Discovery of New AMPA Antagonist, Perampanel, Implication of Role in Seizure Disorders

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Contents

Chapter I:

Preface

The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor.

Glutamate is a principal excitatory neurotransmitter in central nervous system (CNS). It causes strong excitation via activation of receptors at post synaptic membrane and elicits action potential when accumulation of receptor activity is occurred. It is also well recognized that glutamate receptor has a significant role in synaptic plasticity. It is therefore very important neurotransmitter in signal processing in CNS. Perturbation of glutamatergic neurotransmission could elicit various symptoms or diseases including seizures, abnormal movements, sensory abnormality, neuronal degeneration etc (Meldrum, 2000). For the prevention of trouble in glutamatergic neurotransmission, variety of receptors and transporters works in synapse (Featherstone, 2010).

Glutamate receptors are divided into two subclasses. First subclass is metabotropic glutamate receptors. These receptors are G-protein coupled receptor and have eight subtypes. The other one is ionotropic glutamate receptors which have ion channel in its structure. Binding of glutamate in ligand binding site of ionotropic glutamate receptors opens the channels directly. This receptor subclass includes three subtypes. They are named based on name of extrinsic ligands. The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor is one of subtype of glutamate receptors. Remaining two receptors are N-methyl-D-aspartate (NMDA)- and kainate-type receptor. AMPA receptor distributes whole central nervous system and also peripheral nerves and tissues. The most of AMPA receptor in CNS localized at post synaptic neuronal membrane, especially post synaptic density. Channel gating kinetics of AMPA receptor is very fast and rapidly desensitized. It means AMPA receptor is machinery for very fast excitatory

synaptic transmission. The AMPA receptor is homo- or hetero-tetramer of receptor subunits named GluA1, A2, A3 and A4 (Mansour et al., 2001). Among four subunits, GluA2 is the determinant of Ca^{2+} impermeability of receptor (Hollman et al., 1991). GluA2 containing receptor is major receptor species, especially in glutamatergic projection neruons. In general GluA2 lacking Ca^{2+} permeable receptors exist in GABAergic interneurons (Geigaer et al., 1995; Janssen et al., 1998). It is also confirmed that composition of subunits can be altered in various neurons and synapsis according to physiological and pathological conditions (Janssen et al., 1998; de Lanerolle et al., 1998; Kessels and Malinow, 2009; Kobylecki et al., 2013; Liu and Zukin, 2007; Liu et al., 2010).

GluA subunits have two alternative splice variants called Flip and Flop, which exhibit different channel properties. The expression levels of each splice variant are altered during development (Jakowec et al., 1998; Monyer et al., 1991), and change in response to various diseases or conditions (Kobylecki et al., 2013; Acosta et al., 2012; Eastwood et al., 1997; Gitai et al., 2010; Guan et al., 2003; Kamphuis et al., 1992; Seifert et al., 2004). In addition, splice variants can change sensitivity to allosteric modulation of AMPA receptors (Partin et al., 1994; Quirk and Nisenbaum, 2003). AMPA receptors may also be associated with auxiliary subunits, such as transmembrane AMPA receptor regulatory proteins (TARPs), cornichon homolog (CNIH)-2, CNIH-3, cystine-knot AMPA receptor modulating protein 44 (CKAMP44), synapse differentiation-induced gene 1 (SynDIG1), suppressor of Lurcher (SOL)-1, SOL-2 and germ-cell-specific gene 1-like (GSG-1L) (Sumioka, 2013; Straub and Tomita, 2012). These auxiliary subunits can affect receptor kinetics and membrane trafficking of receptors, and their presence can change compounds from specific competitive AMPA receptor antagonists to partial agonists (Menuz et al., 2007; Huganir and Nicoll, 2013). Overall, receptor stoichiometry, splice variants and different auxiliary subunits can control the function of AMPA receptors according to the location of

the receptor, cell types, and condition of the cells.

In terms of function, the AMPA receptor also has a significant role in the plasticity of synaptic strength. In general, synaptic strength is determined by trafficking of AMPA receptors into the post-synaptic active zone (Huganir and Nicoll, 2013,). Activity-dependent insertion of the GluA1-containing AMPA receptor, followed by activation of NMDA receptors, is believed to be an initial step in synaptic potentiation. The resulting insertion of GluA2-lacking AMPA receptors into synapses leads to the stabilization of the synapse (Huganir and Nicoll, 2013; Plant et al., 2006). As the increased GluA1 expression, and presence of Ca^{2+}-permeable receptors, has been confirmed under different conditions (Liu and Zukin, 2007; Chen et al., 2013; Hanley, 2014; Rajasekaran et al., 2013; Silverdale et al., 2010; Whitehead et al., 2013), AMPA receptors might be important in the progression of diseases, as well as their role in established diseases.

AMPA receptor in disease.

The AMPA receptor has very fundamental function in excitatory synaptic neurotransmission. Change of AMPA receptor mediated synaptic transmission could alter the function of neuronal network and causes various kind of symptoms and/or pathological changes. Impairments in synaptic plasticity have been implicated in multiple neuropsychiatric disorders (Citri and Malenka, 2008), supporting a potential role for therapeutic approaches to target AMPA receptors. In addition, given the role of glutamate as an excitatory neurotransmitter, conditions characterized by overexcitation of the CNS may represent putative therapeutic targets for AMPA receptor antagonists. This section provides an overview of AMPA receptors functions in the pathogenesis of epilepsy.

Epilepsy.

Epileptic seizures are characterized by the disordered, rhythmic and synchronous firing of neurons in the brain, which results in transient behavioral changes and is often referred to as 'epileptiform activity' (McNamara, 1994). The balance of excitatory and inhibitory neural activity is crucial to maintaining neural network stability (Chagnac-Amitai and Connors, 1989), and studies in hippocampal slices from guinea pigs have indicated that, in the presence of an antagonist of inhibitory GABA signalling, activation of just a single neuron can in turn activate multisynaptic excitatory pathways and ultimately lead to widespread synchronous discharge across a large population of neurons (Miles and Wong, 1983). It indicated importance of GABAergic system as a main player of inhibitory neuronal system. On the other hand, main machinery promoting neuronal excitability seems glutamatergic neuron. Variety of evidence suggested importance of AMPA receptor in pathophysiology of epilepsy. For example, domoic acid is a marine neurotoxin that causes neuronal toxicity through activation of AMPA and kainate receptors (Costa et al., 2010; Larm et al., 1997). In humans, domoic acid can produce amnesic shellfish poisoning, with severe intoxication leading to seizures, causing neuronal damage with a sequela of short-term memory loss (Costa et al., 2010; Teitelbaum et al., 1990). In 1995, Cendes et al. first reported a case of domoic acid intoxication in a patient who developed status epilepticus (SE) and subsequent spontaneous temporal lobe epilepsy. Such neuronal toxicity caused by domoic acid is very similar to the spontaneous seizures observed in the experimental kainic acid-induced rodent model of SE (Cendes et al., 1995; Lothman 1991). Overall, observations with domoic acid and kainate support a hypothesis that overactivation of AMPA receptors could have a role in the progression of hippocampal damage in temporal lobe epilepsy and refractoriness to drugs.

Findings of animal studies are consistent with a role for AMPA receptor activation in the development of epilepsy, including a study of the effects of Thorase, an AAA+ ATPase, which regulates the internalization of AMPA receptors. Genetic deletion of Thorase in mice resulted in increased cell surface expression of AMPA receptors, as well as death from a seizure-like syndrome between post-natal days 19 and 25 in most animals (Zhang et al., 2011).

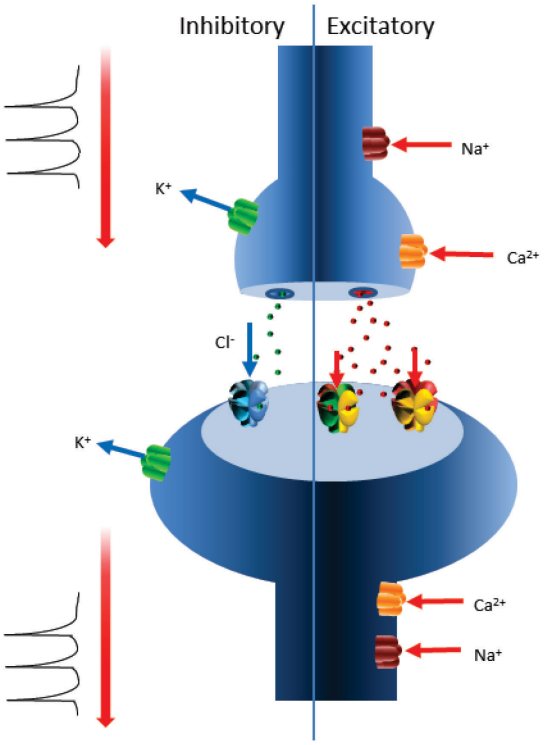
In humans, a pathophysiologic role for AMPA receptors in epilepsy has been indicated by a pharmacologic study of slices of lateral amygdala from patients with medically intractable temporal lobe epilepsy. In this study, blockade of AMPA receptors, but not NMDA receptors, inhibited interictal-like electrical activity, suggesting that AMPA receptors may have a role in abnormal electrical activity in the epileptic brain (Graebenitz et al., 2011). Supporting evidence was provided by an autoradiograph study, which showed an increased density of AMPA receptors in brain slices from patients with epilepsy (Graebenitz et al., 2011; Hosford et al., 1991).

Studies in the human epileptic brain have also looked at AMPA receptor subunit expression: Ying et al (1998) demonstrated that expression of the GluA1 receptor subunit is elevated in the epileptic hippocampus, and positively correlates with axonal sprouting. Increased expression of the GluA1 subunit suggested an increase in levels of the homomeric GluA1 receptor, which exhibits high receptor conductance compared with the GluA2-containing Ca^{2+}-impermeable heteromeric receptors (Coombs et al., 2012; Swanson et al., 1997). Of note, increased expression of GluA2-lacking Ca^{2+}-permeable receptors is thought to occur before neuronal degeneration (Liu and Zukin, 2007; Grossman et al., 1999). This is similar to the process of synaptic plasticity, during which insertion of the GluA1 homomeric receptor is also observed (Liu and Zukin, 2007) and, therefore, AMPA receptors may have a significant role in the pathophysiology of epilepsy: not only the

expression of seizures, but also the progression of epilepsy.

Above noted evidence indicated that targeting AMPA receptors is a logical therapeutic approach to the development of new antiepileptic drugs.

While the evidences indicating relationship between AMPA receptor and epilepsy existed abundantly. Therapeutic drugs for the treatment of epilepsy (antiepileptics (AEDs)) were not renewed. It was because of complexity of pharmacokinetic interaction of AEDs and its multiple mode of action. Second generation of AEDs were introduced in end of 20th century but major concept of drug discovery research was reduction of complexity in pharmacokinetic interactions and of safety (Stefan and Feuerstein, 2007). Major AEDs were categorized into channel modulators, GABA enhancers, presynaptic modulator or compound with multiple targets (Fig. 1). There was no selective postsynaptic glutamate antagonist before perampanel.



Symbol	Target Molecule	Antiepileptics
	Sodium channel (Na^+)	Carbamazepine Oxacarbazepine Eslicarbazepine Phenytoin Lamotrigine Lacosamide Rufimnamide
	Calcium channel	Gabapentin Pregabalin Ethosuximide
	Potassium channel	Ezogabine/Retigabine (KCNQ2–5 (Kv7.2–7.5) opener)
	Glutamate GABA	Levetiracetam (SV2A, Presynaptic modulator) Tiagabine (GABA reuptake inhibitor) Vigabatrin(GABA transaminase inhibitor)
	$GABA_A$ receptor	Benzodiazepine Barbiturate
	AMPA receptor	Perampanel
	NMDA receptor	–
	Multiple target molecules	Sodium Valproate（Na^+, GABA, Glutamate) Topiramate（Na^+, Kainate, AMPA, $GABA_A$) Felbamate (Na^+, $GABA_A$, NMDA)

Figure 1. Mode of action of Antiepileptics

Chapter II:

Creation of New AMPA antagonist

Abstract

This Chapter focuses on the discovery and preclinical profiling of perampanel as AMPA receptor antagonist. Many attempts were made to obtain drug inhibiting AMPA receptors for the treatment of various diseases but failed in the most of cases due to safety issue related to pharmacological effect and physicochemical property of chemical template. Requirement of new chemical template was considered essential to discover druggable AMPA antagonist. Chemical template of perampanel was discovered through High-throughput screening (HTS). HTS hit compound was inhibited AMPA-induced intracellular Ca^{2+} increase in cortical neurons but not displaced [^{3}H]AMPA binding suggesting non-competitive inhibition. Medicinal chemistry focused on the structure modification to achieve increase of intrinsic activity and improve metabolic stability. Here we discovered a series of 1,3,5-triaryl-1H-pyridin-2-one derivatives as antagonists of AMPA receptors. The structure–activity relationships for this series of compounds were investigated by manipulating individual aromatic rings located at positions 1, 3, and 5 of the pyridone ring. This culminated in the discovery of 2-(2-oxo-1-phenyl-5-pyridin-2-yl-1,2-dihydropyridin-3-yl)benzonitrile (perampanel), a novel, non-competitive AMPA receptor antagonist that showed potent activity in an in vitro AMPA-induced Ca^{2+} influx assay (IC_{50} = 60 nM) and in an in vivo AMPA-induced seizure model (minimum effective dose = 2 mg/kg po). [^{3}H]perampanel binding assay was developed for elucidation of mode of action of perampanel revealed non-competitive antagonism similar to known non-competitive antagonists.

Introduction:

Drug discovery research targeting glutamate receptors

Research into the role of AMPA receptors in health and diseases in the late 1980s and into the 1990s was facilitated by the refinement of selective experimental agonists and antagonists for AMPA, kainate and NMDA receptors (Rogawski, 2011). However, research into the therapeutic potential of AMPA receptor antagonists was hampered by the challenging physical properties of the compounds investigated and their poor pharmacokinetic profiles.

The first selective antagonists of non-NMDA ionotropic glutamate receptors were the quinoxalinedione derivatives, including CNQX and NBQX (Honore et al., 1988). These competitive antagonists bind to the glutamate-binding sites on the AMPA receptor, preventing glutamate from binding and activating the channel. However, they have limited selectivity for AMPA receptors over kainate receptors (Randle et al., 1992) and their physical properties prevented clinical development: for example, CNQX has good water solubility but limited ability to penetrate the blood-brain barrier (BBB). NBQX has poorly water solubility and low BBB permeability resulting precipitates in the kidneys (Rogawski, 2011; Weiser 2005).

Next to be developed were the noncompetitive AMPA receptor antagonists. These agents do not prevent the binding of glutamate to the glutamate-binding site by their actions at a separate site in the receptor complex. The prototype noncompetitive AMPA receptor antagonist was the 2,3-benzodiazepine compound GYKI-52466, which shows modest intrinsic antagonistic effect, but good oral bioavailability and in vivo efficacy, indicating good BBB penetration (Weiser, 2005). GYKI-52466 was followed by more potent 2,3-benzodiazepines; one of these compounds, talampanel (GYKI-53773 also known as

LY300164), progressed through preclinical investigation, and entered clinical trials for epilepsy and ALS (Chappell et al., 2002; Pascuzzi et al., 2010). Talampanel demonstrated significant reduction of seizure frequency in patient with refractory partial onset seizure (Chappell et al., 2002). However, further development was suspended, mainly because of suboptimal pharmacokinetic profile: talampanel has a half-life of approximately 6 hours, necessitating 3-times daily dosing, and its metabolism is inhibited by the antiepileptic drug, sodium valproate (Langan et al., 2003). Short half-life time of talampanel might cause high incidence of ataxia (Chappell et al., 2002). It also increased complexity of talampanel as therapeutic drug.

An additional challenge in developing therapeutic AMPA receptor antagonists arises from the fundamental role of this receptor in CNS function. How can this receptor be targeted and inhibited without disrupting essential processes? Such concerns were perpetuated by animal studies, in which both competitive and noncompetitive AMPA receptor antagonists generally had neurological side effects at doses that were anticonvulsant (Yamaguchi et al., 1993). Furthermore, in the clinical setting, the competitive AMPA antagonist ZK200775 was associated with strong CNS-depressant effects on consciousness when evaluated in patients suffering from acute ischaemic stroke, leading to study termination (Walters et al., 2005). However, despite these challenges, the therapeutic potential and promise of AMPA receptor antagonists remained a very real and enticing prospect.

Representative AMPA antagonists were listed in Figure 2. Problems of these AMPA antagonists were basically associated with structure related chemical property and pharmacokinetics. In addition the most of attempt of drug discovery research was started from known chemical template. In general, it is common approach to improve structure related problem but it has also limitation to overcome issues completely. We considered

that novel chemical structure is required to overcome the problem of existing AMPA antagonists. In addition, compound with new structure might have different mode of action. It enables us to find compound with different relationship between effects and side effects.

High throughput screening and identification of seed compound

Two high-throughput screening campaigns were run to discover a new chemical template for an AMPA receptor antagonist: the [^{3}H]AMPA binding assay and the neuroprotection assay against AMPA-induced rat cortical neuronal cell death. The neuroprotection assay identified several promising compounds, including 2,4-diphenyl-4H-[1,3,4]oxadiazin-5-one, which was selected for further development based on its dissimilarity to known AMPA receptor antagonists, as well as its potential for chemical modification. AMPA antagonistic effect of 2,4-diphenyl-4H-[1,3,4]oxadiazin-5-one was 9.17 μM (IC_{50} in AMPA-induced Ca^{2+} influx assay) (Fig. 3). Following identification of drug seed compound, medicinal chemistry effort was continued to discover druggable compounds. In this chapter, I will describe path from hit to final compound in drug discovery research and character of perampanel.

Materials and Methods

Reagents.

Reagents were purchased from the following sources: GYKI52466, N-methyl-D-aspartate (NMDA) and MK-801 from Sigma/RBI (St Louis, MO, USA); (RS)-AMPA from Tocris Cookson Inc. (Ellisville, MO, USA); tetrodotoxin (TTX) from Sankyo (Tokyo, Japan); and fura-2-AM from Dojin Chemical (Tokyo, Japan). Perampanel was synthesized by Eisai Co., Ltd. (Tokyo, Japan).

Measurements of intracellular free calcium concentration.

The cerebral cortex was excised from embryonic day 18 (E18) Wistar rats (Charles River Japan, Kanagawa, Japan) and dissociated by incubation for 30 min at 37°C in Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3.3 mM D-glucose, 0.25% trypsin, 0.2 mg/mL DNase I, 50 units/mL penicillin and 50 µg/mL streptomycin. Cells were resuspended in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and plated at a density of 1×10^5 cells/well on 96-well test plates on an astrocyte feeder layer. Cultures were maintained without exchange of medium for 7–17 days at 37°C in a 5% CO_2/95% air incubator (Abe et al., 1990).

Changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]i$) were measured in rat cortical neurones using the fluorescent Ca^{2+} indicator dye fura-2, as previously described (Fischer et al., 2000). Cells were incubated with fura-2 AM (10 µM) in a 5% CO_2/95% air incubator at 37°C for 2 hours and washed with Ca^{2+} assay buffer (140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 3 mM $CaCl_2$, 24 mM D(+)-glucose, 10 mM HEPES, 1 µM MK801; pH 7.4 adjusted with NaOH for AMPA). Changes in $[Ca^{2+}]i$ were determined by fluorimetry (Fluorescence

Drug Screening System, Hamamatsu Photonics, Shizuoka, Japan) by measuring changes in the fluorescence emission ratio of fura-2 after consecutive excitations at 340 and 380 nM wavelengths. AMPA(1 μM) was used for drug screening. For the characterization of perampanel, AMPA (2 μM) and NMDA (100 μM) were used to stimulate specific receptor subtypes, and the inhibitory effects of perampanel, GYKI52466, and MK-801 on these responses were assessed. Ca2+ assay buffer without $MgCl_2$ or MK-801 was used in assays involving NMDA.

Liand binding assay.

Ligand binding assays were conducted in extracts or membrane fractions obtained from various tissues and from cell lines expressing the respective targets. A radioactively labeled ligand specific for each target was used at a concentration approximately equivalent to the ligand's Kd, and perampnel was added at a concentration of 1.25 μM. In the positive controls, ligands with known Kis for each target were added (listed as "reference compound" in the table 3). After incubation for a period of time sufficient to allow equilibrium binding, the ratio of bound and unbound radioactivity was measured. Binding without perampanel was used as control. From this ratio the % inhibition of binding by perampanel was determined. Data are the result of two experiments.

In vivo antagonism of AMPA receptor (AMPA-induced seizure).

Male ddY mice 4 or 5 weeks old obtained from Japan SLC, Inc (Shizuoka, Japan) were used in the study. Test compounds or perampanel were suspended in 0.5% MC and were orally administered 1 h before each test. AMPA was dissoleved in 0.9% NaCl at 0.4 mM. Intra-cerebral ventricular infusion AMPA was made according to the method described by Turski (1992). Briefly, 30 gauges needle was used and introduced at coronal suture of mice

skull. Depth of insertion was controlled by stopper at 3.5 mm from skull surface then mice are released in the arena to allow spontaneous activity. AMPA was infused with syringe pump (Model CMA100; Carnegie Medicine AB, Stoclkholm, Sweden) at the rate of 5 μl/min. Duration until initial sign of clonic seizure was measured as endpoint. Mice that did not show clonic seizure within 180 seconds were recorded as having a 180-second latency time. The lowest dose providing statistically significant prolongation of latency was defined as the MED.

In vitro metabolism study in liver microsomes.

Hepatic intrinsic clearance (CLint) was calculated from the substrate disappearance rate in liver microsomes. Each compound (0.1 μM) was incubated with a reaction mixture (150 μL) consisting of mouse, rat, or human liver microsomal protein (0.2 mg/mL) in 100 mM potassium phosphate buffer (pH 7.4) and 0.1 mM EDTA. After pre-incubation for 5 min at 37°C, the enzyme reaction was initiated by adding an NADPH-generating system (0.33 mM β-NADP+, 8 mM glucose-6-phosphate, 6 mM $MgCl_2$, and 0.1 unit/mL glucose-6-phosphate dehydrogenase). After incubation of the microsomal matrix for 15 min at 37°C, 150 μL internal standard (1 μM propanolol in methanol/acetonitrile [3/7, v/v]) was added to the reaction mixture and mixed vigorously. After centrifugation, the supernatant was subjected to LC/MS/MS analyses in positive ion mode with multiple reaction monitoring.

For the characterization of perampanel, similar assay but slightly different asaay system was used. Liver microsomes from human, rat, dog, and monkey were purchased from Xenotech, LLC (Lenexa, Kansas, USA). Microsomes (0.5 mg/mL microsomal protein) were incubated with perampanel (0.03 μg/mL), EDTA (0.1 mM), an NADPH-generating system (0.33 mM β-NADP+, 0.8 mM glucose 6-phosphate, 0.1 unit/mL glucose 6-phosphate dehydrogenase, 6 mM $MgCl_2$), and phosphate buffer (100 mM; pH 7.4) for 20

min at 37°C, and reactions were terminated by addition of acetonitrile. The residual concentration of perampanel was determined by liquid chromatography–tandem mass spectrometry (LC-MS/MS) with multiple reaction monitoring operated in the positive ionization mode.

Pharmacokinetic parameters.

For charactaerization of pharmacokinetics, perampanel was administered as follows: to fasted male Sprague Dawley rats (n = 4), intravenously (bolus in 0.25 M HCl in saline) or orally (in 0.33 M HCl) at 1 mg/kg; to fasted male beagle dogs (n = 3), intravenously (bolus in 0.1 M HCl in saline) or orally (in 0.1 M HCl) at 0.1 mg/kg; and to fasted male cynomolgus monkeys (n = 4), intravenously (bolus in 0.1 M HCl in saline) or orally (in 0.1 M HCl) at 0.03 mg/kg. Blood samples were collected from rats via the jugular vein (0.25 mL), and from dogs via the cephalic vein (1 mL), and from monkeys via the cephalicfemoral vein (1 mL and 0.5 mL, respectively) using heparinized syringes. For all species, timepoints for blood collection were pre-dose, 5 min, 15 min, 30 min, 1, 2, 4, 6, 8 and 24 hr after intravenous dosing, and 15 min, 30 min, 1, 2, 4, 6, 8 and 24 hr after oral dosing. Samples from monkey were also taken at 12 h. Plasma was obtained by centrifugation and deproteinized using methanol: 60% perchloric solution (500:1 v/v). The plasma concentration of perampanel was determined by high-performance liquid chromatography with fluorescence (HPLC-FL). Pharmacokinetic parameters for perampanel were calculated by model independent analysis (WinNonlin, Pharsight, North Carolina, USA). For assessment of brain penetration of brain samples were obtained from male ddY mice and male Sprague−Dawley rats after oral administration. Cerebrospinal fluid (CSF) samples were collected from mice by cisternal puncture after intraperitoneal administration. Brain was homogenized in two volumes of water, and the brain

homogenates and CSF samples were processed and analyzed in a manner similar to that for plasma samples.

Radiolabeled perampanel binding assay.

Rat forebrain membranes were prepared as previously described (Balannik et al., 2005). Forebrains of Sprague Dawley rats were homogenized in ice-cold 0.32 M sucrose containing 0.1 mM EGTA (pH 7.4) and centrifuged at 1000 x g for 10 min. The supernatant was centrifuged at 30,000 x g for 20 min and the resulting pellet was lysed in 1 mM EGTA/Tris-HCl (pH 8.0) and centrifuged at 30,000 x g for 20 min to collect the membranes. This lysis and centrifugation step was repeated, and the pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.4). Membranes were sonicated and washed three times, resuspended in 50 mM Tris-HCl buffer (pH 7.4) and stored at -80ºC. Before use, aliquots were thawed and sonicated, washed twice in 50 mM Tris-HCl buffer and resuspended at 0.5 mg/mL with 50 mM Tris-HCl buffer (pH 7.4).

Specific and non-specific binding of [^{3}H]perampanel to rat forebrain neuronal membranes was measured in the presence and absence of various glutamate receptor agonists and antagonists. Briefly, membranes and compounds were mixed with 50 nM [^{3}H]perampanel (specific activity 1.92TBq/mmol) and incubated for 1.5 hours at 4°C. Samples were filtered onto Whatman GF/B glass-fiber filters presoaked in 0.3% polyethyleneimine and washed three times with 2 mL ice-cold 50 mM Tris-HCl buffer (pH 7.4) using a Brandel M-30R cell harvester. Radioactivity was quantified by liquid scintillation counting. Non-specific binding was determined by incubation with 15 µM perampanel. Specific binding was determined by subtraction of non-specific binding from total binding.

Statistical analysis

For in vitro studies, values for Ca^{2+} response and radioligand binding were expressed as mean ± SEM (standard error of the mean). IC_{50} values were determined by regression analyses. The differences between the control group and the perammpanel groups were analyzed by non-parametric one-way analysis of variance (ANOVA) followed by the Dunnett type multiple comparison test. A value of $p<0.05$ (2-sided) was considered statistically significant. Statistical analysis was conducted using the software package, SAS 6.12 (SAS Institute Japan Ltd., Tokyo, Japan).

Results

Identification of Perampanel.

The 2,4-diphenyl-4H-[1,3,4]oxadiazin-5-one was a selected hit compound in high throughput screening with neuronal cell death assay. It was not shown displacement of [^{3}H]AMPA binding therefore hit compound was considered noncompetitive antagonist. This compound has relatively small molecular size therefore it was considered that addition of chemical moiety for the improvement of properties as a drug could be accepted.

The first significant milestone in medicinal chemistry was replacement of oxadiazinone to pyridone. It reduced AMPA antagonistic activity but subsequent insertion of aromatic ring to the position 1 of pyridone ring significantly improved both AMPA antagonist activity and metabolic stability in liver microsome. Improve of metabolic stability was considered due to masking effect of the acidic hydrogen by insertion of another phenyl group to the pyridone template. This 1,3,5-triaryl-1H-pyridin-2-one template showed suitable chemical tractability for further medicinal chemistry effort. It was also a reason of selection as drug lead compound.

Additional optimization of the 1,3,5-triaryl-1H-pyridin-2-one template was conducted by focusing on manipulation of the individual aromatic rings located at positions 1, 3, and 5 of the pyridone ring to yield a series of potent and selective noncompetitive AMPA receptor antagonists. Finally 2 - (2-Oxo-1-phenyl-5-pyridin-2-yl-1,2-dihydropyridin-3-yl)benzonitrile (Perampanel) was identified as potent AMPA antagonist (Fig. 4, Table 1).

Characterization of perampanel.

As a results of medicinal chemistry effort, perampanel (2-(2-Oxo-1-Phenyl-5-Pyridin-2-yl-1,2-Dihydropyridin-3-yl)Benzonitrile) was identified as an orally active, non-

competitive AMPA antagonist (Fig. 5). Pharmacological actitivty of perampanel will be described in this part.

AMPA receptor antagonism.

Rat cortical neurones were used to determine the effects of perampanel on AMPA receptor function, as measured by AMPA-induced increases in $[Ca^{2+}]i$. Perampanel inhibited AMPA-induced increases in $[Ca^{2+}]i$ in a concentration-dependent manner (IC_{50} 93 nM vs. 2 μM AMPA; 95% CI, 40–150 nM) (Fig. 6A).

Perampanel shifted dose response curve of AMPA-induced Ca^{2+} influx at 0.3 μM but suppressed maximal effect of AMPA at 1 μM. This behavior of antagonist often observed in non-competitive modulator of receptor (Fig. 6B).

We assessed binding site of perampanel using [^{3}H]perampanel. The Kd for [^{3}H]perampanel binding to rat forebrain membranes was 59.8 ± 5.2 nM and the Bmax was 3.2 ± 0.1 pmol/mg (n = 4) (Figure 7). The glutamate receptor agonists glutamate (1 mM) and AMPA (0.1 mM), and the competitive glutamate receptor antagonist NBQX (0.1 mM) did not significantly reduce [^{3}H]perampanel binding to rat forebrain membranes (Figure 8A). However, [^{3}H]perampanel binding was affected AMPA receptor channel gating status. Application of AMPA and cyclothiazide to AMPA receptor biased channel gating state toward open. In this condition affinity of perampanel to AMPA receptor was reduced (Fig. 8, Table 2). The other non-competitive AMPA antagonists, “CP465,022” and “GYKI52466” also showed similar change of affinity related to channel gating status (Balannik et al., 2005). As expected, [^{3}H]perampanel binding was displaced by the noncompetitive AMPA receptor antagonists CP465022 (IC_{50} 21.1 ± 1.4 nM; Ki 11.2 ± 0.8 nM; mean ± SEM; n = 4) and GYKI52466 (IC_{50} 23.3 ± 1.9 μM; Ki 12.4 ± 1 μM; mean ± SEM; n = 4) (Fig. 9).

Selectivity.

In rat cortical neuron culture, application of NMDA can cause Ca^{2+} influx through NMDA receptor. The effects of perampanel on NMDA receptor activity induced by NMDA (100 μM) were determined. Perampanel inhibited NMDA-induced increases in $[Ca^{2+}]i$ by 18%, but this change was not statistically significant even at the highest concentration tested (30 μM). In contrast, the noncompetitive NMDA receptor antagonist MK801 (1 μM) markedly inhibited NMDA-induced increases in $[Ca^{2+}]i$ by approximately 85% (Figure 10).

Effect on other physiologically important molecules like receptors, enzymes and transporters were confirmed using ligand binding assay. It was conducted at Novescreen using validated method in their laboratory. Perampanel at 1.25 μM did not exert more than 50% displacement in binding assay. It strongly suggested that perampanel does not interact with other physiologically important molecules (Table 3).

In vivo AMPA antagonism.

Perampanel prolonged latency to seizure onset. Significant prolongation of latency was observed at 2 mg/kg and above (Fig. 11). It indicated oral availability and AMPA tanatagonistic effect in *in vivo*.

Pharmacokinetics.

Pharmacokinetic parameters for perampanel in rat, dog, and monkey are summarized in Table 5. Values for half-life, area under the curve (AUC) and bioavailability of perampanel were higher in dog and monkey than in rat; clearance values were lower in dog and monkey than in rat. Brain-to plasma concentration ratios were evaluated in mice and rats. The ratios were 1.06 and 1.14 in mice and rats, respectively. Furthermore, the cerebrospinal fluid (CSF) concentration was measured in mice, and the CSF concentration

to unbound plasma concentration ratio was calculated to be 1.14.

In vitro metabolic stability of perampanel.

Metabolic stability of perampanel in the presence of rat, dog, monkey, or human liver microsomes was examined in vitro. The residual amount of perampanel remaining after 20 minutes of incubation with rat, dog, monkey, or human microsomes was 69.9%, 54.6%, 85.3%, and 100.2%, respectively (n = 2).

Discussion and Conclusion

High-through put screening had been conducted to obtain novel chemical template of AMPA antagonist. Among hit compound in HTS, 2,4-diphenyl-4H-[1,3,4]oxadiazin-5-one was selected as start compound for medicinal chemistry. It was not bind AMPA receptor in [^{3}H]AMPA binding assay therefore this compound inhibit AMPA receptor non-competitively.

Modification of chemical structure aiming to improve intrinsic activity and metabolic stability discovered 1,3,5-triaryl-1H-pyridin-2-one template. Further derivatization of position 1,3,5 aromatic ring enable us to find 2 - (2-Oxo-1-phenyl-5-pyridin-2-yl-1,2-dihydropyridin-3-yl)benzonitrile (Perampanel).

In in vitro studies, perampanel potently inhibited AMPA-induced increases in intracellular [Ca^{2+}]i in cultured rat cortical neurones, but did not significantly inhibit MK801-sensitive NMDA-induced Ca^{2+} responses, suggesting that perampanel is a selective AMPA receptor antagonist. Selectivity of perampanel for AMPA receptors over NMDA receptors may be an important feature clinically as NMDA receptor antagonists are known to produce psychoactive effects, including schizophrenia-like symptoms and cognitive impairment (Meldrum and Rogawski, 2007).

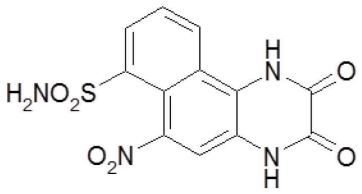
Although perampanel inhibited AMPA-induced functional responses, [3H]perampanel binding to rat forebrain membranes was only slightly reduced by high concentrations of glutamate, and was unaffected by the competitive AMPA receptor antagonist NBQX. Furthermore, perampanel at a concentration of 1.25 μM did not inhibit [^{3}H]AMPA binding. These data suggest a noncompetitive interaction of perampanel with the AMPA receptor. In addition, perampanel binding was reduced in a concentration-dependent manner by the selective noncompetitive AMPA receptor antagonists GYKI52466 and CP465022,

suggesting a common binding site between perampanel and these compounds. In line with these findings, other studies have demonstrated common binding sites between noncompetitive AMPA receptor antagonists on the AMPA receptor. CP465022 and GYKI53655 are reported to share a binding site at the interface between the glutamate binding core and the channel region of the AMPA receptor (Balannik et al., 2005). Binding to this site is thought to stabilize the resting state of the channel and disrupt channel opening in response to agonist binding (Balannik et al., 2005).

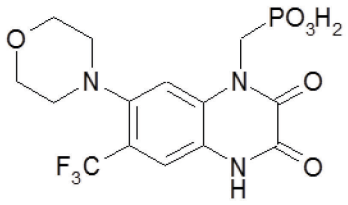
Perampanel showed oral efficacy in AMPA-induced seizure model. It indicated that perampanel act as AMPA antagonist also *in vivo*. Effective dose of peramapanel in this animal model was relatively low. It suggested that perampanel could have good pharmacokinetic properties in animal. Actually prampanel showed favorable pharmacokinetic profile including brain penetration in animals.

Prediction of pharmacokinetics in human is important in drug discovery research. For the purpose, metabolic stabilities in mice, rats, dogs, monkeys and human microsomes were examined. The results clearly indicated that metabolic rate in human should be very low suggesting long half life time. It is well known that sedative drugs often show tolerance in repeated use (Zapantis and Leung, 2005). Gradual increase of plasma concentration due to long half life time expected to help development of tolerance in clinical use.

Competitive AMPA antagonist



NBQX



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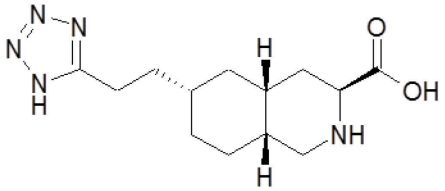
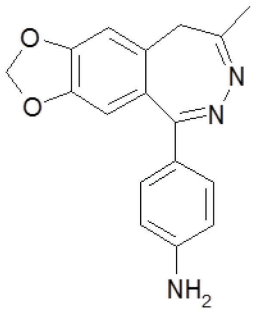
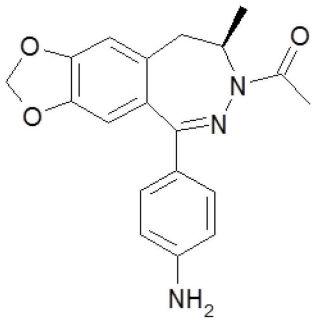


Tezampanel

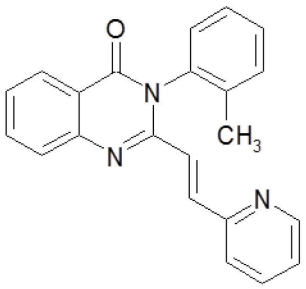
Non-competitive AMPA antagonist



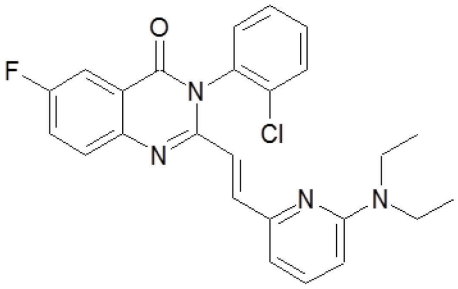
GYKI52466



Talampanel



Piriqualone



CP-465,022

Figure 2. Kown AMPA antagonists

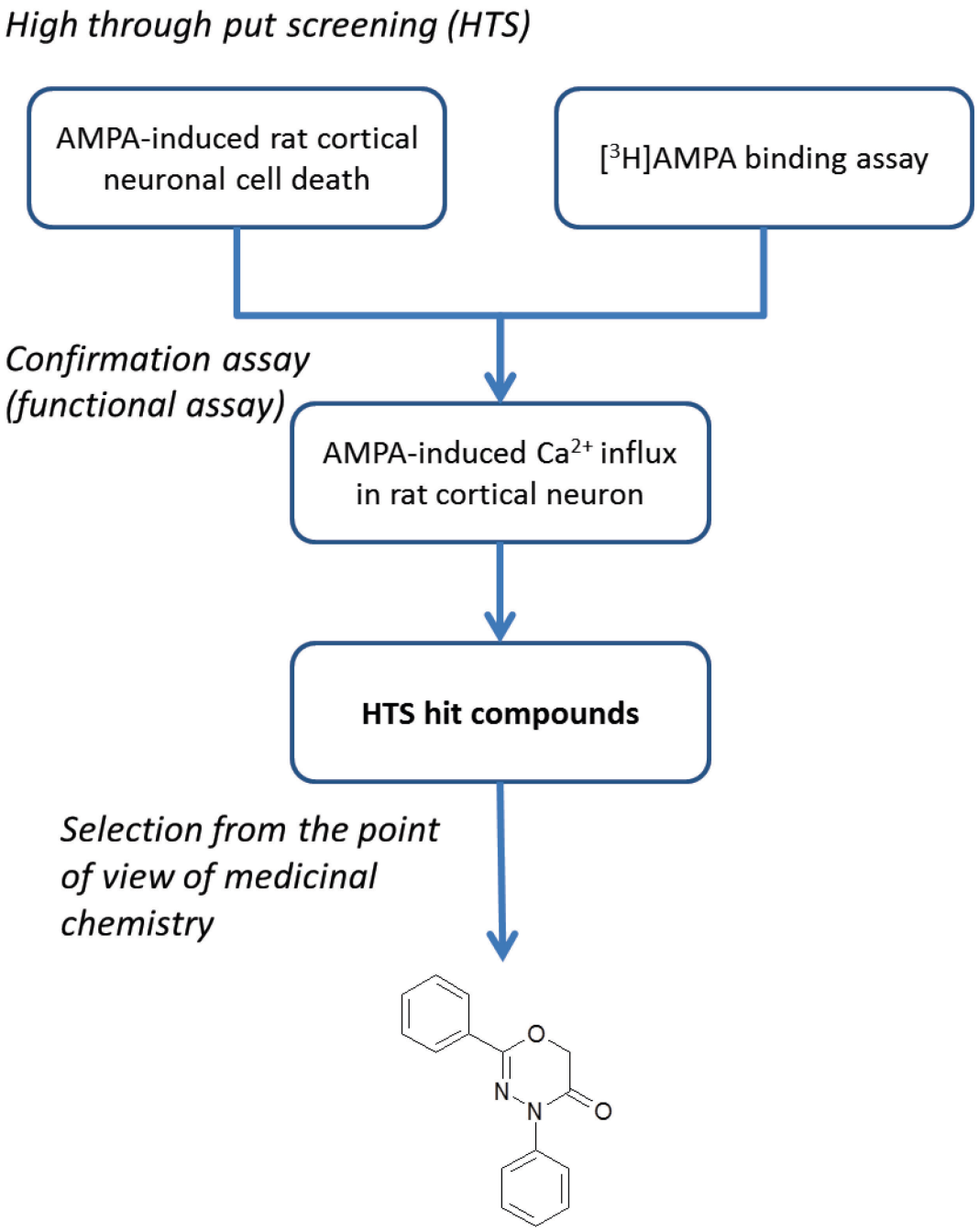


Figure 3. Screening flow finding for new chemical template.

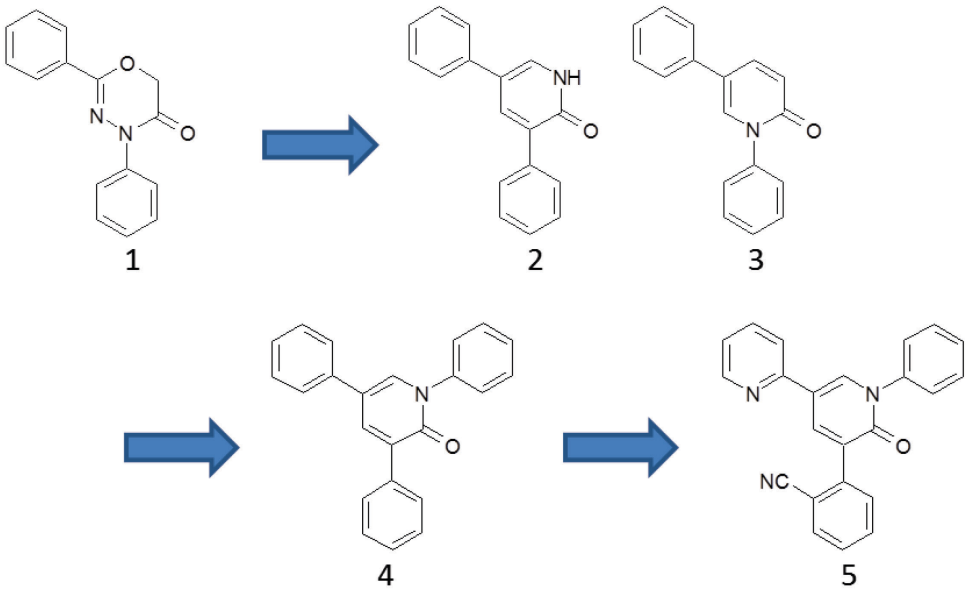
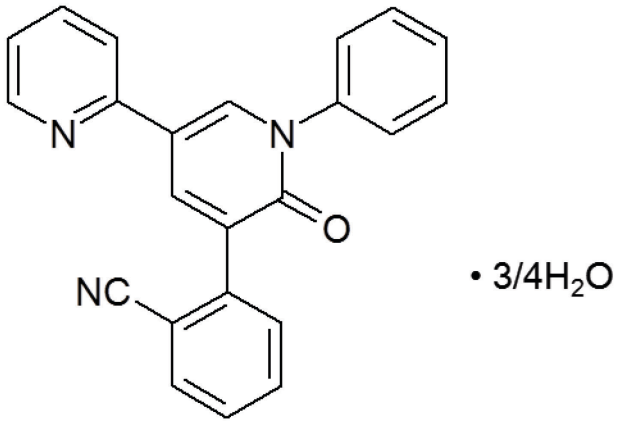


Figure 4. Flow of medicinal chemistry from hit compound of high throught put screening to perampanel.

Table 1. Inhibitory activity against AMPA-induced Ca2+ influx and metabolic stability in liver microsome.

Compound No.	AMPA IC_{50} (μM)	Clint (μL/min/mg protein)
1	9.17 ± 1.44	H; 0.305 ± 0.003 M; 1.158 ± 0.075 R; 0.476 ± 0.010
2	57.72 ± 7.14	H; 0.371 ± 0.003 M; 1.230 ± 0.000 R; 0.822 ± 0.019
3	69.54 ± 8.99	H; 0.114 ± 0.003 M; >1.535 R; 0.486 ± 0.031
4	1.08 ± 0.17	H; 0.045 ± 0.001 M; 0.148 ± 0.005 R; 0.052 ± 0.003
5	0.06 ± 0.01	H; 0.009 ± 0.000 M; 0.020 ± 0.000 R; 0.108 ± 0.003

H; Human, M; Mouse, R; Rat



INN:	Perampanel
Chemical name (IUPAC):	2-(2-oxo-1-phenyl-5-pyridin-2-yl-1,2-dihydropyridin-3-yl)benzonitrile
Molecular formula:	C23H15N3O • 3/4H2O
Molecular weight:	362.90 (3/4 hydrate) 349.38 (anhydrous)

Figure 5. Perampanel.

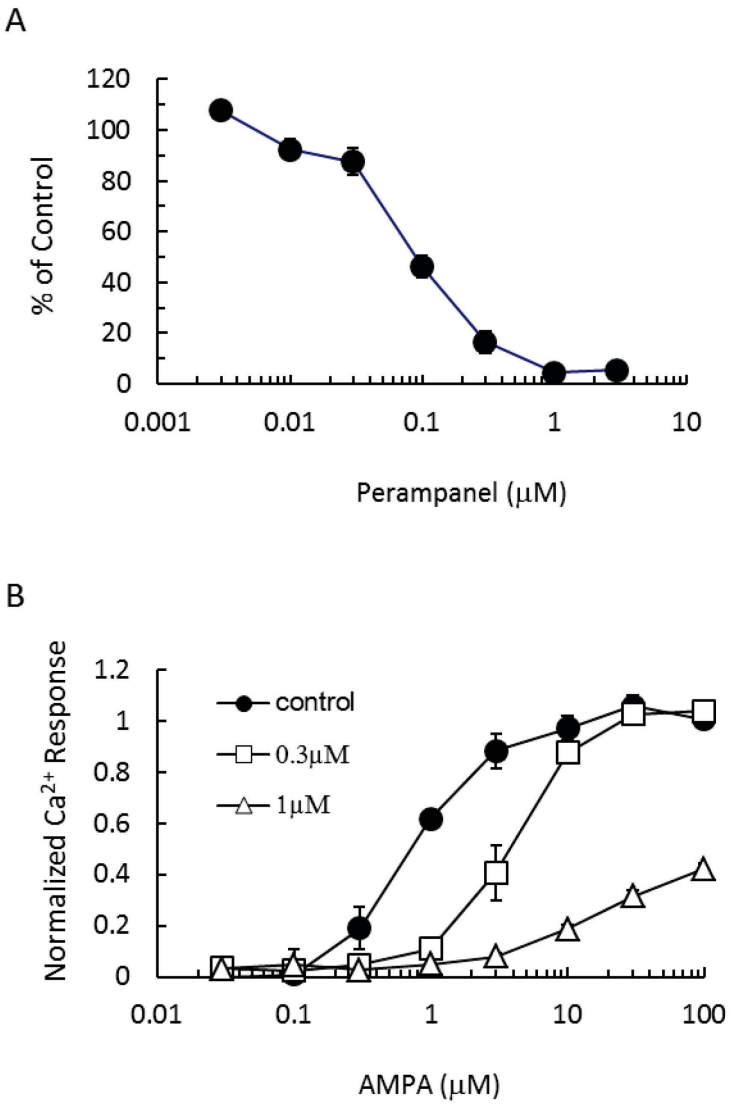
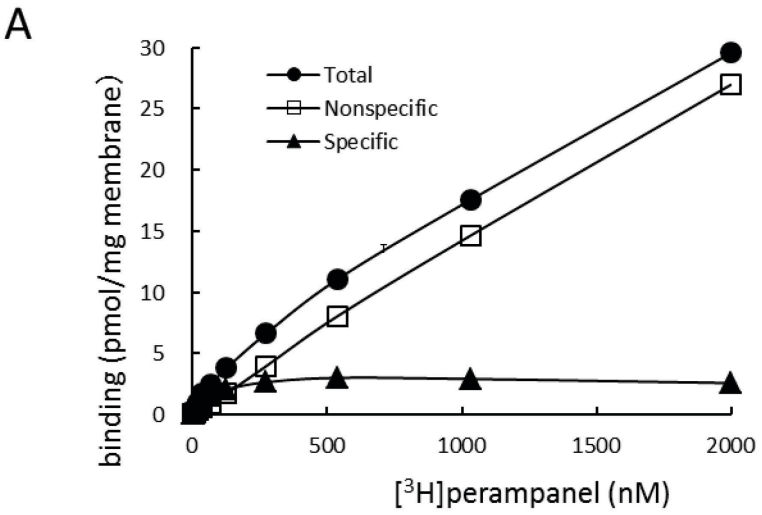
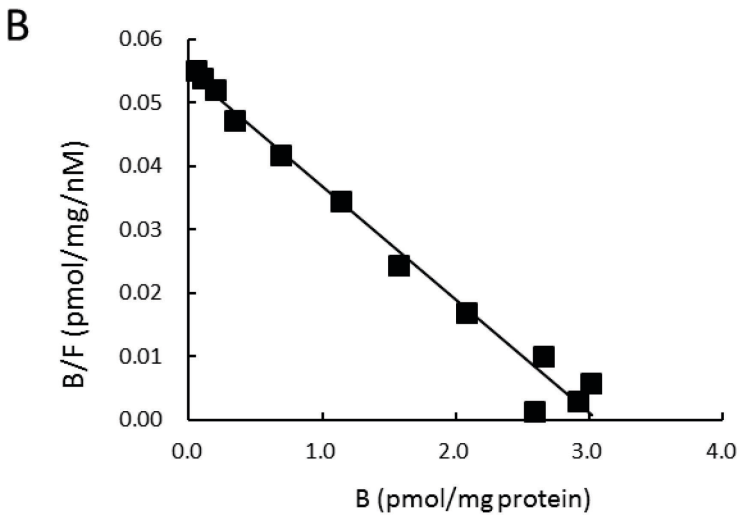


Figure 6. AMPA antagonistic activity of perampanel in AMPA-induced Ca^{2+} influx assay.
A. Inhibition of AMPA-induced Ca^{2+} influx by perampanel in cultured rat cortical neuron. Perampanel inhibited Ca^{2+} influx induced by 2 μM of AMPA with an IC_{50} value at 93 nM. B. Perampanel at 1 μM dampened peak Ca^{2+} response of AMPA.





Kd (nM)	Bmax (pmol/mg)
59.8 ± 5.2	3.2 ± 0.1

Figure 7. [3H]perampanel binding.
A. [3H]Perampanel binding. Filled circle indicated total binding. Open square indicate nonspecific binding displaced with 15 μM perampanel. Filled triangle indicated specific binding of [3H]perampanel binding assay.
B. Schatchard plot of [3H]perampanel binding.

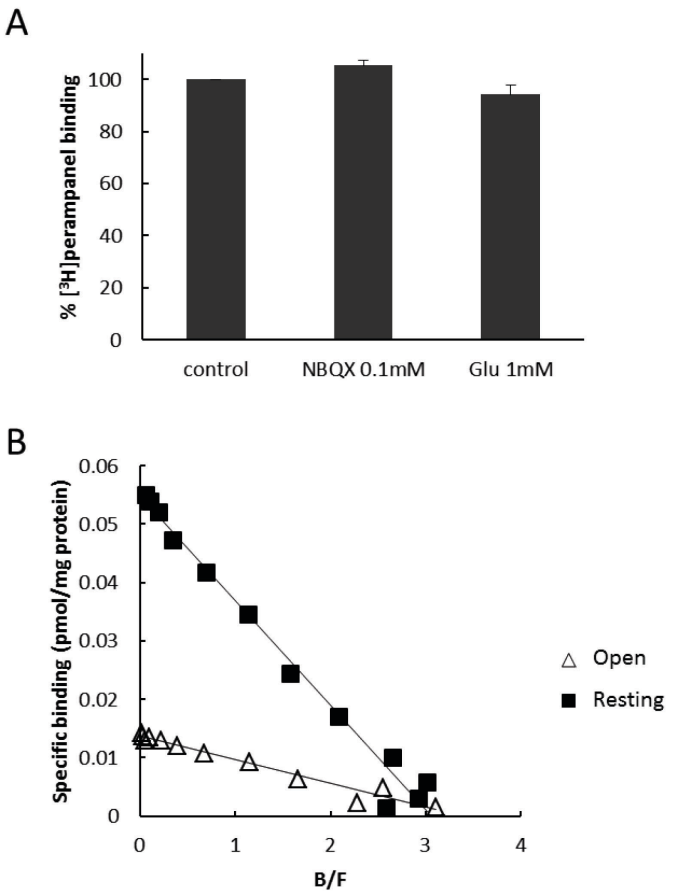


Figure 8. [3H]Perampnael Binding, interaction with competitive agonist/antagonist binding site.
A. Perampanel binding was not displaced by excessive amount of competitive antagonist and agonist. (n=3)
B. Affinity of perampanel was affected by ion channel state (close or open). Filled square is control perampanel binding (close state). Open triangle indicate perampanel binding with 100 μM AMPA and 100 μM cyclothiazide (open state).

Table 2. Channel state dependent change of [3H] perampanel binding.

Channel gating status	Stimulant	N	Kd (nM)	Bmax (pmol/mg)
Close	vehicle	4	59.8 ± 5.2	3.2 ± 0.1
Open	AMPA+CTZ	3	268.2 ±10.8	3.6 ± 0.2

Data are representaed as mean ± SD.
AMPA; 100 μM, Cyclothiazide (CTZ); 100 μM

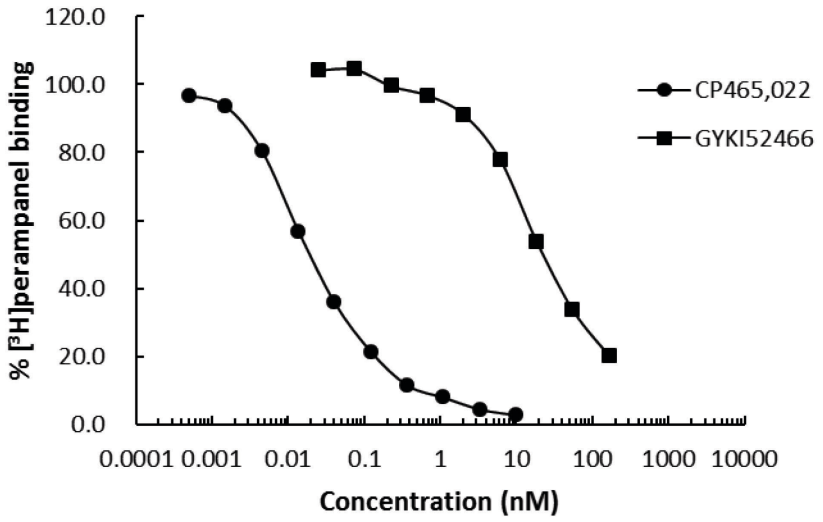


Figure 9. Displacement of [3H]Perampanel binding with known non-competitive AMPA antagonists. Calculated values for each compound is following.
CP465022, IC_{50} 21.1 ± 1.4 nM; Ki 11.2 ± 0.8 nM; n = 4
GYKI52466, IC_{50} 23.3 ± 1.9 μM; Ki 12.4 ± 1 μM; n = 3
Data are represented as mean ± SEM.

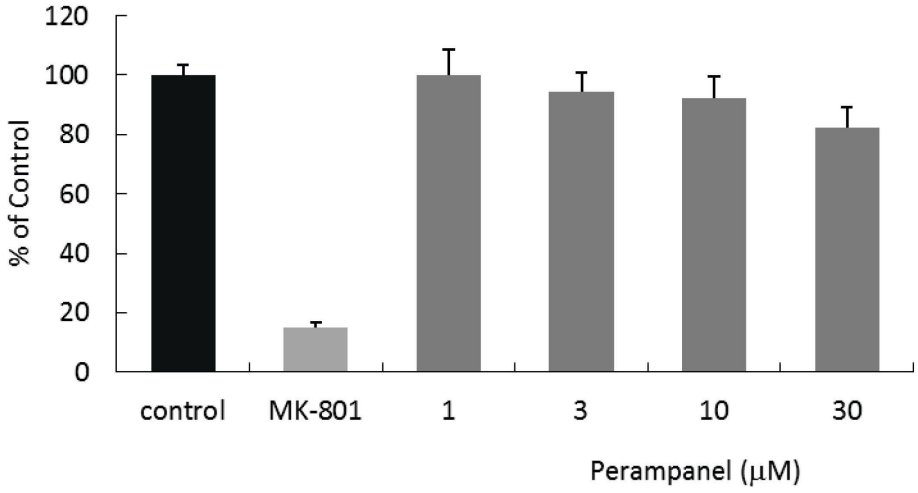


Figure 10. inhibitory effect of perampanel against NMDA -induced Ca^{2+} influx.
Inhibition of NMDA-induced Ca^{2+} influx by perampanel in cultured rat cortical neuron(n=10). Perampanel inhibited Ca^{2+} influx by 18% at 30 μM. Inhibitory effect was not reached statistical significance.

Table 3. Inhibitory effect of perampanel on binding with various ligands.

Targets	Radioligand	Reference Compound	Ki (M) Ref. Cpd.	% Inhibition by Perampanel (1.25 μM)
AMPA	[^{3}H]AMPA	(+/-)AMPA Br	3.90 E-8	5.45
Kainate	[^{3}H]Kainic acid	Kainic acid	1.12 E-8	7.74
NMDA	[^{3}H]CGP 39653	NMDA	2.09 E-6	6.54
NMDA-glycine	[3H]MDL-105,519	MDL-105,519	6.37E-9	7.85
Glycine strychnine sensitive	[^{3}H]Strychinine	Strychinine Nitrate	1.71 E-8	16.91
GABA A, agonist	[^{3}H]GABA	GABA	6.78 E-9	10.13
GABA A, benzodiazepine	[^{3}H]Flunitrazepam	Clonazepam	1.31 E-9	24.27
GABA B	[^{3}H]CGP 54626A	(+/-)Baclofen	1.01 E-6	-2.73
Adenosine, nonselective	[^{3}H]NECA	NECA	2.90 E-9	-8.56
Adrenergic, α1	[^{3}H]7-MeOxy-Prazosin	Phentoramine	2.85 E-9	-15.00
Adrenergic, α2	[^{3}H]RX 821002	Phentoramine	2.39 E-9	14.12
Adrenergic, β	[^{3}H]DHA	Alprenolol HCl	2.53 E-9	12.31
Norepinephrine transporter	[^{3}H]Nisoxetine	Desipramine HCl	1.29 E-9	6.34
Dopamine transporter	[^{3}H]WIN 35,428	GBR12909	2.26 E-8	-9.48
Dopamine, nonselective	[3H]Spiperone	Spiperone HCl	6.76 E-10	-5.95
Histamine, H1	[^{3}H]Pyrilamine	Triplolidine HCl	5.53 E-9	3.38
Histamine, H2	[^{125}I]Aminopotentidine	Tiotidine	7.12 E-9	12.59
Histamine, H3	[^{3}H]N-α- MeHistamine	N-α- MeHistamine	7.91 E-10	21.65
Melatonin	[^{125}I]-2-Iodomelatomin	2-Iodomelatomin	7.31 E-11	11.44
Serotonin, transporter	[^{3}H]Citalopram, N-Methyl	Imipramine HCl	1.52 E-8	16.88
Serotonin, nonselective	[^{3}H]LSD	Methysergide maleate	8.23 E-9	7.02
Muscarinic, M1 (human)	[^{3}H]Scoporamine, N-Methyl	(-)Scoporamine, MeBr	4.91 E-11	4.78
Muscarinic, M2 (human)	[^{3}H]Scoporamine, N-Methyl	(-)Scoporamine, MeBr	1.36 E-10	24.94
Muscarinic, nonselective CNS	[^{3}H]QNB	Atropine sulfate	3.29 E-10	14.0
Muscarinic, nonselective, peripheral	[^{3}H]QNB	Atropine sulfate	1.83 E-10	0.78
Nicotinic	[^{3}H]Epibatidine	(+/-)epibatidine	5.77 E-11	4.10
Opiate, nonselective	[^{3}H]Naloxone	Naloxone HCl	1.93 E-9	28.60
Sigma, nonselective	[^{3}H]DTG	Haloperidol	3.51 E-9	35.41
Acetylcholinesterase	Acetylcholine	Eserine	9.68 E-7	17.32
Choline acetyltransferase	[^{14}C]Acetyl Coenzime	MNEP	2.08 E-9	-10.79
Glutamic acid decarboxylase	[^{14}C]Glutamic acid	AminoOxy acetic acid	4.99 E-10	9.32
MAO-A	[^{14}C]5HT	Ro 41-1049 HCl	6061 E-9	24.94
MAO-B	[^{14}C]Phenylethylamine	Ro-16-6491 HCl	1.28 E-8	-1.00
Estrogen	[^{125}I]3,17B-Estradiol, 16α	17-B-Estradiol	9.54 E-11	6.17
Testosterone	[^{3}H]Methyltrienolone	Methyltrienolone	3.81 E-9	25.86
Calcium, L	[^{3}H]Nitrendipine	Nifedipine	2.10 E-10	9.83
Calcium, N	[^{125}I]Conotoxin GVIA	ω-Conotoxin GVIA	1.21 E-11	16.00
Potassium, ATP sensitive	[^{3}H]Glibenclamide	Glibenclamide	3.05 E-10	-5.45
Potassium, Ca^{2+} activated, VI	[^{125}I]Apamin	Apamin	3.98 E-11	-7.43
Potassium, Ca^{2+} activated, VS	[^{125}I]Charibdotoxin	Charybdotoxin	6.10 E-10	-16.79
Sodium, Site 1	[^{3}H]Saxitoxin	Tetrodotoxin	9.44 E-9	12.68
Sodium, Site 2	[^{3}H]Batrachotoxin A 20-α-Benzo	Aconitine	8.06 E-7	14.80
NOS	[^{3}H]NOARG	NOARG	3.29 E-8	-8.02
Leukotriene B4	[^{3}H]LTB_4	LTB_4	5.07 E-10	6.60
Leukotriene D4	[^{3}H]LTD_4	LTD_4	1.23 E-9	3.59
Thromboxane A2	[^{3}H]SQ 29,548	Pinane-thromboxane	8.27 E-8	-0.89
Corticotropin Releasing Factor	[125I]Tyr0-oCRF	Tyr0-oCRF	3.66 E-9	40.86
Oxytocin	[^{3}H]Oxytocin	Oxytocin	8.18 E-9	26.39
Platelet Activating Factor, PAF	[^{3}H]Hexadecyl, PAF	C16 PAF	4.23 E-9	-1.83
Thyrotropin Releasing Hormone, TRH	[^{3}H]-(3MeHis2)TRH	(3MeHis2)TRH	9.52 E-8	0.49
Angiotensin II, AT1	[^{125}I]-(Sar1-Ile8) Angiotensin II	Angiotensin II	7.05 E-9	0.51
Angiotensin II, AT2	[^{125}I]Thr4-Angiotensin II	Angiotensin II	3.72 E-10	-1.08
Bradykinin, BK2	[^{3}H]Bradykinin	Bradykinin TFA	5.90 E-10	-4.72
Cholecystokinin, CCK1	[^{125}I]CCK-8	CCK-8 (sulfated)	1.79 E-11	21.13
Cholecystokinin, CCK2	[^{125}I]CCK-8	CCK-8 (sulfated)	1.19 E-10	16.76
Endothelin, ET-A	[^{125}I]Endothelin-1	Endothelin-1	2.58 E-10	-5.16
Endothelin, ET-B	[^{125}I]Endothelin-1	Endothelin-1	3.54 E-10	11.58
Galanin	[^{125}I]Galanin	Galanin (Porcine)	4.21 E-10	7.16
Neurokinin, NK1	[^{3}H]Substance P	Substance P	1.33 E-8	7.50
Neurokinin, NK2 (Human)	[^{125}I]NKA	Neurokinin A	8.94 E-10	10.11
Neurokinin, NK3	[^{125}I]Eledoisin	Eledoisin	7.21 E-9	23.98
Vasoactive intestinal peptide	[^{125}I]VIP	VIP	5.82 E-10	3.39
Vasopressin 1	[^{3}H]Vasopressin-1 antagonist	Arg8-Vasopressin	8.89 E-9	-11.58

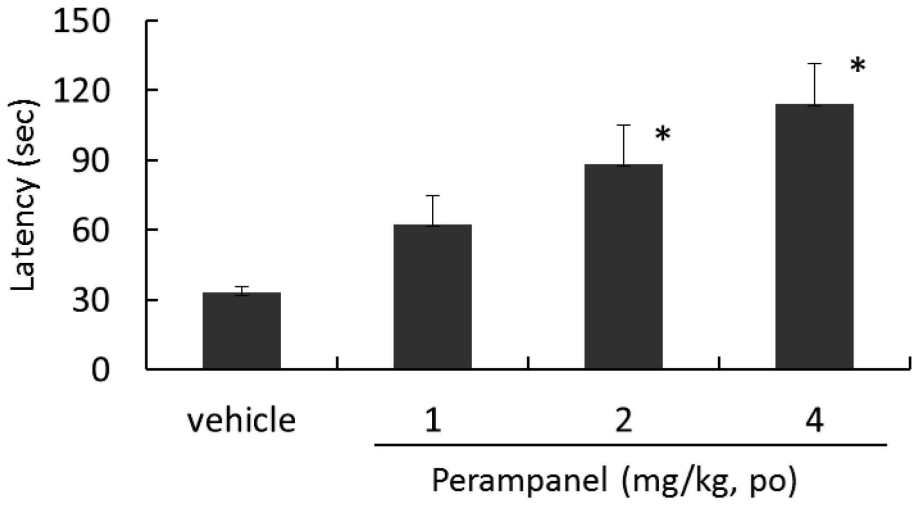


Figure 11. Perampanel antagonized in vivo AMPA receptor agonist effect.
Perampanel or vehicle were administered one hour before test.
Data are represented as mean ± SEM (n=9-10). * for $p<0.05$ vs vehicle (Dunnett's test)

Table 4. Pharmacokinetic parameters for perampanel in rat, dog, and monkey.

	Rat		Dog		Monkey	
Route	i.v.	p.o.	i.v.	p.o.	i.v.	p.o.
Dose (mg/kg)	1	1	0.1	0.1	0.03	0.03
T_{max} (hr)	-	1.00 (0.50-1.00)	-	0.50 (0.25-0.50)	-	1.50 (1.00-2.00)
C_{max} (ng/mL)	-	100±17	-	26±4	-	13±1
$T_{1/2}$ (hr)	1.36±0.15	1.67±0.13	6.87±0.72	5.34±0.73	6.94±0.59	7.55±0.46
MRT (hr)	1.29±0.14	3.19±0.20	5.78±0.75	4.88±0.34	7.66±1.87	7.69±0.39
AUC (ng×hr/mL)	836±96	386±25	132±15	70±5	175±28	118±20
CL (mL/hr/kg)	1239±125	-	780±90	-	185±28	-
Vss (mL/kg)	1560±122		4424±461	-	1813±256	-
B.A. (%)	-	46.1	-	53.5	-	74.5

CL, total clearance; Cmax, maximum concentration; MRT, mean residence time; Tmax, time of occurrence for peak concentration; Vss, volume of distribution at steady state.
Values of Cmax, T1/2, MRT, AUC, CL, and Vss are shown as mean ± SEM; Tmax is shown as median (range).

Chapter III:

Charaterization as therapeutic agents for epilepsy

Abstract

Treatment refractory epilepsy is one of a target for drug discovery research. It is considered that AMPA receptor has contribution on pathophysiology of treatment refractory epilepsy. Perampanel is the novel non-competitive AMPA antagonist. To assess the activity of perampanel as antiepileptic drug, Activity of orally administered perampanel was examined in amygdala-kindled rats and in mice exhibiting audiogenic, maximal electroshock (MES)–induced, pentylenetetrazole (PTZ) –induced, or 6 Hz-induced seizures. In mice, perampanel showed protective effects against audiogenic, MES-induced, and PTZ-induced seizures (ED_{50}s 0.47, 1.6, and 0.94 mg/kg, respectively). Perampanel also inhibited 6 Hz electroshock-induced seizures without changing efficacy by increasing stimulus intensity. In amygdala-kindled rats, perampanel significantly increased afterdischarge threshold ($p < 0.05$ vs. vehicle), and significantly reduced motor seizure duration, afterdischarge duration, and seizure severity recorded at 50% higher intensity than afterdischarge threshold current ($p < 0.05$ for all measures vs. vehicle). In genetic absence epilepsy in rats from Strasbourg (GAERS), perampanel up to 10 mg/kg did not show significant effect. Perampanel caused dose dependent motor impairment in both mice (TD_{50} 1.8 mg/kg) and rats (TD_{50} 9.14 mg/kg), as determined by rotarod tests. In mice, the protective index (TD50 in rotarod test/ED50 in seizure test) was 1.1, 3.8, and 1.9 for MES-induced, audiogenic, and PTZ-induced seizures, respectively. These data suggest that perampanel is an orally active, noncompetitive, selective AMPA receptor antagonist with potential as a broad spectrum antiepileptic agent.

Introduction

Epilepsy is a common neurologic disorder that manifests as recurrent seizures and affects approximately 0.5–1% of the general population(Abu Saleh and Stephen, 2008; Ngugi et al., 2010). While many second- and third-generation antiepileptic drugs (AEDs) have been introduced into clinical practice over the past 20 years, there still remains a significant unmet medical need to control seizures, as evidenced by the 15–35% of all patients with epilepsy who fail to achieve long-term remission (Shorvon and Goodridge, 2013). In order to best address this unmet need, AED research must keep abreast of advances in technology and basic science. To this end, the technical and methodological issues of AED development have been discussed recently as part of several working groups (French et al., 2013; Galanopoulou et al., 2013; Wilcox et al., 2013).

The development of AEDs with modes of action (MOAs) based on a pathophysiologic understanding of the condition may represent a rational strategy. With this in mind, the underlying pathophysiologic condition in seizure disorders is believed to be the imbalance of excitatory and inhibitory neuronal activity (Lason et al., 2013; McCormick and Contreras, 2001). Glutamate and gamma-aminobutyric acid (GABA) are the major neurotransmitters that contribute to excitatory and inhibitory activities in the central nervous system (CNS), and both have been implicated in seizure development. As described, AMPA receptor has significant contribution in the pathophysiology of epilepsy and could have a contribution on development of epilepsy. Therefore antagonist of AMPA receptor could have a significant role in the treatment of epilepsy. It is known that efficacy in preclinical seizure models could be translated into efficacy in human patients. Anti-seizure effect of perampanel is described for the consideration of efficacy profile in human.

Materials and Methods

Generalized tonic-clonic seizures (MES-induced seizures).

Electroshock seizures were induced in 4-week-old male ddY mice obtained from Japan SLC, Inc (Shizuoka, Japan). Animals were dosed orally with perampanel (0.75, 1.06, 1.50, or 2.12 mg/kg), carbamazepine (9.2, 13, 18.4, or 26 mg/kg), sodium valproate (284, 400, 567, or 800 mg/kg) or vehicle 1 hour prior to electrical stimulation. All drugs were suspended or dissolved in 0.5% methylcellulose solution. Electrical stimulus (80 V) was applied to both corneas for 0.4 sec. An occurrence of electroshock-induced tonic extension (TE) was defined as a hindlimb TE lasting >1 sec following stimulation. ED_{50} and 95% confidence intervals (CI) for TE inhibition were calculated by regression analyses.

Absence or myoclonic seizures (Pentylenetetrazole (PTZ)-induced seizures) .

PTZ-induced seizures were generated in 9-week-old male ICR mice obtained from CRJ, Inc. (Kanagawa, Japan). Animals were dosed orally with perampanel (0.75, 1.5, or 3 mg/kg), carbamazepine (100 mg/kg), sodium valproate (150, 300, or 600 mg/kg), or vehicle 30 min prior to initiation of seizures. All drugs were suspended or dissolved in 0.5% methylcellulose solution. Seizures were then induced by subcutaneous injection of PTZ (90 mg/kg). An occurrence of PTZ-induced seizure was defined as a seizure of at least 3 sec duration within 30 min of PTZ administration.

Reflex seizures (Audiogenic seizures).

Audiogenic seizures were induced in 3-week-old male DBA/2J mice obtained from CLEA Japan, Inc. (Tokyo, Japan). Mice were dosed orally with perampanel (0.3, 1, or 3 mg/kg), carbamazepine (1, 3, or 10 mg/kg), sodium valproate (30, 100, or 300 mg/kg), or vehicle 1

hour prior to initiation of seizures. All drugs were suspended or dissolved in 0.5% methylcellulose solution. Mice were then habituated for 1 min in an observation box and exposed to sound stimulation (11 kHz, 115 dB) for 1 min or until TE occurred. An occurrence of sound-induced seizure was defined as a hindlimb TE lasting >1 sec during sound stimulation.

Psychomotor seizures (6-Hz electroshock-induced seizures).

Perampanel (0.5, 1, 2 or 4 mg/kg) or vehicle (0.5% methylcellulose) was orally administered to 9-week-old male ICR mice 1 hour prior to test. An electrical stimulus (6 Hz, 0.2 msec rectangular pulse, 3 sec duration, 32 or 44 mA) was applied to both corneas and an occurrence of seizure was defined as appearance of immobility or stun, forelimb clonus, twitching of the vibrissae, and an elevated (or Straub) tail (Barton et al., 2001).

Motor coordination (Rotarod test).

Six-week-old male Sprague Dawley rats and 8-week-old male ICR mice were obtained from Charles River Japan, Inc. (Kanagawa, Japan). Animals were dosed orally with perampanel (2, 4, 8, or 16 mg/kg for rats; 0.5, 1, 2, or 4 mg/kg for mice) or vehicle, 1 hour prior to being placed on a rotating rod (rotation speed 6 rpm for rats, 8 rpm for mice), and the time animals were able to remain on the rod was recorded. Trial were conducted twice for each animal. Animals that failed to remain on the rotarod for 120 sec in both trials were classified as motor-uncoordinated.

Complex partial seizures (Rat amygdala-kindling model) .

Eight-week-old male Sprague Dawley rats obtained from SLC, Inc. (Shizuoka, Japan) were anaesthetized with pentobarbital (50 mg/kg) and a tri-polar electrode was inserted

into the amygdala complex using stereotaxic surgery. After a recovery period of 1 week, threshold current of afterdischarge was determined using a 25% ascending stimulation paradigm from 0.01 mA. Rats were stimulated at the threshold current once daily until ≥3 consecutive stage 5 seizures were observed. Animals that showed stable threshold current of afterdischarge for at least 3 consecutive stimulations were selected for further experiments.

Threshold current of afterdischarge was determined using an ascending stimulation schedule prior to administration of either perampanel or vehicle. A second threshold determination was performed 1 hour following oral administration of perampanel (1, 1.25, 2.5, 5, or 10 mg/kg) or vehicle (0.5% methylcellulose). Afterdischarge duration, motor seizure duration (time with stage 4 and 5 seizure), and seizure severity (Racine 1972) were then determined in each rat using a stimulus that was two steps (or 50%) stronger than the afterdischarge threshold current.

Racine severity scale;

Score 1: immobility, facial clonus, eye closure, twitching of vibrissae, stereotyped sniffing

Score 2: head nodding, severe facial clonus

Score 3: unilateral forelimb clonus

Score 4: rearing often accompanied by bilateral forelimb clonus

Score 5: continuous rearing and falling accompanied by secondary generalized clonic seizure

Spontaneous absence seizure model (genetic absence epilepsy in rats from Strasbourg (GAERS)).

Eight GAERS from the French breeding colony (Université d'Orléans, France, 250-

350g) were implanted with 5 monopolar stainless steel electrodes positioned over the frontal and parietal cortices, in both sides of the brain, under generalized anaesthesia (xylazine 0.5 ml/kg i.m. + ketamine 100 mg/kg, i.p.). The rats were equipped with a female connector fixed on the skull to allow chronic EEG recordings. Rats were injected systemically with vehicle, E2007 (1, 3 and 10 mg/kg/5ml, per p.o., dissolved in 0.5N HCl) and valproate (200 mg/kg/5ml, i.p., dissolved in saline) in a random order (one week between two injections) so that each animal was its own control. Digital EEG recordings were performed 20 min before the injection and during 120 min after the injection with fronto-parietal derivations. The general behaviour of the animals was observed during the recording period and rats were constantly monitored by an investigator to ensure that they did not fall asleep.

Statistical analysis.

In animal studies, ED_{50} (effective dose causing 50% reduction in seizures), TD_{50} (dose causing 50% reduction in motor coordination) and 95% CIs were calculated by regression analyses. Two-way ANCOVA (analysis of covariance) was used to assess overall effect of treatment, and Dunnett's post test was used to compare differences between treatment groups in amygadala kindling study. Two-way ANOVA for repeated measures followed by Wilcoxon test for paired data was used in GAERS study. Differences between treatment groups were considered significant if the p-value was less than 0.05.

Results

Generalized tonic-clonic seizures (MES-induced seizures).

MES is animal model of human tonic-clinic seizure. This model was also utilized for primary screening model for the discovery of anticonvulsant. Perampanel clearly inhibited occurrence of tonic extension of hindlimb (ED_{50} 1.6 mg/kg) suggesting potential utility in tonic-clonic seizure type (Table 5).

Absence or myoclonic seizures (Pentylenetetrazole (PTZ)-induced seizures).

PTZ model is considered animal model of absence or myoclonic seizure. It is also utilized for the evaluation of compound for characterization. Perampanel also inhibited occurrence of myoclonic seizure with an ED_{50} value at 0.94 mg/kg (Table 5).

Reflex seizures (Audiogenic seizures).

Audiogenic seizure is animal model of reflex seizure and often utilized for the drug screening. Perampanel also inhibited this seizure with and ED_{50} value at 0.47 mg/kg (Table 5).

Psychomotor seizures (6-Hz electroshock-induced seizures).

The 6-Hz seizure is drug resistant seizure model especially for channel blockers. It is considered animal model of psychomotor seizures. Drug efficacy in this model is decreased when stimulus intensity is increased. Perampanel, given orally 1 hour prior to test, protected mice from 6 Hz electroshock-induced seizures in a dose-dependent manner (Figure 4). The ED_{50} value for perampanel was similar at 32 mA (ED_{50} 2.1 mg/kg; 95% CI, 1.4–2.9) and 44 mA (ED_{50} 2.8 mg/kg; 95% CI, 2.0–4.2) stimulus intensities (Figure 4A).

Carbamazepine (20 mg/kg), phenytoin (10 mg/kg) and valproate (100 mg/kg) each further reduced the incidence of seizures in the presence of perampanel (Fig. 12).

Effect on motor coordination (Rotarod test).

The effect of perampanel on motor coordination was determined using the rotarod test. Perampanel caused dose-dependent motor impairment in both mice (TD_{50} 1.8 mg/kg; 95% CI, 1.4–2.8; n = 9 per group) and rats (TD_{50} 9.14 mg/kg; n = 8 per group). In mice, the protective index, defined as TD_{50} in rotarod test/ED_{50} in individual seizure tests, was 1.1, 3.8, and 1.9 for MES-induced, audiogenic, and PTZ-induced seizures, respectively (Table 5).

Complex partial seizures (Rat amygdala-kindling model).

Amygdala kindling is animal model of complex partial seizures. Perampanel, given orally 1 hour prior to test, significantly increased afterdischarge threshold in amygdala-kindled rats at 10 mg/kg (Fig. 13), and significantly decreased motor seizure duration recorded at 50% higher intensity than after discharge threshold current stimulation at 5 mg/kg or higher (Fig. 14C). Perampanel at 10 mg/kg also significantly decreased afterdischarge duration and seizure severity at 50% higher intensity than after discharge threshold current stimulation (Fig. 14A and 14B).

Spontaneous absence seizure model (genetic absence epilepsy in rats from Strasbourg (GAERS)).

Valproate injected at the dose of 200 mg/kg remarkably reduced SWD during the 100 minutes following injection (reduction between 72 and 95% compared to reference period). Wilcoxon test for paired data revealed a significant effect between 0 and 100 min post

injection for cumulated duration of SWD, number of SWD (Fig. 15). After 100 min post-injection level of SWD return to values not significantly different from vehicle. On the other hand, oral perampanel (1, 3 and 10 mg/kg) failed to changed parameters of the study (Fig. 15). No gross modifications of the animals' behaviour or EEG activity were observed at tested doses.

Disucssion and Conclusion

Perampanel inhibited seizures in both MES and PTZ models. The most of anticonvulsant utilized in the treatment for partial onset seizures inhibited MES but limited number of anticonvulsant works in PTZ model. It is generally considered that only the compound with clinical efficacy in myoclonus or absence seizure could inhibit seizures in PTZ model. In addition perampanel showed efficacy in audiogenic seizure. The results suggested that peramapnel could show broader clinical efficacy in patients with epilepsy.

Seizures induced by 6 Hz electroshock are classically considered resistant to phenytoin and other inhibitors of voltage-dependent ion channels, with AED activity being closely linked to the stimulation intensity (Barton et al., 2001). Phenytoin and lamotrigine show only a partial inhibitory response when stimulation intensity is increased from 22 mA to 32 mA in this model, whereas many other AEDs lose effectiveness when stimulation intensity increases further to 44 mA (Barton et al., 2001). In a test of seven established AEDs (phenytoin, carbamazepine, clonazepam, phenobarbital, ethosuximide, trimethadione and valproic acid) and five new generation AEDs (lamotrigine, levetiracetam, felbamate, tiagabine and topiramate), only levetiracetam and valproate showed activity in the 6 Hz model at 44 mA stimulation intensity, and in both cases, efficacy of the compounds was lower at 44 mA than at 32 mA stimulation intensity, as shown by higher ED50 values (Barton et al., 2001). In contrast, perampanel showed similar effectiveness at 32 mA and 44 mA stimulation intensities in our studies.

Drug effects in amygdala kindling model have been shown to be predictive of effects on complex partial seizures with secondary generalization in humans. Perampanel increased afterdischarge threshold and significantly reduced motor seizure duration, afterdischarge duration, and seizure severity in the rat amygdala-kindling model. Thus, perampanel

inhibited both secondary generalized seizures (seizure score ≥4) and focal seizures (score ≤3). Although most AEDs are reported to inhibit amygdala-kindled seizures, their effects differ significantly. Phenytoin, for example, appears to suppress focal seizures, but does not inhibit the spread of afterdischarges and the development of secondary generalized seizures (Ebert et al., 1997). In contrast, some NMDA receptor antagonists have been shown to block only generalized seizures in this model (Barton & White, 2004). AMPA antagonists, on the other hand, block both the focal and generalized components (Hara et al., 2006), consistent with characteristic broad spectrum antiseizure effects of these compounds. AMPA receptors are thought to play a critical role in the propagation of seizures (Namba et al., 1994; Rogawski & Donevan, 1999), and are also likely to be involved in their initial triggering (Tortorella et al., 1997). Interestingly, afterdischarge persisted in some perampanel-treated animals despite complete inhibition of behavioral seizures, suggesting that perampanel may have a greater inhibitory effect on propagation of seizures than on their initiation.

Morimoto at al.(1997) described that increased stimulus intensity could make a condition that would be less sensitive to AEDs. Peramapnel reduced seizure severity in the condition with 50% higher stimulus intensity than after discharge threshold. It may suggest that perampanel inhibit severer seizure condition compared to others.

Efficacy in GAERS showed difference from PTZ study. Difference of end point between two animal models (behavior or EEG) may affect the results. However, at least perampanel did not worsen SWD in GAERS. Some times AEDs could causes aggravation of seizure in patient with specific type of seizures. The most well-known case is aggravation of myoclonic or absence seizure by carbamazepine (Sazgar and Bourgeois, 2005). This clinical phenomenon is also translated into animal model of absence seizure (Russo, 2011). Patients with epilepsy often have multiple seizure types. No seizure

aggravation was confirmed by the treatment with perampanel across animal models. It may connect to reduction of risk for patients experiencing seizure aggravation or appearance of new seizure types.

Results from Rotarod test suggested that perampanel could cause motor impairment close the dose showing antiseizure effects. Similar effects on motor coordination have been reported for both noncompetitive (e.g. GYKI52466) and competitive (e.g. NBQX) AMPA receptor antagonists, suggesting a mechanism associated with AMPA receptor blockade (Yamaguchi et al., 1993). The window between antiseizure effects and motor dysfunction, however, is different across preclinical seizure models, making predictions of clinical therapeutic index difficult. Furthermore, AEDs such as gabapentin and pregabalin cause central nervous system (CNS) depressant effects in patients (Arroyo et al., 2004; Arroyo & Lesser, 1993), despite preclinical studies that indicated a very wide therapeutic margin (Dalby & Nielsen, 1997; Vartanian et al., 2006), whereas valproate shows a narrow therapeutic margin in rodent models (Barton et al., 2001), but does not show strong CNS depressant side effects (Mattson et al., 1992). Thus, clinical therapeutic index of AEDs cannot reliably be inferred from preclinical studies.

Efficacy of perampanel in various seizure models are summarized in table 6. Perampanel showed antiseizure effect in most of seizure models and showed efficacy in severe seizure condition in 6Hz and amygdala kindling model. These could support utility in severe seizure condition like refractory partial onset seizure. It was proven in clinical studies. Regulatory authorities approved perampanel as adjunctive treatment of partial onset seizure.

Table 5. Effect of drugs on audiogenic, MES-, PTZ-induced seizure and motor coordination (rotarod test) in mice.

Drug	Seizure test ED_{50}, mg/kg (95% CI)			Rotarod Test TD_{50}, mg/kg (95% CI)
	MES	Audiogenic	PTZ	
Carbamazepine	21 (16–45)	6.1 (4.1–9.0)	>100	ND
Sodium valproate	460 (290–600)	160 (93–280)	350 (260–470)	ND
Perampanel	1.6 (1.3–1.9)	0.47	0.94 (ND)	1.8 (1.4–2.8)
Perampanel protective index (TD_{50}/ED_{50})	1.1	3.8	1.9	

MES, maximal electroshock; PTZ, pentylenetetrazole; ND, not determined.
Seizure tests (n=10 animals/group) and rotarod test (n=9 animals/group)

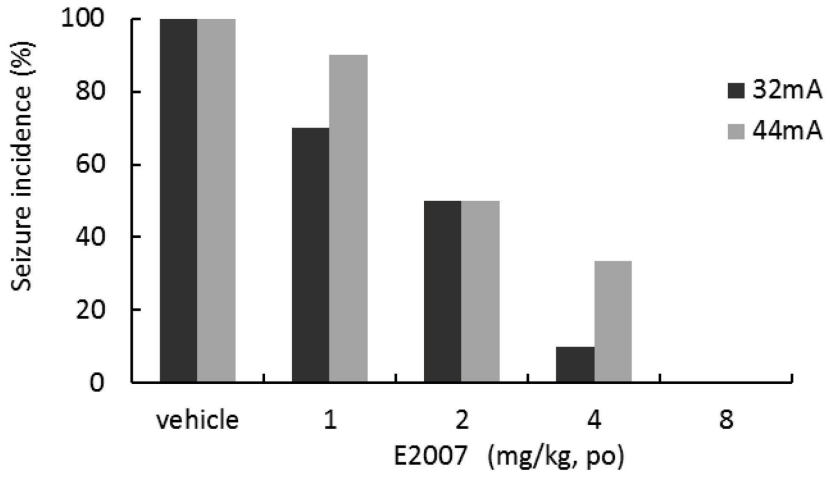


Figure 12. Effect of perampanel on 6-Hz electroshock-induced seizure.
The 6-Hz electroshock-induced seizure is considered as animal model of refractory psychomotor seizures. Perampanel or vehicle were orally administered one hour before test. The 6-Hz electroshock-induced seizure was evaluated with different stimulus intensities (32 mA, 44 mA, n=9 -10). Perampanel exerted anti-seizure effect in both stimulus intensity with similar ED_{50} values (32 mA, ED_{50} 2.1 mg/kg; 95% CI, 1.4–2.9; 44 mA, ED_{50} 2.8 mg/kg; 95% CI, 2.0–4.2)

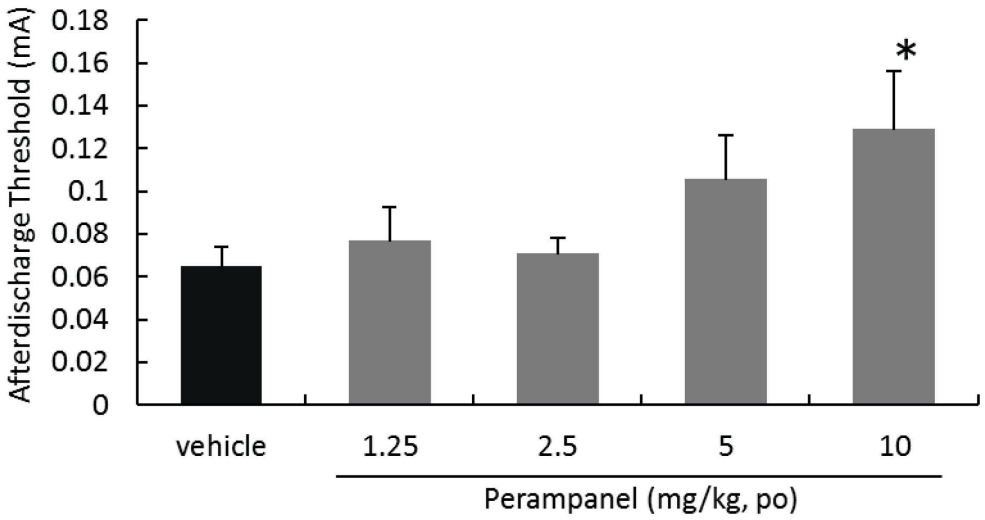


Figure 13. Effect of perampanel on seizure threshold in amygdala kindling model. Seizure threshold was determined by applying ascending electrical stimuli to the model. Appearance of more than 3 seconds after discharge was designated threshold of seizure.
* for $p<0.05$ vs vehicle (ANCOVA followed by Dunnett's test)

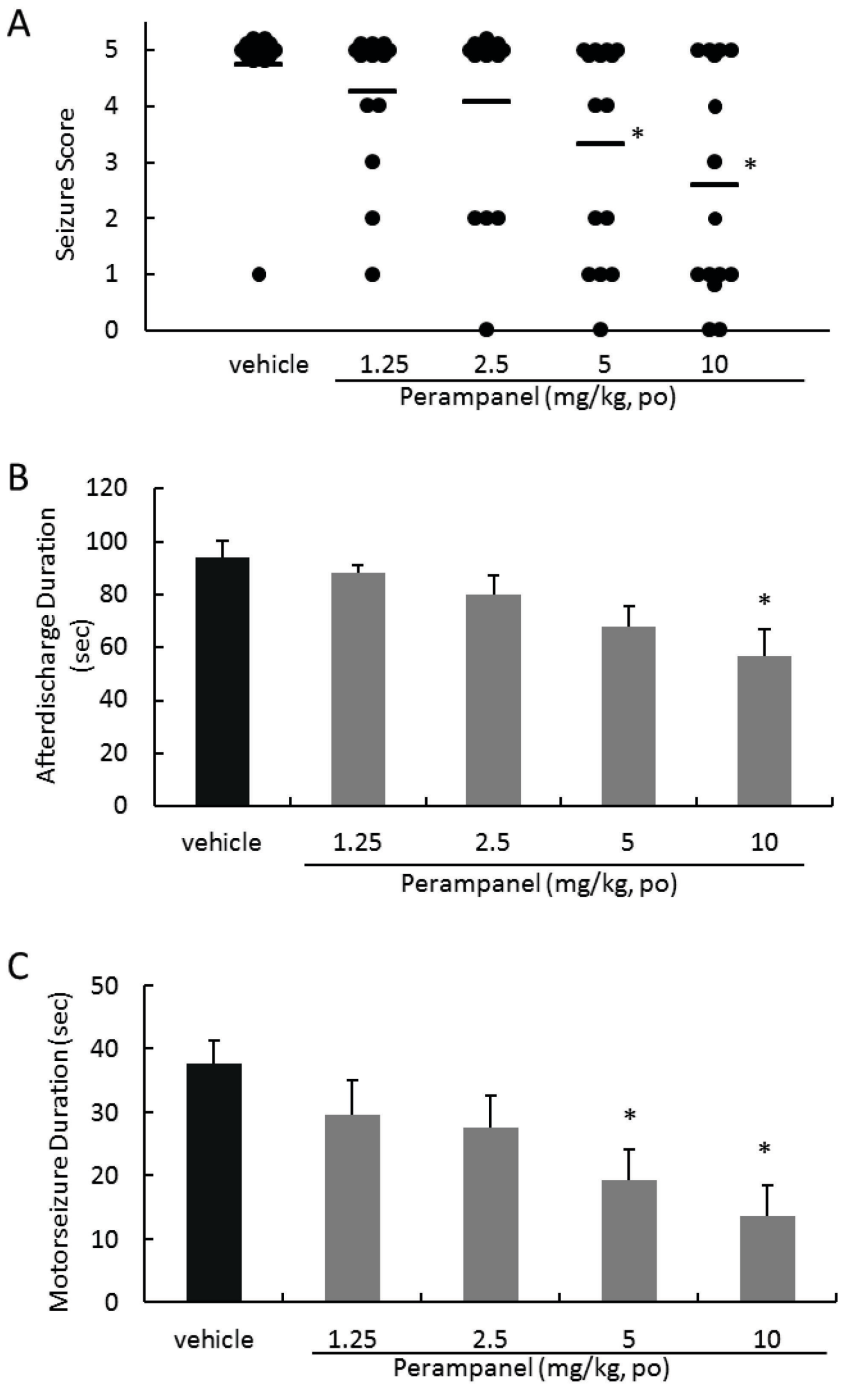


Figure 14. Effect of perampanel on seizure severity in amygdala kindling model. Seizure was elicited with electrical stimuli 50% stronger than threshold of seizure. Seizure severity according to Racine's score (A), afterdischarge duration (B) and motor seizure duration (C, duration of score 4 and 5 seizure) was determined.
* for $p<0.05$ vs vehicle (ANCOVA followed by Dunnett's test)

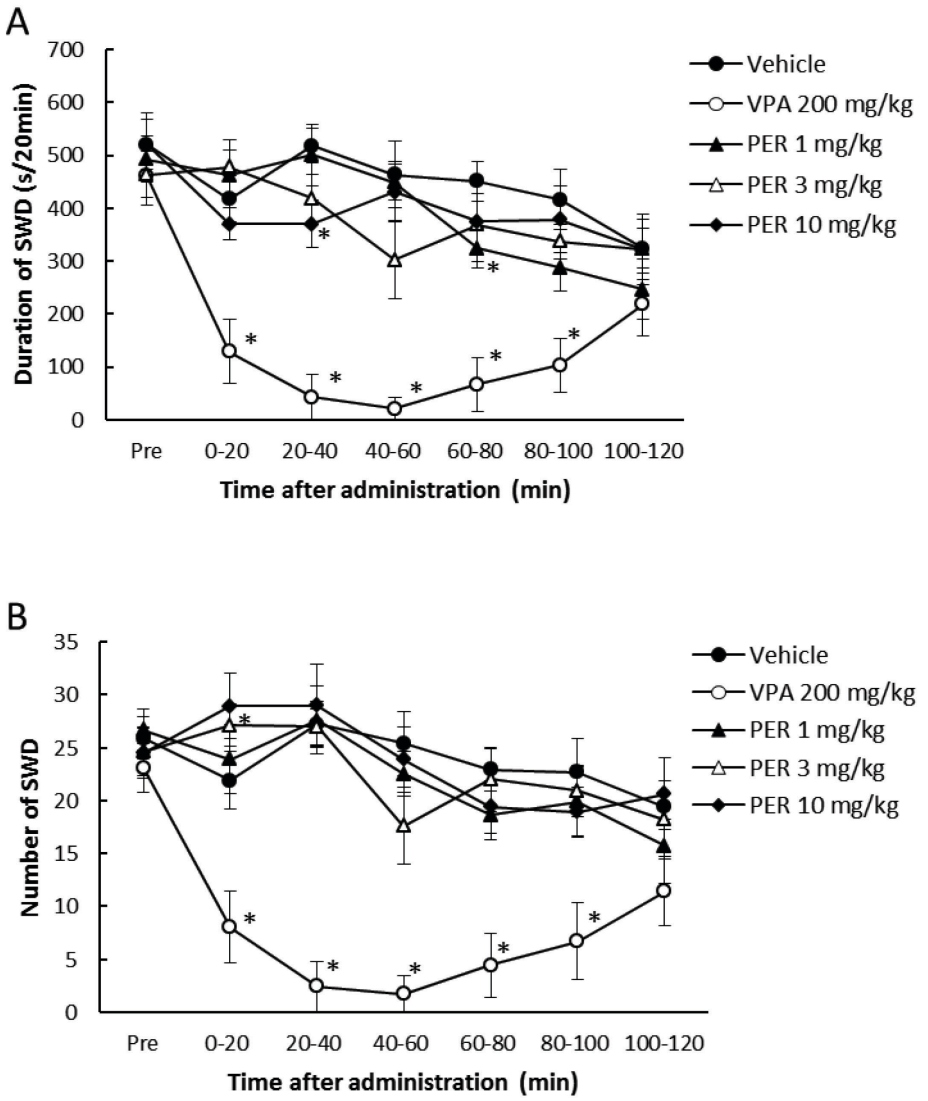


Figure 15. Effect on spike wave discharge (SWD) in GAERS.
A. Cumulative duration of SWD (s/20 min). B. Number of SWD (/20 min).
Data are represented as mean ± SEM (n=8). * for $p<0.05$ vs vehicle (Wilcoxon test).
VPA; Sodium Valproate, PER; Perampanel

Table 6. Effect of perampanel in various seizure models

	Seizure type	Animal model	Species	Perampanel (mg/kg)
Partial onset seizure	Psychomotor	6-Hz seizure	Mouse	2.2[a]
	Complex partial	Amygdala kindling	Rat	10[b,c]
				5[b,d]
Generalized seizure	Tonic-clonic	Maximal electroshock	Mouse	1.6[a]
	Myoclonic/absence	Pentylenetetrazole	Mouse	0.94[a]
	Absence	GAERS	Rat	>10[b]
	Reflex	Audiogenic	Mouse	0.47[a]

[a], ED_{50}; , [b], minimum effective dose; [c], seizure threshold; [d], seizure severity

Chapter IV:

Therapeutic treatment in Status epilepticus

Abstract

The efficacy of diazepam, and the alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor antagonists, perampanel and GYKI52466 was assessed in a lithium-pilocarpine status epilepticus (SE) model. Combination therapy of diazepam and perampenl was also evaluated. SE was induced in rats using lithium chloride, scopolamine methyl bromide, and pilocarpine. Diazepam 10, 20, or 40 mg/kg , or perampanel 1, 2.5, 5, or 8 mg/kg were administered intravenously at 10 or 30 min after seizure onset, and GYKI52466 50 mg/kg , or combinations of diazepam 2.5–5 mg/kg and perampanel 0.5–1 mg/kg, were administered intravenously at 30 min after seizure onset. Diazepam 20 mg/kg terminated seizures (based on electroencephalography and assessment of behavioral seizures) in 2/6 rats at 10 min and 0/6 rats at 30 min (ED_{50}: 10 min, 30 mg/kg; 30 min, not determined). Perampanel 8 mg/kg terminated seizures in 6/6 rats at both 10 and 30 min (ED_{50}: 10 min 1.7 mg/kg; 30 min, 5.1 mg/kg). GYKI52466 50 mg/kg terminated seizures in 2/4 rats at 30 min. Co-administration of diazepam 5 mg/kg and perampanel 1 mg/kg terminated seizures in 9/9 rats at 30 min. In conclusion, perampanel and GYKI52466 provided efficacy in a lithium-pilocarpine SE model at 30 min after seizure onset, when SE was refractory to diazepam, supporting the therapeutic potential of AMPA receptor antagonists for refractory SE. The dose of perampanel and diazepam required to terminate seizures was much reduced by combination therapy, suggesting synergy.

Introduction

Status epilepticus (SE) is a prolonged, self-sustained seizure that is associated with substantial mortality and morbidity (Hui et al., 2003; Shneker and Fountain, 2003; Chin et al., 2004). It is considered that SE is one of the most critical conditions among neurological emergency.

Treatment of SE is started with denzodiazepines. If efficacy is limited, additional AEDs is used for the termination of seizure. However, SE is often refractory to treatment, increasing the risk of poor outcomes (Rossetti et al., 2005; Novy et al., 2010; Hocker et al., 2013; Sutter et al., 2013). In a study of adults with SE, 22.6% of cases did not respond to first- or second-line treatments, and these cases were associated with higher mortality rates than non-refractory SE (39% versus 11%) (Novy et al., 2010). Refractory SE (RSE) can necessitate referral to an intensive care unit and treatment with anesthetizing AEDs, such as midazolam, propofol, or barbiturates, to help prevent severe acute systemic and long-term neuronal consequences (Meierkord et al., 2006; Meierkord et al., 2010; Brophy et al., 2012).

SE is thought to be a consequence of dysfunction in the neuronal machinery required for the termination of seizures: specifically, a loss of inhibitory γ-amino butyric acid (GABA) neuronal activity coupled to sustained glutamate-mediated excitatory activity (Naylor et al., 2005; Chen and Wasterlain,, 2006; Naylor, 2010; Deeb et al., 2012). In the lithium-pilocarpine rat model of SE there is a functional loss of post-synaptic GABAA receptors approximately 1 h after seizure onset and this is associated with internalization of the receptor to the cytoplasm (Naylor et al., 2005). Loss of GABA-mediated inhibitory activity may also be a result of changes in chloride homeostasis, as the inhibitory effects of $GABA_A$ receptors are mediated by chloride flux (Chen and Wasterlain, 2006; Deeb et al.,

2012). Anesthetizing AEDs that enhance GABA activity are currently used to treat RSE (Meierkord et al., 2006; Meierkord et al., 2010), but novel approaches targeting non-GABAergic mechanisms may help to improve treatment outcomes.

In animal studies, AMPA antagonist was shown efficacy of seizure termination in refractory condition to benzodiazepine (Pitkanen et al., 2007; Fritsch et al., 2010; Langer et al., 2011; Rajasekaran et al., 2012) .

In a study of AMPA receptor-mediated neurotransmission in a lithium-pilocarpine rat model of SE, RSE was associated with a selective reduction in surface expression of the GluA2 subunit of the AMPA receptor on hippocampal membranes and an increase in GluA2 subunit internalization rates (Rajasekaran et al., 2012). This resulted in calcium-permeable GluA2-lacking AMPA receptors with distinct biophysical characteristics, and continued neurotransmission. This synaptic plasticity may be an important pathophysiologic change in SE leading to subsequent neurodegeneration and increased mortality and morbidity. Therefore, inhibition of AMPA receptor activity may have potential as a therapeutic approach for the treatment of RSE.

These suggested that perampanel could be an option to terminate status epilepticus. Here, efficacy of perampanel in Li-pilocarpine model of status epilepticus which is animal model of benzodiazepine refractory SE. In addition, pharmacodynamic interaction with diazepam was examined

Materials and Methods

Animals.

Male Sprague Dawley rats (Charles River Laboratories, Kanagawa, Japan) were used for seizure termination studies. All animals weighed weighing 240–400 g were housed in cages in a controlled environment (constant temperature 22 ± 1°C; humidity 50–60%; 12-h dark/light cycle [lights on 07:00 to 19:00 h]) with free access to food (MF diet; Oriental Yeast Co., Tokyo, Japan) and water. All experiments were approved by the Committee for the Welfare of Laboratory Animals of Eisai Co., Ltd.

Reagents.

Lithium chloride (Wako Pure Chemical Industries, Osaka, Japan), pilocarpine (Wako Pure Chemical Industries), and scopolamine methyl bromide (Sigma-Aldrich, Tokyo, Japan) were dissolved in 0.9% sodium chloride solution. Diazepam (Wako Pure Chemical Industries), perampanel (Eisai Co., Ltd, Kashima, Japan), and GYKI52466 (Sigma-RBI, St Louis, MO, USA) were prepared in 1:1:1 (v/v) distilled water, dimethyl sulfoxide, and polyethylene glycol 300.

Surgical procedures for implantation of electrodes.

Rats were acclimatized for at least 1 week prior to surgery. On the day of surgery, rats were anesthetized with pentobarbital 50 mg/kg (Somnopentyl injection; Kyoritsu Seiyaku, Tokyo, Japan) administered intraperitoneally (i.p.) and surface electroencephalography (EEG) electrodes (XR2C-2011-N; Omron, Kyoto, Japan) were positioned epidurally in the skull using stereotaxic surgery. One electrode was placed over the right somatosensory cortex (2.5 mm posterior from the bregma and 3.0 mm lateral to the midline) according to

the coordinates of Paxinos and Watson (2007), with another reference electrode placed over the right cerebellum. Electrodes were fixed to the skull with acrylic dental cement. After electrode implantation, rats were returned to their home cage and allowed to recover.

Induction of status epilepticus.

At least 1 week after implantation of EEG electrodes, and 16–24 h before pilocarpine treatment, rats were treated with lithium chloride 3 mEq/kg i.p. On the day of testing, rats were placed in acrylic boxes and baseline EEG was recorded (Lab Charts® 7 v7.2; AD Instruments, Sydney, Australia) for at least 10 min. Rats were then injected with scopolamine methyl bromide 5 mg/kg i.p. and pilocarpine 30 mg/kg i.p. The dose and timing of scopolamine methyl bromide injection was selected to confer inhibition of the peripheral side effects of pilocarpine (salivation, diarrhea, lacrimation), which was not achieved with the standard regimen of scopolamine methyl bromide 1 mg/kg, administered 30 min prior to pilocarpine. In addition, the selected regimen of scolpolamine methyl bromide appeared to reduce the potential for respiratory problems, which occasionally resulted in animal death, apparently due to the aspiration of saliva, when the standard regimen of scopolamine methyl bromide was used prior to the administration of high doses of perampanel or diazepam. The higher dose used here slowed seizure onset, but did not change the subsequent course of SE. Seizure onset was designated as the first spike train in EEG recording (not as the start of SE).

Drug treatment.

Diazepam, perampanel, and GYKI52466 were administered by bolus intravenous (i.v.) injection to the rat tail vein after seizure onset.

Diazepam has previously been shown to terminate or attenuate kainic acid-induced SE in rodents when administered 5 min to 2 h after seizure onset at doses of 20–25 mg/kg i.p. (Pitkanen et al., 2007; Fritsch et al., 2010); therefore a dose range of 10–40 mg/kg i.v. was selected for this study. Diazepam doses of 10, 20, or 40 mg/kg i.v. were administered to groups of six rats at 10 min after seizure onset. Doses of 20 or 40 mg/kg i.v. were also administered to six or seven rats, respectively, at 30 min after seizure onset.

In pilot experiments using the lithium-pilocarpine rat model of SE, perampanel 8 mg kg-1 consistently terminated seizures and was well tolerated; therefore, a maximum dose of 8 mg kg-1 i.v. was selected for this study. Perampanel 1, 2.5, 5, or 8 mg/kg i.v. was administered at 10 min after seizure onset to groups of six rats each. Perampanel doses of 2.5 or 5, 5 or 8 mg/kg i.v. were also administered at 30 min after seizure onset, and a dose of 8 mg/kg i.v. was administered at 30, 60, or 90 min, all to groups of six rats.

Assessment of seizure termination.

Seizures were considered terminated if EEG spike activity was abolished, EEGs were spike-free at 30 min after drug dosing, and there was a lack of behavioral seizures. Behavioral seizures were classified according to Racine (1972): stage 1 – immobility, eye closure, twitching of vibrissae, sniffing, facial clonus; stage 2 – head nodding associated with more severe facial clonus; stage 3 – clonus of one forelimb; stage 4 – rearing, often accompanied by bilateral forelimb clonus; stage 5 – all of the above plus loss of balance and falling, accompanied by generalized clonic seizures.

Statistical analyses.

Doses required to terminate seizures in 50% of animals (ED_{50}) values and their statistical comparisons were calculated by computer probit analysis using SAS version 9.3

(SAS Institute, Tokyo, Japan).

Results

Lithium-pilocarpine-induced status epilepticus.

Approximately 15–30 min after pilocarpine administration, rats exhibited a train of spikes in EEG recordings that grew progressively larger (seizure onset; Fig. 16). Continuous EEG spikes and behavioral generalized seizures developed within 10 min of seizure onset, indicating establishment of SE, and were maintained for at least 180 min. Administration of vehicle (i.v.) did not affect the continuous EEG spikes.

Diazepam monotherapy.

When administered 10 min after seizure onset, diazepam terminated seizures at an ED_{50} of 30 mg/kg (95% confidence interval [CI], 17–130 mg kg-1; Table 1). A dose of 20 mg/kg i.v. terminated EEG seizures in three two of six rats when administered at 10 min (Fig. 17A), and also conferred strong muscle relaxation, such that no visible behavioral seizures persisted. However, when administered 30 min after seizure onset, diazepam 20 mg/kg i.v. failed to terminate EEG seizures, and a higher dose of 40 mg/kg was only effective in one of seven rats (Table 7, Fig. 17B).

Perampanel monotherapy.

Perampanel terminated seizures at an ED_{50} of 1.7 mg/kg (95% CI 0.3–3.8 mg/kg) when administered 10 min after seizure onset, and 5.1 mg/ kg (95% CI 4.9–5.2 mg/kg) when administered at 30 min (lack of overlap of 95% CI values indicates a significant reduction in efficacy between 10 and 30 min; Table 8). In contrast to diazepam, perampanel 8 mg /kg i.v. immediately terminated seizures in six of six rats whether administered at 10 or 30 min (Fig. 18).

Combination therapy.

Diazepam 20 mg/kg, perampanel 8 mg/kg, and GYKI52466 50 mg/kg caused strong CNS depressant effects (immobility, loss of righting reflex) during observation in all rats, with higher doses of diazepam and perampanel compromising respiration in some cases. Therefore, in an attempt to reduce CNS inhibition, the combination of lower doses was explored.

When diazepam 5 mg/kg i.v. was administered in combination with perampanel 1 mg/kg i.v. at 30 min after seizure onset, seizures were terminated in all rats (n = 9, Fig 19, Table 9). At lower doses, seizures were terminated in four of six rats (diazepam 2.5 mg/kg, perampanel 1 mg/kg) and two of six rats (diazepam 5 mg/kg, perampanel 0.5 mg/ kg) (Table 9).

Discussion and Conclusion

In this lithium-pilocarpine rat model, continuous EEG spikes, indicative of the development of SE, were observed within 10 min of seizure onset. Diazepam i.v. terminated seizures at an ED_{50} of 30 mg/kg when administered 10 min after seizure onset. However, when administered at 30 min after seizure onset, no animals responded to diazepam 20 mg/kg, and only one of seven animals responded to diazepam 40 mg/kg, suggesting that refractoriness to benzodiazepine had started to develop by this time point. It is well recognized that pharmacoresistance to benzodiazepines develops rapidly in the lithium-pilocarpine rat model, and the current results appear to be in accordance with this. For example, diazepam 20 mg/kg was able to stop SE in the lithium-pilocarpine rat model when administered at the early stage of discrete electrographic seizures, but became less effective when administered at later stages of SE (Walton and Treiman, 1998). Similarly, it has been reported that diazepam i.p., at doses of up to 20 mg/kg, provided some efficacy at 10 minutes after the development of a stage 3 seizure in the lithium-pilocarpine rat model, but not at later time points (Jones et al., 2002).

In contrast, perampanel i.v. terminated seizures at an ED_{50} of 1.7 mg/kg when administered 10 min after seizure onset, and continued to provide efficacy at 30 min with an ED50 of 5.1 mg/kg. A dose of perampanel 8 mg/kg terminated seizures in all animals at 30 min after seizure onset. These data are in accordance with previous studies of AMPA receptor antagonists in animal models of SE other than pilocarpine model (Pitkanen et al., 2007; Fritsch et al., 2010; Langer et al., 2011; Rajasekaran et al., 2012) and support the potential efficacy of agents with this mechanism of action in benzodiazepine-refractory SE.

Previous research using animals models of SE has indicated that repeated i.p. injections of GYKI52466 are required to terminate seizures (Fritsch et al., 2010), but a

single i.v. injection was sufficient to cease seizures in this study. This discrepancy may reflect the different administration routes, since i.v. administration may be expected to increase plasma concentrations more rapidly, and to a greater extent, than i.p. administration.

SE has been associated with internalization of $GABA_A$ receptors to the cytoplasm at just 1 h after seizure onset (Naylor et al., 2005). In accordance with this, evidence from animal models also suggests that plastic changes in GABAA receptor function occur rapidly during the development of SE (Feng et al., 2008). Such functional changes have previously been associated with decreases in the sensitivity of SE to benzodiazepines over time in the lithium-pilocarpine rat model (Walton and Treiman, 1988; Kapur and Macdonald, 1997; Feng et al., 2008), as was observed with diazepam at the 30 min time point in the present study. In contrast, while expression of the AMPA receptor subunit GluA2 has been found to be reduced in SE, similar receptor function may be provided by GluA2-lacking AMPA receptors (Rajasekaran et al., 2012). This is consistent with our findings that AMPA receptor antagonists continue to provide efficacy for the treatment of SE at 30 min and beyond.

However, the efficacy of perampanel appeared to wane over time, as the ED_{50} value for the termination of seizures was greater when perampanel was administered 30 min after seizure onset than when it was administered at 10 min. Given these timings, and the potential interactions of the GABA and AMPA systems indicated by the synergistic effects of co-administering perampanel and diazepam, such a decline in efficacy may be associated with the early changes in GABAA receptor function.

Diazepam 20 mg/kg, perampanel 8 mg/kg, and GYKI52466 50 mg/kg caused strong CNS depressant effects (immobility, loss of righting reflex) in all rats. Although such effects may be justified by the termination of SE, it is important to explore approaches to

improve tolerability while maintaining efficacy. High doses of AEDs have been associated with substantial toxicity in animal models (Morimoto et al., 1997), and we therefore hypothesized that administration of lower doses of perampanel might be useful in optimizing safety outcomes. Combination therapy was explored as an option to reduce dosing because synergistic effects have previously been reported with AEDs in the lithium-pilocarpine model, including diazepam in combination with N-methyl-D-aspartate receptor antagonists (Rice and DeLorenzo, 1999; Martin and Kapur, 2008). We report that seizures were consistently terminated in the lithium-pilocarpine rat model by the co-administration of low doses of diazepam (5 mg/kg) and perampanel (1 mg/kg) at 30 min after seizure onset, similar to the efficacy observed at this time point with perampanel 8 mg/kg alone. Although CNS depressant effects were observed with both approaches, recovery of righting reflex occurred more quickly with the low-dose combination therapy. These results indicate synergistic effects that may reduce the required therapeutic dose of perampanel in the presence of diazepam, conferring improved safety outcomes. Similarly, a subclinical dose of the AMPA antagonist LY-300164 has been shown to significantly inhibit seizures in amygdala-kindled seizure models when combined with low-dose benzodiazepines, but the combination did not cause the motor impairment or memory deficits observed with higher-dose benzodiazepine monotherapy (Borowicz et al., 1999; Borowicz et al., 2000).

Oral perampanel has been found to reduce the incidence of 6-Hz electroshock-induced seizures in mice at doses of 1–8 mg/kg, with lower doses required to achieve similar efficacy when co-administered with carbamazepine, phenytoin, or valproate. However, in our lithium-pilocarpine SE model, there were no such interactions between perampanel 2 mg/kg and phenytoin 50 mg/kg (n = 4), and only a weak interaction between perampanel 2 mg/kg and valproate 300 mg/kg (seizures terminated in 2/6 rats; data not shown).

Therefore, synergistic effects may depend on the seizure type and condition, and the interactions between perampanel and diazepam observed in the present study may be specific to benzodiazepine-resistant SE.

As yet, there are no established therapies for RSE that directly attenuate neuronal excitation through the inhibition of glutamate receptors, with current guidelines recommending the use of anesthetizing AEDs that enhance GABA activity (Meierkord et al., 2006; Meierkord et al., 2010). However, studies using the lithium-pilocarpine rat model support the further investigation to elucidate a role of AMPA receptor antagonists in the treatment of RSE.

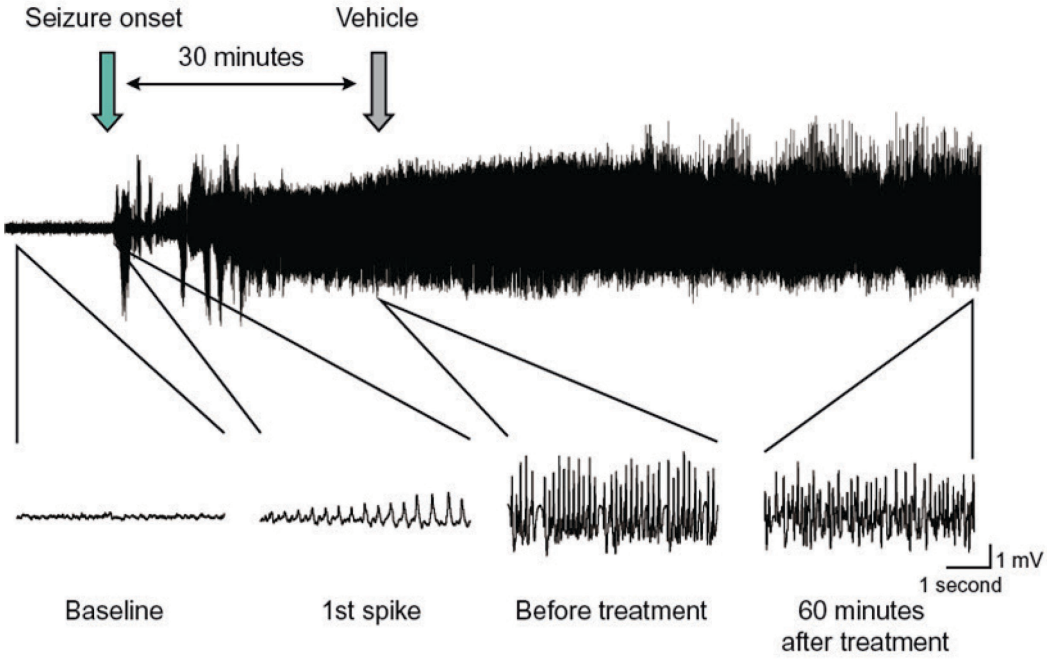


Figure 16. Representative electroencephalogram (EEG) of Lithium - Pilocarpine model of status epilepticus and outline of drug treatment.
First spike train was designated as onset of seizure. Drugs was administered intravenously at designated timing after seizure onset. This EEG trace was from the rat treated with vehicle. No major effect on EEG was observed. Seizure activity was persisted during experiment.

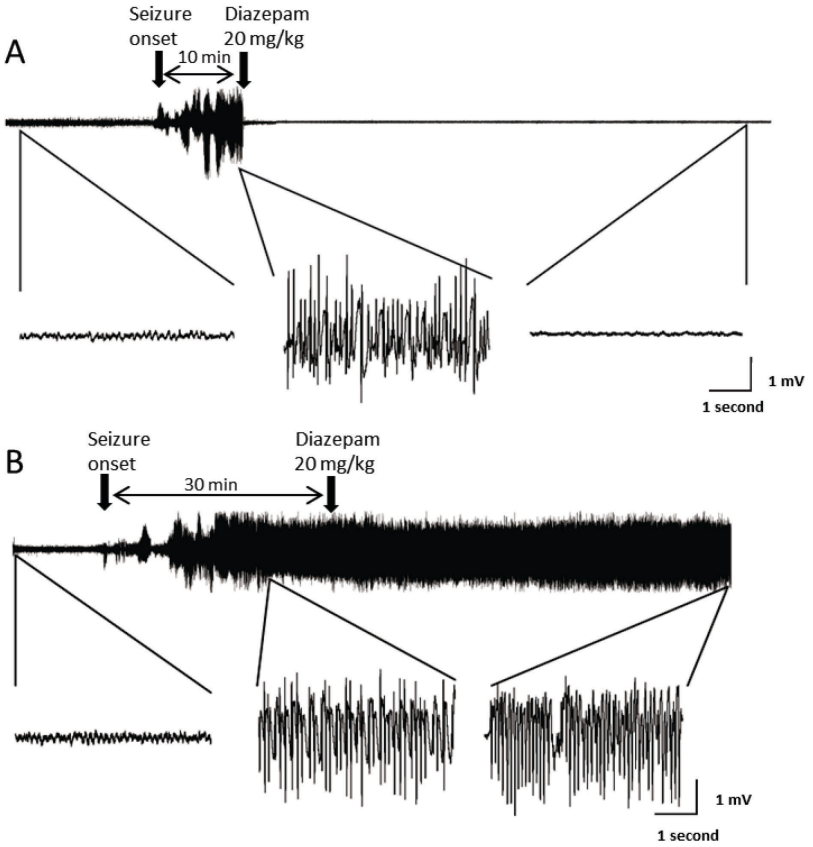


Figure 17. Efficacy of diazepam on EEG seizure in Li-Pilocarpine status epilepticus. A. Representative EEG trace from the rat treated with diazepam 10 minutes after seizure onset. Diazepam terminated EEG seizure soon after treatment in some rats. B. Diazepam treatment at 30 minuets after seizure onset did not terminate EEG seizure in all tested rats.

Table 7. Number of treatment responder after diazepam treatment.

Diazepam (mg/kg, iv)	Responder/Total	
	10 min after seizure onset	30 min after seizure onset
10	0/6	
20	3/6	
40	4/6	1/7
ED_{50}	26	ND
(95 % CI)	(14-96)	

CI, confidence interval; ED_{50}, dose required to terminate seizures in 50% of animals; iv, intravenous; ND, not determined.

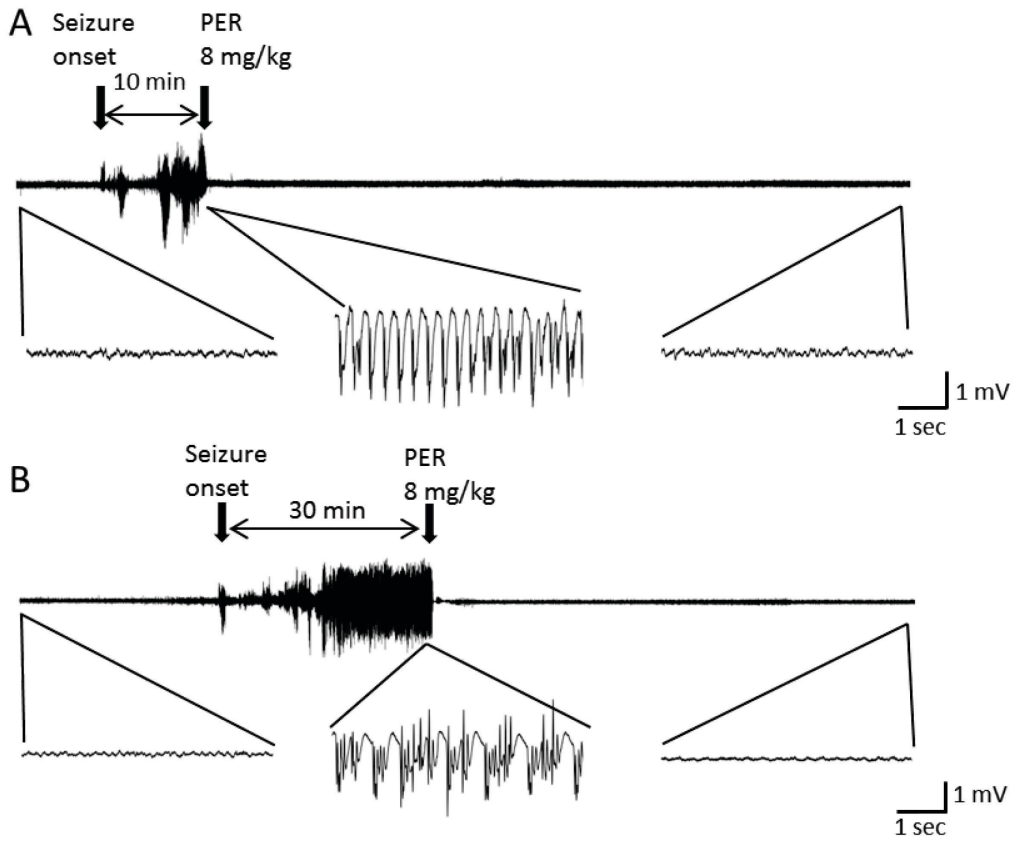


Figure 18. Efficacy of perampanel on EEG seizure in Li-Pilocarpine-induced status epilepticus.
Perampanel at 8 mg/kg successfully terminated EEG seizure at 10 minutes (A) and 30 minutes (B) after seizure onset.
PER, perampanel

Table 8. Number of treatment responder after perampanel treatment

Perampanel (mg/kg, iv)	Responder/Total	
	10min after seizure onset	30min after seizure onset
1	1/6	
2.5	5/6	0/6
5	5/6	2/6
8	6/6	6/6
ED_{50}	1.7	5.1
95% CI	0.3-3.8	4.9-5.2

CI, confidence interval; ED_{50}, dose required to terminate seizures in 50% of animals; iv, intravenous.

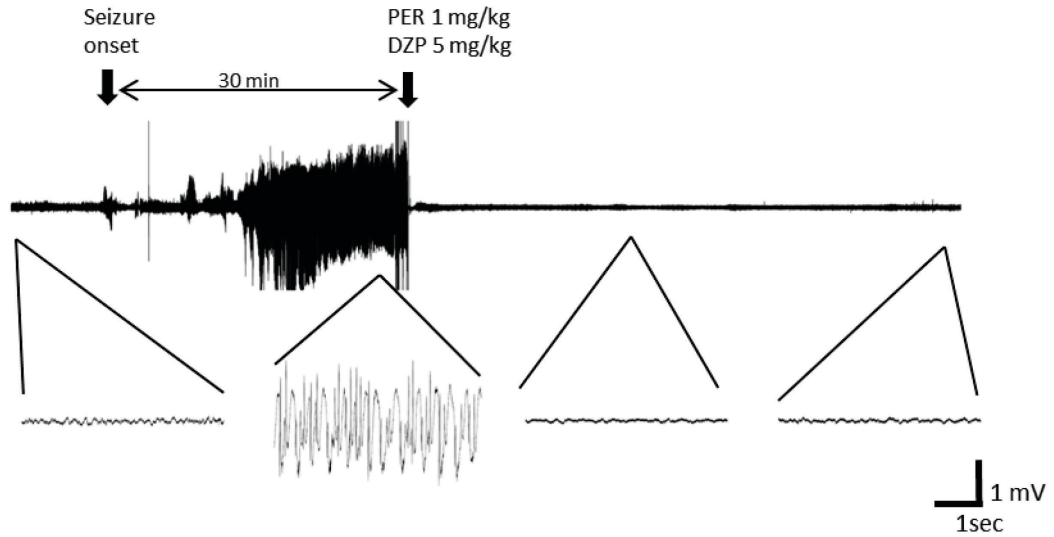


Figure 19. Representative trace of EEG seizure in Li-pilocarpine –induced seizures. Perampanel and diazepam was co-administered at 30 minutes after seizure onset.
PER, perampnael; DZP, diazepam

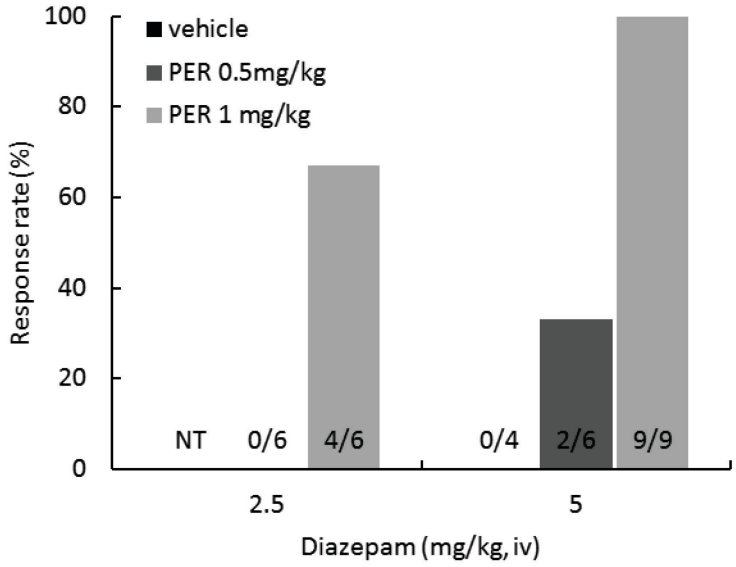


Figure 9. Effect of combined treatment with perampanel and diazepam on lithium-pilocarpine-induced seizures when administered intravenously 30 min after seizure onset.
PER, perampnael

Chapter V:

Conclusion

Perampanel is the first market available AMPA antagonist claimed for the treatment of partial onset seizure adjunctive to other AEDs. Problem of AMPA antagonist in drug discovery and development was narrow therapeutic window as an extension of pharmacological effect in addition to chemical structure related drawbacks. From the middle of 1980's, pharmaceutical companies had been tried to discover new AMPA antagonist for the treatment of various neurological diseases. However the most of discovered chemical structure had problems as CNS drug. Problem of competitive antagonist was poor blood brain barrier permeability resulting very high peripheral concentration connecting to peripheral safety issues. Prototype non-competitive antagonist showed favorable blood brain barrier permeability but short half life time in living body emphasized pharmacology related side effects at therapeutic doses. New chemical structure with favorable property as CNS drug was required to maximize the potential of target mode of action. We found new chemical template by high through put screening. It was a compound with small molecular weight therefore we believed that it had a large room to modify chemical structure to improve activity and property as drug. Medicinal chemistry effort identified 1,3,5-triaryl-1H-pyridin-2-one structure which showed potent AMPA antagonist property and good chemical tractability. It led discovery of perampanel (2-(2-oxo-1-phenyl-5-pyridin-2-yl-1,2-dihydropyridin-3-yl)benzonitrile). Perampanel showed strong *in vitro* activity against AMPA receptor and good AMPA antagonistic effect in animal. Furthermore, perampanel showed favorable metabolic stability in assay with human liver microsome.

AEDs are generally utilized for the prevention of seizure attack. Pharmacology studies

in seizure models indicated that perampanel have broader antiseizure profile than most of other AEDs. It is favorable to reduce the risk of paradoxical exacerbation of seizure in epilepsy patients. It is also demonstrated that perampanel could protect the animal from the severer seizure. These characters seemed favorable to treat the patients with refractory epilepsy. However, therapeutic window between antiseizure effect and motor impairment indicated narrow therapeutic margin similar to known AMPA antagonists. While, metabolic stability in human liver microsome assay and pharmacokinetic parameters in animal suggested that perampanel could have very long half-life time in human. It was considered beneficial for development of tolerance in human like other CNS drugs. We considered that this could balance issues between tolerability and efficacy. This idea was proven through clinical study in epilepsy and approved as an AED in many countries.

Perampanel also showed seizure terminative effect in animal model of refractory SE. It is quite similar property to other AMPA antagonists. There is an idea to treat refractory SE with drug combination therapy to improve outcome. It was recognized that combination therapy with benzodiazepine and ketamine could show pharmacodynamic interaction (Rice and DeLorenzo, 1999; Martin and Kapur, 2008) however combination of benzodiazepine and AMPA antagonist had not been demonstrated yet. Combination therapy of perampanel with diazepam exerted very prominent seizure terminative effect than that of monotherapy. Combination of perampanel and diazepam could be considered a reliable treatment for seizure termination in drug refractory SE. Further evaluation of this possibility is warranted.

Chapter VI:

Acknowledgement

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Chapter VII:

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