. In response to NEPP11, cells mount a more robust electrophilic counterattack, represented by a series of responses that detoxify the products of oxidative stress [8–9]. In fact, NEPP11 activates not only an electrophilic counterattack but also a milder GSH depletion than DOX, inducing net protective effects at relatively low concentrations, but causing toxicity outside these narrow boundaries. Investigations into this limited ability of EP2-type electrophiles to protect cells against oxidative stress, led to the discovery that the predominant protective activity is mediated by reaction of the EP with a critical thiol in the cytoplasmic protein Kelch-like ECH-associated protein 1 (Keap1), causing it to release the transcription factor Nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Nrf2 is then free to enter the nucleus for subsequent activation of ARE-mediated transcription of phase 2 antioxidant enzymes [1–4]. The development of NEPP11 represented an early attempt to contribute to this antioxidant protection with minimum toxic effects, as required for the development of a drug that is effective against neurodegenerative diseases yet clinically tolerated [9–12]. However, NEPP11, as an EP2 category electrophile, still resulted in a moderate degree of GSH depletion. Note that the difference between EP2 and EP3 compounds is the degree of GSH depletion in the face of a strong electrophilic counterattack. We found that EP3 compounds result in less GSH depletion but still do exert some toxic effect. The reduction in toxic side effects, hence, becomes more critical than a further increase in the protective effect in terms of clinical tolerability and druggability.

Thus, we reasoned that development of a pro-drug, which is innocuous prior to activation and does not react with GSH thiol, would be the most-promising strategy to follow for creating an effective yet tolerated treatment for oxidative stress [12]. For this reason, we have focused our attention on geometric isomers of hydroquinone in electrophilic cores of terpenoids and flavonoids [8–12]. We have studied the protective effects of carnosic acid (CA) and carnosol (CS), derived from the herbs rosemary and sage, because they are only converted to their active forms in response to pathological oxidation. In other words, they function in a pro-drug-like manner *in vivo* [8–12].

In practical terms, EP3 compounds may represent desirable drug candidates because they have broad therapeutic dose ranges and minimal potential side effects [9–12]. One of the predominant actions of EP3s is to engage the electrophilic counterattack response, via activation of the Keap1/Nrf2/ARE cascade, without depleting GSH; in fact, to the contrary, EP3s dramatically increase GSH levels by transcriptional upregulation of the enzyme's synthetic machinery via Nrf2 activation. Hence, EP3 compounds are capable of inducing protective effects with a broad therapeutic window [9–12]. The essential question considered here is to define the parameters essential for EP3 compounds that make them viable drug candidates. In this review, we will establish the specific chemical and biological bases for selection of candidate hydroquinone geometric isomers as a means for focusing development of effective drug therapies. We will present the case that these parameters closely relate to the 'pro-drug-like features' described above [12]. While space allows us to

present only a few examples of such drugs, we do hope that this monograph will spur and guide further research in this field. Our hypothesis is that two concepts will guide the development of these compounds for future anti-oxidative drug development: 1) the compounds should be pro-electrophilic drugs (PEDs) [9–12]; and 2) the compounds should represent pathologically-activated therapeutics (PATs) [21–23]. Having made this case, we will then outline the prospects for use of such compounds for a variety of neurodegenerative diseases.

(3) Importance of geometric isomers of hydroquinones

We first considered molecules that are simple hydroquinones because their electrophilic core contains the key to understanding the concepts of PEDs and PATs [12]. We previously reported that the cyclical structure of a hydroquinone represents one of the chemical requirements for generating a compound that possesses neuroprotective qualities without serious side effects [8–12]. Next, using a simple hydroquinone, we asked which positions on the ring (i.e., which geometric isomers) were critical for phenol placement in order to generate the most effective neuroprotective drug. We found that *ortho*- and *para*-hydroquinones were the critical electrophilic cores for effective drug development [24–27]. Emerging evidence for this conclusion includes the following: Molecules containing *para*- and *ortho*-hydroquinone (diphenol) moieties are associated with Nrf2 activation in the brain and subsequent transcriptional activation of phase 2 enzymes (Fig. 2) [24–27]. The finding that *ortho*-hydroquinones and *para*-hydroquinones, but not *meta*-hydroquinones, induce antioxidant-response element (ARE)-driven gene expression suggested that the oxidative ability of these compounds is essential for ARE inducer activity [24–27]. Accordingly, ARE activation correlates well with the tendency of each compound to donate an electron pair to oxygen [24–27]. Importantly, *meta*-hydroquinone can convert to semiquinone but not to quinone and thus cannot react with Keap1 to cause S-alkylation. *Ortho*- and *para*-hydroquinones, in contrast, can be oxidized to their quinone form by the $\text{Cu}^{2+}/\text{Cu}^{+}$ - recycling system [27], and thus react via covalent binding to Keap1 to activate the Keap1/Nrf2 pathway [24–27].

In addition to the Keap1/Nrf2 pathway [1–3], the HSP90/HSF-1 transcriptional pathway [28] provides effective redox regulation by activating endogenous gene networks that are involved in antioxidant defense [11, 29]. Several activators of Nrf2 that act via reaction with Keap1 also covalently bind to cysteine residues of HSP90 to activate HSF-1, thereby inducing molecular chaperones, including additional HSPs [11, 29]. These findings suggest that S-alkylation of critical cysteine residues by EPs can activate both of these transcriptional pathways, contributing to electrophilic counterattack [11, 12]. Since neurons have little capacity for redox regulation, these backup systems become very important in the nervous system during periods of oxidative stress [11, 12].

After electrophilic activation, the transcription factors Nrf2 and HSF-1 bind to the antioxidant response element (ARE) and heat-shock factor response element (HSE), respectively, to initiate transcription of genes whose products provide neuroprotection against oxidative and nitrosative insults [1–3, 11, 28, 29]. Various geometric isomers of electrophilic compounds (hydroquinone (1), catechol (2), or resorcinol (3)) were examined for ARE and HSE activation (Fig. 3A and B). To explore the chemical properties of these compounds in an appropriate biological context, we used HT22 cells, a mouse hippocampal neuronal cell line [25]. As expected, since quinone formation closely correlated with transcriptional activation, the *meta*-isomer did not activate but the *para*- and *ortho*-isomers did activate the ARE in HT22 cells transfected with cDNAs containing a reporter for ARE transcriptional activity. Additionally, while the *para*-isomer activates the HSE, the *ortho*- or *meta*-isomers were ineffective in this regard. The ARE and HSE ‘activation profile’ for

these three compounds was 1,4-hydroquinone > catechol > resorcinol. Importantly, resorcinol did not activate the ARE or HSE to any significant degree.

HT22 cells also represent a widely-used *in vitro* system to model neuronal cell death after oxidative insult [12]. Glutamate (5 mM) induces oxidative glutamate toxicity via GSH depletion [25]. Importantly, the protective effects of the isomers against oxidative glutamate were consistent with their order of the transcriptional activation (Fig. 3C). Specifically, while the addition of each compound significantly protected the cells against oxidative glutamate toxicity, the potency of the effect was again 1,4-hydroquinone > catechol > resorcinol. In summary, we conclude that, at least for these simple compounds, their beneficial effect is consistent with chemical activation to the quinone form, with the *ortho*-subtype (1 in Fig. 3D) being most active, the *para*-form (2) somewhat less active, and the *meta*-form (3) least active.

(4) Lessons from investigation of dopamine oxidation

We next considered the effects of dopamine (DA) oxidation because this compound is a relatively hydrophilic catechol, and neurons containing DA are lost in Parkinson's disease (PD). DA metabolism is relevant here for the following reasons:

1. The brain is sensitive to oxidative and nitrosative damage [30–32].
2. Increased levels of oxidative stress within specific brain regions (due to generation of excessive reactive oxygen species (ROS) and reactive nitrogen species (RNS)) leads to selective neurodegeneration [31–35].
3. Catechol metabolism in neuronal cells is a major regulator of neuronal redox balance [33].
4. Oxidation of DA itself has been hypothesized to be a contributory factor to PD [36, 37], although this point has remained contentious.

There are two distinct potential pathways for the oxidation of DA in neurons (Fig. 4) [36–39]. A critical point in these metabolic pathways is whether DA quinone is toxic [36, 37] or protective [38, 39]. Both scenarios are based on the same chemical reaction with DA hydroquinone being oxidized by Cu^{2+} (or potentially another transition metal) to form DA quinone. The reduced Cu^+ is then reoxidized to Cu^{2+} by oxygen. Under Scenario 1, DA quinone is a toxic substance that induces severe oxidative stress in neurons [36, 37] because DA quinone reacts readily with cellular nucleophiles, such as reduced thiol groups of GSH, small peptides and protein cysteinyl residues.

In contrast, under Scenario 2, DA quinone is a protective substance [38, 39] that activates the Keap1/Nrf2 pathway through S-alkylation of targeted cysteines on Keap1 [1–4]. Recent papers show that DA and other catecholamines produce electrophilic quinones that activate the Keap1/Nrf2 pathway and can thus protect neurons against oxidative stress [38, 39]. These findings may represent a more general phenomenon given reports on other hydroquinones [24–27]. It should be noted that the toxic as well as the predominant protective effects of DA quinone appear to be independent of DA receptor activation and require high concentrations of DA quinone, which are indeed found under pathological conditions [36]. In fact, either protection or toxicity may result depending on the circumstances. One possible explanation is that DA quinone is toxic to neurons [36, 37] but protective to astrocytes [39]. Because of their high sensitivity to oxidative stress [30, 31], DA quinone appears to be directly toxic to neurons. In contrast, at least in some cases, DA quinone appears to be better tolerated by astrocytes and may activate their Keap1/Nrf2 pathway [39]. Thus, astrocytes may protect themselves and in turn nearby neurons in a non-cell autonomous fashion, perhaps by generating protective molecules such as GSH and

neurotrophic factors after Nrf2 transcriptional activation [39]. Nonetheless, as discussed further below, an EP that is preferentially neuroprotective and cannot injure neurons would, in our opinion, be preferred. In that case, the potential consequences of toxic side reactions could be avoided entirely. The complex action of quinones teaches us that the exact nature and location of the response is critical for the prediction and interpretation of the biological actions by EPs.

(5) CA Quinone as a protective compound

Similar to DA quinone, CA quinone may potentially be involved in both protective and toxic pathways. CA has polyphenol groups and transfers two protons and two electrons to oxidants such as oxygen or peroxide; this results in oxidation of CA to a quinone [40–43]. Under Scenario 1, the protective action is mediated by donation of protons and electrons, while the quinone form is the toxic substance. Scenario 1 is derived from three basic concepts concerning the biological actions of polyphenols that had been postulated in prior publications:

1. The two protons and two electrons in the reaction can lead to a protective action.
2. The Quinone form product of the reaction is a toxic substance.
3. The protective action is correlated with the number of phenolic hydroxyl groups.

However, several recent lines of evidence suggest that point #3 is not correct. A comparison of various chemical derivatives demonstrates that the number of hydroxyl groups does not correlate with the protective effect [44–46]. Instead, these reports showed that the type of geometric phenol isomer is a much more important determinant of protection than is the actual number of hydroxyl groups [44–46]. Incorporating this evidence concerning the relationship between geometric phenol isomers and Nrf2-targeted antioxidant action [1–4] has allowed us to propose a new model for the protective antioxidant actions of CA and CS [10]. Recently, we proposed to broaden this interpretation [9–11] to encompass the biological actions of additional if not all polyphenols [12].

The core concepts of this new interpretation consist of the following four points:

1. The Quinone form constitutes the major protective substance.
2. Transfer of the two protons and two electrons constitute a minor protective pathway.
3. The hydroquinone represents a pro-electrophile form, preceding the formation of quinone.
4. The specific geometric isomer (e.g., *para*- or *ortho*-hydroquinone) is an important determinant of neuroprotective activity.

Under Scenario 1, CA reacts with an oxygen radical in a 1:1 manner, and the resulting oxidized molecule is toxic unless it is reduced by a specific recycling system [40]. In contrast, under Scenario 2, the phenolic form of CA is protective due to the highly amplified antioxidant actions of Phase 2 enzymes that result from Nrf2 transcriptional activation. Due to this transcriptional activation and thus long-lasting effect, even transient treatment with CA can initiate sustained antioxidant activity [10, 41–43]. Hence, Scenario 2, but not Scenario 1, can explain the potent and prolonged protective effects observed after treatment with CA [10, 41–43]. We note that CA and CS, in addition to their possessing *ortho*-hydroquinone cores, share several chemical characteristics, such as 1) they are both diterpenes and 2) they are both pro-electrophilic [10–12]. We focus on the third feature in this review

because prior work has indicated that the pro-electrophilic properties contribute to the observed protective effects of these compounds in the virtual absence of side effects [12].

An important question concerns the observation that CA and CS are clinically tolerated and safe, and yet catechols and DA can be toxic. In other words, although catechols and DA have a cyclical structure with an electrophilic core, similar to CA and CS, why are catechols considered to be toxic substances and thus not useful as potential PEDs? The first potential explanation concerns their redox potential. The oxidation potential of catechol (528 mV) is much higher than that of CA (345 mV), as determined by cyclic voltammetry, suggesting that CA can convert to the active quinone form much more readily than catechol. The second potential explanation concerns the hydrophobic microenvironment of the target Cys(151) on Keap1 [47, 48], which is the apparent target of CA [10]. Because of a stretch of hydrophobic amino acids surrounding this cysteine, molecules of strong hydrophilic nature cannot readily access the critical cysteine, resulting in modest activation of the Keap1/Nrf2 pathway at best. Considering the hydrophobicity of each compound, CA (LogP = 4.905) and CS (LogP = 4.336) are both hydrophobic, but hydroquinone (LogP = 1.295), as well as catechol (LogP = 1.295), L-dopa (−2.089) and DA (0.773) are all relatively hydrophilic (data from ChemBank: <http://chembank.broadinstitute.org/welcome.htm>). Thus, CA and CS can access the Cys(151) on Keap1 more efficiently than small hydroquinones. A third potential explanation is the hydrophilic microenvironment of the Cys residue of GSH. By virtue of this feature, catechol binds to the Cys residue of GSH more readily than CA, resulting in predominant reaction with and hence depletion of GSH by catechol but not by CA. By similar reasoning, most small hydroquinones (catechol, methyl-catechol, methyl-hydroquinone, L-dopa and DA) also readily deplete GSH and are thus excluded as candidate therapeutic PEDs because depletion of GSH would result in increased vulnerability to oxidative stress rather than in protection.

(6) Linear and cyclical EPs

Here, we divide neuroprotective EPs (designated EP2 and EP3 in Fig. 1) into two major categories, linear and cyclical EPs, as shown in Fig. 6, based upon their core electrophilic chemical structure. This is one of, but not the exclusive, criteria since catechol is a cyclical EP but not in the EP3 group because small hydroquinones do not have as broad a therapeutic index. In another words, a minor population of cyclical EPs constitute the EP3 group. Linear EPs include NEPP11 [49–51] and bardoxolone methyl [52], while cyclical EPs include CA and CS, and can contain *ortho*- or *para*-hydroquinone as the group that becomes the active electrophile [10–12]. Interestingly, bardoxolone methyl, which advanced to a phase 2 human clinical trial in the United States, has a linear electrophilic core structure [52]. Bardoxolone methyl is a very powerful activator of the Keap1/Nrf2 pathway, but this compound failed in clinical trials because of serious side effects [52], possibly due to the nitrile enone group on its electrophilic core (Fig. 6). We propose that there are at least two possible means to potentially overcome the side effects of linear EPs caused by GSH depletion: one is simplification of the chemical structure to an organic acid, exemplified by the recently FDA-approved drug (for multiple sclerosis (MS)) dimethyl fumarate (DMF or BG-12, trade name Tecfidera®); the other is cyclization of the alkyl chain to form a dihydroquinone, as in cyclical EPs such as CA and CS [9–12]. DMF is a linear EP (α,β -unsaturated carbonyl) similar to NEPP11 and there is controversy in the literature regarding its effect on GSH [53–60]. Some groups report that DMF decreases GSH levels [55–57], while others report increased levels [58, 59]. Still others report that GSH levels increase after transiently decreasing [60]. These contradictory results may be due to the nature of DMF as a linear EP (EP2 in Fig. 1); it may result in activation of the Keap1/Nrf2 pathway but also S-alkylation of GSH thiol [12]. In point of fact, we have shown that NEPP11 has features very similar to

DMF in this regard [8, 9]. The critical differences between DMF and NEPP11/bardoxolone methyl, however, may be hydrophobicity and molecular mass [12]. DMF is highly hydrophilic, while NEPP11/ bardoxolone methyl are hydrophobic; DMF is a very simple molecule, while NEPP11/bardoxolone methyl have complicated structures [12]. We have been concentrating on cyclization of chemical structures as the other measure to overcome the side effects of EPs due to GSH depletion [12], as discussed below.

Cyclical EPs are not electrophilic in and of themselves but are instead converted to electrophilic “quinones” in the presence of Cu^{2+} and oxygen. They can then form an adduct with cysteine thiol, for example, on Keap1 protein or GSH, by chemical reaction that falls under the rubric of S-alkylation [9–12]. The cyclical structure of the EP is also an intrinsic characteristic of the *pro-drug* properties of the EP3 group of compounds (cyclical EPs) in contrast to the active *drug* properties of the EP2 group (linear EPs) [10–12]. The fact that linear EPs (EP2) are very reactive drugs represents a serious flaw in their use as potential therapeutic agents because systemic administration can result in reaction with and hence depletion of thiol substrates, such as GSH, prior to their reaching the intended target in the brain or elsewhere [9, 10]. It would be far better to have pro-electrophilic compounds (represented by the EP3 group of compounds) that remain non-reactive until converted to an electrophile by the very oxidative insult that they will then counteract via activation of the Nrf2 pathway at or near the pathological site of intended action [12]. One reason for the advantage of conversion to the active electrophilic form at the intended site of action is that the oxidative stress present there will have already have severely depleted GSH. Hence, after conversion to the active form, the PED will react with the active thiol of Keap1 to activate Nrf2 rather than with the thiol of GSH. This concept is considered in further detail in the ensuing section.

(7) Pro-electrophilic drugs (PEDs)

Compounds that become active electrophiles after oxidation represent what we have termed a “Pro-Electrophilic Drug or PED,” [10–12] and we have shown that CA is an example of this type of compound (Fig. 6). *Para*- and *ortho*-hydroquinones, but not *meta*-hydroquinones, are neuroprotective pro-drugs since they become EPs (i.e., quinones) upon oxidation, but most importantly they are not active EPs until they are converted in this manner [10–12, 24–27]. Thus, oxidative damage itself converts the pro-drug, which is relatively innocuous, to an active neuroprotectant [21–23]. PEDs possess a definitive advantage for human drug development over other EPs because pro-drugs should manifest fewer clinical side effects [10–12]. For example, one problem with current EPs is that they react with and hence deplete GSH, which can contribute to cell damage even in normal tissue, rather than offering protection [10–12]. We have demonstrated that cyclical EPs, representing a subset of PEDs, appear to have two clear-cut advantages over linear EPs and similar compounds: (i) lower potential for toxicity and hence fewer clinical side effects due to conversion of the drug to an active EP by the very oxidative insult that it will then counteract at the pathological site, and (ii) effective penetrance into brain tissue given their chemical structure [12]. Moreover, when the hydroquinone form is oxidized to a quinone, it becomes more hydrophobic and will then tend to stay in the injured tissue rather than leave [12].

Additionally, some proteins contain active cysteine thiol(s), which can be easily converted to thiolate anion(s) if surrounded by basic amino acids [9]. Such cysteine residues are even more reactive with electrophilic compounds, and, in fact, many but not all EPs, including CA, appear to be more reactive with Keap1(Cys151) than GSH [47, 48]. In other words, low concentrations of CA quinone preferentially react with Keap1 over GSH and can thus contribute to activation of cell defense systems while sparing GSH [12]. Moreover, in cells

undergoing oxidative stress, GSH is generally depleted, as it is a first line of defense against oxidative stress [12]. Thus, in stressed cells where PEDs are converted to the active quinone electrophilic form, the resulting EP would be even more likely to react with Keap1(Cys151) since GSH is already depleted [12]. In summary, PEDs are converted from an electrophilic precursor (or pro-electrophilic) compound to an electrophilic/active form, which stimulates the neuroprotective Keap1/Nrf2 pathway [12].

(8) Candidate PEDs

In Fig. 7, we present several possible PEDs that are candidate therapeutics against neurodegenerative diseases. The quinone form of each of these cyclical EPs can activate the electrophilic counterattack response, which includes activation of the Keap1/Nrf2 pathway [12]. Each compound has been reported to induce phase 2 enzymes via activation of the Nrf2/ARE pathway [61–63] and to protect neuronal cells against oxidative stress. CA and TBHQ protected the rodent brain in various models of neurodegenerative diseases [10, 64, 65]. In particular, neuroprotective diterpene-type PEDs such as CA and CS [10], triterpene-type PEDs such as strongylophorine (STR) 8 from sponge [66], and synthetic compounds such as TBHQ [64, 65] and D1 [11], are quite striking in their ability to trigger the Keap1/Nrf2 pathway. Flavonoid-type PEDs, such as eriodictyol and fisetin, also manifest neuroprotective effects, although their action may occur via other pathways, including direct anti-oxidant effects [44–46]. All of these compounds have *para*-hydroquinone (TBHQ, D1 and STR8) or *ortho*-hydroquinone (CA, CS, fisetin and eriodictyol) as their electrophilic core and thus have potential PED properties [12].

Interestingly, PEDs such as CA and TBHQ are frequently used EPs in neuroscience experiments as agents for neuroprotection via activation of the Keap1/Nrf2 pathway [61–63]. Also, fisetin and eriodictyol, although they may in fact work via additional mechanisms as well, were identified from a vast number of flavonoids by the screening efforts of the Maher and Schubert group [44–46]. This body of data suggests the potential effectiveness of PEDs as neuroprotective agents, and suggests that natural compounds such as terpenes and flavonoids may be useful in this regard. Furthermore, we and others have recently shown that *para*-hydroquinones (*para* > *ortho* > *meta* isomers) form electrophilic compounds with increased electrophilicity and thus display maximal activation of the Nrf2/ARE pathway. Thus, we feel that these drugs may have the greatest clinical potential as neuroprotectants [11, 12]. Importantly, however, the ultimate success of a given PED in the clinic will depend on its balance between anti-oxidant effect and clinical tolerability [10]. Only clinical studies in humans will be able to determine eventual outcome, but the approach does appear to be a promising one. Given the result that *para*-dihydroquinone is a more potent activator than *ortho*-, at least within the same chemical context, we feel justified to move forward under the assumption that *para*-hydroquinone is a better electrophilic core than *ortho*- or *meta*-isomers for activation of Nrf2-targeted antioxidant pathways [11–12].

(9) Redox regulation by Cu²⁺/Cu⁺ recycling

Next, we describe the chemical justification that a subset of Pro-Electrophilic Drugs (PEDs) actually represent a Pathologically Activated Therapeutic (PAT). PEDs have the potential to become electrophilic, i.e., they are oxidized to the active quinone form to trigger an electrophilic counterattack via the Keap1/Nrf2 pathway. A Cu²⁺/Cu⁺ recycling system (Fig. 8) is critical to enhance quinone formation from PEDs [27]. It is important to emphasize that PEDs are preferentially activated in the face of oxidative stress rather than under physiological conditions (labeled A and B in Fig. 8) [21–23]. This occurs because quinone formation is under the influence of the cellular redox state, and in particular the Cu²⁺/Cu⁺ recycling system. While in many tissues Cu²⁺/Cu⁺ is present at micromolar concentrations,

in some tissues, including kidney and liver, its concentration exceeds 100 $\mu\text{mol/l}$ [27]. The redox-active transition metal copper can catalyze oxidative activation of a number of phenolic compounds via $\text{Cu}^{2+}/\text{Cu}^+$ cycling [27]. Under nonpathological conditions, CA hydroquinone is very slowly oxidized to CA quinone [10], whereas this is much faster in the presence of $\text{Cu}^{2+}/\text{Cu}^+$ recycling, suggesting that this may be a rate-limiting factor for CA quinone formation. The recycling systems labeled A and B in Fig. 8 differ in their driving force (their oxidative power as electron acceptors). The oxidative power of O_2 is the driving force of cycling system A, while B uses other ROS/RNS such as $\text{H}_2\text{O}_2/\text{ONOO}^-$. Because H_2O_2 has a much larger driving force than O_2 [27], the cycling rate of B is much greater than that of A. Furthermore, because protons can enhance the driving force of B, the acidosis present under pathological conditions may further increase flux via pathway B [34, 35]. These chemical dynamics provided by oxidative stress and acidosis indicate that CA quinone formation will occur much more rapidly under pathological conditions than under physiological conditions. It is these chemical conditions that afford the potential for generating a Pathologically-Activated Therapeutic (PAT) drug, as described in the next section [21–23].

(10) Pathologically-Activated therapeutics (PATs)

Given the redox regulation of the $\text{Cu}^{2+}/\text{Cu}^+$ recycling system, cells undergoing oxidative stress will preferentially generate active EPs from PEDs while normal cells do not possess the necessary condition to generate EPs from PEDs, making the PEDs relatively innocuous in normal cells not facing oxidative stress [17–19]. However, after conversion to the active form, PEDs take advantage of an important property of EPs, namely, their superiority to classical antioxidant molecules because of the sustained action and amplification of EPs via transcription-mediated signaling pathways [9–12].

In terms of developing a clinically-tolerated drug, we sought to learn principles from other recent ventures in successful drug development in the CNS. Along these lines, the development of the FDA- and European Union-approved drug memantine, an *N*-methyl-D-aspartate (NMDA) receptor antagonist, against dementia was in part based on the principle that drugs should interact with their target only during states of pathological hyperactivation, and not interfere with the target if it functions normally [15, 16, 21]. Such drugs therefore exhibit little interference with normal physiological functions. Drugs that have been developed using this strategy have been designated *Pathologically-Activated Therapeutics* (or PAT drugs) since a “pat” is a gentle tap and therefore may be better tolerated clinically [15, 16, 21]. PEDs are candidates to be PAT drugs because conversion to the active quinone form is redox-controlled and thus enhanced by the presence of the ROS that is causing the oxidative stress [12].

As stated above, the best PED compounds that are candidate PATs appear to remain relatively innocuous in their pro-drug form and only become active at the site of oxidative stress when converted from their hydroquinone to quinone form [12]. We believe that this chemical conversion is the key to understanding the molecular mechanism of the protective yet well tolerated effect afforded by PEDs [12]. During a number of neurodegenerative insults, oxidative stress plays a critical role in disease progression [30–32]. Published studies have demonstrated that this pathological insult can be used to activate pro-electrophilic compounds via their oxidation, which occurs specifically in the target tissue, so neuroprotection is provided where it is needed [10–12]. This approach thus represents a novel strategy against neurodegenerative disorders that could activate electrophilic drugs via pathological activity [10–12].

(11) Electrophilic counterattack

In response to EPs, some cells mount an ‘electrophilic counterattack,’ a system that detoxifies electrophiles and removes them immediately [9–14]. The electrophilic counterattack usually lies relatively dormant, with only mild baseline activity, but becomes activated by EPs [9]. This electrophilic counterattack can prevent neurodegeneration and tumor growth because it eliminates not only electrophiles but also ROS [9]. Talalay [1] was the first to introduce this concept and termed the phenomenon ‘chemoprevention’ in view of its cancer-combating properties. Several chemopreventive agents are electrophilic and increase cellular resistance to oxidative stress [1, 2]. This form of chemoprevention often entails a transcription-based mechanism involving the Keap1/Nrf2/ARE transcriptional pathway and the consequent induction of phase 2 antioxidant genes [1–7]. The phase 2 genes that encode enzymes representing a coordinated response to EPs include glutamyl cysteine ligase modifier subunit (GCLM), glutamyl cysteine ligase catalytic subunit (GCLC), GPX, glutathione peroxidase (GPX); NADPH-dependent GSSG reductase (GR), glutathione-S-transferase (GST), hemoxygenase-1 (HO-1), NADPH quinone oxidoreductase 1 (NQO-1), ATP-dependent reductase, sulfiredoxin 1 (SRXN1), peroxiredoxin 2 (PRX2), thioredoxin (TRX), thioredoxin reductase (TRXR), Na⁺ - independent cystine-glutamate exchanger (xCT), and ATP-binding cassette, sub-family C (ABCC) [1–4]. In addition to generating GSH, these enzymes also represent an *endogenous* antioxidant response, for example, by detoxifying peroxides and other forms of ROS.

As introduced above, in neurons EPs manifest two disparate actions: a neurotoxic effect, mediated by a decrease in total cellular reductive capacity, and an electrophilic counterattack through the induction of phase 2 enzymes [9]. These enzymes can be upregulated by the transcription factor Nrf2 acting coordinately with a battery of cytoprotective proteins [9–12]. This elaborate network of protective mechanisms allows eukaryotic organisms to counteract the damaging effects of oxidants and other electrophiles, which represent a major class of agents that are thought to contribute to the pathogenesis of cancer, atherosclerosis, neurodegeneration, as well as normal aging [9]. Therefore, the Keap1/Nrf2 signaling pathway has become an attractive target for the prevention and treatment of oxidative stress-related diseases [61–63]. Over the last few decades, numerous Nrf2 inducers have been developed and some of them, such as bardoxolone, have undergone human clinical trials [52–54]. Since modulation of Nrf2 has been shown to occur in animal models of several neurodegenerative disorders [64, 65], activation of Nrf2 has been suggested as a therapeutic avenue for several of these conditions, including Alzheimer’s disease (AD), PD, multiple sclerosis (MS), and amyotrophic lateral sclerosis [61–63]. The success of DMF, a member of the EP2 group of Fig. 1, as an FDA-approved drug for MS, suggests that Nrf2 activators may be clinically-tolerated drugs in the CNS [53, 54]. DMF appears to have a safety and efficacy profile that would make it a first-line agent against MS, and tolerability data to date seem reasonable [53, 54]. Moreover, DMF reportedly also has anti-inflammatory effects [52, 53]. Recently, DMF was reported to activate the Keap1/Nrf2 pathway and increase cellular resistance to oxidative damage in primary neurons [58]. Accordingly, DMF increases cellular redox potential, glutathione levels, ATP levels, and mitochondrial membrane potential in a concentration-dependent manner [58–60]. If these neuroprotective actions can be applied to other targets such as AD, PD and Huntington’s diseases [67], DMF, CA, or other similar agents may represent drug candidates that can be used against a variety of chronic neurodegenerative diseases through activation of the Keap1/Nrf2 pathway, as shown in Fig. 9.

(12) GSH as a major effector of cell protection

Through the study of various EPs, we suggested that the level of intracellular GSH is a key in determining whether an EP will be protective or toxic to neurons [12, 19, 20, 68, 69]. EPs can exert opposite effects on the level of GSH: they can directly react with GSH to deplete it, or they can induce phase 2 enzymes that synthesize GSH (via electrophilic counterattack) [12]. If GSH depletion outweighs electrophilic counterattack, then the EPs will be toxic by rendering the cells more susceptible to oxidative and nitrosative stress [12]. Thus, preferential activation of electrophilic counterattack, while minimizing the GSH depletion, has been proposed as an effective therapeutic strategy against neurodegeneration [12]. We feel that PEDs are a viable solution to effect electrophilic counterattack and manifest the following attributes: (i) neuroprotection is transcription dependent and thus requires pretreatment; (ii) the protective compounds can generate electrophiles; (iii) the compounds increase essential cellular redox factors such as GSH; and (iv) the compounds induce the expression of phase 2 enzymes through the Keap1/Nrf2/ARE transcription factor pathway [51, 52, 61–63].

The Keap1/Nrf2/ARE transcriptional pathway provides an effective 'backup system' for cysteine-based redox regulation (provided predominantly by GSH) via activation of endogenous gene networks that are involved in antioxidant defense [42, 43, 64, 65]. S-alkylation of critical cysteine residues by PEDs activates this transcriptional pathway, representing an electrophilic counterattack [12]. Since neurons have little redox reserve, this backup system becomes very important in the nervous system [42, 43, 64, 65]. Because EPs, including CA and CS, are effective activators of the Keap1/Nrf2/ARE pathway, they can induce the expression of a set of metabolic antioxidant enzymes, called 'phase 2 enzymes,' which includes enzymes that synthesize GSH. In fact, GSH synthesis represents one of the main effector systems of the electrophilic counterattack that is activated by CA and CS, and GSH-based redox regulation is an effective frontline defense to combat oxidative and nitrosative stress [10–12, 42, 43].

(13) Astrocyte- and neuron-mediated neuroprotection

Interestingly, the cellular distribution of linear EPs and cyclical EPs in the brains of mice treated with these compounds appears to be different and suggests that there are astrocyte-mediated neurotrophic actions of CA (Fig. 10) [10, 57, 58]. Linear EPs, including NEPP11, appear to act preferentially on neurons based on our earlier findings that NEPP11 accumulates in neurons as opposed to astrocytes; consequently, NEPP11 induces phase 2 enzymes in neurons, including HO-1 [8–9]. In contrast, CA appears to preferentially act on astrocytes, as evidenced by the fact that biotin-labeled CA preferentially accumulates in astrocytes [10]. In view of these findings, we proposed that linear EPs such as NEPP11 exert direct protective effects on neurons [8], while cyclical EPs/PEDs such as CA exert non-cell autonomous effects via astrocytes to protect neurons [10]. In all likelihood paracrine or non-cell autonomous effects are the most plausible mechanism for neuroprotection by most PEDs, and candidate mediators of neuroprotection that are released from astrocytes have been identified by microarray analysis [64]. For example, CA potentially releases nerve growth factor (NGF) protein from glioblastoma cells and primary astrocytes [75–79]. Release of other non-cell autonomous, potentially neuroprotective molecules may include additional neurotrophins, such as brain-derived neurotrophic factor (BDNF) or ciliary neurotrophic factor (CNTF).

Another possible candidate for the action of PEDs is the production of GSH since CA and TBHQ potentially induce GSH synthesis in astrocytes [64, 65]. Importantly, tissues undergoing oxidative stress generally involve both neurons and astrocytes, so astrocytes

contiguous to stressed neurons may also be expected to have undergone insult and initiated protective actions via an electrophilic counterattack response [70, 71]. In fact, in a number of neurodegenerative diseases, it has become increasingly evident that it is the non-neuronal cells or astrocytes that mediate both protective and toxic effects on neurons. Thus, the effects of PED treatment on astrocytes may in fact be a more important mechanism than activation of transcriptional pathways in neurons themselves [10].

The role of the astrocyte and the mechanisms underlying the neuroprotective components that confer resistance to neurons has been reported by Johnson and Murphy's groups [39, 64, 65]. They have shown, based on *in vitro* data using TBHQ, that increased GSH secretion from astrocytes following Nrf2–ARE activation is the primary factor leading to neuroprotection of CNS neurons [64, 65]. The preferential activation of Nrf2 in astrocytes leads to more efficient GSH synthesis and thus higher GSH content in astrocytes than neurons [64, 65]. Increased production and secretion of GSH by astrocytes improves the antioxidant status of cocultured neurons and protects them from oxidative insults [64, 65]. Secreted GSH can protect neurons by acting as an antioxidant in the extracellular compartment and/or boosting GSH levels in neurons by increasing the availability of precursors for GSH synthesis [64, 65].

p62/ZIP is another possible candidate for the action of PEDs [72–75]. p62/ZIP proteins anchor various signaling platforms, including members of the Trk and Keap1/Nrf2 pathways. Importantly, induction of p62/ZIP is regulated by Nrf2 [72–75]. Thus, p62/ZIP and Nrf2 may be essential for each other's activity in biological systems, including neurons and astrocytes [72–75]. This notion is supported by results from p62 gene-knockout mice, which display an AD-like phenotype, including age-dependent neurofibrillary tangles, memory deficits, loss of synaptic plasticity, and accumulation of polyubiquitin [72].

PEDs such as CA also enter neurons and have direct effects on the Nrf2/ARE pathway in these cells as well as in astrocytes [10]. In neurons, CA induces phosphorylation of Trks and p62/ZIP, both of which positively activate the NGF signaling pathway [75]. Since CA induces expression of both NGF and GSH in primary astrocytes, it is likely that these and other trophic agents are released by the astrocytes to act on contiguous neurons in the brain [77–79]. Therefore, these prior reports suggest that CA can activate non-cell autonomous as well as cell autonomous effects on neurons (Fig. 10) [12].

(14) Potential for Carnosic Acid (CA)/Carnosol (CS) in clinical practice

Rosemary (*Rosmarinus officinalis*) is widely used and commercialized, not only as a culinary herb but also as an antioxidant in foods, nutritional supplements, and cosmetics [80–87]. The plant also has been used in traditional folkloric medicine as a natural healing remedy with alleged therapeutic effects such as prevention of neurodegenerative and cardiovascular diseases, inflammation disorders, and reduction in cancer risk [80–87]. Recently, biopharmaceutical attention has focused on rosemary due to the presence of active chemical substances that may act as potential drugs [85, 86]. Rosemary is known to contain a variety of polyphenols, including caffeic acid, rosmarinic acid, luteolin, verbenone, genkwanin, carnosic acid (CA) and carnosol (CS). CA and CS have been identified as the predominantly active compounds in rosemary [42, 43]. For example, rosemary leaves contain approximately 5% CA+CS by dry weight, and this fraction is estimated to account for >90% of the antioxidant-inducing activity [40]. Thus, Takahashi et al. [42] and Tamaki et al. [43] reported that CA and CS are the protective compounds among the various rosemary-derived compounds. Moreover, CA and CS share similar biological actions although CA is more potent than CS in various model systems [42, 43]. It should be noted that CA and CS are capable of both directly scavenging free radicals [40] and indirectly

increasing endogenous cellular antioxidant defenses via activation of the Nrf2 transcriptional pathway, as well as potentially via multiple other mechanisms [10, 11, 79, 92–94]. We feel, however, that activation of the Nrf2 pathway represents a major mechanism of action for CA and CS, particularly in the central nervous system (CNS).

CA and CS are *ortho*-diphenolic diterpenes with an abietane carbon skeleton containing hydroxyl groups at positions C-11 and C-12. CS has a lactone moiety across the B ring, while CA has a free carboxylic acid group [10, 41]. CA manifests significant protective effects in a cerebral artery occlusion model in mice (10), ameliorates obesity and hepatic steatosis in ob/ob mice [88, 89], inhibits microglial activation by lipopolysaccharide [90], inhibits adipocyte differentiation of 3T3-L1 cells [42], and abates light-induced retinal degeneration in rats [91]. In addition, CA and CS have been reported to display beneficial effects against acute and chronic inflammation, cardiovascular diseases, obesity, and cancer [94, 95], inhibition of prostaglandin synthesis [96], skin inflammation [97], p38 protein kinase activation [98], antiangiogenesis [99], protection against cisplatin [100], induction of neurotrophins [101], protection in an Alzheimer's disease model [102], inhibition of NF- κ B [103], inhibition of 5-lipoxygenase [104] and protection against dieldrin-induced degeneration of dopaminergic neurons [105]. For these reasons, we expect there to be intense activity in the coming years in an attempt to move these compounds or similar agents into clinical trials of specific patient populations, particularly for the CNS.

(15) Conclusions

The level of GSH can reflect differential effects of compounds in the EP1, EP2 and EP3 categories. Specifically, compounds in the EP1 class generally decrease GSH via S-alkylation, while EP2 compounds display more variable effects on GSH levels depending on concentration and cell type. In contrast, the EP3 class of agents increases GSH levels, predominantly via Nrf2-mediated transcriptional activation of glutathione synthetic enzymes. These differential actions on cellular GSH levels are critically related to the EP chemical structure. The chemical and biological characteristics of the EP3 class of drugs is closely connected to the electrophilic core structure (*ortho*- and *para*-hydroquinones). Because of the favorable features of this class of structures, we have been concentrating on the study of EP3 compounds such as CA and CS. CA has an *ortho*-hydroquinone group and can transfer two protons and two electrons to oxidants such as oxygen or peroxide; this reaction results in oxidation of CA to a quinone. CA and CS, compounds, which are found in the herb rosemary (*Rosmarinus officinalis*), are not themselves electrophilic but in response to oxidation become electrophilic, and then activate the Keap1/Nrf2/ARE transcriptional pathway to synthesize endogenous anti-oxidant 'phase 2' enzymes. Thus, the very oxidative insult that these drugs are designed to combat represents the chemical stimulus that converts the compound to its active form. The chemical basis for PED and PAT drugs is embodied in the *ortho*- and *para*-hydroquinone electrophilic core of the molecule, which is oxidized by the Cu²⁺/Cu⁺ cycling system. Importantly, this cycling pathway is under stringent regulation by the cell redox state. We propose redox-dependent quinone-formation as the core mechanism driving the formation of PED and PAT drugs. In fact, redox-dependent generation of the active form of a drug from the "pro-form" distinguishes this therapeutic approach from traditional EPs such as DMF and curcumin. The pro-drug quality of PED/PAT compounds is a key factor for generating drugs that are clinically tolerated and yet useful for combatting neurodegenerative diseases.

ABBREVIATIONS

ABCC ATP-binding cassette, sub-family C

ARE	antioxidant response element
CA	carnosic acid
CS	carnosol
DA	dopamine
DMF	dimethyl fumarate (also known as BG-12)
DOX	doxorubicin (also known as adriamycin)
EP	electrophile
GCLM	glutamyl cysteine ligase modifier subunit
GCLC	glutamyl cysteine ligase catalytic subunit
GSH	glutathione
GST	glutathione-S-transferase
H₂O₂	hydrogen peroxide
HO-1	hemeoxygenase-1
HSE	heat-responsive element
HSF-1	heat-shock factor-1
HSP	heat-shock protein
Keap1	Kelch-like ECH-associated protein 1
MS	multiple sclerosis
NEPP	neurite outgrowth-promoting prostaglandin
NGF	nerve growth factor
NQO1	NADPH quinone oxidoreductase 1
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
PAT	pathologically activated therapeutic
PD	Parkinson's disease
PED	pro-electrophilic drug
RNS	reactive nitrogen species
ROS	reactive oxygen species
STR	strongylophorin
TBHQ	<i>tert-butyl</i> hydroquinone
xCT	Na ⁺ -independent cystine-glutamate exchanger

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Highlights

- We propose two novel types of clinically-tolerated drugs.
- Pro-Electrophilic Drugs (PEDs) and Pathologically-Activated Therapeutics (PATs).
- PEDs/PATs can manifest pro-drugs because of the presence of an ortho- or para-hydroquinone.
- PEDs are converted by oxidative stress to electrophiles, which activate the Nrf2/ARE pathway.
- PEDs produce anti-oxidant phase 2 enzymes to combat cellular redox stress.

Electrophile-Cell Interactions

Fig. 1. Electrophile-Cell Interactions

A. Dose-response curves. Because all EPs have two opposing actions on cells, toxic and protective, these compounds manifest an inverse U shaped dose-response curve. There are three distinctive groups of EPs in terms of the broadness of their therapeutic index: EP1, none; EP2, limited; EP3, broad. **B. Three distinctive groups of EPs.** The size of the arrows indicates the relative strength of the opposing effects of GSH depletion vs. Electrophilic counterattack in response to EP treatment.

Chemical conversion of geometric isomers

A. Para-Hydroquinone (hydroquinone)

B. Ortho-Hydroquinone (catechol)

C. Meta-Hydroquinone (resorcinol)

Fig. 2. Chemical conversion of geometric isomers

Geometric isomers of hydroquinones donate an electron and proton to Cu^{2+} and are thus converted to a semiquinone. Cu^+ is reoxidized by an oxygen molecule. *Para*- and *ortho*-hydroquinones (A and B) convert to quinones, but *meta*-semiquinone (C) is not converted to a quinone form. Apparently for this reason, *para*- and *ortho*-hydroquinones, but not *meta*-hydroquinone, can activate the Keap1/Nrf2/ARE transcriptional pathway.

Fig. 3. Effects of various geometric isomers of hydroquinones on biological systems

A and B. ARE and HSE activation. HT22 cells transfected with the reporter constructs, ARE(GST-Ya)- or pTK-hHSP70-luciferase vector, were treated with vehicle or 2 μ M hydroquinone geometric isomers 1, 2 or 3 (as indicated in Fig. 2). After 24 h, activity was measured by reporter gene assay, as described previously [8–11]. Values are mean $\pm$ SD; * p < 0.05, ** p < 0.01. **C. Protective effects of geometric isomers.** HT22 cells were seeded onto 24-well plates at a density of 4×10^4 cells/cm². After a 5-h incubation, the various isomers of the compounds were added. One hour later, the cells were exposed to glutamate for 24 h and then subjected to a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) viability assay, as described previously [106–109]. High concentrations (5 mM) of glutamate induce oxidative stress in HT22 cells due to GSH depletion, a process termed “oxidative glutamate toxicity” [110]. The various geometric isomers of hydroquinone compounds offer varying degrees of protection from this oxidative stress. Values are mean $\pm$ S.D. (n = 4) and represent percentage of control (in the absence of glutamate exposure). **D. Antioxidant actions of geometric isomers.** Summary of biological activity of the three geometric isomers in activating the Nrf2 cellular antioxidant defense system. “Antioxidant activity by electron donation” represents reductive power originated from the chemical reactions described in Fig. 2. “Antioxidant activity by Nrf2 activation” represents phase 2 induction by S-alkylation of Keap1 and Nrf2 activation. Note that ‘Total antioxidant actions’ represents the sum actions of “Antioxidant activity by electron donation” and “Antioxidant activity by Nrf2 activation.”

Conversion of DA to DA Quinone

Fig. 4. Conversion of DA to DA Quinone

In Scenarios 1 and 2, DA quinone can be a toxic or protective substance, respectively.

Circles show pro-electrophilic *ortho*-hydroquinone (left) and electrophilic *ortho*-quinone (right).

Conversion of CA to CA Quinone

Fig. 5. Conversion of CA to CA Quinone

In Scenarios 1 and 2, CA quinone can be a toxic or protective substance, respectively.

Circles show pro-electrophilic *ortho*-hydroquinone (left) and electrophilic *ortho*-quinone (right).

Linear and Cyclic EPs

Linear EPs

Cyclical EPs

Fig. 6. Two Distinctive Groups of Electrophiles

A. Linear EPs. Linear EPs are exemplified by NEPP11 [8] and bardoxolone methyl [48]. Circles highlight the linear electrophilic cores of the compounds. **B. Keap1 Activation by Cyclic EPs.** Catechol-type CA is oxidized to a quinone form, with the carbon at position 14 [C(14)] becoming electrophilic (*). This CA quinone is subject to nucleophilic attack by the cysteine thiol of Keap1 to form an adduct. The Keap1-CA adduct results in release of Nrf2 protein from the Keap1/Nrf2 complex. Nrf2 can then be translocated into the nucleus, where it activates transcription of phase 2 enzymes via ARE transcriptional elements of the cognate genes. These phase 2 enzymes improve the redox state of neurons, contributing to an endogenous anti-oxidant defense system. Note that quinone formation is enhanced under oxidative stress, as described in the text. (*) indicates electrophilic carbons in NEPP11 [8], bardoxolone methyl [52] and CA [10] (the electrophilic carbons are subject to nucleophilic attack by cysteine thiols such as Cys151 on Keap1).

Candidate PEDs **Fig. 7. Candidate PEDs**

PEDs, as proposed here, consist of two types, *ortho*- and *para*-hydroquinones. Circles highlight the electrophilic cores of the structures.

Redox Regulation of $\text{Cu}^{2+}/\text{Cu}^+$ Recycling

Fig. 8. Redox-regulation of $\text{Cu}^{2+}/\text{Cu}^+$ recycling

Because H_2O_2 is a much better acceptor of electrons than O_2 , the $\text{Cu}^{2+}/\text{Cu}^+$ recycling system is enhanced in the pathological state.

The Keap1/Nrf2 pathway

Fig. 9. The Keap1/Nrf2 pathway participates in electrophilic counterattack

The Keap1/Nrf2 pathway constitutes an electrophilic counterattack triggered by EPs, including DMF and CA. Note that EPs activate the pathway by triggering S-alkylation on the regulatory thiol of Keap1 protein.

Astrocyte and neuron-mediated neurotrophic actions by CA

Fig. 10. Astrocyte- and neuron-mediated neurotrophic actions of CA

CA preferentially acts on astrocytes, leading to the release NGF and GSH from astrocytes, both of which exert trophic actions on neurons [76–78, 64, 65]. CA is also thought to act directly on neurons to upregulate TrkA, ERK1/2 and p62/ZIP via Nrf2 activation [12, 75, 79]. CA-activated Nrf2 then induces p62/ZIP expression, which plays an important role in mediating neurotrophic actions [12, 75, 79].