Perspectives on the Development of Antioxidant Antiepileptogenic Agents

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Abstract: Epilepsy is a chronic disorder of abnormal electrical activity in the brain characterized by recurrent unprovoked seizures. Currently used pharmaceutical agents do not treat the underlying disease process, and a significant proportion of epileptic patients are refractory to current therapies. Therefore there is a strong need for additional therapeutic agents, especially those that address the underlying disease process of epileptogenesis. The redox potential of cells is maintained by an appropriate balance between pro- and anti-oxidative molecules; oxidative stress and increases in toxic reactive oxygen species occur when this balance shifts towards oxidation. Neural tissues are especially sensitive to oxygen levels, and oxidative stress is thought to be involved in epileptogenesis. Increases in reactive oxygen species occur in response to sustained neuronal electrical activity and seizures. Therefore antioxidants have been suggested as therapeutic design strategies for the treatment and modulation of epilepsy. This minireview focuses on several key antioxidants and agents involved in defending against oxidative stress that may be targets for new antiepileptogenic drug design, including direct-acting antioxidants, Nrf2-activating agents, and prolyl-4-hydroxylase inhibitors. A description of the necessary physico-chemical properties and a summary of animal models that are thought to be useful for developing antiepileptogenic agents are presented.

Keywords: Animal models, Antiepileptogenic, Antioxidant, Antioxidant response element, Hypoxic response element, Nrf2, Prolyl-4-hydroxylase, Reactive Oxygen Species.

INTRODUCTION

Epilepsy is a chronic disorder of abnormal electrical activity in the brain characterized by recurrent unprovoked seizures [1]. It manifests as a collection of disorders which vary widely in etiology, appearance, and severity. The epilepsies are common and often quite devastating disorders, affecting ~2.5 million people in the US [2]. Treatment with traditional antiepileptic drugs (AED) may reduce symptoms of seizures but does not influence the course of the disorder. Despite some benefit of AED treatment, approximately 30-35% of treated patients will be refractory [1]. These two factors indicate the need for additional therapeutic agents.

Classification of epilepsies due to mode of seizure onset has focused on two main divisions: partial (focal) seizures (originate in neural networks limited to one hemisphere) and generalized seizures (originate in bilateral networks) [3]. The knowledge of the pathophysiology of seizures has increased substantially in the past few decades, and the reader is referred to review articles for more details [4, 5]. Epilepsy is thought to be caused by some predisposing factor(s) (eg. genetic, age, environmental) and/or insult (eg. head trauma, status epilepticus, febrile seizure) that trigger the initial seizure. After this first event, brain neuronal circuitry undergoes changes that may eventually produce a condition of chronic, spontaneous seizures, ie epilepsy. Epilepto-genesis is a term for which there is no universally accepted definition [6]. At a minimum, it refers to the time period between the initial insult and spontaneous, recurring seizures. More recently it has been extended to include both this initial latency period up to the point of epilepsy, as well as the progression of events that occur after epilepsy has been established [7-9]. Epileptogenesis has been described as a continuous process that extends much beyond the first spontaneous recurrent seizure [10]. This view of epileptogenesis expands the opportunities in which new acting agents may be tested. Antiepileptogenic agents therefore would have such activities as decreasing seizure frequency and severity, increasing responsiveness to traditional AEDs, and decreasing pharmacoresistance [9]. They would have the potential to be given for a finite period of time until their reparative action has been completed and then discontinued. Providing neuroprotective actions would slow or prevent the pathological neuronal alterations that eventually lead to a state of chronic seizures. Thus, antiepileptogenic agents provide clear advantages in the treatment of epilepsy and promise of better clinical outcomes and have been the focus of new drug development. These antiepileptogenic agents are likely to be different from more traditional AEDs [9, 11], and various animal models have been utilized to identify potential new agents [12].

Many molecular changes and signaling cascades are involved in seizure generation and development of epilepsy, including elevated intracellular calcium, apoptosis, production of free radical reactive oxygen species (ROS), uncoupling of mitochondria, and stimulation/induction of enzymes, to name just a few. Many of these molecules and systems involved in the regulation of neuronal activity have been studied as potential targets for new agents and excellent

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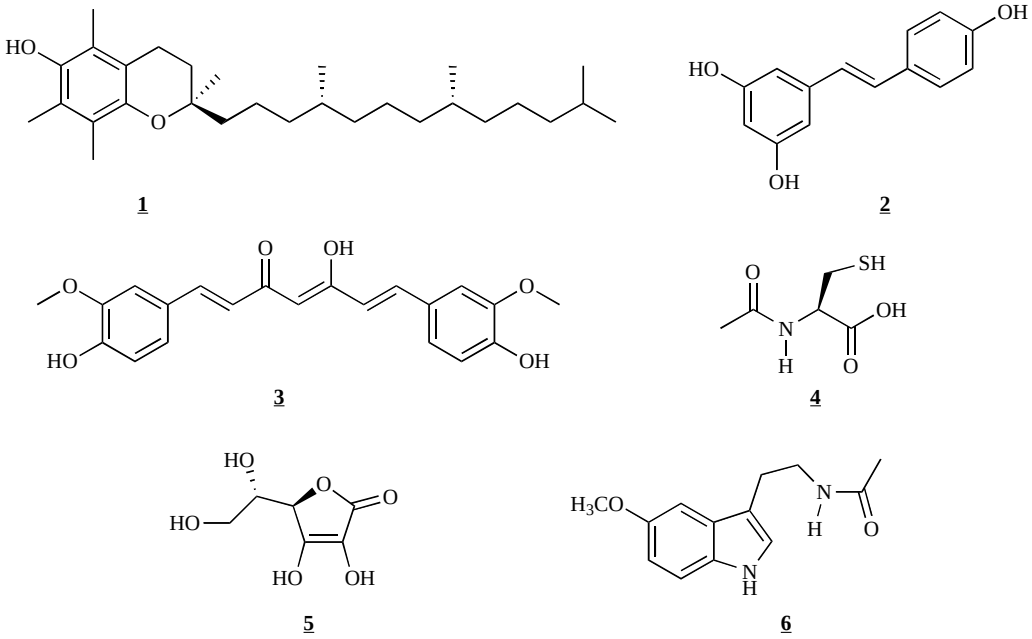
reviews exist [8, 13, 14]. Oxidation, the loss of electrons, is a biochemical process influenced by pro-oxidant and anti-oxidant compounds that maintain an appropriate balance under normal conditions [15]. Oxidative stress occurs when the balance is tipped in the favor of oxidation. This imbalance causes the accumulation of various toxic compounds generally referred to as free radicals, which are any molecule or element with an unpaired electron. Free radicals are highly reactive species that can damage lipids, proteins and nucleic acids and cause dysfunction. ROS are free radicals that contain oxygen, such as hydroxyl, nitric oxide and superoxide. Neural tissue is especially sensitive to oxygen levels, and oxidative stress is thought to have a significant role in the development and propagation of epilepsy and other neurodegenerative conditions [15-17]. Antioxidants have been suggested for therapeutic design strategies in the treatment and modification of epilepsy [8, 18]. This mini-review will focus on three types of agents known to be involved in the oxidative stress response, and from which targets for the development of new antiepileptogenic drugs are likely: 1) small molecule antioxidants, 2) antioxidant response element inducers, and 3) prolyl-4-hydroxylase inhibitors. A description of the physico-chemical properties that would be required for any of these proposed new agents is provided. Finally a brief summary of common animal models used to screen for antiepileptogenic agents is presented.

SMALL MOLECULE ANTIOXIDANTS

Small molecule antioxidants can be generally defined as molecules with the ability to quench, or reduce, ROS, which are a type of highly reactive radical. There are many examples of small molecule antioxidants, but we focus our discussion on those with known neuroprotection in seizure or epilepsy animal models. We will present their mechanism of antioxidant action, evidence for use in epilepsy, and potential modifications to enhance antioxidant and antiepileptogenic activity. Examples of modifications will be limited to several that are more likely to be successful and show good therapeutic promise. The list of antioxidants with activity in seizure or epilepsy animal models includes vitamin E type molecules, resveratrol and other phenols, N-acetyl cysteine and other thiol-containing molecules, ascorbic acid (vitamin C) and related tetronic acids, and melatonin and other alkaloids (Fig. **1**, compounds **1**-**6**). The development of new antiepileptogenic antioxidants would come from enhancing the potency of these known compounds by the addition of chemical functional groups. These new groups could contribute additive or synergistic antioxidant activity, enhance blood brain barrier (BBB) penetration, or improve pharmacokinetic properties. Also, the addition of functional groups may confer a new, non-antioxidant mechanism of action that is known to be involved in treating epilepsy.

There are various antioxidant mechanisms utilized by small molecules. Vitamin E type molecules, such as **1** α-tocopherol, typically contain a hydroxylated benzopyran and are highly lipophilic. The antioxidant activity comes from the ability of the hydroxybenzopyran to react with and quench ROS. Resveratrol, **2**, is a conjugated aromatic compound with a conjugated bridge between two aromatic rings. The antioxidant activity is due to the ability to delocalize a radical over an extended conjugated framework, thus stabilizing the radical. However, the stabilized aryloxy radical may also serve as a pro-oxidant by reacting with hydrogen peroxide and related oxygen species resulting in generation of ROS. Thus, resveratrol and related compounds can be either anti-oxidant or pro-oxidant, depending on physiological conditions [19]. Curcumin, **3**, is a related polyphenol with a similar antioxidant mechanism as resveratrol and may also be pro-oxidant under certain



1 **2** **3** **4** **5** **6**

Fig. (1). Small molecule antioxidants with known neuroprotection in seizure or epilepsy animal models. **1** α-tocopherol, **2** resveratrol, **3** curcumin, **4** N-acetyl cysteine, **5** ascorbic acid, **6** melatonin.

conditions. In addition to direct antioxidant action, curcumin also enhances the ability to induce various genes that are involved in responding to oxidative stress (see next section). The compounds in the thiol-containing group are similar to the major endogenous antioxidant peptide, glutathione, and the antioxidant activity comes from the reducing activity of the thiol group. This sulfhydryl provides an electron to ROS, causing ROS reduction and therefore decreased reactivity. The oxidized glutathione product that is generated (glutathione disulfide) is reduced by the action of glutathione reductases. N-acetyl cysteine, **4**, is a simplified mimic of glutathione with a similar antioxidant mechanism utilizing the thiol group to reduce ROS. Ascorbic acid, **5**, is a classic antioxidant that can react with ROS in a stoichiometry of 2:1 to yield dehydroascorbic acid. Melatonin, **6**, is an example of an endogenous biochemical hormone that is also a direct hydroxyl radical scavenger. The antioxidant mechanism of melatonin is uncertain, but most likely involves a combination of proton transfer, electron transfer, and sequential electron proton transfer [20].

All of the above small molecule antioxidants have shown various beneficial effects in some experimentally-induced seizure models or *in vitro* studies. α-Tocopherol has been shown to decrease lipid peroxidation and nitrite content in pilocarpine-induced seizures, and to increase catalase and superoxide dismutase activities [21]. Ascorbic acid or α-tocopherol suppressed behavioral seizure episodes and decreased brain damage in pilocarpine seizures [22]. Pretreatment with vitamin C before pilocarpine-induced seizures increased the latency to first seizures and decreased the mortality of rats, and also decreased lipid peroxidation [23]. Curcumin pretreatment reduced hippocampal cell death from kainic acid-induced seizures [24]. Resveratrol was associated with reductions in severity of kainic acid-induced seizures [25]. N-Acetyl cysteine has demonstrated an ability to suppress epileptogenesis in a combined fluid percussion brain injury/pentylenetetrazole model [26], probably due to the direct antioxidant effect and increase in cellular glutathione levels [27]. Melatonin has shown protective functions in various animal seizure models [28].

Despite these animal and *in vitro* results, the promise of small molecule antioxidants for the treatment of epilepsy in humans has yet to be realized [17]. One potential reason is that these chemical antioxidants suffer from a significant stoichiometric problem, namely the quenching of a low molecular weight radical (17 atomic mass units (amu) for hydroxyl radical or 32 amu for superoxide radical) by a higher molecular weight small molecule (chemical antioxidants range from 200 to 400 amu) in a stoichiometric reaction ratio of 1:1 or 2:1 unless the antioxidant can be regenerated enzymatically (see below). This direct chemical mechanism therefore requires a large amount of antioxidant to be present to have a significant effect, which may be difficult to achieve in central nervous system (CNS) tissues. Additionally, these antioxidants would also likely be reactive towards ROS in other tissues such as the heart, kidney and liver, further reducing the availability of the antioxidant to the CNS. Finally, there is evidence to suggest that small concentrations of ROS provide beneficial physiological functions in vascular cells [29], and antioxidant therapy could potentially cause adverse events in these particular situations.

Therefore, modifications to the approach of direct antioxidants as therapy need to be considered. It is difficult to circumvent the stoichiometry of direct antioxidants. However, existing small molecule antioxidants may be chemically modified in such a way as to incorporate an additional therapeutic action. General strategies for combining multiple activities in the same pharmacophore have been recently reviewed [30], and the use of virtual screening may be helpful in this regard [31]. A single entity capable of acting on multiple targets has several advantages over a traditional polypharmacy-based approach; lower risk of drug-drug interactions, less complex pharmacokinetics, increased efficacy, reduced adverse effects, and increased patient adherence [30]. One proposed method to enhance the efficacy of direct acting antioxidants is to combine multiple antioxidant activities in the same molecule. A simple method would be to tether two different pharmacophores possessing potent yet distinct antioxidant activities. Such an approach has recently been done with two antioxidants, coenzyme Q (CoQ) and lipoic acid [32]. Lipoic acid is a type of thiol antioxidant that works through a thiol/disulfide mechanism. These researchers combined the antioxidant functional groups of CoQ and lipoic acid into derivatives such as **7** (Fig. **2**), that retained both types of antioxidant activities, as well as other activities [32]. It remains to be determined whether these or other multi-targeted antioxidants have efficacy against epilepsy or other neurological pathology.

Melatonin, in addition to having antioxidant properties, is also known to suppress excitatory glutaminergic neuronal activity. A metabolite, kynurenic acid, also has antioxidant and anticonvulsant properties [28]. Melatonin is perhaps best known for CNS activity that involves the regulation of sleep cycles and circadian rhythm. There are three different melatonin receptors, MT1, MT2, and the recently identified MT3 receptors [33]. Agonism at MT1/MT2 receptors is thought to be the mechanism for inducing sleep. Unfortunately, melatonin has an extremely short half-life and is limited as a pharmaceutical agent. These factors prompted the search for melatonin analogs that retain agonist activity, and lead to the development of ramelteon, **8**, a non-selective MT1/MT2 agonist that is FDA-approved for insomnia. Development of melatonin agonists for CNS applications remains a major area of interest for the pharmaceutical industry, and several selective agonists have been described [34]. Recently, ramelteon has been shown to have anticonvulsant effects in an animal model of epilepsy [35]. Although melatonin has shown benefit in various animal epilepsy models [28], the evaluation of melatonin analogs in these models is sparse. In addition to enhancing receptor agonism activity, melatonin anlogs have been synthesized with enhanced antioxidant potential. The indole ring system was substituted with a phenylhydrazine derivative, **9**, that enhanced the antioxidant activity [36]. These new analogs have not yet been tested in animal models of epilepsy. Evaluation of melatonin-type antioxidants with selective or non-selective agonist activities may demonstrate potential in animal models of epileptogenesis, and the combination of reasonable pharmacokinetics, antioxidant activity, and

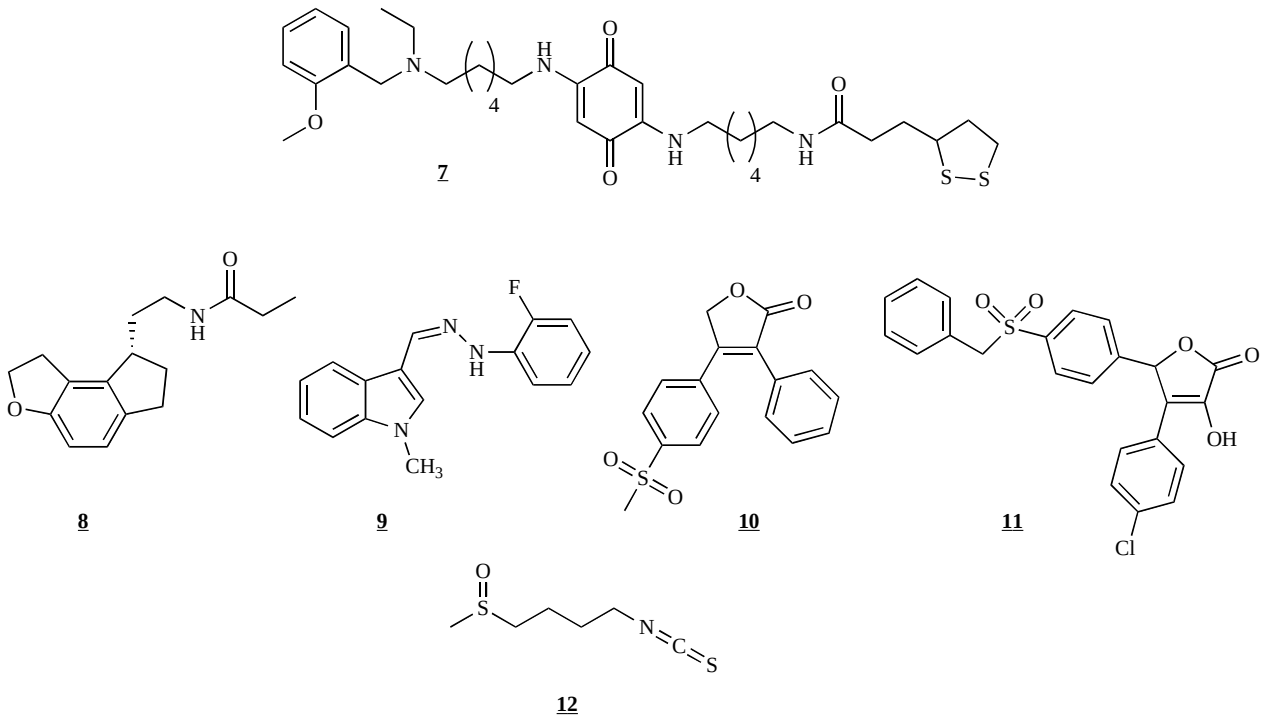


Fig. (2). **7** CoQ - lipoic acid conjugate [32], **8** ramelteon, **9** indole phenylhydrazide [36], **10** rofecoxib, **11** hydroxyfuranone [38], **12** sulforaphane.

melatonin receptor agonism may yield a new class of antiepileptogenic agents.

The potent antioxidant, ascorbic acid, has also demonstrated anti-seizure activity and neuroprotective effects in animals [22, 23]. For improvement, the existing ascorbic acid structure may be modified to incorporate additional beneficial antiepileptogenic activities such as anti-inflammation. The inflammatory process is clearly recognized as a contributor to the development of epilepsy [6, 8, 14]. An obvious drug target for inflammation remains cyclooxygenase, the enzyme responsible for producing multiple eicosanoids from arachadonic acid that are involved in pro-inflammatory actions. Inhibition of cyclooxygenase is the main mechanism of action of aspirin and other non-steroidal anti-inflammatory drugs. The cyclooxygenase inhibitor rofecoxib, **10**, has been shown to decrease seizure severity, to reverse the increases in lipid peroxidation, nitrite levels and myeloperoxidase levels, and to significantly restore reduced (ie. non-oxidized) glutathione levels in a kindling model of epilepsy [37]. These neuroprotective and antioxidant actions make cyclooxygenase inhibitors reasonable candidates to combine with small molecule antioxidants to potentially enhance activity. Several new hydroxyfuranone compounds that are related to ascorbic acid have been recently synthesized that are substituted by methylsulfonylphenyl, such as **11** [38]. These substituted aromatic groups are commonly found in non-steroidal anti-inflammatory drugs such as rofecoxib. These new hydroxyfuranone compounds retain antioxidant and anti-inflammatory activities as assessed by *in vitro* and *in vivo* methods respectively, and two compounds were especially promising as agents that protect against inflammatory tissue damage [38]. The *in vivo* activity shown by these compounds was peripheral, and it remains to be determined if these compounds will cross the BBB, a requirement if they are to be effective as antiepileptogenic agents. In this regard, transporter systems are known to be involved in carrying ascorbic acid into CNS tissue, suggesting that these analogs or related molecules may also penetrate the BBB.

ANTIOXIDANT RESPONSE ELEMENT INDUCERS

The primary biological antioxidant mechanism is provided by ROS-detoxifying enzymes such as superoxide dismutase, glutathione-S-transferase, glutathione peroxidase, and catalase, and the role of these enzymes in neurodegenerative diseases has been reviewed [39]. However, the effectiveness of these biological proteins when administered systemically is extremely poor due to their instability, poor absorption, and poor BBB transport. There have been determined efforts to develop nucleic acid vectors to deliver enzymes to the CNS through gene therapy [40], but success remains elusive. Small molecular mimics of these enzymes that retain catalytic activity have begun to be tested in models of epilepsy, but it is not known whether they reduce disease severity or progression [39].

An additional approach is to develop agents that increase the biosynthesis of these enzyme antioxidants through gene regulation. The simultaneous induction of numerous antioxidant enzymes occurs through the antioxidant response element (ARE), and the reader is referred to more detailed reviews of this pathway [41, 42]. Briefly, the transcription factor Nrf2 is normally bound by a cytoplasmic inhibitor, Keap-1, and prevented from nuclear translocation and transcription activities (Fig. **3**). However when Keap-1

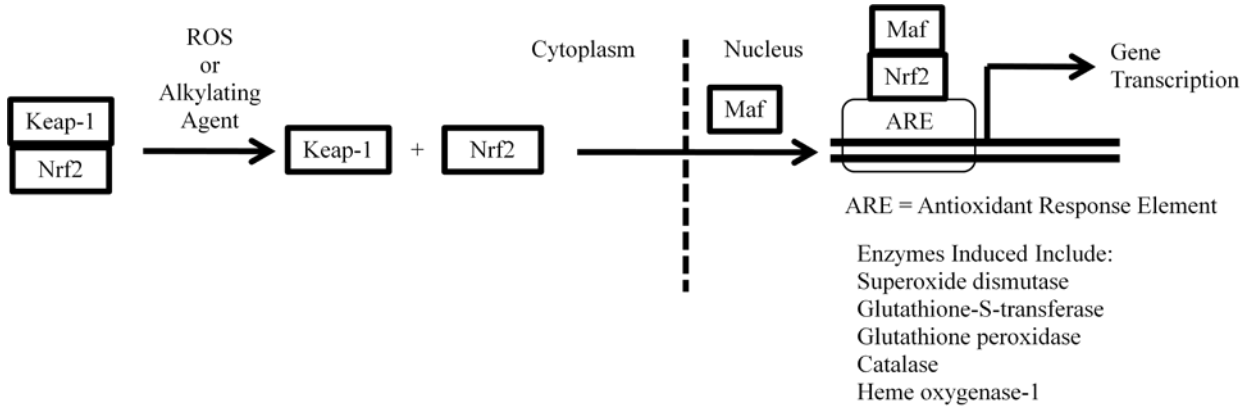


Fig. (3). Cartoon showing antioxidant response element (ARE) pathway. Oxidative conditions trigger release of Nrf2 from Keap-1, allowing nuclear transport of Nrf2. Maf is one potential partner to form an active dimer with Nrf2 to stimulate ARE-driven genes.

Abbreviations: Nrf2 = Nuclear Factor (Erythroid-derived 2) like-2 protein; Keap-1 = Kelch-like ECH-associating protein 1; Maf = musculoaponeurotic fibrosarcoma oncogene homolog, a leucine zipper transcription factor.

becomes oxidized by ROS, Nrf2 is released and can then enter the nucleus. There it dimerizes with different partners and stimulates AREs that drive the transcription of many different genes involved in responding to the oxidative stress, including the enzymes listed above. Thus, agents that activate ARE have been sought as chemoprotectants for a wide variety of disease conditions including neurodegeneration and epilepsy [43, 44].

There are numerous agents that activate the Nrf2/Keap-1/ARE pathway, and all known activators are electrophilic agents that react with and oxidize certain key cysteine residues of Keap-1 [43]. The polyphenol curcumin in its pro-oxidant form is thought to be a ROS generator that oxidizes Keap-1, and so promotes the release of Nrf2 [45]. Curcumin is also known to increase the expression of Nrf2 [46], and this may provide another mechanism to overcome the binding capacity of Keap-1. Another Nrf2 activator is the isothiocyanate sulforaphane **12**, which is thought to directly alkylate critical thiol groups on Keap-1, thus resulting in release of Nrf2 [47]. Many agents that are known to induce ARE have shown potential as therapies in various neurodegenerative diseases [43, 44].

The majority of these known agents are of plant or plant-derived origins, suggesting the continued exploration of these sources. Pharmacognosy has long been a major avenue of research in medicinal chemistry, providing many useful pharmacological agents from digoxin to paclitaxel. Ethnopharmacology and traditional Chinese medicine (TCM) have provided numerous sources of lead compounds based on long-term human medicinal use, and have recently begun to be evaluated in limited clinical trials. An advantage of investigating TCM is that the active compounds are very often phenols and flavonoids, classes of compounds with known ARE-inducing activity [43]. Another is that isolation of the active compound is generally easier than synthesizing de novo a new compound. Various TCM extracts have been shown to contain ARE-inducing activity [48]. Therefore TCM is a reasonable area in which to investigate new ARE-inducing agents. There have also been efforts to screen synthetic libraries to identify ARE activators with some success [49].

Specific TCM products currently under investigation for ARE-inducing potential include ginseng, Si-Wu-Tang, and *Ginkgo biloba*. Extracts of ginseng have long been suspected of possessing medicinal potential, and a recent review indicates that both Nrf2 activation and anti-inflammatory activity may be involved in the proposed activity of ginseng extracts [50], and others have shown protection of ginsenosides against kainate-induced neurotoxicity [51]. Si-Wu-Tang is a well-known phytoestrogen herbal complex that has been traditionally utilized for regulation of menstrual cycles and other women's health issues. Using gene expression microarray analysis of Si-Wu-Tang-treated breast cancer cells, the pathway most significantly increased by Si-Wu-Tang was Nrf2, and increased ARE transcriptional activity was also confirmed [52]. Extracts of *Ginkgo biloba* have been shown to activate the Nrf2 pathway with subsequent induction of heme oxygenase-1 and increased vascular heme oxygenase activity [53]. Thus there is good evidence for TCM-derived products to be sources for Nrf-2 activating agents. Once individual compounds are identified, they can be evaluated for CNS penetration and application to antiepileptogenic therapy.

HYPOXIC RESPONSE ACTIVATION AND PROLYL-4-HYDROXYLASE INHIBITORS

Oxygen homeostasis is critical for cell survival and the body has evolved many mechanisms to maintain appropriate tissue and cellular oxygen concentrations. There are many and complex compensatory mechanisms to respond to hypoxia, a condition of low oxygen levels. One compensatory mechanism utilizes hypoxia inducible factors (HIF), which are transcription factors that regulate the expression of hypoxia-induced genes. There are about 60 such genes identified, being involved in glycolysis, angiogenesis and vascular tone, erythropoiesis, iron homeostasis, apoptosis, and proliferation [54]. CNS tissue is especially sensitive to hypoxia, and the HIF system is thought to have an important role for neuronal survival. The

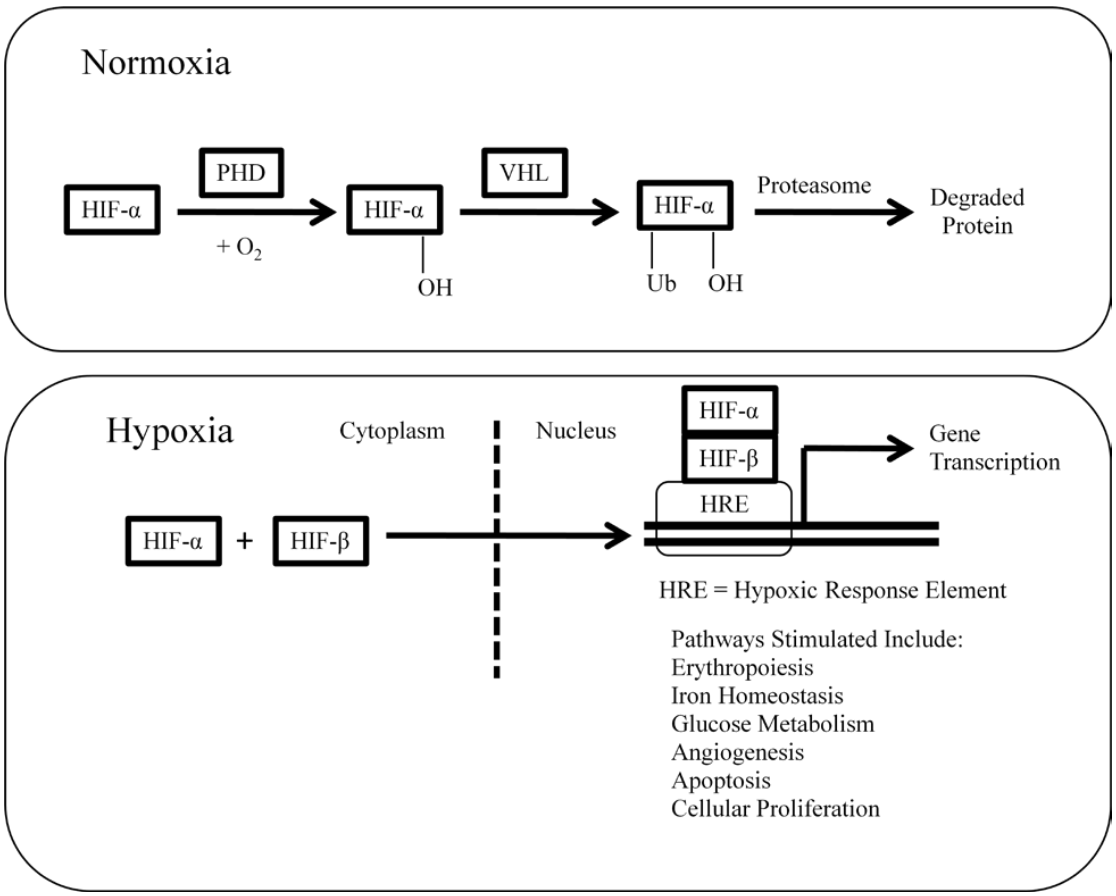


Fig. (4). Cartoon showing Hypoxia Induced Factor (HIF) pathway. Under normoxia, PHD hydroxylates HIF-α, targeting it for proteasomal degradation via a VHL-ubiquitination dependent process. During hypoxia HIF-α accumulates and enters the nucleus, forming an active dimer with HIF-β that stimulates HRE-driven genes. See text for details.

Abbreviations: VHL = Von Hippel Landau protein; Ub = ubiquitin; PHD = prolyl-4-hydroxylase.

reader is referred to reviews of the HIF pathway especially as it pertains to the CNS [54-56]. Briefly, the main factors are HIF-α and HIF-β, which are constitutively expressed proteins. Under normal oxygen conditions HIF-β is stable, but HIF-α is hydroxylated by prolyl-4-hydroxylase (PHD) and thereby targeted for proteasomal degradation. Under hypoxic conditions HIF-α is not hydroxylated by PHD, is not targeted for degradation, and is able to accumulate and form a dimer with HIF-β. The dimerized proteins serve as the master regulator for the HIF pathway by binding and activating the hypoxic response elements (HRE) upstream of the numerous hypoxia-induced genes (Fig. **4**). There is certainly a connection between hypoxia and the development of seizures and risk for epilepsy. The most common cause of pediatric seizures is hypoxic encephalopathy and various animal models of hypoxia-induced seizures exist [57]. Tang and colleagues examined the RNA expression profiles of rat brains after various insults, including ischemia and kainate-induced seizures. They found that many genes induced by ischemia were also induced by kainate [58]. Patients with brain tumors frequently present with seizures, and tumor-related hypoxia and acidity may be a major factor contributing to epileptic activity after tumor removal [59]. Thus it is reasonable to pursue HIF modifying-therapies for epilepsy.

As stated above, oxygen homeostasis is a complex and intricate process. Mitochondria consume the majority of oxygen in a cell, and the electron transport chain normally produces ROS. While it might be expected that during hypoxia ROS would decrease, this has not always been experimentally verified. In fact, a major effect of hypoxia is the increased generation of ROS from mitochondria [reviewed in 60]. Recently three independent groups provided genetic evidence that mitochondrial electron transport chain is required for HIF stabilization [60]. It is thought that mitochondria serve as oxygen sensors during hypoxia, and that PHD act as oxygen sensors during anoxia when the mitochondrial electron transport chain is not functional [60, 61]. Thus hypoxia produces mitochondrial-derived ROS that prevent the hydroxylation of HIF-α, through an as yet unknown mechanisms [60].

One of the genes up-regulated by HIF is erythropoietin (Epo). The role of kidney-derived Epo in the regulation of red blood cell production as a normal response to hypoxia is well known. However, hypoxia-induced increases in Epo have been described in other tissues including brain, and electroconvulsive seizures are also known to induce Epo [62]. There are many reports of the neuroprotective effects of Epo [63, 65], and recently it is described as having

antiepileptogenic effects [66-69]. Epo pretreatment delayed the onset and decreased the severity of kainate-induced status epilepticus (SE) and was effective if administered 24 hours prior to SE, but not 30 minutes prior [68]. Furthermore, a single dose of Epo provided protection for at least three days. These results indicate that Epo was not acting as a conventional AED, leading the authors to suggest that it may increase the expression of other protective genes [68]. In another report, investigators administered Epo immediately after cessation of SE induced by lithium-pilocarpine, and then daily for seven days. Epo provided multiple protective effects, including significant decreases in neuronal cell loss, microglial activation, and BBB disruption. Importantly, it was shown to decrease the frequency and duration of later-developing spontaneous seizures, further supporting a role in anti-epileptogenesis [69]. Additionally, Epo has been shown to activate the Nrf2 pathway and to ameliorate several adverse effects of traumatic brain injury, leading these authors to suggest that much of the beneficial effect of Epo is due to its stimulatory effect on Nrf2 signaling [70]. Although Epo was administered exogenously, this report raises the possibility of an endogenous cross-talk between the HIF and the ARE pathways. Stimulation of HIF during hypoxia induces Epo, which may then act to stimulate the Nrf2/ARE pathway.

Known adverse effects of Epo can occur due to increased blood viscosity from erythropoiesis. Therefore efforts to eliminate the erythropoietic function of Epo have been attempted. Carbamylated erythropoietin is a derivative that does not affect hematocrit but retains potent cell survival and function-enhancing effects [71]. It is currently being investigated for a variety of CNS indications, including traumatic brain injury, ischemic stroke, and spinal cord injury [72]. Another modified Epo lacking hematocrit effects is asialoerythropoietin, the product of recombinant human Epo that has been enzymatically de-sialylated with neuraminidase. This Epo derivative has shown neuroprotective activity against cerebral ischemia, spinal cord compression, and sciatic nerve crush when administered intravenously to rodents, as well as the ability to penetrate the BBB [73]. These authors report that a single exposure to Epo shortly before injury was able to produce protective effects several days later, which suggests that Epo increases the expression of protective genes. The fact that Epo is neuroprotective in a variety of hypoxic, hypoglycemic, and excitotoxic *in vitro* models and *in vivo* models, and has shown promise in pilot clinical studies of acute ischemic stroke and other neurological conditions [72], provide strong evidence for pursuing Epo as an antiepileptogenic therapy.

One mechanism for stimulating the HIF pathway would be through the inhibition of PHD which would prevent HIF-α degradation, and so generate the active HIF dimer. This would then result in the induction of a number of genes, including Epo and stimulation of the ARE response, all of which may be beneficial for treating epileptogenesis. PHDs are a family of 2-oxoglutarate (2-OG) dependent hydroxylases that utilize non-heme iron in the catalytic site, and require oxygen [55]. Various PHD isoforms exist, and specific functions of each are not yet completely known [54]. The crystal structure of PHD-2 has recently been solved [74], providing crucial information for the development of PHD inhibitors, and revealing specific molecular interactions between the 2-OG ligand, the critical active site iron, and Arginine-383 that is involved in 2OG recognition. Another residue, Tyrosine-303 appears to form a hydrogen bond with a critically positioned water molecule that is also important in 2-OG cofactor recognition.

Investigational small molecule PHD inhibitors exist (Fig. **5**, compounds **13**-**18**) and the majority of these mimic the 2-OG ligand [75-79]. All of these compounds possess a bi-dentate iron chelating motif utilizing either nitrogen or oxygen to form a five or six membered ring with the active site PHD iron. Derivatives of compound **13** are ~ 10 fold more potent against PHD1 and PHD3 than against PHD2, suggesting that the development of selective inhibitors may be possible [75]. Pyrazolopyridines, such as compound **14**, were shown to be potent PDH inhibitors capable of stabilizing HIF-α and increasing the expression of a HRE-driven gene in cells [76]. Caffeic acid phenethyl ester (CAPE), **15**, an active component of honeybee propolis extract, was shown to inhibit PHD, stabilize HIF-α, and induce the activity of several HRE-driven genes [77]. This report suggests that the non-ionic arginine recognition motif, the phenethyl ester moity of CAPE, possesses PHD inhibitory activity without requiring ester hydrolysis. Interestingly, CAPE is also reported to increase the activity of the Nrf2/ARE pathway [80], thus indicating that a single molecule is capable of activating two major nuclear response element pathways. The other inhibitors listed in Fig. (**5**) were also shown to stabilize HIF-α and increase the activity of HRE-driven genes. Compound **16** is orally bioavailable [78]. Ciclopirox, **17**, is a known iron sequestering agent with a different mechanism of inhibition than the 2-OG ligand mimics. It inhibits PHD by denying the enzyme access to the essential iron cofactor [79]. As such, therapeutic application may not be straight forward. Compound **18**, based on *in silico* calculations, lead the authors to suggest that it may be selective for PHD over other iron-containing enzymes such as asparaginyl hydroxylase and 12-lipoxygenase [79]. Finally, the investigational PHD inhibitor FG-2216 whose structure from the pharmaceutical company Fibrogen has not been publically disclosed, is in clinical trials and has demonstrated a greater than 10-fold increase in plasma Epo in anephric patients [81].

Future drug development of PHD inhibitors would need to address BBB penetration. Ideally, the agent would be rapidly transported into the CNS to prevent unnecessary peripheral activity. Additionally, these agents should meet minimal structure activity relationships for interaction with the catalytic site, which poses additional challenges. These would include the recognition of iron, hydrogen bond accepting functionality for the critically positioned water molecule, and preferably a non-ionic mechanism of arginine recognition to facilitate BBB transport. Functional groups with the ability to accept two hydrogen bonds from an arginine, such as a sulfone, methoxyacetyl, or potentially even an isoxazole ring may be useful in the development of a non-ionic PHD inhibitor. An obvious alternative to a non-ionic arginine recognition motif would be a prodrug designed to mask a properly positioned carboxylic acid or

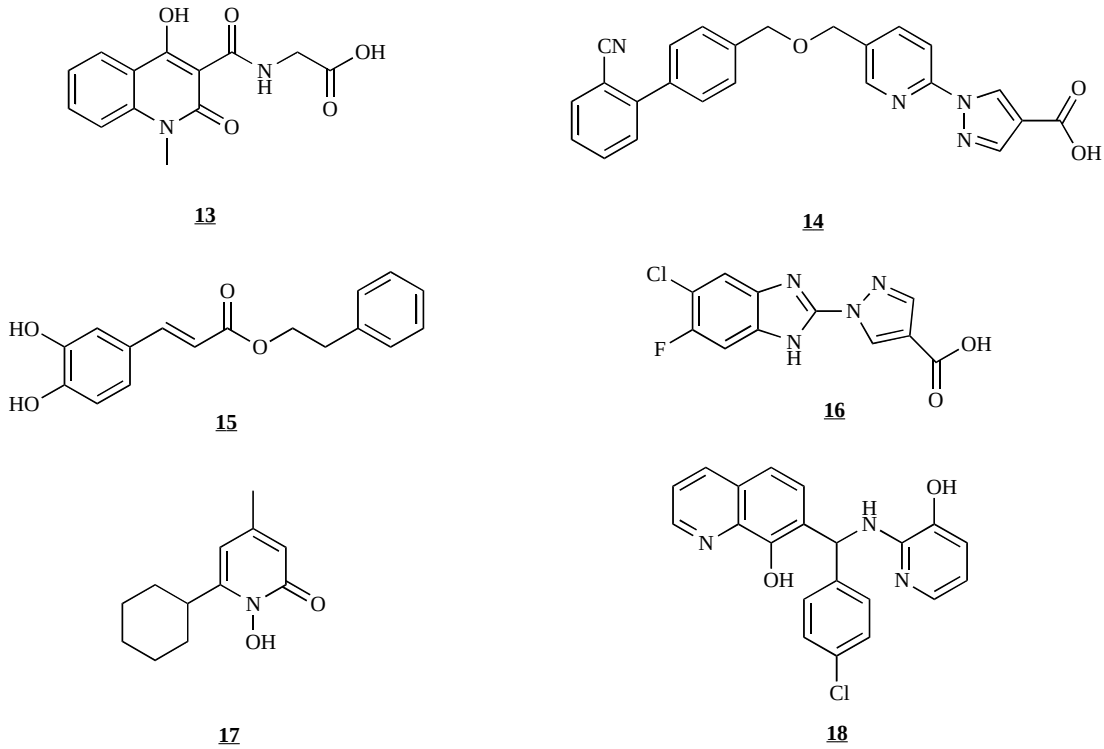


Fig. (5). Prolyl-4-hydroxylase inhibitors: **13** Amgen quinolone [75], **14** Proctor & Gamble pyrazolopyridine [76], **15** caffeic acid phenethyl ester [77], **16** JNJ-42041935 [78], **17** iron binding agent ciclopirox [79], **18** branched oxyquinlines [79].

equivalent that can be metabolized to the active drug species after penetrating the BBB. For example, PHD inhibitors could be esterified to remove the anionic charge resulting in a neutral molecule with enhanced BBB penetration. Due to the known connection between hypoxia and the risk for seizures, the development of agents that respond to hypoxia, such as non-specific and specific PHD inhibitors, may provide new therapeutic agents for antiepileptogenesis.

PHYSICAL-CHEMICAL REQUIREMENTS FOR AGENTS

While parenteral route of administration is sometimes utilized in the treatment of seizures, the vast majority of therapies are oral, and our discussion is limited to this route. There are general requirements for any orally active drug that were developed in response to combinatorial chemistry "hits", and these are summarized in "Lipinski's Rule". This empirical rule consists of a set of guidelines that describe the properties of many orally acting drugs. "Lipinski's Rule" states that a drug candidate should have a lipophilicity with a LogP value ≤ 5, a molecular weight ≤ 500 amu, no more than 10 hydrogen bond acceptors, no more than 5 hydrogen bond donors, and no more than 10 rotatable bonds. Lipophilicity is normally measured using the octanol/water partition coefficient LogP, octanol providing a good approximation of lipid membranes. Agents that partition preferentially into the octanol layer have a positive LogP, agents that partition primarily into the water layer have a negative value, and agents with equal concentrations in both phases have a LogP value of zero. Agents with a LogP > 5 tend to have poor aqueous solubility that can hamper drug development efforts. Drug candidates with a molecular weight > 500 amu have problems with passive diffusion, and excessive hydrogen bonding tends to result in poor lipophilicity and potential interactions with multiple targets resulting in unintended activities. Molecular volume is a function of molecular weight, structure, and conformational flexibility, and sometimes can be simplified to counting the number of rotatable bonds, since it has been shown that oral bioavailability in rats is decreased if the number of rotatable bonds > 10.

In addition to oral absorption, these agents must also pass through the BBB and so more criteria are needed. Guidelines for this purpose exist, and significant efforts have been underway for some time to develop an accurate, interpretable, fast, and robust method for calculating BBB penetration [82, 83]. To date, most BBB penetration models utilize lipophilicity, polar surface area (PSA) and the number of rotatable bonds. Since 1967 it has been recognized that for optimal CNS penetration, a LogP between 1.5 and 2.7 is important [84]. Molecules with a LogP < 1.0 to 1.5 tend to have poor BBB penetration [83]. Molecular weight is another important criterion, with CNS active agents typically having a molecular weight < 400 amu, although in some cases a more generous, but perhaps less accurate guideline of 470 to 500 amu can be used. Polar surface area has recently been recognized as a very important parameter for predicting BBB penetration, and can be readily calculated by most commercial computational medicinal chemistry programs. A general recommendation for CNS penetrating agents is that the PSA should be < 70Å, and definitely < 90Å. Above 90Å, poor CNS penetration is observed in many situations [82]. For molecular flexibility, BBB penetrating agents should

have 7 or fewer rotatable bonds [82]. Charge is also a well-known determinant of BBB penetration, with neutral and positively charged molecules being beneficial for CNS penetration, and negative charge(s) being deleterious [83]. Consequently, molecules with a mechanism of action inside the CNS typically should have a pKa between 7.5 and 10.5, although active uptake of certain anions such as ascorbic acid are well characterized [85]. Incorporation of these physico-chemical requirements is likely to be essential for the development of future antiepileptogenic drug candidates, and the primary challenge for the development of BBB-penetrating PHD inhibitors will be to keep the PSA below 70Å and the pKa above 7.5, while still maintaining the abilities to chelate iron and hydrogen bond with Arginine-383 and Tyrosine-303.

ANIMAL MODELS

Background

Animal models have been extensively used in the development of AEDs, and continue to be very valuable tools. The two classical models, maximal electric shock (MES) and pentylenetetrazole (PTZ), measure a drugs ability to inhibit the generation of an acute seizure event and have helped discover many AEDs that are still widely used [86]. However, these acute models tend to find new agents with similar mechanisms and they fail to find agents that utilize different mechanisms. Another acute electric shock protocol, the 6Hz model developed in the 1950s [87], has more recently been suggested to be of value to identify novel agents with mechanisms that may be different from established AEDs [88]. Another limitation of acute models is that the animals have normal brain physiology prior to the induced seizure, whereas seizures in human epilepsies come from an altered CNS substratum. Therefore recent emphasis has focused on animal models that more closely resemble human epilepsy, especially those that mimic epileptogenesis [6, 8, 86, 89, 90].

The NIH/NINDS conducted a workshop to evaluate models of epilepsy and provide guidance on their usefulness. Two models were recommended to screen agents for antiepileptogenesis: SE-induced recurrent seizures, and kindling [12]. The post SE model uses various chemical (especially pilocarpine and kainic acid, see below) or electrical treatments to produce SE, which is followed by a latent period before the occurrence of spontaneous seizures. In the kindling model, repetitive excitatory stimuli are given over a prescribed time, frequency, and intensity. After a latent period, spontaneous seizures may develop [12]. These models are chronic, meaning that recurrent seizures occur. The latent period is a seizure-free time during which there is a continuation of the pathological changes that lead to epilepsy. This latency provides an opportunity to give drugs that can be assessed for their ability to prevent the occurrence of spontaneous seizures, or to lessen the severity or frequency of spontaneous seizures that do occur. These models have characteristics that more closely mimic human epilepsy than do the acute MES, PTZ, and 6Hz models. Overviews of the two models recommended to screen for antiepileptogenic agents are presented.

Post SE Model

Status epilepticus may be induced by chemical or electrical methods. Two of the most common chemical-induced methods (pilocarpine and kainic acid) will be presented. The reader is referred to a more complete review of other chemical and electrical-induced SE methods [91].

Pilocarpine-induced SE is a frequently used model to screen drugs for effectiveness during epileptogenesis, and reviews of this model exist [92, 93]. Briefly, pilocarpine, a muscarinic agonist, induces in rats a progression of three phases; initial seizures that develop into acute SE, followed by a seizure-free latent period, and culminating in the spontaneous generation of chronic, recurrent seizures that remain for the life of the animal. The features of the chronic recurrent seizures resemble partial complex seizures in humans. The SE period is accompanied by widespread damage and cell loss to various regions of the brain; this process of neurodegeneration continues and extends to other brain regions during subsequent weeks and months after SE. Secondary effects of the drug-induced damage from seizure-induced glutamate release are thought to include axonal sprouting, increased rate of neurogenesis and gliosis, and synaptic remodeling. Thus this model is useful to study the effect of agents to prevent the occurrence of chronic seizures and decrease their severity and frequency. The latent period provides an opportunity to test agents for their antiepileptogenic potential. Furthermore, changes in ROS occur in these animals. The chronic phase has been shown to have significantly elevated hippocampal lipid peroxidation and nitrite content over saline treated animals, indicating an increase in ROS [94]. Furthermore, catalase and superoxide dismutase activities were also significantly increased [94]. Others have shown persistent impaired mitochondrial and tissue redox status as well as increase in ROS [95, 96]. These data provide support for use of this model to also measure effects of potential anti-oxidant agents. Recently, small molecule antioxidants were shown to inhibit seizures and damage induced by pilocarpine [21-23].

Kainic acid (KA), an agonist at inotropic glutamate receptors, is another chemoconvulsant that is often used to induce SE and provide a model for epileptogenesis [10, 97]. The kainate model is similar to the pilocarpine model in the phases that are produced [98, 99]. KA is a potent neurotoxin and well known to induce behavioral and electrophysiological seizures with lesions that resemble those found in humans with temporal lobe epilepsy [100]. Glutamate is the most prevalent excitatory neurotransmitter in the CNS. There are two main classes of glutamate receptors; inotropic, which are ion channels, and metabotropic, which are G-protein coupled receptors. KA binds with high affinity to a separate family of glutamate ionotropic receptors that have been named kainate receptor. The seizures induced by KA are thought to originate in the CA3 region of the hippocampus, from which there can be spreading and neuronal damage [97, 100]. Changes in free radical production and mitochondrial dysfunctions have been shown to occur after KA-induces seizures [101-103], suggesting that this model may be useful to test antioxidant agents. Recently the AED zonisamide has been shown to

increase the antioxidant ability in the hippocampus after KA-induced seizure activity [104].

Due to its direct neurotoxicity KA has also been used as a neurodegenerative model to mimic disorders such as Alzheimer's disease, Parkinson disease and multiple sclerosis [105]. It may be difficult to distinguish between the direct toxic effect of KA and the damage that occurs due to seizure activity and excess glutamate, and this has been a criticism of this model to study epileptogenesis [90]. However, others provide evidence that hippocampal CA3 damage after KA administration is due to seizure activity *per se*, and not a result of direct toxicity [97].

In general, there is high mortality associated with the systemic administration of chemoconvulsants to induce SE [106, 107]. This presents not only as a procedural limitation (fewer animals available for study), but also raises some concerns about the model and its interpretation. There may be a bias introduced in the remaining surviving animals in that their seizures were less severe [90]. Further there does not seem to be a positive correlation between the dose of chemoconvulsant and mortality. Goffin and others reported that with 55% mortality from pilocarpine-induced SE, that the average dose of pilocarpine in survivors tended to be higher than the dose in those that died [107]. There is a wide range in the sensitivity of animals to chemoconvulsants. There is also variability among animals to develop spontaneous seizures, and of their frequency once developed [92]. These issues have been at least partially addressed by adjusting the amount of chemoconvulsant given based on observations of the animal, giving drugs to lessen peripheral adverse effects, and giving drugs to abort the SE [106-108]. Also, the administration of potentiating chemical agents may be helpful. Pretreatment of animals with lithium chloride in the pilocarpine model and with ouabain in the KA model potentiates the effects of the convulsant agents and allows for lower doses to be administered, greater consistency in animal responses, and decreased mortality [91, 99, 109].

Kindling Model

As first defined by Goddard and others, the kindling model applied repeated, subthreshold electrical stimulation to the limbic region [110]. Initially there was no induced motor activity and electrographic evidence of afterdischarge was variable. With repeated stimulation, minimal motor activity was induced that progressively increased to convulsive seizures. Thus the metaphor of 'kindling', that small bits of tinder continuously added eventually result in a big fire, may seem appropriate. Reviews of this model have been published [111-113]. It is characterized by progressive seizure susceptibility during repetitive focal seizure discharges [114]. The initial focal stimulation extends to other regions and involves neural networks. Kindling produces relatively permanent effects on the nervous system; triggered seizures occur months after animals have become fully kindled [113]. It generates behavioral and electrographic changes that are similar to partial complex seizures of temporal origin in humans [114]. Kindling is most often induced by electrical stimulation, however chemoconvulsant-induced kindling has also been described [115, 116].

Spontaneous seizures have been shown to develop in this model [117] but this is not universally seen and it is used primarily as an evoked-seizure model [12, 118]. This lack of spontaneous seizures has caused some to question the applicability of this model to epilepsy [112]. However, even if suppression of *induced* seizures is the main outcome, kindling still provides a chronic model with a clear progression of neuronal pathology involving seizure production and severity and accompanying brain damage [86, 114]. These factors likely contributed to its recommendation as a model to study the epileptogenesis process [12].

Kindling model has an advantage over post-SE models in that the seizure occurs at a specific time designated by the experimenter; therefore observational and electroencephalograph (EEG) data will not be missed, as may occur if seizures are spontaneous. This also eliminates the need for continuous video EEG monitoring that has been suggested to be necessary in post SE models to accurately measure the initiation of seizure activity [107]. In general, the damage done to the brain is less severe than in post-SE models. Kindling requires the stereotaxic, surgical placement of the electrodes in specific brain regions, a method that is not needed to deliver systemic chemoconvulsants.

SUMMARY

Current treatments for epilepsy do not address the underlying pathological process, and one third of epileptic patients remain refractory to therapy. These factors show the need for new therapeutic agents that address the epileptogenic process. The role of antioxidants as antiepileptogenic agents is extremely promising. The initial insult to the brain that initiates the pathology of epilepsy can be mitigated by the action of antioxidants, thus potentially preventing the disease entirely. Even if protection is not complete, they can decrease the severity and frequency of spontaneously occurring seizures that develop. This effect would have the benefit of decreasing the use and dose of traditional AEDs, and therefore decreasing their adverse events. Modification of existing antioxidants and design of new compounds that are capable of responding to cellular oxidative stress have produced agents that are in various stages of assessment. Potential new antioxidant therapeutic agents are small molecule antioxidants, Nrf2 activating agents, and prolyl-4-hydroxylase inhibitors. These agents will need to meet physico-chemical requirements for oral bioavailability and BBB penetration and to show activity in various animal models of epileptogenesis before human trials are considered. Significant effort is being directed toward these agents due to their high therapeutic potential for epilepsy and other CNS pathologies, and the prospects for the development of new antioxidant antiepileptogenic agents look promising.

CONFLICT OF INTEREST

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PATIENT CONSENT

Declared none.

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