Pharmacological Strategies for Neuroprotection in Traumatic Brain Injury

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Abstract: Traumatic brain injury affects over a million Americans annually, but pharmacological therapy remains limited. Current standards of care in acute, subacute and chronic phases of injury are primarily supportive. This review discusses pharmacological strategies and future directions in patient treatment emphasizing pleiotropic agents targeting inflammation, oxidative damage, and glutamate excitotoxicity.

INTRODUCTION

Despite intense efforts to prevent primary traumatic brain injury (TBI) in the USA with stringent enforcement of speed limits, seatbelt laws, and helmet laws, it remains a significant cause of mortality and morbidity with devastating socioeconomic consequences. Trauma is the leading cause of mortality between the ages of 15 and 44 years, with head injury accounting for the majority of these deaths. In the USA, over 5 million people live with TBI-related disability or impairment, and the average cost of initial treatment of severe TBI is estimated at 150 thousand dollars per patient. Altogether, annual costs for TBI in the USA are estimated to be 60 billion dollars [1]. Although motor vehicle collisions (MVCs) account for most injuries, of recent interest are mild concussive brain injury seen in sports venues, and TBI sustained in military conflicts in Iraq and Afghanistan. In addition to these mechanisms of injury in young adults, older individuals suffering falls, and physical abuse of pediatric patients both contribute to the number of TBI patients. Current treatment for TBI is limited, and includes only temporizing measures such as decompressive neurosurgery and supportive medical care. Development of effective pharmacological agents continues to be an area of intense study, and numerous classes of agents are neuroprotective in preclinical studies. However, clinical studies have been discouraging. The paucity of treatment options is, in large part, due to difficulties encountered when translating discoveries in animal models to clinically efficacious therapies in the heterogeneous human population suffering TBI. Thus, there continues to be an urgent need for effective pharmacological intervention aimed at neuroprotection in the immediate setting of TBI, and cognitive rehabilitation in the long term.

SEVERE TBI AND MULTISYSTEM TRAUMA

The complex injury pattern encountered in severe trauma creates significant challenges for implementation of neuroprotective treatment, as life-threatening concomitant injuries often accompany TBI in MVCs as well as combat-related injuries. The presence of multisystem trauma, which includes injuries to the thoracic and/or abdominal cavities, requires a general assessment of the potential benefit of neuroprotection in the context of compromised cardiovascular status. Mannitol will reduce brain edema, for example, but can lead to life threatening hypotension, cerebral ischemia and, in extreme cases, cardiovascular collapse when administered intravenously and rapidly in the presence of severe blood loss or neurogenic shock. Since Emergency Medical Technicians are charged with cardiovascular resuscitative efforts in severe trauma patients, neurological injury is typically not addressed until the patient reaches the emergency department. Table **1** shows the 15-point Glasgow Coma Scale (GCS), the most widely recognized assessment of neurologic severity in trauma cases. Though far from ideal (reviews, [2, 3]), it allows clinicians to quickly categorize neurological injury as mild (13-14), moderate (9-12) or severe (3-8), and is a reasonable predictor of cognitive recovery and one year survival (review, [4]).

Severe head injury patients (GCS 3-8 after resuscitation) are those in whom neurosurgery, invasive intracranial monitoring and therapies aimed at rapidly reducing intracranial pressure (ICP) are used. Cerebral edema and ICP tend to peak within 72 hours after injury. Sustained ICPs greater than 20 mm Hg contribute to morbidity, and can lead to brain death from complete brainstem herniation. Cerebral autoregulation is often impaired, presenting challenges in patient management because efforts to reduce ICP can lead to systemic hypotension thereby decreasing cerebral perfusion pressure (CPP). Mild and moderate TBI (GCS 9-14) are different entities, as increased ICP and disruption of cerebral blood flow and autoregulation are not primary issues. Therefore, there are enhanced opportunities to focus on neuronal rescue and regeneration strategies, as these patients are more likely to suffer from subtler cognitive deficits. Although they constitute the majority of head injuries, the mild and moderate groups have been underrepresented in clinical trials, raising the possibility that appropriate administration of currently available pharmacological agents will improve outcome.

PRIMARY AND SECONDARY INJURY

An understanding of the dynamic pathophysiology of brain injury is vital in the development of appropriate thera-

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Table 1.

Glasgow Coma Scale	
Best Eye Response (4)	
No eye opening	1
Eye opening to pain	2
Eye opening to verbal command	3
Eyes open spontaneously	4
Best Verbal Response (5)	
No verbal response	1
Incomprehensible sounds	2
Inappropriate words	3
Confused, disoriented	4
Orientated	5
Best Motor Response (6)	
No motor response	1
Extension to pain (decerebrate posturing)	2
Flexion to pain (decorticate posturing)	3
Withdrawal from pain	4
Localizing pain	5
Obeys Commands	6
Interpretation	
13-14 mild brain injury	
9-12 moderate brain injury	
3-8 severe brain injury	

peutic strategies. For example, stroke trials have addressed the progression of cerebral ischemia to devise a treatment algorithm; assessment for hemorrhage followed by the administration of tissue plasminogen activator (TPA) within 3 hours of symptom onset [5, 6]. There is ample evidence for significant benefit if thrombolytics are administered within this 3 hour therapeutic window, whereas detrimental effects are observed if TPA is given after this time period. A similar time-dependent protocol is needed for TBI patients, which would enable clinicians, for example, to take advantage of the differential activation and modulation of NMDA receptor (NR) subunits to provide both neuroprotection and enhance neuronal recovery. Primary TBI, or the result of the direct initial insult can be difficult to address, but the complex cascade of biochemical changes involved in secondary injury offers diverse treatment strategies (reviews, [7-9]). This review will discuss promising therapies targeting inflammation and excitotoxicity, which are reciprocally interacting processes, in the development and progression of secondary brain injury. Particular emphasis is placed on the rational design of drugs targeting inflammation and glutamate toxicity. Other reviews offer a more comprehensive listing of neuroprotective agents being tested in preclinical and clinical trials of TBI [10-13].

TARGETING INFLAMMATION

Uncontrolled inflammation exacerbates neuronal damage after neurological insults. Traumatic brain injury initiates a massive and complex inflammatory response, which contributes to the direct release of neurotoxic mediators, dysregulation of cerebral blood flow, and disruption in the blood brain barrier (BBB) with subsequent edema (review, [9]). Both the primary and secondary stages of injury lead to the release of proinflammatory cytokines, prostaglandins (which are regulated by kinins, cyclooxygenase and leukotrienes), free radicals, and activation of the complement cascade. In particular, the adhesion of leukocytes and platelets to the microvasculature, disruption of the BBB, and cerebral edema further reduce tissue perfusion and contribute to secondary brain damage [14]. Several arachidonic acid derivatives lead to platelet aggregation, vasodilation and oxygen free radical production with lipid peroxidation. The loss of cellular integrity from ionic shifts results in cytotoxic edema, and vasogenic edema often develops due to cerebral capillary endothelial damage. The cascade of events in inflammatory tissue is similar to that seen in ischemic reperfusion injury. Although making use of this complex cascade to treat TBI may appear daunting, it provides a number of targets for controlling trauma-induced inflammation in the early stages of injury. Recent TBI trials have attempted to identify safe and effective anti-inflammatory agents, including bradykinin receptor antagonists, statins, and novel pleiotropic agents.

Cerebral contusion damages the BBB, leading to activation of kinin-kallikrein system (KKS), brain edema and microcirculatory disturbances. The KKS is important in both initiation and potentiation of the inflammatory response in TBI [15]. In vascular endothelial cells, glia, and neurons, kinins, in particular bradykinin (BK), stimulate the production and release of eicosanoids, cytokines, nitric oxide, free radicals, thereby contributing to a toxic increase in intracellular calcium. Kinins activate two types of G-protein coupled receptors, B1 (inducible) and B2 (constitutive), and it is activation of the B2 receptor that contributes to inflammatory damage. Its role in the development of neurological deficit and the inflammation-induced secondary damage resulting from diffuse TBI was elegantly demonstrated using mice rendered null for the B2 receptor [16]. These studies and others led to the characterization and use of B2 receptor antagonists in severe TBI patients, exemplified by the small peptide Bradycor (Fig. **1**, also called deltibant or CP-0127). Bradycor was produced from disulphide linkage of two kinin peptide molecules with a homobifunctional linker [17]. In single-blind and multicenter randomized placebo-controlled trials, Bradycor decreased intracranial pressure and death rate, while improving performance in neuropsychological tests [18, 19]. Anatibant (LF16-0687 or XY2405) was subsequently developed as a B2 receptor antagonist to achieve greater potency and bioavailability. It is derived from the quinoline family of B2 antagonists, modified to contain a central sulfonamide linker (review, [20]). As a small molecule, non-peptide inhibitor, Anatibant has the advantage of improved access through the BBB. It was shown to be safe

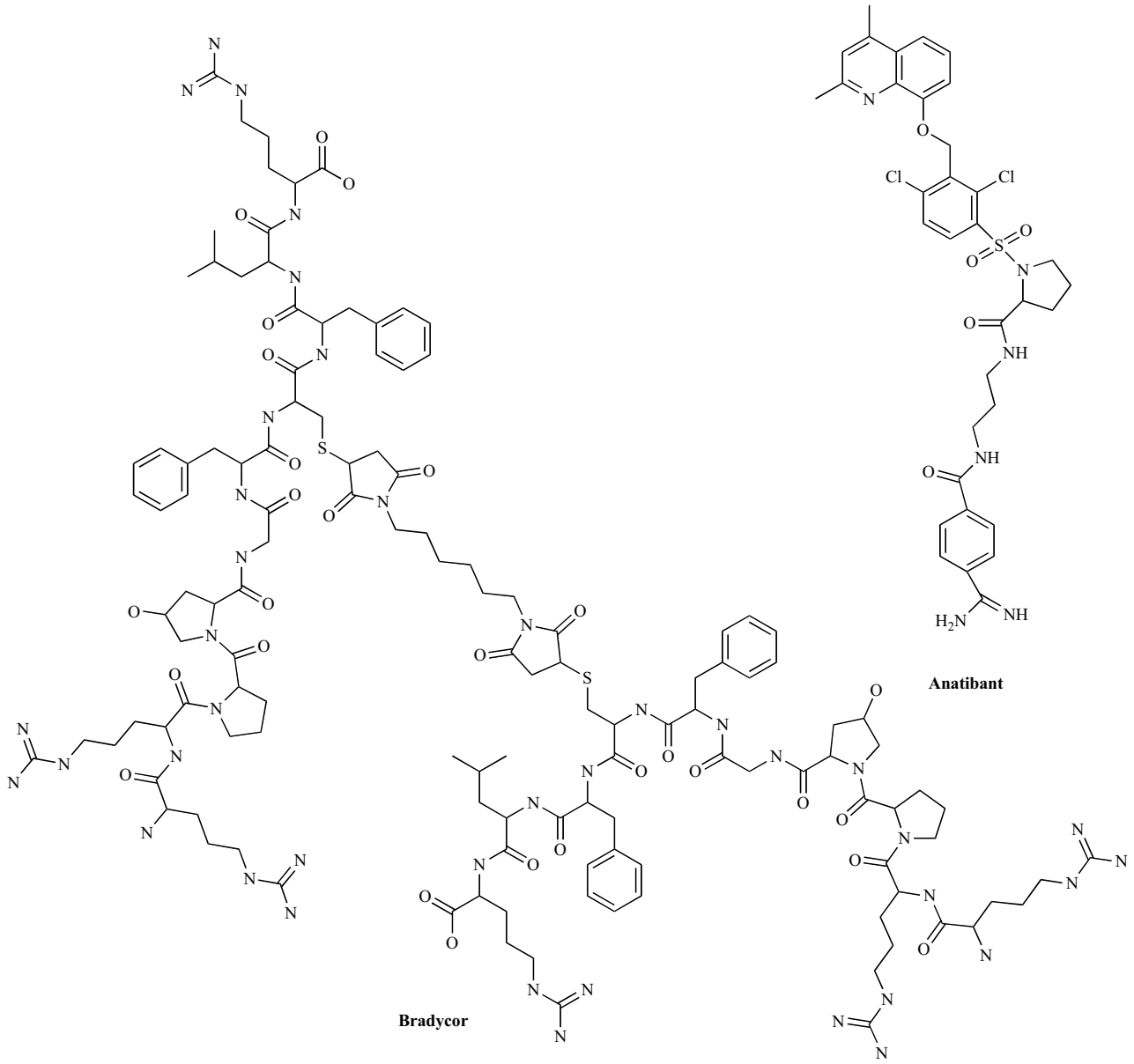

Fig. (1). Chemical structures of selected B2 receptor antagonists used in clinical trials. **Bradycor** = D-Arginyl-L-arginyl-L-prolyl-trans-4-hydroxy-L-prolyl-S-(1-(6-(3-mercapto-2,5-dioxo-1-pyrrolidinyl)hexyl)-2,5-dioxo-3-pyrrolidinyl)-L-cysteinyl-L-phenylalanyl-L-seryl-D-phenylalanyl-L-leucyl-L-arginine (5-5')-sulfide with D-arginyl-L-arginyl-L-prolyl-trans-4-hydroxy-L-prolyl-L-cysteinyl-L-phenylalanyl-L-seryl-D-phenylalanyl-L-leucyl-L-arginine. **Anatibant** = N-[3-[(4 carbamimidoylbenzoyl)amino]propyl]-1-[2,4-dichloro-3-[(2,4-dimethylquinolin-8-yl)oxymethyl]phenyl]sulfonylpyrrolidine-2-carboxamide.

and effective in animal models of TBI where it reduced brain edema, improved long-term neurological functional recovery and reduced the inflammatory response [16, 21]. Phase I clinical trials in healthy volunteers and patients with TBI demonstrated favorable clinical safety, tolerability and pharmokinetic profiles [22]. Phase II trials have been initiated in the UK and South Africa where it is anticipated that Anatibant may prove to be more clinically efficacious than Bradycor.

Cytokines such as interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNFα) are potent enhancers of inflammatory reactions *via* activation of glia, capillary endothelial cells and blood elements (macrophages and neutrophils) and *via* enhanced expression of multiple downstream inflammatory factors. TNFα and IL-1β also participate actively in BBB breakdown. Major increases in the transcription of inflammatory mediators occur within minutes of injury and, thus, are important targets that should be silenced as early as possible after injury. Statins are 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors (Fig. **2**) that may prove beneficial in this regard. Mevalonic acid is a product of HMG-CoA reductase and the precursor for key

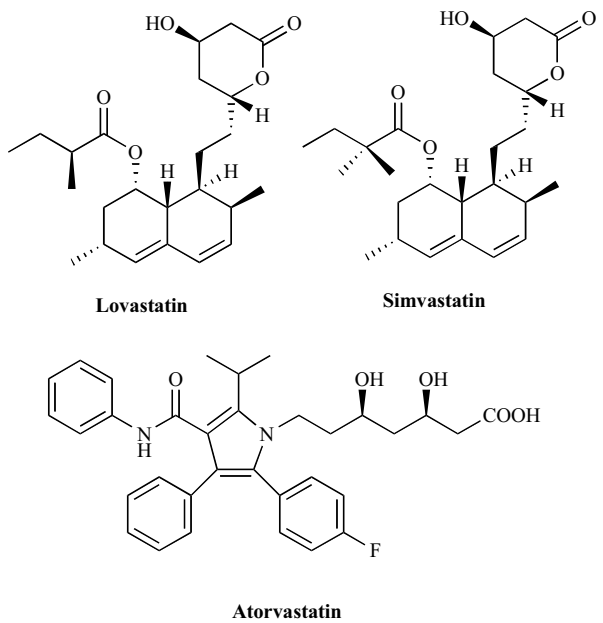

Fig. (2). Chemical structures of selected statins used in clinical trials. **Lovastatin** = [(3R,7S,8S,8aR)-8-[2-[(2S,4S)-4-hydroxy-6-oxooxan-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl] 2-methylbutanoate. **Simvastatin** = [(3R,7S,8S,8aR)-8-[2-[(2S,4S)-4-hydroxy-6-oxooxan-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl] 2,2-dimethylbutanoate. **Atorvastatin** = (3R,5R)-7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-propan-2-ylpyrrol-1-yl]-3,5-dihydroxyheptanoic acid.

cellular isoprenoid compounds in addition to cholesterol. By inhibiting the production of one or more of these compounds, statins have been shown to exert anti-inflammatory effects in the setting of acute brain injury. Examples include lovastatin, a natural product from fungi, and simvastatin, its 2,2-dimethyl butyrate analogue [23]. They are widely used to inhibit cholesterol biosynthesis, and currently favored over fibric acid derivatives in patients at risk for atherosclerosis. Additionally, both are neuroprotective *in vitro* and *in vivo* because they reduce production of inflammatory mediators including IL-1β and TNFα. Statins also improve cerebral blood flow by selective upregulation of endothelial nitric oxide synthase (NOS). For example, in a rat model of closed cortical injury, lovastatin improved histological and functional outcome [24]. Similarly, simvastatin and atorvastatin reduced functional neurological deficits, degeneration of hippocampal neurons, suppressed inflammatory cytokine mRNA expression in brain parenchyma, and improved cerebral blood flow in a mouse model of TBI [25]. Although these drugs are members of the same class, they differ in pharmacokinetic properties; atorvastatin is fully synthetic and structurally distinct, and has a longer half-life (Fig. **2**). They remain to be tested in the clinic.

Of emerging interest are pleiotropic agents that reduce inflammation and oxidative damage through multiple mechanisms. Of particular note are agonists of peroxisome proliferator-activated receptors (PARP), a family of nuclear receptors consisting of α, β(δ) and γ subtypes that regulate lipid metabolism and glucose homeostasis (review, [26]). Agents in the fibrate family, derivatives of 2-phenoxy-2-methylpropionic acid, including fenofibrate and WY14643, have reemerged for clinical use in brain injury (Fig. **3**). Both were neuroprotective in preclinical trials of brain injury due to activation of PARPα [27, 28]. Fenofibrate promoted neurological recovery, reduced edema and lesion volume in a fluid percussion model of TBI in rats by reducing inflammatory enzymes iNOS, cyclooxygenase 2 and matrix metallopeptidase 9. It also decreased four markers of oxidative stress [29]. Since fenofibrate is currently approved for use in hyperlipidemia, its clinical profile is well established, and it may prove to be beneficial in clinical TBI trials. Activation of PARPγ also mitigates inflammation arising from neurological insults. In particular, the thiazolidinediones, rosiglitazone and pioglitizone, are neuroprotective in animal models of neuronal injury by virtue of their agonist activity on PARPγ (review, [30]). Like fenofibrate, they are already FDA approved, in this case for use in diabetic patients. Interestingly, although rosiglitazone has ten-fold greater affinity for the receptor than pioglitizone, it is not significantly more effective. This has been attributed to the fact that pioglitizone crosses the BBB more readily, and appears to activate both PARPα and PARPγ. Finally, synthetic cannabinoid receptor agonists and endocannabinoids, discussed below, also activate PARPα and PARPγ (review, [26]).

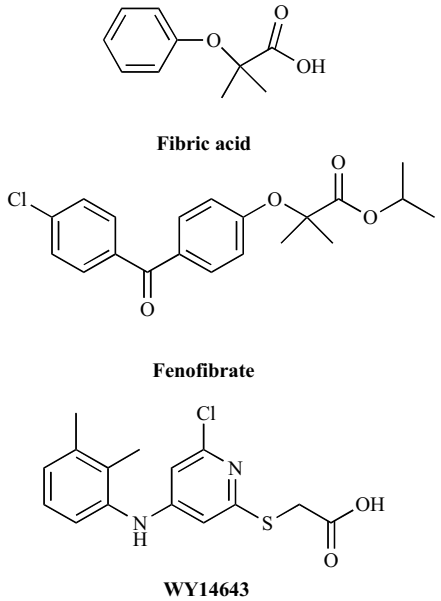

Fig. (3). Chemical structures of selected members of the fibrate family of neuroprotectants. **Fibric acid** = 2-methyl-2-(phenoxy) ropanoic acid. **Fenofibrate** = propan-2-yl 2-[4-(4-chlorobenzoyl) henoxy]-2-methylpropanoate. **WY14643** = 2-[4-chloro-6-[(2,3-dimethylphenyl)amino]pyrimidin-2-yl]sulfanylacetic acid.

The principles of rational drug design have been applied to apolipoprotein E (apoE) mimetic peptides and these, too, show significant neuroprotection in TBI in rodents *via* multiple mechanisms. ApoE is the major apolipoprotein produced in the brain in response to injury, and mediates several components of the anti-inflammatory cascade. On this basis, Vitek and colleagues designed an apoE-mimetic peptide that interacts with the ligand-binding region in ApoE (amino ac-

ids 133-149; [31]), called COG133. In a mouse model of TBI, COG133 improved neurofunctional outcome while reducing neuronal death when administered intravenously within 30 minutes post injury [32]. COG1410 was subsequently produced by shortening the length of the peptide (amino acids 138-149) and introducing aminoisobutyric acids at positions 140 and 145 to enhance potency and efficacy. In the same TBI mouse model, it improved neurofunctional outcome while reducing microglial activation and neuronal death when administered within 2 hours post injury [33]. Altogether, this novel strategy warrants study in TBI clinical trials and is likely to be beneficial in other neurological disorders.

TARGETING NMDA RECEPTORS:

NR antagonists are perhaps the most intensely investigated class of neuroprotective agents. During physiological synaptic transmission, NRs gate calcium entry in response to glutamate release thereby activating neuronal survival and plasticity programs [34, 35]. However, pathological release of glutamate, as observed during the secondary phase of TBI, results in aberrant, excitotoxic NR activation leading to excessive calcium influx and accumulation within neurons [15]. Upon surpassing cellular physiological tolerances, intracellular calcium induces free radical production *via* NR-associated NOS, mitochondrial dysfunction and dysregulation of calcium-dependent signaling pathways, exacerbating inflammation. Ultimately, accumulated damage from these pathways and others results in cell death *via* apoptosis or necrosis [36, 37]. A significant proportion of neuronal death is preventable *via* antagonism of NRs in multiple experimental excitotoxicity paradigms both *in vivo* and *in vitro* [38-42]. Consequently, intense research efforts are invested in the development of clinically efficacious NR antagonists. Since diverse brain injuries such as ischemic stroke, cerebral hemorrhage and chronic neurodegeneration are all characterized by an NR-mediated excitotoxic component, these compounds would have wide-ranging clinical applicability beyond that of TBI alone. Unfortunately, despite much anticipation of success, human trials of NR antagonists have largely failed with compounds demonstrating no positive effect on clinical outcomes and/or intolerable side effects [43-45].

Recent drug development strategies place emphasis on the production of uncompetitive, open-channel antagonists of NRs. This class of pharmacophore requires an NR to be in the open state thereby exposing channel pore binding sites. Theoretically, pathological glutamate release leads to an increase in antagonist binding efficacy as a greater population of activated NRs present available binding sites [36, 46]. Development of a pore topographic model by Bolshakov and colleagues using structure activity relationships of organic cations, demonstrates three binding regions within the NR pore lining domains (M1-M4): (1) a shallow binding site in the vestibule whose occupation inhibits gating and trapping, (2) a deep hydrophobic site and (3) a deep nucleophilic site below the constriction forming the calcium selectivity filter [47] (Fig. **4**). By binding at sites 2 and 3, open channel antagonists permit gate closure at site 1 and become entrapped within the channel. This results in highly effective antagonism of NR activity as "trapping blockade" inhibits both the current and any subsequent receptor activations [36, 47].

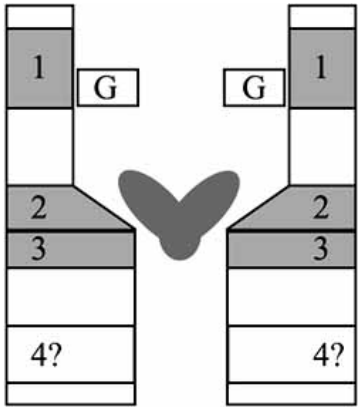

Fig. (4). Topographic model of NR pore. Three binding regions within the NR pore lining domains (M1-M4) are shown: (1) a shallow binding site in the vestibule whose occupation inhibits gating and trapping, (2) a deep hydrophobic site and (3) a deep nucleophilic site below the constriction forming the calcium selectivity filter. Open channel antagonists bind at sites 2 and 3 and permit gate closure at site 1. Thus, they become entrapped within the channel (after Bolshakov and colleagues, [47]).

Efficient interaction with sites (2) and (3) is achieved by V-shaped compounds consisting of two hydrophobic wing regions that likely bind site 2 and a small monocationic terminal amine group at the vertex able to penetrate the selectivity filter and bind site 3 [47-49]. MK-801 (dizocilpine), CNS-1102 (cerestat, aptiganel) and dextrorphan are uncompetitive, open-channel blockers that adhere to this structural paradigm and are highly effective NR antagonists (Fig. **5**). In animal studies, both MK-801 and CNS-1102 effectively decrease contusion and infarct volume in experimental models of cortical trauma and ischemia, respectively [38, 50-52]. Dextrorphan is effective at mitigating ischemic damage fol-

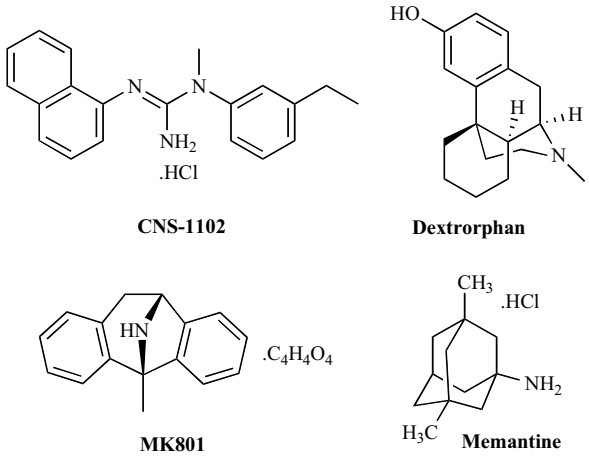

Fig. (5). Chemical structures of selected uncompetitive, open-channel NR blockers. **CNS-1102** = 1-(3-ethylphenyl)-1-methyl-2-naphthalen-1-ylguanidine hydrochloride. **Dextrorphan** = 17-methyl-9a,13a,14a-morphinan-3-ol. **MK-801** = ((5R,10S)-(+)-5-Methyl-10, 11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate. **Memantine** = 3,5-dimethyladamantan-1-amine hydrochloride.

lowing middle cerebral artery occlusion in rats [53]. However, despite their efficacy in animal models, attempts to realize the clinical potential of uncompetitive, open-channel blockers have been wrought with failure over the past decade. A notable example was abandonment of the clinical development of MK-801 as a therapeutic agent for epilepsy and anxiety due to cognitive side effects [43]. CNS-1102 failed phase II clinical trials for stroke due to increased patient mortality [45]. Intolerable side effects such as confusion, stupor, somnolence, cerebral edema and hemodynamic instability were all observed in patients receiving MK-801, CNS-1102 or dextrorphan [43, 45, 54, 55]. CNS-1102 infusions given to healthy volunteers caused a similar profile of side effects in a dose dependent fashion [56]. Cognitive side effects were likely a manifestation of inhibition of normal NR synaptic activity. All three of these compounds are high affinity NR antagonists with slow off-rates; they bind the channel pore tightly and remain there for long periods of time [36, 57, 58]. Consequently, drug accumulation leads to the simultaneous blockade of critical synaptic functions in healthy tissue and pathological receptor activation at the site of injury. This is evident in experimental models in which MK-801 inhibits NR activity during synaptically-induced long-term potentiation (LTP) and excitotoxic NMDA stimulation at its IC_{50} [59]. Regarding the development of neuroprotective NR antagonists, it is apparent that achieving optimal receptor-drug kinetics in conjunction with sub-optimal receptor blockade is the ultimate goal to develop successful therapy.

Memantine (Namenda) is the current model for NR antagonists with desirable kinetic properties. Experimentally, memantine prevents neuronal loss after injury induced by cortical impact in rats [40, 60]. Importantly, memantine prevents cell death while preserving normal NR function; at its IC_{50} synaptic LTP in slice culture is not inhibited while NMDA induced excitotoxicity is; and it restores the NR mediated migratory ability of stressed cerebellar granule cells in pontine explants while still exerting neuroprotective effects [40, 59]. It is the ability to inhibit aberrant NR activation while permitting normal receptor functions that contributes to memantine's clinical tolerability and success for the long-term treatment of Alzheimer's disease [61, 62]. This unique ability is related to the kinetic properties of the compound. Memantine is a low affinity antagonist with an IC_{50} of 2.3μM compared to 0.14μM and 1.3μM of MK-801 and dextrorphan, respectively [57]. Dwell time within the channel is also low, ~ 5 seconds as compared to 3.3 minutes for MK-801 [57]. As a result of its low channel affinity, memantine likely requires an increase in NR channel openings to effect pore binding, a condition prevalent under excitotoxic conditions such as those experienced during TBI. This can be demonstrated experimentally by an increase in receptor blockade proportional to stimulation by increasing concentrations of NMDA [36, 63]. The low dwell time prevents drug accumulation and the permanent blockade of synaptic activity. These two properties account for memantine's ability to inhibit abnormal NR activity while preserving synaptic function, contributing to a favorable ED_{50} as compared to high affinity antagonists in rat models [57]. Favorable kinetic properties of compounds like memantine likely result from variations of substituents composing the hydrophobic wing regions and their interactions with the deep hydrophobic pore binding site (Fig. **5**). For instance, varying functional groups on the ring structures of amino-adamantanes greatly influences the receptor binding kinetics [57]. The parent compound of CNS-1102, N,N`-diarylguanidine, can also undergo functional group substitution to alter NR affinity. While previous research focused on developing tri- and tetra-substituted derivatives optimized for high NR affinity, lower affinity compounds were largely ignored [58, 64]. Perhaps revisiting some of these structures could uncover antagonists with a kinetic profile similar to that of memantine as potential therapeutic agents in this class. Although agents like memantine remain untested in the treatment of acute TBI, their properties predict the potential to be well-tolerated neuroprotective agents.

In concert with the search for effective uncompetitive antagonists, a significant effort is currently invested in the development of subunit selective NR antagonists. A functional NR is composed of two NR1 subunits and a dimer of one (or two) of four NR2 (A-D) subunits (review, [65]). Approximately 50% homologous, structural differences between NR2 subunits confer distinct pharmacological and signaling properties to the NR complex [65-72]. Exploiting the contrasting functions of NRs containing NR2A and NR2B subunits using selective antagonists and agonists to provide neuroprotection has garnered significant attention [73, 74]. Central to this approach is evidence that predominantly extrasynaptic NR2B-containing receptors promote neuronal death in response to cell stress while synaptic populations composed primarily of NR2A-containing NRs are linked to survival mechanisms [70, 75, 76]. Consistent with this, the use of the NR2B antagonist CP 101, 606 (Traxoprodil) in rat models of focal ischemia and cortical compression successfully decreased infarct volume and enhanced functional recovery [77, 78]. Enhancing the activity of NR2A-containing NR complexes with the glutamate co-agonist glycine had similar neuroprotective effects in a rat model of ischemia, and some evidence was also provided for synaptic toxicity through NR2B [39]. Clinical trials of CPP 101, 606, the only NR2B antagonist tested in humans with acute TBI, have had mild success. Unlike the high affinity uncompetitive antagonists, CPP 101, 606 appears to be devoid of severe psychotropic side effects that limited their clinical study. Long-term infusions of up to 72 hours were well tolerated without any adverse events. However, like all classes of NR antagonists tested to date in the setting of acute neuroprotection, clinical benefit appears to be small or not significant [79-81].

After years of research into the structure and function of the NR and the development of numerous effective antagonists one question remains; why has neuroprotective therapy failed to deliver benefit in the setting of acute excitotoxicity, such as that experienced during TBI? The cause of this problem may not be our pharmacopoeia but rather, not optimizing its usage to correlate with the physiological processes of brain injury. Theoretically, glutamate release induced by the primary injury in TBI peaks quickly, within the first minutes to hours of injury. This translates into a small effective window for the administration of antagonists like memantine that would block pathological overaction of both synaptic

and extrasynaptic populations of NRs by extracellular glutamate. Consistent with this, a study by Biegon and colleagues measuring NR activation by quantitative autoradiography of MK-801 binding reveals an early transient increase in receptor activation within first 15 minutes of focal ischemic injury. At ~60 minutes postinjury, this is followed by a decline in receptor activity and sustained NR hypoactivity for up to seven days in injured tissue [82]. During this period, stimulation of NR activity with D-cycloserine promotes cognitive and motor recovery and restores LTP in hippocampal slices [83]. Similarly, a study performed by Liu and colleagues demonstrated decreased infarct volume in response to antagonism of NR2B-containing NRs during an ~4.5 hours period, during which high extracellular glutamate levels would likely access the extrasynaptic NRs. Following the closure of this therapeutic window, only the potentiation of survival-promoting "synaptic" NR2A containing NRs reduced infarct size [39]. The dual nature of different NRs in toxicity and protection may account for clinical failure of broad NR antagonists and point to the need for additional development of subunit-selective agents to be used in a defined temporal sequence. In human trials of CPP 101,606, patients did not receive drug infusions until 8 hours postinjury at the earliest, possibly placing drug administration outside the therapeutic window to prevent cell death [79-81]. NR2A agonism to enhance survival pathways post-injury has not been attempted in humans, but based on current research data this will likely be attempted in the future. Overall, the use of NR antagonists still holds great promise in the treatment of excitotoxic cell injury despite previous failures. As current research suggests, success will likely mean learning to modulate receptor activity throughout the timeline of TBI rather than simply blocking its activity. Inclusion of patients with mild to moderate TBI, where the injury cascade can be better defined, should also prove beneficial.

TARGETING THE CANNABINOID SYSTEM

Cannabinoid agonists are a separate class of agents being explored as therapy for TBI (reviews, [84-87]). They have been shown to exert both anti-inflammatory and anti-excitotoxic activities. N-arachidonoylethanolamine (anandamide or AEA) and 2-arachidonoylglycerol (2-AG), analogues of arachidonic acid, are the predominant and best-characterized endogenous cannabinoids (Fig. **6A**). They are produced on demand from lipid precursors in response to neural activity and function as retrograde messengers to regulate neurotransmitter release in a variety of synaptic circuits (review, [88]). In the CNS, the major known effector is the type 1 cannabinoid receptor (CB1) [89] which is enriched in presynaptic nerve terminals and linked to $G\alpha_{i/o}$. CB1 activation leads to inhibition of adenylyl cyclase and, ultimately, inhibition of neurotransmitter release (reviews, [90, 91]). Direct endocannabinoid effects on presynaptic calcium and potassium channels also contribute to decreased neurotransmission. Notably, CB1 is localized in neocortex, hippocampus, cerebellum and basal ganglia, and its binding profiles are similar in humans and rodents [92, 93]. Endocannabinoids can inhibit neurotransmitter release for tens of seconds, providing a physiological feedback mechanism for neurons to self-regulate the strength of their synaptic inputs (reviews, [94, 95]). For example, depending on whether glutamatergic or GABAergic terminals are involved, this process has been termed depolarization-induced suppression of excitation or inhibition, respectively. Accordingly, depolarization-induced suppression of excitation at overactive glutamatergic synapses would contribute to the therapeutic potential of cannabinoid agonists in TBI and other neurological insults characterized by glutamate excitotoxicity. There is also compelling evidence that cannabinoids exert neuroprotection through distinct mechanisms, some unrelated to the CB1 receptor, and others as yet undiscovered. For example, recent data indicate that activation of CB1 mediates neurogenesis and neural progenitor proliferation in response to excitotoxic injury in hippocampus [96]. In microglia, CB1-mediated induction of a mitogen activated protein kinase phosphatase limits the inflammatory response [97]. As mentioned above, there is also emerging evidence that endocannabinoids (and CB1 agonists such as HU-210 and WIN-551212) exhibit neuroprotective effects *via* activation of PARP receptors in cell nuclei (review, [26]).

Over forty years ago, Δ^9-tetrahydrocannabinol (THC) was identified as the active ingredient in *Cannabis sativa* [98]. Decades later investigators established that the central effects of THC are mediated primarily by CB1 receptors, including hypothermia, catalepsy, analgesia and hypoactivity (at higher doses). Plant or phytocannabinoids are oxygen-containing aromatic hydrocarbons that lack nitrogen, and the structural characterization of THC provided a basis for synthesis and functional analysis of a variety of synthetic analogues (review, [99]). For example, it was possible to determine the stereoselectivity of cannabinoids, a property expected of ligands that transduce their response by binding to a biological receptor. A pair of enantiomers known as HU-210 and HU-211 (dexanabinol or sinnabidol) serves to demonstrate that cannabinoids are highly stereospecific [100]. HU-210 and 211 are referred to as classical cannabinoids because they are structurally similar to THC (Fig. **6B**). Introduction of a hydroxyl group at C-11 and replacement of the n-pentyl side chain with a 1,1-dimethyl heptyl side chain in THC enhances cannabimimetic activity. Behavioral testing in mice, rats and pigeons revealed that HU-210 is ~100 times more psychoactive than THC. In contrast, HU-211 lacks cannabimimetic activity even when used at 1000 times the dosage of its psychoactive enantiomer. Specifically, the potency ratio for HU-210:HU-211 exceeded 1000 for inhibition of cAMP, and 1500 for binding to the CB1 receptor [101]. Earlier reports that the stereochemical requirements for cannabimimetic activity were rather low have been attributed to the presence of impurities, namely the (-)-(3R, 4R) enantiomer, in preparations of HU-211 [100, 101].

Because it lacks cannabimimetic i.e., psychotropic activity at CB1 receptors, HU-211 is actually preferred over HU-210 as therapy for TBI. It is a pluripotent agent that acts as an uncompetitive NMDA receptor antagonist [102], an inhibitor of cytokine production [103], and a free-radical scavenger likely due to its multi-ring backbone with conjugated double bonds and phenolic moieties [104]. Similar to memantine, it also lacks psychotropic effects associated with several NR blockers (review, [49]). Because it does not activate CB1 receptors, HU-211 does not induce cannabimimetic side-effects such as cognitive, affective, sensory or somatic

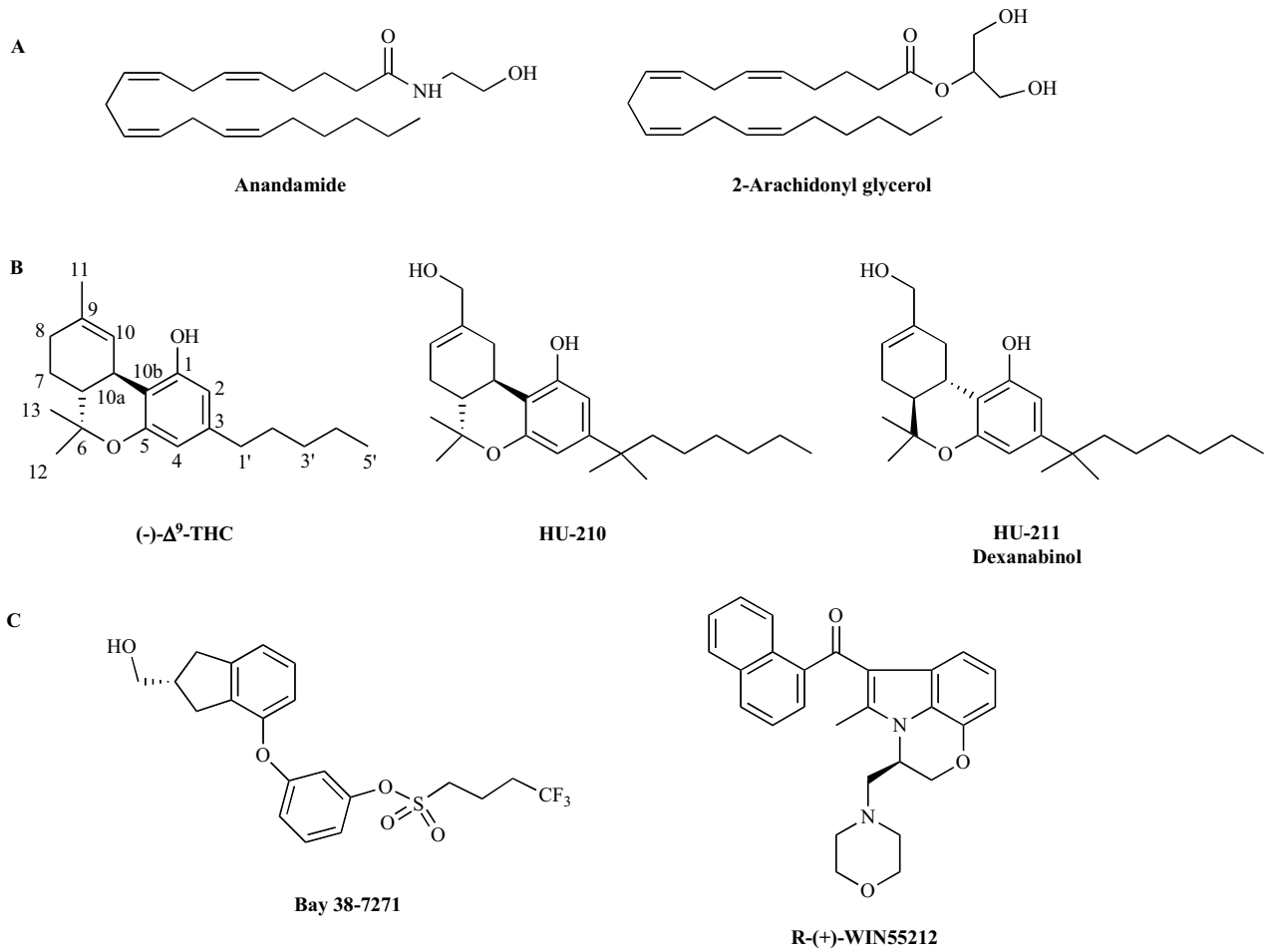

Fig. (6). Chemical structures of selected endocannabinoids, classical cannabinoids and non-classical cannabinoids. **Anandamide** = (5Z,8Z,11Z,14Z)-N-(2-hydroxyethyl)icosa-5,8,11,14-tetraenamide. **2-Arachidonoylglycerol** = 1,3-dihydroxypropan-2-yl (5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraenoate. **Δ9-tetrahydrocannabinol** = (6aR,10aR)-6,6,9-trimethyl-3-pentyl-6a,7,8,10a-tetrahydrobenzo[139]chromen-1-ol. **HU-210** = (6aR,10aR)-9-(hydroxymethyl)-6,6-dimethyl-3-(2-methyloctan-2-yl)-6a,7,10,10a-tetrahydrobenzo[139]chromen-1-ol. **HU-211** = (6aS,10aS)-9-(hydroxymethyl)-6,6-dimethyl-3-(2-methyloctan-2-yl)-6a,7,10,10a-tetrahydrobenzo[139]chromen-1-ol. **Bay 38-7271** = (-)-(R)-3-(2-hydroxymethylindanyl-4-oxy)phenyl-4,4,4-trifluoro-1-sulfonate. **WIN55212** = (R)(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl) methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-yl]-(1-naphthalenyl)methanone mesylate.

disturbances. In a weight-drop model of closed head injury in rats, HU-211 improved both motor and cognitive function with a therapeutic window of 4 hours post injury [105]. Phase I and II clinical trials demonstrated its safety and evidence supporting an improved intracranial pressure profile in severe TBI patients [106]. Though not significant, there was also evidence of improved neurological outcome for HU-211 recipients using the Glasgow Outcome Scale. Results of a phase III randomized, placebo-controlled, clinical trial confirmed the safety of HU-211, but showed no significant efficacy in TBI patients [107]. However, all recipients were severe TBI patients, leaving open the possibility that HU-211 may be therapeutically useful in those with mild or moderate injuries. Also, development of an analogue that is less lipophilic and therefore more soluble in aqueous solutions could improve efficacy by enhancing delivery.

Development of non-classical CB1 agonists that exert neuroprotection at lower doses than those that elicit cannabimimetic side effects is also of interest. In the early 1990s, the aminoalkylindoles were introduced as full CB1 agonists that were not based on the structure of THC, with *R*-(+)-WIN55212 (Fig. **6C**) being the best characterized [108, 109]. In hippocampal cultures, *R*-(+)-WIN55212 prevented excitotoxicity in a CB1-dependent manner [110] and there is also evidence for antiinflammatory/antioxidative effects *via* activation of nuclear PARPs (review, [26]). Of recent interest in TBI, a diarylether sulfonylester designated as BAY 38-7271 or KN38-7271 that demonstrates high affinity for CB1 receptors is under study because its neuroprotective actions are achieved at lower doses than its psychomimetic effects (Fig. **6C**). Drug discrimination, hyptothermia, and *in vitro* binding assays all indicate that BAY 38-7271 is a full CB1 agonist, being slightly less potent than HU-210 and substantially more potent than Δ^9 THC [111, 112]. It demonstrated significant neuroprotection against an acute subdural hematoma (SDH) model of TBI in the rat [111]. It has a therapeutic window of at least five hours, and also reduced intracra-

nial pressure and brain water content when assayed 24 hours post-SDH injury, suggesting multiple mechanisms of action [113]. Neuroprotection with BAY 38-7271 is abolished by a CB1 selective antagonist, suggesting that at least some of its therapeutic benefit is due to suppression of glutamate release from presynaptic terminals, and suppression of inflammation through microglia CB1 receptors. As mentioned, other beneficial CB1-mediated effects are likely to be identified as research in this area progresses. Importantly, the neuroprotective effects are unrelated to hypothermia, and occur at doses that do not elicit cannabimimetic side effects. A Phase I study demonstrated that the drug was well tolerated and safe in healthy male volunteers [113]. The favorable therapeutic window and efficacy of BAY 38-7271 at doses well below those eliciting cannabinoid-like side effects are encouraging, and Phase II clinical trials for the treatment of TBI are underway, with results expected in late 2008 (KeyNeurotek Pharmaceticals). In addition, further investigation of structurally similar compounds appears warranted. For example, an analogue designated as BAY 59-3074 containing 2-cyano-3 trifluoromethyl phenoxy moieties is a partial agonist at CB1 receptors, and based on preclinical studies in rat, holds promise for the treatment of diverse chronic pain [114].

A different strategy is to better utilize the neuroprotective effects of endocannabinoids by developing agents that inhibit their metabolism. Endocannabinoid generation is significantly increased in a variety of brain injury paradigms including TBI [115], possibly as part of a compensatory neuroprotective in response to injury. When administered exogenously, endocannabinoids significantly reduce the extent of brain damage [116]. The neuroprotective effects of endocannabinoids appear to be mediated by CB1 receptors, as mice rendered null for CB1 exhibited little spontaneous recovery after closed head injury compared to control mice [117, 118]. Thus, by preventing their metabolism, the protective actions of the endocannabinoids can be further mobilized, particularly in regions sustaining injury. A brief schematic diagram of endocannabinoid synthesis and degradation is reviewed in Fig. (**7**), with a focus on anandamide/AEA, an affinity-driven agonist with greater efficacy at CB1 (K_i 61 nM) than CB2 (K_i 1930 nM) receptors. For a thorough and recent review of endocannabinoid metabolism, see Basavarajappa [119]. Briefly, the precursor of AEA is *N*-arachidonylphosphatidylethanolamine (N-ArPE). Production of this precursor is mediated by a calcium-dependent N-acyltransacylase. Accordingly, a synaptic NMDA receptor-driven pathway can stimulate endocannabinoid synthesis [120, 121] as expected under conditions of glutamate excitotoxicity. N-ArPE is cleaved by a specific phospholipase D (PLD), designated as *N*-acylphosphatidylethanolamine (NAPE)-specific PLD, generating AEA and phosphatidic acid. NAPE-PLD is a member of the zinc metallohydrolase family and is structurally and functionally distinct from other known PLDs [122]. After release in the synapse, AEA acts as a retrograde messenger to inhibit neurotransmitter release from presynaptic terminals *via* activation of CB1 and effects on calcium and potassium channels. It is rendered inactive by enzymatic hydrolysis, primarily by a membrane bound fatty acid amide hydrolase (FAAH). Consistent with this, mice rendered null for FAAH demonstrated 10-15-fold increases in AEA and related ethanolamines [123, 124], and exogenous administration of AEA elicited behaviors expected of CB1 agonists [123]. In general, there is good complementarity between anatomical expression of CB1 and FAAH [125], but regions of divergence are presumably available as targets for cannabinoid-like drugs that engage non-CB1-mediated neuroprotective mechanisms.

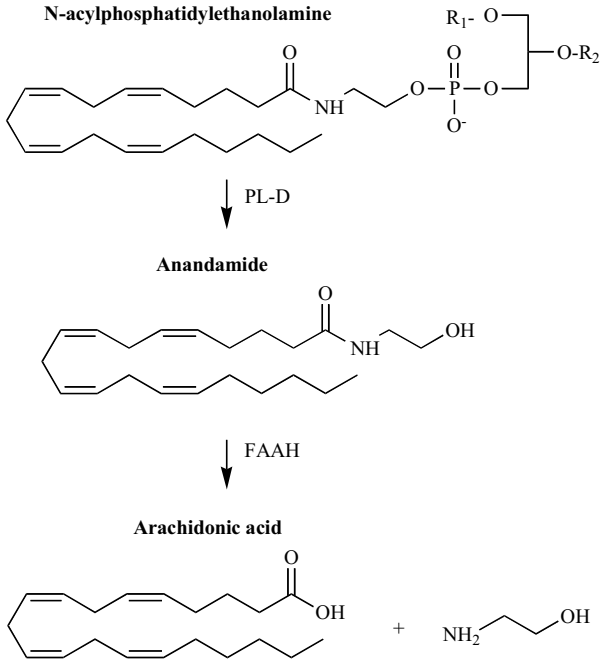

Fig. (7). Simplified schematic diagram showing the role of FAAH in anandamide degradation.

FAAH has been crystallized and shows several structurally unusual features [126] that lend themselves to design of specific inhibitors. In particular, the core catalytic domain is composed of a serine-serine-lysine triad (S241-S217-K142) that distinguishes it from the serine-histidine-aspartate triad usually observed in other serine hydrolases, thus offering opportunities for development of highly selective inhibitors. It also contains domains that appear to mediate access to both cytoplasmic and membrane compartments that may facilitate catalytic turnover. First generation FAAH inhibitors, such as phenylmethylsulfonyl fluoride (PMSF), are fatty-acid based compounds that resemble the structure of anandamide (Fig. **8**). Palmitylsulfonyl fluoride (AM374) is an irreversible inhibitor of FAAH, a weak CB1 ligand, and substantially more potent than PMSF in preventing anandamide hydrolysis [127]. In rats, it prolonged damage-induced elevation of anandamide in several brain areas, protected against kainate-induced hippocampal damage, and improved functional recovery when injected intraperitoneally immediately after kainate administration [128]. It has been argued, however, that AM374 and other fatty acid sulfonyl fluorides lack the necessary selectivity and bioavailability to be therapeutically beneficial in humans (review, [129]). A potentially promising second generation agent is cyclohexlcarbamic acid 3'-carbamoylbiphenyl-3-yl-ester, commonly referred to as KDS-4103 or URB597. It is more structurally complex (Fig. **8**; review, [129]). KDS-4103 is a

highly potent and selective inhibitor of FAAH that does not interact substantially with CB1 receptors, a host of non-cannabinoid brain receptors and channels, or other cannabinoid-related targets at doses that effectively raise brain anandamide levels in rats and mice. KDS-4103 is being explored as an anxiolytic and antidepressant, and has not been used in TBI studies. It enhanced hypothermia in response to a sub-threshold dose of anandamide in a CB1 dependent fashion, yet did not influence body temperature when administered alone. Furthermore, this effect was not observed in mice rendered null for FAAH, pointing to a selective action on the enzyme. Altogether, these studies indicate that development of selective FAAH antagonists for use in TBI is warranted. For detailed chemical considerations, Boger and associates have performed extensive analyses of the structure-activity relationships that determine the potency of FAAH inhibitors [130-134].

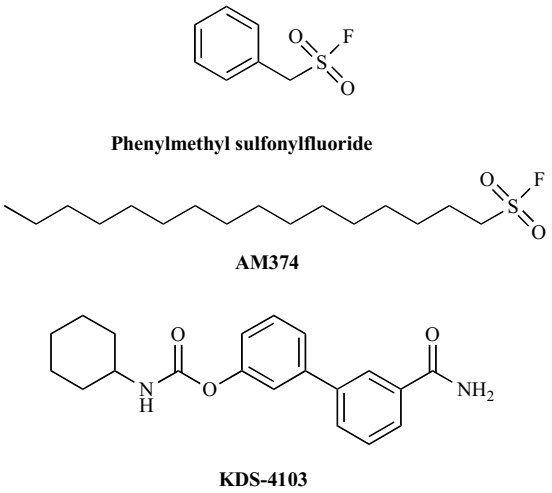

Fig. (8). Chemical structures of selected FAAH inhibitors. **Phenylmethylsulfonyl fluoride** = phenylmethanesulfonyl fluoride. **AM374** = hexadecane-1-sulfonyl fluoride. **KDS-4103** = [3-(3-carbamoylphenyl)phenyl] N-cyclohexylcarbamate.

A distinct pathway of inactivation is reuptake by a specific transport molecule or facilitated diffusion. Characterization of these less understood pathways for endocannabionoid release and reuptake could provide additional opportunities for drug development. For example, the putative transport inhibitor N- (4-hydroxyphenyl)-arachidonamide (AM404) used in combination with AM374 produced additive, CB1-mediated neuroprotection against hippocampal damage when coinjected with an excitotoxin [135]. Similar strategies can be applied to metabolism of 2-AG, though one study has questioned the contribution of this endocannabinoid to neuroprotection after brain injury (e.g., [136]). In this case, both FAAH and monoacylglycerol lipase may need to be targeted as both contribute to 2-AG inactivation (review, [137]).

CONCLUSIONS

Numerous and diverse pharmacological agents are neuroprotective in TBI in preclinical but not clinical trials. To effectively treat TBI in humans, several considerations are warranted. First, it is necessary to further characterize neuropathological distinctions between mild, moderate and severe TBI that can be quickly used by the clinician to categorize the injury, including the onset on injury. Such characterizations must include relevant pathophysiologic cascades and timing of activation of these cascades relative to the primary damage. Second, having done this, it is critical to systematically determine which agents or combinations of agents are likely to provide benefit based on an in-depth knowledge of their mechanisms of action and pharmacokinetic profiles. To date, the majority of clinical trials have involved patients with severe TBI and drugs are often administered at times exceeding several hours post injury. Possibly, the patients most likely to benefit from agents showing preclinical benefit are those with moderate or mild injuries. As discussed, the therapeutic window for several available agents lies within minutes or a few hours post injury, and investigators have achieved some successes in modifying existing compounds to extend their potencies and therapeutic windows. Finally, improved trial design and analysis may reveal clinical benefits not seen using current methods [10, 138].

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ABBREVIATIONS

2-AG	=	2-arachidonoylglycerol
HMG-CoA	=	3-hydroxy-3-methyl-glutaryl-CoA
apoE	=	Apolipoprotein E
BBB	=	Blood brain barrier
BK	=	Bradykinin
CPP	=	Cerebral perfusion pressure
FAAH	=	Fatty acid amide hydrolase
GCS	=	Glasgow Coma Scale
IL-1β	=	Interleukin
ICP	=	Intracranial pressure
LTP	=	Long term potentiation
MVCs	=	Motor vehicle collisions
AM404	=	N-(-hydroxyphenyl)rachidonamide
NAPE	=	*N*-acylphosphatidylethanolamine
AEA	=	N-arachidonoylethanolamine
N-ArPE	=	*N*-arachidonylphosphatidylethanolamine
NOS	=	Nitric oxide synthase
NR	=	NMDA receptor
AM374	=	Palmitylsulfonyl fluoride
PARP	=	Peroxisome proliferator-activated receptors
PMSF	=	Phenylmethylsulfonyl fluoride
PLD	=	Phospholipase D

SDH = Subdural hematoma

TPA = Tissue plasminogen activator

TBI = Traumatic brain injury

TNFα = Tumor necrosis factor

CB1 = Type 1 cannabinoid receptor

THC = Δ^9-tetrahydrocannabinol

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