การประเมินฤทธิ์ต้านชักของสาร
เอน-(พารา-อะมิโนเบนโซอิล)-1, 2, 3, 4-เตตระไฮโดรควิโนลีน

นางสาวนุชจรีย์ ภู่ทับทิม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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EVALUATION OF ANTICONVULSANT EFFECT OF N- (*p*-AMINOBENZOYL)-1,2,3,4-TETRAHYDROQUINOLINE

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การวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ต้านการชักของสารเอน-(พารา-อะมิโนเบนโซอิล)-1,2,3,4-เตตระไฮโดรควิโนลีน ซึ่งเป็นอนุพันธ์ตัวใหม่ของอะเมลโตไลด์ที่สังเคราะห์ขึ้น ในด้านประสิทธิภาพ ความเป็นพิษเฉียบพลัน และพิษต่อระบบประสาทส่วนกลาง นอกจากนี้ยังได้ทำการศึกษาผลของสารทดสอบต่อตัวรับ NMDA ที่ถูกเหนี่ยวนำให้แสดงออกบนผิวของไข่กบ *Xenopus laevis* โดยการฉีด mRNA

จากการศึกษาฤทธิ์ต้านการชักของสารทดสอบทั้งสอง พบว่าเอน-(พารา-อะมิโนเบนโซอิล)-1,2,3,4-เตตระไฮโดรควิโนลีนมีฤทธิ์ต้านการชักในหนูถีบจักรที่ถูกเหนี่ยวนำให้ชักทั้งจากการกระตุ้นสมองด้วยกระแสไฟฟ้าและสารเคมีเพนทิลีนเตตระซอลได้ ในขณะที่อะเมลโตไลด์มีฤทธิ์ต้านการชักในหนูถีบจักรที่ถูกเหนี่ยวนำให้ชักโดยการกระตุ้นด้วยกระแสไฟฟ้าเท่านั้น เอน-(พารา-อะมิโนเบนโซอิล)-1,2,3,4-เตตระไฮโดรควิโนลีนมีประสิทธิภาพต้านการชักน้อยกว่าอะเมลโตไลด์ โดยขนาดของสารทดสอบทั้งสองที่สามารถต้านการชักในหนูถีบจักรได้จำนวนครึ่งหนึ่ง (ED_{50}) เท่ากับ 13.33 และ 0.96 มิลลิกรัม/กิโลกรัม (มก/กก) น้ำหนักตัว ตามลำดับ สารทดสอบทั้งสองสามารถต้านการชักจากการกระตุ้นสมองด้วยกระแสไฟฟ้าได้นานถึง 9 ชั่วโมงหลังจากให้สารทดสอบ อย่างไรก็ตาม ค่า ED_{50} ก็เพิ่มขึ้นด้วย ในด้านความปลอดภัย เมื่อดูจากค่าขอบเขตความปลอดภัยสัมพัทธ์ (Relative safety margin, LD_{50}/ED_{50}) ของเอน-(พารา-อะมิโนเบนโซอิล)-1,2,3,4-เตตระไฮโดรควิโนลีนและอะเมลโตไลด์ซึ่งเท่ากับ 44.97 และ 65.28 ตามลำดับ แสดงให้เห็นว่าอะเมลโตไลด์มีความปลอดภัยมากกว่า ในการทดสอบความเป็นพิษต่อระบบประสาทส่วนกลางโดยวิธี Rotorod test พบว่าขนาดของสารทดสอบที่ทำให้เกิดความเป็นพิษต่อระบบประสาทส่วนกลางในหนูถีบจักรจำนวนครึ่งหนึ่ง (TD_{50}) ของเอน-(พารา-อะมิโนเบนโซอิล)-1,2,3,4-เตตระไฮโดรควิโนลีนและอะเมลโตไลด์เท่ากับ 37.28 และ 7.18 มก/กก น้ำหนักตัวตามลำดับ เมื่อนำค่าดังกล่าวมาคำนวณหาค่าดัชนีปกป้อง (Protective index, $PI=TD_{50}/ED_{50}$) พบว่าอะเมลโตไลด์มีค่าดัชนีปกป้องสูงกว่าเอน-(พารา-อะมิโนเบนโซอิล)-1,2,3,4-เตตระไฮโดรควิโนลีน อย่างไรก็ตาม จากการศึกษาอัตราการเคลื่อนไหวและอัตราการเพิ่มระยะเวลาการหลับในหนูถีบจักรที่ได้รับบาร์บิทูเรต คาดว่าเอน-(พารา-อะมิโนเบนโซอิล)-1,2,3,4-เตตระไฮโดรควิโนลีนในขนาดที่มีผลการรักษาน่าจะมีผลกดระบบประสาทส่วนกลางน้อยกว่าอะเมลโตไลด์ เมื่อนำสารทั้งสองมาศึกษาผลต่อกระแสไฟฟ้าที่ถูกเหนี่ยวนำโดย NMDA ในไข่กบ *Xenopus laevis* พบว่า ทั้งเอน-(พารา-อะมิโนเบนโซอิล)-1,2,3,4-เตตระไฮโดรควิโนลีนและอะเมลโตไลด์ไม่เหนี่ยวนำให้เกิดกระแสไฟฟ้าหรือการเปลี่ยนแปลงค่าศักย์ไฟฟ้าใดๆของเยื่อหุ้มเซลล์ไข่กบ แต่สารทดสอบทั้งสองสามารถยับยั้งการเปลี่ยนแปลงทางไฟฟ้าโดยการเหนี่ยวนำจาก NMDA ได้อย่างมีนัยสำคัญทางสถิติ โดยค่าความเข้มข้นที่ทำให้เกิดการยับยั้งครึ่งหนึ่ง (IC_{50}) ของเอน-(พารา-อะมิโนเบนโซอิล)-1,2,3,4-เตตระไฮโดรควิโนลีนและอะเมลโตไลด์เท่ากับ 0.12 และ 0.10 ไมโครโมลาร์ ตามลำดับ ซึ่งผลในการยับยั้งต่อตัวรับ NMDA ดังกล่าวมานี้ แม้จะยังไม่ทราบถึงกลไกการออกฤทธิ์ที่ชัดเจน แต่ก็น่าจะเป็นปัจจัยที่ทำให้สารทั้งสองออกฤทธิ์ต้านชักได้ในสัตว์ทดลอง

จากการศึกษาในครั้งนี้อาจสรุปได้ว่า เอน-(พารา-อะมิโนเบนโซอิล)-1,2,3,4-เตตระไฮโดรควิโนลีนเป็นอนุพันธ์ของอะเมลโตไลด์ที่ออกฤทธิ์ต้านชักได้กว้างและมีขอบเขตความปลอดภัยสัมพัทธ์ต่ำกว่าอะเมลโตไลด์ แต่อย่างไรก็ตามในขนาดที่มีผลการรักษา เอน-(พารา-อะมิโนเบนโซอิล)-1,2,3,4-เตตระไฮโดรควิโนลีนน่าจะมีผลอันไม่พึงประสงค์ในการกดระบบประสาทส่วนกลางน้อย จากการศึกษาผลของสารนี้ต่อตัวรับ NMDA บนผิวไข่กบ แสดงให้เห็นว่าฤทธิ์ต้านชักในสัตว์ทดลองของสารทดสอบ ส่วนหนึ่งน่าจะเป็นผลสืบเนื่องจากการยับยั้งที่ตัวรับ NMDA การปรับปรุงโครงสร้างของเอน-(พารา-อะมิโนเบนโซอิล)-1,2,3,4-เตตระไฮโดรควิโนลีนเพื่อให้มีความปลอดภัยมากขึ้นโดยที่ยังมีคุณสมบัติต้านการชักที่กว้างอาจนำไปสู่การค้นพบอนุพันธ์ตัวใหม่ของอะเมลโตไลด์ซึ่งมีคุณสมบัติทางเภสัชวิทยาและพิษวิทยาที่น่าพึงพอใจ

ภาควิชา เภสัชวิทยา ลายมือชื่อนิสิต.....

สาขาวิชา เภสัชวิทยา ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา 2545 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4276570233: MAJOR PHARMACOLOGY
KEY WORDS : ANTICONVULSANT / N-(*p*-AMINOBENZOYL)-1,2,3,4-TETRAHYDROQUINOLINE / *XENOPUS LAEVIS* OOCYTES

NUCHJAREEYA BHUTHABTHIM: ANTICONVULSANT EFFECT OF N- (*p*-AMINOBENZOYL)-1,2,3,4-TETRAHYDROQUINOLINE. THESIS ADVISOR: ASSOC. PROF. MAYUREE TANTISIRA, Ph.D. THESIS CO-ADVISOR: ASST.PROF.PATHAMA LEEWANICH, Ph.D., and ASSOC.PROF.BOONYONG TANTISIRA, Ph.D. 76 pp. ISBN 974-17-2177-3.

The purposes of the present study were to investigate anticonvulsant effect of N-(*p*-aminobenzoyl)-1,2,3,4- tetrahydronquinoline (NTQ), a newly synthesized ameltolide analog, with regards to efficacy, acute toxicity, neurotoxicity and some other effects on the central nervous system (CNS). In addition, *Xenopus laevis* expressing system was used to examine the possible pharmacological properties of the test substances on the NMDA receptors expressed in X*enopus laevis* oocytes injected with mRNA.

While NTQ was effective in both Maximal Electroshock Seizure (MES) and Pentylenetetrazole (PTZ) test, ameltolide was exclusively effective in the MES but not PTZ test. When given by an intraperitoneal route in mice, NTQ was less potent than ameltolide, exhibiting the median effective dose (ED_{50}) of 13.33 mg/kg body weight (B.W.) whereas the corresponding value for ameltolide was 0.96 mg/kg B.W. Both compounds exhibited protection against MES at least 9 hours after dosing, however, with an increment of the ED_{50} values. In terms of safety, ameltolide seems to be safer than NTQ as indicated by the relative safety margin (LD_{50}/ED_{50}) of 65.28 for ameltolide and 44.97 for NTQ. The median neurotoxic dose (TD_{50}) established by Rotorod test, were 37.28 and 7.18 mg/kg B.W. for NTQ and ameltolide, respectively. Thus ameltolide seems to possess more favorable protective index ($PI=TD_{50}/ED_{50}$) than that of NTQ. However, based on the results of locomotor activity and potentiation of barbiturate sleeping time tests, NTQ is preferably expected to exert a minor degree of CNS depression in its effective dose. *Xenopus* oocyte with NMDA receptor composing of NR1a and NR2B subunits was used to probe the effect of NTQ and ameltolide on NMDA-induced current. While NTQ and ameltolide did not induce either current or a shift in membrane potential of oocyte, both of them significantly inhibited NMDA-induced current demonstrating IC_{50} of 0.10 and 0.12 μM, respectively. Therefore, it is likely that inhibition of excitation of NMDA receptor may, at least in part, accounted for anticonvulsant activity in animal models of both NTQ and ameltolide, though, precise mechanism of action remains unknown.

In conclusion, the present study identified NTQ as a broad spectrum anticonvulsant agent with lower safety margin and lower protective index than those exerted by ameltolide. However in its effective dose, NTQ is expected to produce less unwanted effect regarding CNS depression. Inhibition of excitation of NMDA receptor (NR1a/NR2B) may explain anticonvulsant effect exhibited by ameltolide and NTQ in animal models. Further structural modification of NTQ to improve its safety profile while preserving broad spectrum property may lead to a discovery of new ameltolide analogs with favorable pharmacological and toxicological properties.

Department	Department of Pharmacolgy	Student's signature……..
Field of study	Pharmacology	Advisor's signature…….
Academic year	2002	Co-advisor's signature…….

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LIST OF ABBREVIATIONS

α	=	Alpha
β	=	Beta
γ	=	Gamma
μ	=	Micro
μl	=	Microlitre
p	=	Para
%	=	Percent
Ca^{2+}	=	Calcium ion
Mg^{2+}	=	Magnesium ion
K^{+}	=	Potassium ion
Cl^{-}	=	Chloride ion
Na^{+}	=	Sodium ion
Zn^{2+}	=	Zinc ion
a.m.	=	ante meridian (before noon)
AMPA	=	Alpha-amino-3-hydroxy-5-methyl - isoxyzole- 4 -propionic acid
B.W.	=	Body weight
cDNA	=	Cloned deoxyribonucleic acid
CNS	=	Central nervous system
CSF	=	Cerebrospinal fluid
^{0}C	=	Degree celsius
DNA	=	Deoxyribonucleic acid
ED_{50}	=	Median effective dose
EEG	=	Electroencephalographic
e.g.	=	exampli gratia (for example)
et.al.	=	et alii (and other)
etc.	=	et cetera (and so on)
GABA	=	Gamma aminobutyric acid
GABA-T	=	Gamma aminobutyric acid transaminase
GAD	=	Glutamic acid decarboxylase
GluRs	=	Glutamate receptors
g	=	Gram

LIST OF ABBREVIATIONS (continued)

IC_{50}	=	Median inhibitory concentration
iGluRs	=	Ionotropic glutamate receptors
ILAE	=	International League Against Epilepsy
i.p.	=	Intraperitoneal
kg	=	Kilogram
L	=	Litre
LD_{50}	=	Median lethal dose
M	=	Molar
MΩ	=	Megaohm
MBS	=	Modified Barth's Solution
MES	=	Maximal Electroshock Seizures
mg	=	Milligram
mGluRs	=	Metabotropic glutamate receptors
min	=	Minute
ml	=	Millilitre
mm	=	Millimetre
mM	=	Millimolar
mV	=	Millivolt
nA	=	Nanoampare
ng	=	Nanogram
nl	=	Nanolitre
NMDA	=	N-methyl-D-aspartate
NSS	=	Normal saline solution
NTQ	=	N-(*p*-aminobenzoyl)-1,2,3,4,tetrahydro quinoline
PEG 400	=	Polyethylene glycol 400
PI	=	Protective index
p.m.	=	post meridian (afternoon)
PTZ	=	Pentylenetetrazole
rev/min	=	revolution per minute
sc.	=	Subcutaneous

LIST OF ABBREVIATIONS (continued)

S.E.M	=	Standard error of the mean
sec.	=	Second
TD_{50}	=	Median toxic dose
VNS	=	Vagus nerve stimulation
VDCCs	=	Voltage dependent calcium channels

CHAPTER I

INTRODUCTION

Epilepsy

Epilepsy is a common neurological disease encompassing a variety of disorders with paroxysms. It is a disorder of brain function characterized by recurrent episodes of neurological or behavioral manifestations, commonly termed seizures that are caused by the abnormally synchronous and excessive discharge of large populations of neurons. It is therefore a chronic neurological condition with permanent pathophysiological features (Avoli, 1997; Hirose et al., 2000).

Electrophysiologically, epilepsy may be defined as an intermittent derangement of the nervous system due presumably to a sudden, excessive, disorderly discharge of cerebral neurons. The discharge results in an almost instantaneous disturbance of sensation, loss of consciousness, impairment of psychic function, convulsive movements, or some combination thereof (Adams et al, 1997).

The epilepsies encompass diverse seizure disorder afflicting as many as 50 million people worldwide. Over two third of all epileptic seizures begin in childhood (mostly in the first year of life), and this is the age period when seizures assume the widest array of forms. The incidence become increasingly common after the age of 60 years and can have a significant impact on functional status. The goal of antiepileptic drug therapy is to control seizures but preserve quality of life. If possible, seizure control should be achieved with one agent given in the lowest effective dosage. Clinical response, rather than drug levels, should guide dosage changes. All antiepileptic drugs can cause dose-dependent sedation and cognitive impairment. Only 80 % of the patients have succeeded in controlling the symptom while 20 % failed to do so. Many forms of epilepsy are intractable to current therapies and there is a pressing need to develop agents

and strategies to not only suppress seizures, but also cure epilepsy (Porter, 1993; Adams et al., 1997; Jones, 2002; Selwa and Velez, 2003). Thus, there is a need for new, more effective anticonvulsant drugs for intractable epilepsy. Furthermore, because of the inadequacy of the currently available anticonvulsant armamentarium with the respect to safety, newly developed drugs should be less toxic than existing drugs (Loscher, 1998).

1. Epileptogenesis

1.1. Pathologic causes

The causes of seizures are many and include the full range of neurologic diseases, from infection to neoplasm and head injury. The latter are rare in early life. The frequency of seizures is not fully known (Adams et al., 1997 and Porter and Meldrum, 2001).

1.2. Genetic causes

In the genesis of the epilepsies, heredity plays an important role through variable role. The heredity element is most clearly discerned in two classic childhood types, absence attacks with 3-per-second spike-and-wave discharges and benign epilepsy of childhood with centrotemporal spikes, both of which are transmitted as incompletely penetrant autosomal dominant traits. Other epileptic syndromes of proven inherited type are benign neonatal familial convulsions (autosomal dominant) and the benign myoclonic epilepsy of childhood (autosomal recessive). Undoubtedly inherited is the tendency to develop simple febrile convulsions, although it is not clear whether the mode of inheritance is polygenic or autosomal dominant (Adams et al., 1997).

Genetic mapping studies of the familial epilepsies have suggested that benign neonatal convulsions be linked to a gene on chromosome 20 (Leppert et al, 1989) and juvenile myoclonic epilepsy to chromosome 6 (Delgado-Escueta et al, 1989).

1.3. Biochemical causes

The flow of ions through highly specialized protein aggregates of neuronal membranes has long been recognized to form the basis of nerve cell excitability. There are a bewildering number of ion channels that depending on their specificity for various ions (e.g., K^+, Na^+, Ca^{2+}, Cl^-), their gating by various ligands (e.g., glutamate, GABA, ACh, serotonin), or their second messengers (e.g., Ca^{2+}, cGMP) (Mody, 1998).

Activation of Na^+ channels underlies the generation of action potentials in central neurons and it is the repetitive firing of action potentials that is an essential condition of epileptic discharges (Stuart and Sakmann, 1995). The drug delays recovery from the inactivation of Na^+ channels, thus preventing high frequency discharges in neurons. The novel anticonvulsant drug, lamotrigine, appears to work in a similar fashion (Lang et al., 1993; Xie et al., 1995).

Voltage-dependent Ca^{2+} channels (VDCCs) are critical for nerve function. By coupling changes in the membrane potential to the influx of the pivotal "second messenger" Ca^{2+}, VDCCs represent the primary route for translating electrical signals into the biochemical events underlying key processes such as neurotransmitter release, cell excitability and gene expression. Originally, VDCCs were classified according to their biophysical and pharmacological characteristics into P/Q-, N-, L-, and T-type. T-type VDCC is believed to provide a pacemaker current in thalamic neurons that generate the rhythmic cortical discharge associated with absence seizures (petit mal). Ethosuximide, which blocks T-type current, is an effective therapy for seizures (Jones, 2002).

Various types of neuronal K^+ current, both voltage-and Ca^{2+} -dependent, are critically involved in dampening neuronal excitability. Their pharmacological antagonism has been used for many years to produce acute experimental models of

epilepsy both *in vivo* and *in vitro* (Schwartzcroin, 1993). However, the little we know about possible chronic alterations in the function of K^+ channels in the epileptic brain is not consistent with the reduction in K^+ channel function contribution to epileptogenesis (Mody, 1998).

Changes in excitatory or inhibitory receptors and inbalance between excitation and inhibition are the most important factors underlying synaptic mechanisms of epileptogenesis (Najm et al., 2001). Gamma-aminobutyric acid (GABA), the principle inhibitory neurotransmitter in the cerebral cortex, maintains the inhibitory tone that counterbalances neuronal excitation. When this balance is perturbed, seizures may ensue. Experimental and clinical study evidence indicates that GABA has an important role in the mechanism and treatment of epilesy (Treiman, 2001). Glutamate is the most common excitatory neurotransmitter in the brain. The α-amino-3-hydroxy-5-methyl-4-isoxazole (AMPA) and N-methyl-D-aspartate (NMDA) receptors are ionotropic subreceptors that directly control membrane channels and are important in the initiation and propagation of seizures. An abnormality of glutamate-mediated neurotransmission may critically contribute to the pathophysiology of seizures and epilepsy (Natsch et al., 1997 and Löscher, 1998).

2. Amino acid neurotransmitters

Several amino acids have gained recognition as major neurotransmitter candidates in the mammalian CNS. On the basis of neurophysiological studies, amino acids have been separated into two general classes: excitatory amino acids (glutamic acid, aspartic acid, cysteic acid, and hemocycteic acid), which depolarize neurons in the mammalian CNS; and inhibitory amino acids (GABA, glycine, taurine, and β-alanine), which hyperpolarize mammalian neurons (Cooper, 1996).

2.1. Inhibitory amino acid neurotransmitters

2.1.1. GABA

GABA is the central inhibitory neurotransmitter for about 40 % of all neurons. Neurotransmission can be influenced by GABA-mediated potentiation of inhibition, by an increase in GABA activity and by GABA substitution. GABA cannot cross the blood-brain and blood-CSF barriers, therefore direct substitution therapy is not possible. GABA is formed within GABAergic axon terminals and released into the synapse, where it acts at one of two types of receptor: $GABA_A$ which controls chloride entry into the cell, and $GABA_B$, which increases potassium conductance, decreases calcium entry, and inhibits the presynaptic release of other transmitters. GABA is a small amino acid derived from glutamate by glutamic acid decarboxylase (GAD) and rapidly removed by uptake into both glia and presynaptic nerve terminals and then catabolized by GABA-transaminase (GABA-T) (Natsch et al., 1997; Hevers and Luddens, 1998; and Treiman, 2001).

$GABA_A$ receptor

The bicuculline-sensitive γ-aminobutyric acid receptor, commonly referred to as the $GABA_A$ receptor, is a multi-subunit receptor-channel complex that can be allosterically modulated by two important classes of drugs, the benzodiazepines and the barbiturates. This receptor is a member of a large superfamily of ligand-gated ion channels that includes the nicotinic-cholinergic, ionotropic glutamate, and glycine receptors. Activation of the $GABA_A$ receptor by GABA agonists results in the opening of the chloride channel. The ensuing influx of chloride anions inhibits the firing of the neurons by causing hyperpolarization. Benzodiazepines increase the frequency of channel opening without appreciably altering the channel conductance or duration of opening. Barbiturates, in contrast, slightly decrease the opening frequency and prolong the duration of opening. The $GABA_A$ receptor can be functionally altered by a variety of compounds that bind to the receptor at several different sites, including the GABA site, the picrotoxin/barbiturate site, the benzodiazepine site, and the steroid site (Figure 1.) (Eugene and Barnes, 1996; and Cooper, 1996).

$GABA_B$ receptor

$GABA_B$ is a G-protein-coupled receptor that can open potassium channels or close calcium channels. $GABA_B$ receptor are presynaptic or postsynaptic. $GABA_B$ – receptor activation may result in different effects depending on their location. Activation of the $GABA_B$ receptor-linked potassium channels results in prolonged hyperpolarization and leads to postsynaptic inhibition. On the other hand, the long duration of the $GABA_B$ –mediated potentials may be responsible for some epileptic effects, as $GABA_B$ agonists, such as baclofen, are reported to exacerbate the spike-wave discharges in generalized epilepsies. Absence seizures are believed to be generated by the interaction between the T-type (transient) calcium current (T-current), the $GABA_B$ induced hyperpolarization, and the intrinsic bursting activities of the relay cells (Najm et al., 2001).

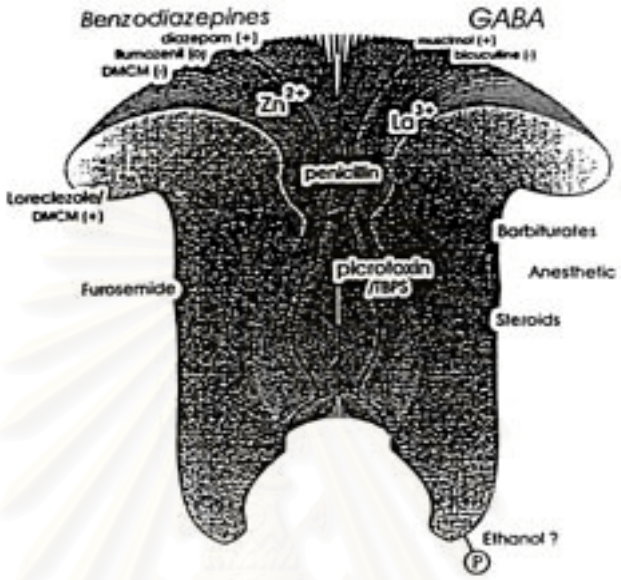

Figure 1. The $GABA_A$ receptor-channel complex as drug target: Aside from GABA and its agonists, e.g. muscimol, or antagonists, e.g. bicuculline, various drugs exert their actions by modulating the $GABA_A$ receptor. These include the clinically relevant benzodiazepines, which on a functional basis can be subdivided into positive modulators like the agonist diazepam and negative modulators (inverse agonist) like the β-carboline DMCM. Further binding sites have been identified for loreclezole, furosemide, and picrotoxin. Sedative barbiturates and stroids as well as various anesthetics also work via the $GABA_A$ receptor. Binding sites for the polycationic ions Zn^{2+} and La^{3+} exist and penicillin at high concentration supposedly is an open channel blocker. In this artistic view, the localization of the drug binding site though not totally arbitrary, do not suggest specific, proven localization of these sites (Hevers and Luddens, 1998).

2.1.2. Glycine

Glycine accomplishes several function as a transmitter in the central nervous system (CNS). As an inhibitory neurotransmitter, it participates in the processing of motor and sensory information that permits movement, vision, and audition. This action of glycine is mediated by the strychnine-sensitive glycine receptor, whose activation produces inhibitory post-synaptic potentials. In some areas of the CNS, glycine seems to be co-released with GABA, the main inhibitory amino acid neurotransmitter. In addition, glycine modulates excitatory neurotransmission by potentiating the action of glutamate at N-methyl-D-aspartate (NMDA) receptors. It is believed that the termination of the different synaptic actions of glycine is produced by rapid re-uptake through two sodium-and-chloride-coupled transporters, GLYT1 and GLYT2, located in the plasma membrane of glial cells or pre-synaptic terminals, respectively (Lopez et al., 2001).

2.2. Excitatory amino acid neurotransmitters

Glutamate receptor (GluRs) mediate most of the excitatory neurotransmission in the mammalian central nervous system (CNS). In addition, they are involved in plastic changes in synaptic transmission as well as excitotoxic neuronal cell death that occurs in a variety of acute and chronic neurological disorder. The GluRs are divided into two distinct groups, ionotropic and metabotropic receptors. The ionotropic receptors (iGluRs) are further subdivided into three groups: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate and N-methyl-D-aspartate (NMDA) receptor channels. The metabotropic receptors (mGluRs) are coupled to GTP-binding proteins (G-proteins), and regulate the production of intracellular messengers. These receptors all incorporate ion channels that are permeable to cation, although the relative permeability to Na^+ and Ca^{2+} varies according to the family and the subunit composition of the receptors (Ozawa et al., 1998 and Meldrum, 2000).

Ionotropic glutamate receptors (iGluRs)

Traditionally, iGluRs have been divided into three major subtypes, AMPA, kainate and NMDA receptors, on the basis of agonist specificities. However, since neither agonist nor antagonist clearly distinguished between AMPA and kainate receptors, they were often collectively referred to as non-NMDA receptors. Cloning studies have demonstrated that they are distinct receptor complexes although they can be activated by the same agonists, notably AMPA receptors are activated by kainate and kainate receptors with certain subunit compositions by AMPA. Excitatory transmission involves actions mediated by one or more combinations of these receptors. AMPA receptor channels are responsible for fast excitatory neurotransmission by the sodium-potassium channel and coexist with NMDA receptors in all synapses. However, the opening of the AMPA channels releases the magnesium block present in the resting stage, and NMDA channels open to Ca^{2+} entry and provide the prolonged phase of the excitatory neurotransmission (Ozawa et al., 1998 and Najm et al., 2001).

The NMDA receptor is a ligand-gated ion channel composed of two different protein subunits named NR1 and NR2. There are four NR2 gene products (NR2A-D) and single NR1 gene product that can be expressed in eight different splice variants that arise from different combinations of a single 5-prime terminal exon insertion or two 3-prime exon deletions. The NR1 subunits form a functional multimeric channel and show all the characteristic properties of an NMDA receptor. In contrast to NR1, members of the NR2 family expressed by themselves or in combination with other NR2 members, never yield functional channels to NMDA or glutamate and, therefore, are electrically silent. However, the combined expression of individual NR2 subunits with NR1 markedly potentiates channel opening and current responses to NMDA or glutamate. NR1 subunits serve as the general component of NMDA receptors and are essential for receptor function and NR2 subunits potentiate the channel activities to yield an increased ionic current (Figure 2.) (Monyer et al., 1992). A distinctive feature of the NMDA receptor is its voltage-sensitive block by Mg^{2+}. This is operative under normal circumstances but is overcome by partial depolarization of the resting membrane

potential. A further specific feature is the need for glycine as a coagonist. Each receptor unit appears to have two glycine and two glutamate binding sites (Laube et al., 1998)

Metabotropic glutamate receptors (mGluRs)

Metabotropic glutamate receptors (mGluRs) are second-messenger-coupled receptors that have diverse effects on the cellular and synaptic properties of nerve cells. Recent evidence suggests that mGluRs may play a role in the production of neuronal plasticity and epilepsy (Cartmell, et al., 1993; Merlin, et al., 1995; and Taylor, et al., 1995). The mGluRs include at least eight receptor subtypes. These subtypes are grouped into three subclasses on the basis of similarities in amino acid sequences, signal transduction mechanisms, and agonist selectivities (Nakanishi, 1994). Group 1 comprises mGluR1 and mGluR5, which are linked via G-proteins to activation of phospholipase C, producing diacylglycerol and inositol triphosphate as second messengers. Group II (mGluR2 and mGluR3) and Group III (mGluR4, mGluR6, mGluR7, mGluR8) are both negatively linked to adenylyl cyclase activation (Chapman, 2000; and Meldrum, 2000). Until recently, the metabotropic ligands available have not had sufficient bioavailability, potency or selectivity to permit a confident assignment of physiologic and pharmacologic properties to the individual metabotropic receptors (Chapman, 2000).

Activation of Group I mGluR enhances neuronal excitability by several mechanisms (blockade of accommodation to a steady current, potentiation of the effects of NMDA and AMPA and depolarization); accordingly, agonists acting on Group I receptors (e.g. 3,5-dihydroxyphenylglycine) have convulsant activity (Ghauri et al., 1996; and Tizzano et al., 1995). Furthermore, Group I antagonists, both those selective for mGluR1 (e.g. AIDA and LY 367385) and for mGluR5 (e.g. MPEP and SIB 1893) have anticonvulsant activity in several experimental seizure model (Thomsen et al., 1994; Chapman, et al., 1999 and 2000).

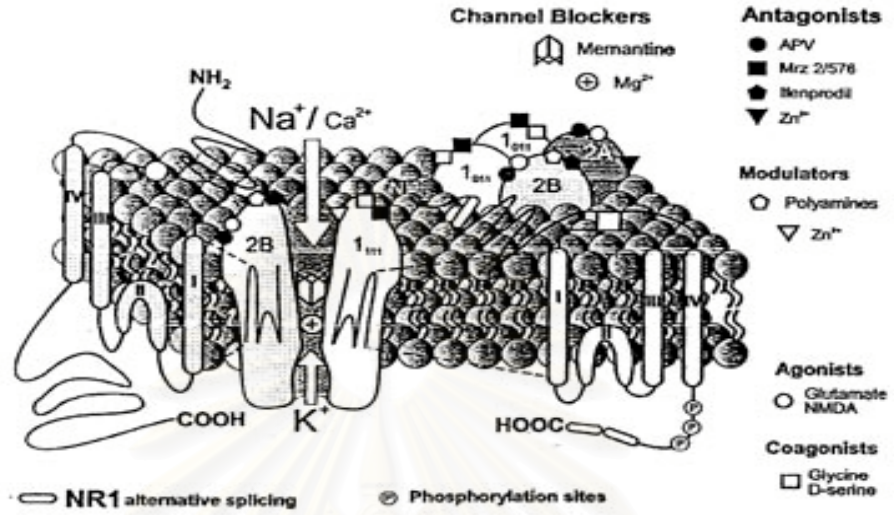

Figure 2. Model showing that NMDA receptors are probably heteromeric assemblies of four subunits. Each subunit has four hydrophobic regions. Functional NMDA receptor complexes are formed by combinations of NR1 and NR2 subunits, which contain the glycine and glutamate recognition sites, respectively. Alternative splicing at three axons, one in the amino-terminal domain (N1) and two in the carboxyl-terminal domain (C1 and C2), generates eight isoforms for the NR1 subunits, NR2A to NR2D. Competitive antagonists such as APV probably bind to one site, which may be distinct from the agonist recognition sites but isosterically coupled in such a way as to allow competitive interactions. Glycine is a coagonist at the glycine$_B$ site and prevent Ca^{2+}-independent receptor desensitization. MRZ 2/576 is an example of a glycine$_B$ antagonist. Polyamines such as spermine and spermidine are positive modulators but also block the channel at higher concentrations. All heteromeric and homomeric NMDA receptor subtype complexes are permeant to Ca^{2+}, Na^+, and K^+. The open NMDA channel is blocked by Mg^{2+} and uncompetitive NMDA channel receptor antagonists, such as memantine and (+) MK-801, in a voltage-dependent manner, although the speed and voltage dependence of this effect depend on the antagonist affinity and the subunit compostion. Ifenprodil is a selective antagonist for NR2B-containing receptors. Zn^{2+} is a potent voltage-independent antagonist at NR2A-containing receptors. In addition, most NMDA receptors are influenced by Zn^{2+} ions in a voltage-dependent manner, as well as by oxidation/reduction and pH (Danysz and Parsons, 1998).

3. Classification of epilepsy

Epilepsy has been classified in several ways: according to their supposed etiology and site of origin, on the basis of their clinical form (generalized or focal), frequency (isolated, cyclic, prolonged, or repetitive), or electrophysiologic correlates.

The classification was first proposed by Gastaut in 1970 and was then refined repeatedly by the Commission on Classification and Terminology of the International League against Epilepsy (1981). This classification based mainly on the clinical form of seizures and its electroencephalographic (EEG) features has been adopted worldwide and is generally referred to as the International Classification of Epileptic Seizures. A modified version of it is reproduced in Table 1.

Table 1

The International Classification of Epileptic Seizures (ICES)* (Najm et al., 2001)

---

1. Partial seizure
   - 1a. Simple partial
     - 1a 1. Motor signs
     - 1a 2. Sensory symptoms
     - 1a 3. Autonomic symptoms or signs
     - 1a 4. Psychic symptoms
   - 1b. Complex partial seizure
     - 1b 1. Simple partial at onset (with or without automatisms)
     - 1b 2. With impairment of consciousness (with or without automatisms)
   - 1c. Partial seizures evolving into generalized seizures
2. Generalized seizures
   - 2a. Absence seizures
     - 2a 1. Typical
     - 2a 2. Atypical
   - 2b. Myoclonic seizures
   - 2c. Clonic seizures
   - 2d. Tonic seizures
   - 2e. Tonic-clonic seizures
   - 2e. Atonic seizures
3. Unclassified epileptic seizures

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*Adapted from Commission on Classification and Terminology of the International League Against Epilepsy: Proposal for revised clinical and electroencephalographic classification of epileptic seizures: Epilepsia 22: 489-501, 1981; with permission.

4. Anti-epileptic therapy

4.1. New therapeutical approaches

4.1.1. Genetic studies

A further perspective for new therapeutic developments in treatment of epilepsy stems from identification of genetic defects (Schmidt, 1997). Advances in molecular genetics and molecular pharmacology may become a source of promise for diseases previously unresponsive to conventional treatment. In the case of epilepsies, identification of mutant genes underlying familial epilepsies may lead to a new pharmacological treatment strategy, through the development of *in vitro* expression systems permitting rapid search for novel drugs, creation of specific animal models based on expression of the precise mutation and correction of disease phenotypes by introducing novel and highly specific genetic information into the person with epilepsy (McNamara, 1994; Allen and Walsh, 1996; and Schmidt, 1997).

4.1.2. The control of neurotransmitters, their receptor on ionic flux

In generalized absences, pharmacological approaches attempt to achieve; (1) NMDA receptor inactivation; (2) $GABA_B$ receptor inactivation; (3) inactivation of low threshold T calcium currents.

In focal epilepsy, with or without secondary generalization, and in primary generalized epilepsy, the pharmacological approaches attempt to achieve; (1) blockade of Na^+ conductance that promotes repetitive voltage-dependent discharges; (2) blockade of voltage-dependent Ca^{2+} currents; (3) inactivation of NMDA receptors and other types of the GluR superfamily; (4) an increase in K^+ conductance; (5) an increase in Cl^- entry; (6) activation of GABAergic receptors; (7) interactions with cyclic nucleotide and PI system (second messenger); and (8) Na^+ , K^+ -ATPase stimulation (Ure and Perassolo, 2000).

4.1.3. Surgical control of epileptic discharges

Surgical procedures are in vogue for treatment of partial epilepsies, temporal lobe epilepsies, unilateral hemispherical lesions, and uncontrollable Lennox syndrome. A global survey on epilepsy surgery which covers a period of ten years, reported by the International League Against Epilepsy (ILAE) provided the following data: In adults: (1) anterior temporal lobectomies were performed in 71% of the surgical patients and there was a reduction or suppression of seizures in almost 90% of them; (2) selective amygdalo hippocampectomies were done in 10% of the intervened patients with marked post-operative improvements in almost 80% of them; (3) frontal lobe resections in another 10% were less effective to render the patients seizure-free; and (4) parietal and occipital resections were done infrequently and their results were a little better than frontal lobe resections but so effective as temporal lobe resections. In children: (1) anterior or complete callosotomies were performed, leading to a marked reduction of seizures, although the children were not completely seizure-free in more than 90% of cases; and (2) hemispherectomies were performed in almost 24% of the patients and their results were much better than the callosotomies, which were performed in order to render the children seizure-free (Engel, 1993 and ILAE commission report, 1997).

Vagus nerve stimulation (VNS) is a novel adjunctive therapy that has recently become commercially available for intractable epilepsy. In 1997, VNS was approved in the United States as an adjunctive treatment for medically refractory partial-onset seizures in adults and adolescents. The VNS system is comprised of a battery generator that delivers software and an interrogating wand. The generator is implanted in the left upper chest and connected to the left cervical vagus nerve via a pair of semi-circular helical electrodes wound around the vagus nerve and wires tunneled under the skin. Surgery is normally completed within 2 hours under general anesthesia and the patient can go home within a few hours postoperatively. Side effects during the use of VNS are usually related the “on” phase of stimulation. Common side effects are cough, hoarseness, voice alteration, and paresthesias. These side effects tend to diminish with

time. Cognitive side effects often seen with antiepileptic drug use are not reported. The precise mechanism of action remains to be elucidated (Schacter and Saper, 1998; Uthman, 2000; Ben-Menachem, 2001; and Vonck et al, 2001).

4.2. Drugs in use

For a long time it was assumed that a single drug could be developed for the treatment of all forms of epilepsy, but the causes of epilepsy are extremely diverse, encompassing genetic and developmental defects and infective, traumatic, neoplastic, and degenerative disease processes, and drug therapy to date shows little evidence of etiologic specificity. Existing antiepileptic drugs provide adequate seizure control in about two-thirds of patients (Porter and Meldrum, 2001).

With respect to mechanisms of action of old drugs, Macdonald has proposed that anticonvulsant drugs can be divided mechanistically into at least three classes based on ability to block sustained high-frequency repetitive firing of action potentials by blockade of voltage-dependent Na^+ channels, to enhance GABAergic inhibiting and to block slow, pacemaker-driven, repetitive firing by blocking T-Ca^{2+} current (Macdonald, 1989; Macdonald and Kelly, 1995; Macdonald and Meldrum, 1995). Macdonald (1989) further suggests that the ability of an anticonvulsant drug to block generalized tonic-clonic seizures and some forms of partial seizures may correlate with the ability of the drug to block sustained repetitive firing, while drugs with a broader spectrum of anticonvulsant activity block both sustained repetitive firing and enhance GABAergic inhibition (Table 2). Anti-absence drugs such as ethosuximide may act via their effect on thalamic T-Ca^{2+}current (Table 2). However, by this concept it is difficult to explain how valproate and benzodiazepines act on absence seizures. Furthermore, the concept ignores several additional cellular mechanisms of the old drugs which could explain the differences in pharmacology between drugs in the same class (Rogawski and Porter, 1990; Löscher and Schmidt, 1994; Schachter, 1995; White, 1997). For instance, while carbamazepine and phenytoin are generally thought to act by their effect on Na^+channels (Macdonald and Meldrum, 1995), an epileptic patient being

resistant to one of these drugs may respond favorably to alternative treatment with the other of the two drugs, clearly indicating that these drugs act by more than one mechanism (Schmidt and Gram, 1995).

Table 2

Proposed cellular mechanisms of action of old drugs (Macdonald, 1989; Macdonald and Kelly, 1995).

Seizure type /anticonvulsant drug	Blockage of voltage-dependent Na^+channels	Potentiation of GABAergic mechanisms	Blockage of thalamic T – type Ca^{2+}channels
Generalized tonic-clonic and partial seizures			
Phenytoin	++	NE	NE
Carbamazepine	++	NE	?
Phenobarbital	+	+	NE
Broad spectrum			
Valproate	++	+	NE
Benzodiazepines	+	++	NE
Absence seizures			
Ethosuximide	NE	NE	+

Effect is indicated by +, ++ = effective; NE = not effective in therapeutically relevant concentrations; ? = no data available (or found).

There are at least three preclinical strategies which are used for development of new anticonvulsant drugs : (1) random screening of newly synthesized chemical compounds of diverse structural categories for anticonvulsant activity in animal model, (2) structural variation of known anticonvulsant drugs and (3) mechanism-based

rational drug development, based on knowledge of the basic pathophysiological events involved in seizures or epilepsy (Löscher and Schmidt, 1994 and Upton, 1994). All three strategies have generated clinically effective anticonvulsant drugs, although many scientists currently believe that the strategies of rational ('modern') drug development has important advantages over the more traditional strategies.

The most important strategies of rational design of anticonvulsant drugs have been (1) enhancement of GABA-mediated neuronal inhibition, (2) diminution of glutamate-mediated neuronal excitation and (3) modulation of Na^+, K^+, and particularly Ca^{2+} ion channels (Löscher and Schmidt, 1994 and Upton, 1994).

New drugs

Gabapentin is an antiseizure drug that was approved by the United States Food and Drug Administration in 1993. The chemical structure of gabapentin is GABA molecule covalently bound to a lipophilic cyclohexane ring that is effective against partial seizure. Gabapentin was designed to be a centrally active GABA agonist, its high lipid solubility aimed at facilitating its transfer across the blood-brain barrier (McNamara, 2001 and Porter and Meldrum, 2001). Gabapentin has been shown to increase GABA turnover in some brain regions of laboratory animals (Löscher et al., 1992), presumably by activation of glutamate decarboxylase (Taylor et al.,1992), and to enhance brain GABA in patients (Petroff et al., 1996b).

Felbamate is a dicarbamate, which was approved by the United States Food and Drug administration for partial seizures in 1993. It is effective in both the maximal electroshock and pentylenetetrazol seizure model. Clinically relevant concentrations of felbamate inhibit NMDA-evoked responses and potentiate GABA-evoked responses in whole-cell, voltage-clamp recordings of cultured rat hippocampal of neurons (Rho et al., 1994). This dual action on excitatory and inhibitory transmitter responses may contribute to the wide spectrum of action of the drug in seizure models. Although it is effective in some patients with partial seizures, the drug causes aplastic

anemia and severe hepatitis at unexpectedly high rates and has been regulated to the status of a third-line drug for refractory cases (McNamara, 2001 and Porter and Meldrum, 2001).

Lamotrigine was developed when some investigators thought that the antifolate effects of certain antiseizure drugs (eg. phenytoin) may contribute to their effectiveness. It is a phenyltriazine derivative, which was approved by the United States Food and Drug Administration in 1994. Lamotrigine blocked sustained repetitive firing of mouse spinal cord neurons and delayed the recovery from inactivation of recombinant Na^+ channels, mechanisms similar to those of phenytoin and carbamazepine. This may well explain lomotrigine's actions on partial and secondarily generalized seizures. Lamotrigine is effective against a broader spectrum of seizures than phenytoin and carbamazepine. The mechanisms underlying its broad spectrum of actions are incompletely understood (Xie et al., 1995 and McNamara, 2001).

Topiramate is a sulfamate-substituted monosaccharide that is structurally different from all other antiseizure drugs. It was approved by the United States Food and Drug Administration in 1996 for partial seizures in adults when used in addition to other drugs. Topiramate blocks repetitive firing of cultured spinal cord neurons, as do phenytoin and carbamazepine. Its mechanism of action, therefore, is likely to involve blocking of voltage-dependent sodium channels. Topiramate also appears to potentiate the inhibitory effect of GABA acting at a site different from the benzodiazepine or barbiturate sites. Topiramate also depresses the excitatory action of kainate on AMPA receptors. It is possible that all three of these actions contribute to topiramate's anticonvulsant effect (McNamara, 2001 and Porter and Meldrum, 2001).

Tiagabine is a derivative of nipectoic acid and was "rationally designed" as an inhibitor of GABA uptake (as opposed to discovery through random screening). It was approved by the United States Food and Drug Administration in 1998 for treating partial seizures in adults when used in addition to other drugs. Tiagabine is an inhibitor of GABA uptake in both neuron and glia. It preferentially inhibits the transporter

isoform 1 (GAT-1) rather than GAT-2 or GAT-3 and increases extracellular GABA levels in the forebrain and hippocampus. It prolongs the inhibitory action of synaptically released GABA. Tiagabine inhibits maximum electroshock seizures and both limbic and secondarily generalized tonic-clonic seizures in the kinding model, results suggestive of efficacy against partial and tonic-clonic seizures clinically (McNamara, 2001 and Porter and Meldrum, 2001).

Vigabatrin (γ-vinyl-GABA) is an irreversible inhibitor of GABA aminotransferase (GABA-T), the enzyme responsible for the degradation of GABA. It apparently acts by increasing the amount of GABA release at synaptic sites, thereby enhancing inhibitory effects. Vigabatrin may also potentiate GABA by inhibiting the GABA transporter. It is effective in a wide range of seizure models. It is useful in the treatment of partial seizures and West syndrome (Porter and Meldrum, 2001).

Levetiracetam is a piracetam analog, which was approved by the United States Food and Drug Administration in 1999 for treating partial seizures in adults when used in addition to other drugs. Levetiracetam exhibits a novel pharmacological profile insofar as it inhibits partial and secondarily generalized tonic-clonic seizures in the kinding model yet is ineffective against maximum electroshock and pentylenetetrazole-induced seizures, findings consistent with effectiveness against partial and secondarily generalized tonic-clonic seizure clinically. The mechanism by which levetiracetam exerts these antiseizure effects is unknown. No evidence for an action on voltage-gated Na^+ channels or either GABA-or glutamate-mediated synaptic transmission has emerged (McNamara, 2001).

Zonisamide is a sulfonamide derivative that was approved by the United States Food and Drug Administration in 2000. Zonisamide inhibits the T-type Ca^{2+} currents. In addition, zonisamide inhibits the sustained, repetitive firing of spinal cord neurons, presumably by prolonging the inactivated state of voltage-gated Na^+ channel in a manner similar to actions of phenyltoin and carbamazepine. Zonisamide inhibits tonic hindlimb extension evoked by maximal electroshock and inhibits both partial and

secondarily generalized tonic-clonic seizures in the kindling model, results predictive of clinical effectiveness against partial and secondarily generalized tonic-clonic seizures. Zonisamide does not inhibit minimal clonic seizures induced by pentylenetetrazol, suggesting that the drug will not be effective clinically against myoclonic seizures. Zonisamide's inhibition of T-type Ca^{2+} currents suggests that it may be effective against absence seizures (McNamara, 2001).

The most commonly employed animal models in the search for new anticonvulsant drugs are the maximal electroshock seizure test and the pentylenetetrazole seizure test (Loscher and Schmidt, 1998). The maximal electroshock test, in which tonic hindlimb seizures are induced by bilateral corneal or transauricular electrical stimulation, is thought to be predictive of anticonvulsant drug efficacy against generalized tonic-clonic seizures, while the pentylnetetrazole test, in which generalized myoclonic and clonic seizures are induced by systemic (usually s.c.) administration of convulsant doses of pentylenetetrazole, is thought to represent a valid model for generalized absence and/or myoclonic seizures in human. In addition, the rotorod test is used to establish motor impairment neurotoxicity in mice and the effect of test substances on barbiturate sleeping time is used to evaluate the depressant effect on central nervous system (CNS) (Löscher and Schmidt,1988).

5. *Xenopus laevis* oocytes expressing system

The *Xenopus laevis* oocyte expression and recording system allowed a detailed analysis of the physiology and pharmacology of neuronal ion channels, transporters and receptors following an injection of the appropriate messenger RNA (mRNA) and allowing a period of incubation to translate the mRNA and appropriately synthesize the relevant protein (Gurden et al., 1971; Clayton and Woodward, 1999; Weber, 1999). Various receptors and ion channels, such as, GABA (Carpenter et al., 1992; Wahl et al., 1993), glycine (Wall et al., 1993), serotonin (Gurdensen et al., 1983., Julins et al., 1988) and NMDA (Moriyoshi et al., 1991) have been functionally expressed after injection of mRNA. The expression of foreign proteins in Xenopus oocytes has many advantages for

electrophysiological measurements. The large size, which easily accommodates manipulations like mRNA injections and electrode penetration, is certainly one aspect. However, the fact that it is possible to obtain cell attached patch clamp recordings and, in particular, recording from macropatches is among the main advantages (Stuhmer, 1992).

Xenopus laevis is often colloquially referred to as the South African clawed frog or, occasionally, toad, and is typified by the presence of up to three claws present on each of the hind limbs of mature frogs. These frogs are members of the family *Papidae* and are classified as Anurans (Verhoeff-de Fremery and Griffin, 1987). The frogs are endemic in South Africa, Boswana, and South West Zimbabwe. The larger frogs (mass 100-200 g) tend to have more oocytes than smaller frogs (<80 g) and of these oocytes a greater proportion will be of developmental stages IV-VI (Smart and Krishek, 1995). Oogenesis in the anuran *Xenopus laevis* can be divided into six stages based on the anatomy of the developing oocyte. Stage I consists of small (50 to 100 μ) colorless oocyte whose cytoplasm is transparent. Their large nuclei and mitochondrial masses are clearly visible in the intact oocyte. Stage II oocytes range up to 450 μ in diameter, and appear white and opaque. Stage I and II are both previtellogenic. Pigment synthesis and yolk accumulation (Vitellogenesis) begins during Stage III. Vitellogenesis continues through Stage IV (600 to 1,000 μ), the oocytes grow rapidly, and the animal and vegetal hemispheres become differentiated. By Stage V (1,000 to 1,200 μ) the oocytes have nearly reached their maximum size and yolk accumulation gradually ceases. Stage VI oocytes are characterized by the appearance of an essentially unpigmented equatorial band. They range in size from 1,200 to 1,300 μ, are postvitellogenic and ready for ovulation (Dumont, 1972). *Xenopus laevis* can produce oocytes of differing stages of maturity all year round. *Xenopus* are aquatic air-breathing animals that will rapidly suffer from dehydration and eventual death if denied access to water. The temperature of the tank water can be maintained by thermostatically controlling the room temperature between 18 and 22 °C and filter pump will keep the water clean from particulate debris. Since *Xenopus* skin is sensitive to chloride ions, the water used to fill the tanks should be taken from stored water contained in large open tanks for at least 24 hours, which allows the chlorine to evaporate (Smart and Krishek, 1995).

Expression of foreign proteins in the *Xenopus* oocyte, either following microinjection with mRNA into the cytoplasm (Gurdon et al., 1971) or of cDNA into the nucleus (Mertz and Gurdon, 1977) has been pioneered by Gurdon and collaborators. The oocyte efficiently transcribes and translates injected genetic information, performs assembly of the protein products, correctly process the nascent polypeptides and targets them to the proper subcellular compartment (Sigel, 1990). The use of Gurden's Xenopus oocyte translation system has allowed the production of neurotransmitter receptors in a foreign cell membrane, following the translation of microinjected mRNA isolated of various sources. This very accessible and relatively simple preparation permits the study of the requirements for receptor-ionophore function, assembly and membrane integration (Houamed et al., 1984). The experimental procedures involved in the functional expression membrane protein in the *Xenopus* oocyte are illustrated in Fig. 3.

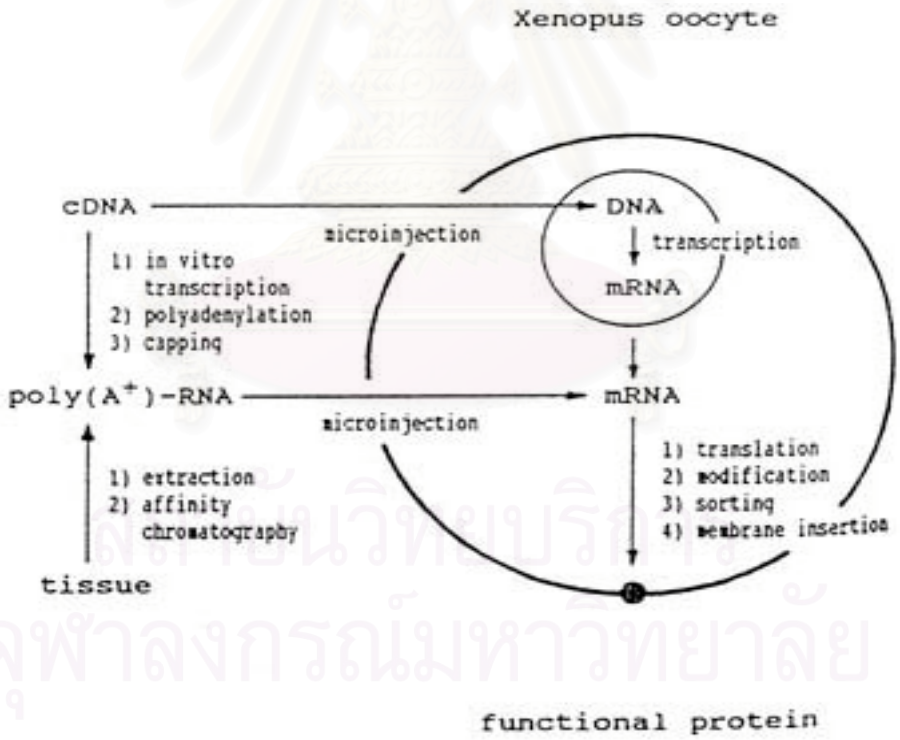


Figure 3. Scheme illustrating functional plasma membrane protein expression in *Xenopus* oocyte.

The fully grown oocyte from *Xenopus laevis* is an extraordinarily large cells with a diameter of 1-1.2 mm which is surrounded by the noncellular, fibrous, vitellin layer and by several layers of follicular cells (Dumont and Brummett, 1978). Their size allows simple handing with ordinary pasteur pipettes whose sharp edges have been fire-polished. The follicular cells maybe removed, either mechanically or by enzymatic procedures to give "naked", "denuded" or defolliculated oocytes. Most experiments are carried out using naked oocytes that are still surrounded by the vitellin layer which provides mechanical stability to the oocyte. For the recording of single ion channel currents using the patch-champ technique, it is also necessary to remove the vitellin layer or to rupture it locally.

It should also be mentioned that the oocyte system exhibits some disadvantages that could complicate the experiments and the interpretation of results (Sigel, 1990). The major drawbacks of the oocytes include seasonal variations reported by some laboratories, biological variance between oocytes of different donors, short life time (around 14 days) and strong temperature dependence.

6. Ameltolide (4-amino-N-(2,6-dimethylphenyl)benzamide , LY2O1116, ADD 75073)

Because of major deficiencies in the efficacies and side-effect profiles of existing antiepileptic medications, there has been a resurgence of interest in development of new classes of antiepileptic drugs. Ameltolide is the most potent 4-aminobenzamide anticonvulsant studied to date (Figure. 4 (a)). It was well absorbed (~94%) from the gastrointestinal tract following oral adminitration. The majority of the radioactivity was excreted in the urine (64.5%), with the remainder of the radioactivity excreted in feces. However, the majority of the radioactivity in the feces can be accounted for by biliary excretion (29%). Analysis of tissues for total ameltolide-derived radioactivity indicated that this compound was rapidly distributed to all tissues. The elimination rate constant from the tissues was also very rapid, with a monophasic decline and a mean half-life of

3.42 ± 0.27 hr. The major route of metabolism was N-acetylation to from 4hydroxylation to from 4-(acetylamine)-N-(2-hydroxymethyl-6-methylphenyl) benzamide (HADMP) (Figure 4(b) and 4 (c)). The elimination of ameltolide from the systemic circulation following intravenous administration was monophasic, with a terminal half-life of 9.4 min. The half-life of ameltolide was 15 min, as calculated between 60 and 120 min after oral administration. The metabolism, disposition, and pharmacokinetics of ameltolide have been studied in rat (Robertson et al., 1991 and Potts et al., 1989).

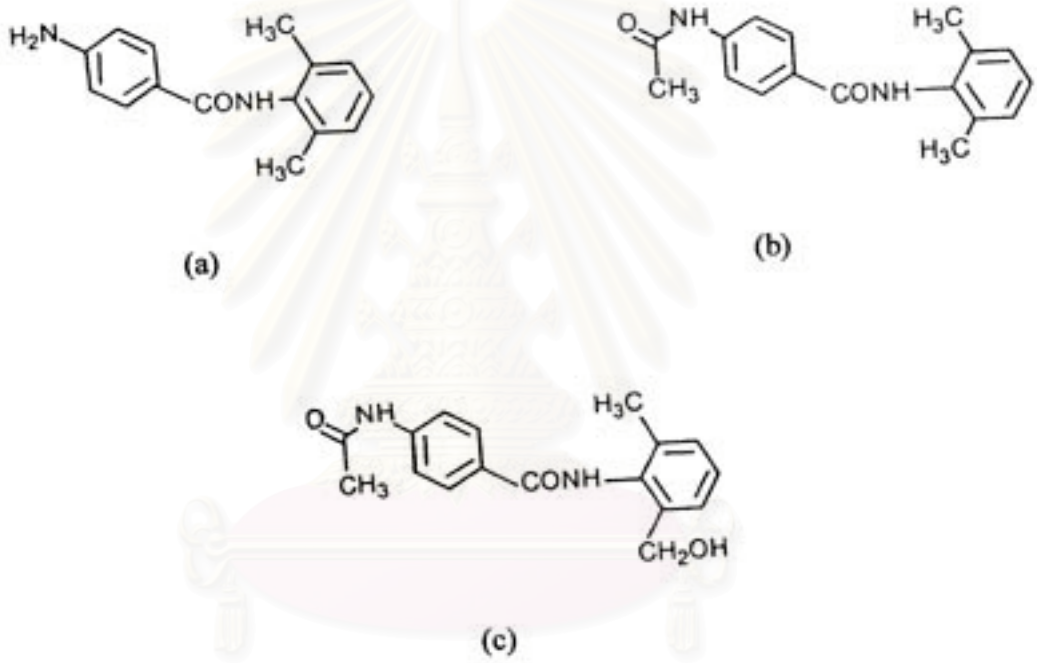


Figure 4. Structural formula of ameltolide (a), ADMP (b), and HADMP (c)

Pharmacology

Ameltolide is a very potent anticonvulsant in the maximal electroshock seizures (MES) model. This compound was effective in nontoxic doses following intraperitoneal (i.p.) administration in mice and oral administration in both mice and rats. Ameltolide was unable to antagonize any of the standard chemically induced seizure. Thus, in these animal models, ameltolide displayed a phenyltoin-like profile. Ameltolide had no pharmacologically relevant effects on hexabarbital-induce sleeping time in mice. On the basis of extensive pharmacological studies, ameltolide is being developed for treatment of generalized tonic-clonic and partial seizures (Robertson et al., 1987 and Clark, 1988).

By considering the values of ED_{50}, TD_{50}/ ED_{50} (Protective index, PI), LD_{50}/ HD_{50} (dose that caused death in 50% of animals/dose at which 50% of animals lost righting reflex) and LD_{50}/ TD_{50} (Relative safety margin), the anticonvulsant activity and neurotoxicity of ameltolide compare favorably with those of prototype anticonvulsants in the same assays (Table 2-4). Ameltolide produces no sign of tolerance with daily administration and does not interact with the hypnotic effects of hexabarbital. The PI values of ameltolide appear to be a good safety margin. All of these results suggest that ameltolide will be an effective anticonvulsant in humans and support development of the compound for the treatment of epilepsy.

Table 3. Minimal neurotoxicity and anticonvulsant potency of intraperitoneally administered ameltolide and some prototype antiepilepic drugs in mice (Clark, 1988).

Substance	Time of test[a](hr)	Rotorod TD_{50} (mg/kg)	ED_{50} (mg/kg) and PI			
			MES	s.c. PTZ	s.c. BIC	s.c. PIC
Ameltolide	½, ½	15.0 (13.3-16.9)	[5.8] 2.6 (2.2-3.1)	[<0.75] No protection up to 20	[<0.75] No protection up to 20	[<0.50] No protection up to 30
PHT	2, 2	65.5 (52.5-72.1)	[6.89] 9.50 (8.13-10.4)	[<0.22] No protection up to 300	[<0.65] No protection up to 100	[<0.65] No protection up to 100
PB	½, 1	69.0 (62.8-72.9)	[3.17] 21.8 15.0-25.5	[5.24] 13.2 (5.87-15.9)	[1.83] 37.7 (26.5-47.4)	[2.51] 27.5 (20.9-34.8)
ESM	½, ½	441 (383-485)	[<0.44] No protection up to 1,000	[3.38] 130 (111-150)	[0.96] 459 (350-633)	[1.82] 243 (228-255)
VPA	¼, ¼	426 (369-450)	[1.57] 272 (247-338)	[2.87] 149 (123-177)	[1.18] 360 (294-439)	[1.10] 387 (341-444)

PHT = phenytoin; PB = phenobarbital; ESM = ethosuximide; VPA = valproate; TD_{50} = dose eliciting evidence of minimal neurotoxicity in 50% of animals; ED_{50} = dose required to produce the desired endpoint in 50% of animal; MES = maximal electroshock seizures; s.c.PTZ = subcutaneous pentylenetetrazole; s.c.BIC = subcutaneous biculline; s.c.PIC = subcutaneous picrotoxin; Protective index (PI = TD_{50}/ ED_{50}) in bracket [ ]; The 95% confidence interval in parentheses ( ); [a]first number = TD_{50}; second number = ED_{50}

Table 4. Minimal neurotoxicity and anticonvulsant potency of orally administered ameltolide and some prototype antiepileptic drugs in mice and rats (Clark, 1988).

Time of test[a] (hr)			TD_{50} (mg/kg)		MES ED_{50} (mg/kg)		PTZ ED_{50} (mg/kg)	
Substance	**Mice**	**Rats**	**Mice**	**Rats**	**Mice**	**Rats**	**Mice**	**Rats**
Ameltolide	½, 1	2, 1	38.3 (31.8-46.0)	458.9 (371.4-548.2)	[9.8] 3.9 (2.9-4.7)	[14.1] 32.5 (29.3-36.1)	[<0.5] No protection up to 80	[<0.9] No protection up to 500
PHT	2, 2	½, 4	86.7 (80.4-96.1)	No ataxia up to 3,000	[9.59] 9.04 (7.39-10.6)	[>100] 29.8 (21.9-38.9)	[<0.29] No protection up to 300	[NA] No protection up to 800
PB	2, 2	½, 5	96.8 (79.9-115)	61.1 (43.7-95.9)	[4.82] 20.1 (14.8-31.6)	[6.68] 9.14 (7.58-11.9)	[7.69] 12.6 (7.99-19.1)	[5.29] 11.6 (7.74-15.0)
ESM	1, ½	2, 2	879 (840-943)	1,012 (902-1,109)	[<0.44] No protection up to 1,200	[<0.84] No protection up to 2,000	[4.56] 193 159-218)	[18.8] 54.0 (45.6-60.9
VPA	2, 1	1, ½	1,264 (800-2,250)	280 (191-353)	[1.90] 665 (605-718)	[0.57] 490 (351-728)	[3.26] 388 (349-439)	[1.56] 180 (147-210)

NA = not applicable; [a]toxicity, MES and s.c. PTZ respectively; other abbreviations as in Table

Table 5. Quantitative toxicity profile of intraperitoneally administered ameltolide and some prototype antiepileptic drugs in mice (Clark, 1988).

Substance	Time of test[a] (hr)	Dose (mg/kg)		
		Lethality (LD_{50})	**Righting reflex (HD_{50})**	**Rotorod (TD_{50})**
Ameltolide	24, ½, ½	160.8 (144-182)	[3.67] 43.8 (38-53)	[10.71] 15.0 (13-17)
PHT	24, 12, 2	230 (216-259)	[1.29] 178 (153-195)	[3.51] 65.5 (52.5-72.1)
PB	24, 1, ½	265 (242-286)	[1.95] 135 (115-117)	[3.84] 69.0 (62.8-72.9)
ESM	24, ½, ½	1,752 (1,607-1,867)	[2.06] 851 (751-918)	[3.98] 441 (383-485)
VPA	24, ¼, ¼	1,105 (1,022-1,254)	[1.25] 886 (821-947)	[2.59] 426 (369-450)

LD_{50} = dose that caused death in 50% of animals;

HD_{50} = dose at which 50% of animals lost righting reflex;

Ratio LD_{50}/ HD_{50} or LD_{50}/ TD_{50} in bracket [ ];

[a]lethality, righting reflex, and rotorod respectively; other abbreviation as in Table

Ameltolide potently inhibited MES-induced seizures in mice and rats, but was ineffective against a variety of chemically induced seizures. This phenytoin-like profile, coupled with a high protective index, suggests that the compound may be suitable for treatment of generalized tonic- clonic and partial seizures in man (Robertson, 1991). It does not increase hexabarbital-induced sleeping time but has a short half-life (3.4 hours) (Potts et al., 1989). Thus, there is a need to improve analogs with a longer duration of anticonvulsant activity and higher potency.

7. N-(*p*-aminobenzoyl)-1,2,3,4-tetrahydroquinoline (NTQ)

In 1994, N-(*p*-aminobenzoyl)-1,2,3,4-tetrahydro-4,8-dimethylquinoline was firstly synthesized as the rigid analogue of ameltolide (Sathit Nirathisai, 1994). The preliminary result in experimental animals indicated that it exhibited anticonvulsant activity against MES test. However, its synthetic approaches are complicate with several steps. In an attempt to extend the structure activity relationship and to improve the synthetic procedures, N-(-aminobenzoyl)-1,2,3,4-tetrahydroquinoline (NTQ) was subsequently synthesized by Assist. Prof. Chamnan Patarapanich, Ph.D. and Miss Tanarat Kietsakorn, B.Sc. (Pharm.) Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

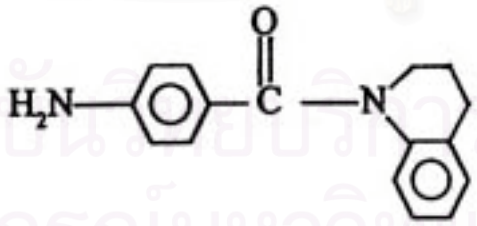


Figure 5. Structural formula of N-(*p*-aminobenzoyl)-1, 2, 3, 4-tetrahydroquinoline, (NTQ).

The main purpose of this study was to evaluate anticonvulsant effect of NTQ in several animal models of epilepsy as well as its adverse effects and acute toxicity. Furthermore, a possible effect of NTQ on NMDA receptor, a neurotransmitter generally known to play an important role in pathogenesis, is also investigated in *Xenopus laevis* oocytes expressing system.

CHAPTER II

MATERIALS AND METHODS

Experimental animals

Anticonvulsant activity and neurotoxicity experiments were performed on male Swiss albino mice weighing 18-25 g obtained from National Laboratory Animal Center, Mahidol University, Nakornpathom, Thailand. The animal were acclimatized in the laboratory in a ventilated room at the ambient temperature of 25°c on a natural light/dark cycle for at least one week prior to the experiments. Food [F.E.Zuellig. (Thailand) Co.,Ltd] and water were provided *ad libitum*. Experiments were completed within a week to minimize the effect of increasing age on seizure susceptibility (Loscher and Nolting, 1991). In all experiments, each animal was used for only once and the experiments were carried out between 9.00 a.m.-7.00 p.m.

The oocytes from *Xenopus laevis* were used to study the effects of ameltolide and N-(*p*-aminobenzoyl)-1,2,3,4-tetrahydroquinoline on NMDA receptor. *Xenopus laevis*, the South African clawed frog (family, Pipidae; subfamily, Xenopinae) obtained from *Xenopus Express*™ , U.S.A. were maintained on a 12 hour light/dark cycle in tanks of dechlorinated water at 18°c and were fed twice a week.

Equipments

1. Autoclave (Iwaki Glass Co., LTD, Japan)
2. Automatic injector (Drummond Scientific Company, Broomall, PA, U.S.A.)
3. Autopipettes (Scorex, Swiss)
4. Basile 7430 activity cage (UGO Basile, Comerico, Italy)
5. Electroshock apparatus with corneal electrodes (King Mongkut Institute of Technology, North Bangkok, Thailand)
6. Fiber illuminator (model C-FID, Nikon, Japan)
7. Gene clamp 500 (Axon Instruments, Foster city, CA, U.S.A.)
8. Macintosh® computer (Performa 6200, Apple computer, Inc., U.S.A.) With Chart™ V.3.2.8 program for data recording system
9. MacLab®/4e (ADInstruments Pty LTD, Australia)
10. pH meter (model 210, ThermoOrion, MA, U.S.A.)
11. Refrigerated centrifuge (model J2-21, Beckman, U.S.A.)
12. Rotorod apparatus (UGO Basile, Comerio, VA, Italy)
13. Sonicator
14. Surgical equipment
15. Virtual-ground bath clamp (Axon Instruments, Foster city, CA, U.S.A.)
16. Vertical micropipette puller (Narishike, Japan)
17. Water bath shaker (Maxi-Shake®, Heto, Japan)

Chemicals

1. Ameltolide (4-amino-N-(2,6-dimethylphenyl)benzamide) was kindly supplied by Assist. Prof. Chamnan Patarapanich, Ph.D. and Miss Tanarat Kietsakorn, B.Sc. (Pharm.) (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University)
2. Ammonium acetate ($C_2H_7NO_2$) (Scharlau, Barcelona, Spain)
3. Calcium chloride dihydrate ($CaCl_2.2H_2O$) (MERCK, Darmstadt, Germany)
4. Calcium nitrate tetrahydrate ($Ca(NO_3)_2.4H_2O$) (MERCK, Darmstadt, Germany)

5. Collagenase (Wako Pure Chemicals Industries, Ltd., Japan)
6. Dimethyldichlorosilane (Wako Pure Chemicals Industries, Ltd., Japan)
7. Dextromethorphan ($C_{18}H_{25}NO.HBr.H_2O$) (SIGMA, U.S.A.)
8. Glycine (SIGMA, U.S.A.)
9. Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) (MERCK, Darmstadt, Germany)
10. Mineral oil
11. mMessage mMachine™ T7, T3 kits (AMBION, U.S.A.)
12. N-methyl-D-aspartic acid (NMDA) (SIGMA, U.S.A.)
13. N-(*p*-aminobenzoyl)-1, 2, 3, 4-tetrahydroquinoline (NTQ) was supplied by Assist. Prof. Dr. Chamnan Patarapanich and Miss Tanarat Kietsakorn (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University)
14. Normal saline Solution (NSS) (General Hospital Products Co., Ltd., Thailand)
15. Penicillin G sodium 1,000,000 units (General Drugs House Co., Ltd., Thailand)
14. Pentobarbital sodium (Nembutal®) (Sanofi (France) Ltd.)
15. Pentylenetetrazole (PTZ) (SIGMA, U.S.A.)
16. Polyethylene glycol 400 (PEG 400) (Witthayasom, Thailand)
17. Potassium chloride (KCl) (MERCK, Darmstadt, Germany)
18. Sodium chloride (NaCl) (MERCK, Darmstadt, Germany)
19. Sodium hydrogen carbonate ($NaHCO_3$) (MERCK, Darmstadt, Germany)
20. Sodium hydroxide (NaOH) (MERCK, Darmstadt, Germany)
21. Streptomycin (General Drugs House Co., Ltd., Thailand)
22. Tris (hydroxymethyl) aminomethane hydrochloride (Tris HCl; $(CH_2OH)_3CNH_2.HCl$) (USB™, OH, U.S.A.)

Preparation and administration of test substances

PEG 400 was used as vehicle for ameltolide and NTQ. Normal saline solution (NSS) was used to dissolve pentobarbital sodium and PTZ. The test substances were injected intraperitoneally (i.p.) except for PTZ, which was given by subcutaneous injection. The volumes of injection in mice were kept at 0.1-0.2 ml for each animal.

Experimental Methods

1. Anticonvulsant activity

1.1. Anticonvulsant activity against MES

MES was elicited by the passage of an alternating current of 55 mA, 50 Hz for 0.2 second through the brain via corneal electrodes after the pretreatment with test substances. The endpoint of the MES test was generalized seizures with tonic hindlimb extension (Loscher and Nolting, 1991; Thompson, 1990).

The test substance was considered to possess anticonvulsant activity if previously described convulsion did not occur within 0.2-0.5 second after the MES was performed.

1.2. Determination of the optimal pretreated time and median effective dose (ED_{50})

The peak time of maximal anticonvulsant activity, which would be subsequently used as the optimal pretreated time, was performed in mice. The test animals were divided into 3 groups according to pretreated time (15, 30 and 60 min). Two subgroups with 4 animals each (NSS and PEG 400, 0.1 ml/25 g B.W.i.p.) were used as control groups. Eight animals per dose and 3-5 doses were used to establish the ED_{50} of ameltolide and NTQ to protect the animals against the

MES test. When the pretreated time was due, MES was performed as previously described in 1.1. The pretreated time that gives the maximal anticonvulsant activity of each test substances was selected and used as optimal pretreated time in other experiments.

1.3. Duration of protection against MES

Mice were divided into 6 groups according to pretreated time (0.25, 0.5, 1, 3, 6 and 9 hr.). Eight animals per dose and 3-5 doses were used to determine the duration of protection against MES of ameltolide and NTQ. When pretreated time was due, MES was performed as previously described in 1.1 and the ED_{50} of ameltolide and NTQ at different times were calculated.

1.4. Anticonvulsant activity against PTZ seizure test

Seizure was induced by a subcutaneous injection of PTZ (70 mg/kg). The end point of this chemoshock test was a generalized clonic seizure with loss of righting reflex within 60 minutes after the injection of PTZ (Loscher et al., 1991).

Two groups with 4 animals each (NSS and PEG 400, 0.1 ml /25 g B.W.i.p.) were used as control groups. Eight animals per dose and 3-5 doses were used to establish the ED_{50} of ameltolide and NTQ to protect the animals against convulsions induced by PTZ. The PTZ test was performed after the optimal pretreated time obtained from 1.2.

2. Neurotoxicity

2.1. Acute toxicity test

Male Swiss albino mice were divided into 6 groups of 5-8 animals each for determination of the median lethal dose (LD_{50}) of ameltolide and NTQ and other effects such as ataxia, sedation and hypnosis within 72 hours after the

intraperitoneal administration of ameltolide (60, 65 and 70 mg/kg B.W.) and NTQ (250, 500 and 1,000 mg/kg B.W.).

2.2. Rotorod test

The rotorod test was modified from the one previously described by Dunham and Miya (1957), carried out with a rod of 3.5 cm diameter, rotating at 16 rev/min. Neurological deficit was indicated by inability of the animals to maintain their equilibrium for at least 1 min on the rotating rod in each of three successive trials. Untreated mice were able to maintain their balance on the rod for several minutes. Substance or vehicle-treated mice, which were not able to maintain their equilibrium on the rod for 1 min, were put back on the rod twice. Only animals, which were not able to remain on the rod for 1 min in each of trials, were considered to exhibit neurological deficit.

2.3. Determination of median neurotoxic dose (TD_{50})

Two groups with 4 animals each (NSS and PEG 400 0.1 ml/25 g B.W.i.p.) were used as control groups. Eight animals per dose and 3-5 doses were used to establish the TD_{50} of ameltolide and NTQ. After the optimal pretreated time was due, rotorod test was performed as previously described in 2.2.

2.4. Locomotor activity test

Basile 7430 activity cage (UGO Basile, Comerio, Italy) was used to assess the locomotor activity of mice which were placed in a box (width 23 cm × length 35 cm × height 20 cm). The cage floor is made of evenly spaced stainless steel bars (3 mm diameter) that are spaced 11 mm apart. Each of the steel bar is insulated from each other. The odd bars are grounded and the even bars are active and wired out in four sets. The bridge that animal breaks with its paw disconnected one or more active bars with ground, thereby producing configurations with change as the animal moves. These changes in configuration are converted into pulses and subsequently count by a counter. The counters were detected at 20 minute

intervals. A baseline locomotor activity of each mouse was established allowing a control period of 40 minutes before the administration of the test substances. Motor activity was measured in the animal cage for a period of 160 min.

Mice were divided into 6 groups of 8 animals each. Two groups (NSS and PEG 400 0.1 ml/25 g B.W.i.p.) were used as the control groups. The other 4 groups, ameltolide 1.0, 10.0 mg/kg B.W.i.p. and NTQ 10.0, 20.0 mg/kg B.W.i.p. were used to test the effects on locomotor activity. After each mouse was placed single in the Basile 7430 activity cage for familiarization with the environment for 40 min, the test substances was given intraperitoneally and the animal was allowed back into the cage immediately in order to record its locomotor activity for another 160 min.

2.5. Barbiturate potentiation test

The effect of test substances on barbiturate sleeping time was used to evaluate the depressant effect on CNS. Pentobarbital sodium, 60 mg/kg, was intraperitoneally injected to the animals at the optimal pretreated time after the administration of the test substances. The inability and ability of the animal to upright itself within 5 sec when being placed on its back in three successive trials was taken as the criteria of loss and recovery, respectively, of the righting reflex (Thompson, 1990).

Mice were divided into 6 groups of 8 animals each. Two groups (NSS and PEG 400 0.1 ml/25 g B.W.i.p.) were used as control groups. The other 4 groups, ameltolide 1.0, 10.0 mg/kg B.W.i.p. and NTQ 10.0, 20.0 mg/kg B.W.i.p. were used to test for the potentiation of barbiturate sleeping time. The test substances were administered at the optimal pretreated time described in 1.2.

3. Preparation of *Xenopus laevis* oocytes

3.1. *In vitro* transcription

Cloned cDNA encoding NMDAR1 subunit, NR1a was the generous gift of Dr. S. Nakanishi (Kyoto University, Kyoto, Japan); and cloned cDNA encoding NMDAR2 subunit, NR2B (generously provided by Dr. P.H. Seeburg (University of Heidelburg, Heidelburg, Germany)) were linearized by digestion with NotI (NR1A), EcoRI (NR2B) and transcribed *in vitro* using T7 (NR1) or T3 (NR2) mMessage mMachine kits (Ambion, TX, U.S.A.).

3.2. *Xenopus* oocytes injections

Oocytes were collected from ovarian lobes of anesthesized *Xenopus laevis* and placed in sterile modified Barth's Solution (MBS; 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.33 mM $Ca(NO_3)_2.4H_2O$, 0.41 mM $CaCl_2.2\ H_2O$, 0.82 mM $MgSO_4.7\ H_2O$, and 7.5 mM Tris HCl, pH 7.6). The layer of follicular cells was removed by collagenase treatment (2 mg/ml in Ca^{2+}- free MBS at 19°c for 2 hours) and then change the collagenase after 1 hour, or when about half of the oocytes are free and replace with fresh collagenase. Incubate for another 1 hour, and the remaining follicular layer was removed manually. Stage V and VI oocytes were maintained in MBS. NMDA receptor mRNA were dissolved in sterile distilled water. Oocytes were injected with NR1 plus NR2 in ratio of 1:4. Twenty-five nanolitres of the final mRNA mixtures (10-20 ng total) was microinjected into the oocytes cytoplasm. The oocytes were incubated in MBS containing 2.5 units/ml penicillin and 2.5 μg/ml streptomycin at 18°c for 1-4 days before recording. The MBS was replaced daily

3.3. Electrophysiological recordings

One to four days after injection, oocytes were placed in a recording chamber (volume 50 μl) and continuously perfused with Mg^{2+}- free MBS at

1.5 ml/min at room temperature (22-25 °c). Oocytes were voltage-clamped by a two-microelectrode voltage-clamp technique. The membrane potential of the oocytes was held at –80 mV using a Gene Clamp 500 (Axon Instruments, Foster city, CA, U.S.A.). The voltage-monitoring electrode was filled with 3M KCl and the current-passing electrode with 3M ammonium acetate and had resistances between 0.5-5 MΩ. Vigorous oocytes possessing negative membrane potential exceeding –20 mV were used for the experiments. Drugs were diluted with Mg^{2+}-free MBS and were applied until the peak of the response was observed (usually for 30 sec or less). A washout period for recovery was 1-15 min, depending on the concentration of drugs applied (Pathama Leewanich, 1998).

4. Calculation and statistical analysis

4.1. For determination of ED_{50}, TD_{50} and LD_{50} groups of 8 mice each were used to test the effect of test substances at various dose until at least 3 points were established between the limit of 0-100 percent response or non response and transform to probit unit by transformation table of Fisher and Yates (Diem and Lentner, 1972). The linear regression method was used to fit a curve between probit unit of response or non-response and dose (log scale) by using Crickcet graph program (Macintosh® computer). The 95 percent confidence interval was calculated by the method of Litfield and Wilcoxon (1949).

4.2. Concentration-response curves were constructed by applying different concentrations of NMDA in random order and were calculated by using the logistic equation, $E = E_{max} \times [drug]/(EC_{50}^{n} + [drug]^{n})$, where E_{max} is the maximal effect, n is the Hill coefficient, and EC_{50} is the concentration of drug producing 50 % of the maximal effect. When drug shows inhibitory effect, IC_{50} is the concentration of drug producing 50 % of the maximal inhibition.

4.3. Statistical analysis was carried out using SPSS program version 9.0. All numerical data are expressed as mean ± standard error of the mean (S.E.M.). Analysis of variance (oneway ANOVA followed by Scheffe test) was used to compare the data between various groups ($p<0.05$).

CHAPTER III

RESULTS

1. Anticonvulsant activity

None of the mice in control groups receiving either NSS or PEG 400 (0.1 ml/25 B.W. i.p.) was protected against MES and PTZ tests. In contrast, NTQ given intraperitoneally was effective in both MES and PTZ models. Ameltolide demonstrated about 13 times greater potency (ED_{50} = 0.96 mg/kg B.W.) than NTQ (ED_{50} = 13.33 mg/kg B.W.) in protection against MES, but was ineffective in PTZ models.

1.1. Anticonvulsant activity against MES

As illustrated in figure 6, intraperitoneally given ameltolide demonstrated a protection against MES in a dose dependent manner exhibiting of ED_{50} of 0.96, 1.6 and 2.29 mg/kg B.W. at the pretreated time of 15, 30 and 60 min, respectively. The corresponding values of ED_{50} exhibited by NTQ were 13.33, 27.32 and 40.68 mg/kg B.W. (Figure 7).

1.2. Duration of protection against MES

In MES test, the ED_{50} of intraperitoneally given ameltolide and NTQ were determined for 9 hours after dosing. The ED_{50} of both ameltolide and NTQ increased as a function of time demonstrating the final ED_{50} of 11.23 and 158.50 mg/kg B.W. at 9 hours (Figure 8).

1.3. Anticonvulsant activity against PTZ

In contrast to NTQ which exerted its protection effect against convulsion induced by PTZ giving the ED_{50} of 1.06 mg/kg B.W. (figure 10),

ameltolide in the doses up to 10 mg/kg B.W. did not protect experimental animals against this chemoshock.

2. Neurotoxicity

2.1. Acute toxicity test

The most frequently adverse effects observed in mice receiving high doses (250, 500 and 1,000 mg/kg B.W.) of NTQ were sedation, ataxia, loss of righting reflex and hypnosis. Lethality was observed within the period of 72 hours, however, death occurred mostly within 24 hours. The median lethal dose (LD_{50}) of ameltolide and NTQ were 62.67 and 599.42 mg/kg B.W., respectively (Figure 11). However, in terms of safety, ameltolide possessed higher relative safety margin (LD_{50}/TD_{50}) than NTQ (65.28 v.s. 44.97, Table 6).

2.2. Rotorod test

Mice in control groups receiving either NSS or PEG (0.1 ml/25 g B.W.) were able to maintain their equilibrium for at least 1 min on the rotating rod in each of three successive trials whereas neurological deficit was noted in mice receiving either ameltolide or NTQ. Apparently, ameltolide demonstrated a lower neurotoxicity than that of NTQ. The TD_{50} at 15 min pretreated time of intraperitoneally given ameltolide and NTQ were 7.18 and 37.28 mg/kg, respectively (Figure 12). This resulted in a higher neuroprotective index (TD_{50}/ED_{50}) of ameltolide than that of NTQ. As depicted in Table 6, the protective indices were 7.48 and 2.80 for ameltolide and NTQ, respectively.

2.3. Locomotor activity test

In comparison to NSS (0.1 ml/25g B.W.), an intraperitoneal administration of PEG (0.1 ml/25 g B.W.), ameltolide (1.0, 10.0 mg/kg B.W.) and NTQ (10.0 and 20.0 mg/kg B.W.) significantly depressed the locomotor activity of

mice. However, no statistically significant difference was noted among the effect of PEG, ameltolide and NTQ (Figure 14 and 15).

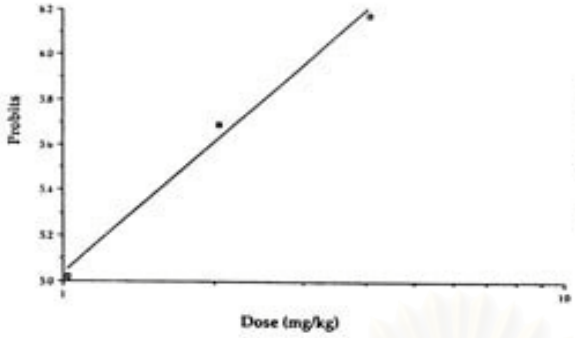
2.4. Barbiturate sleeping time

As illustrated in Figure 16, PEG 400 (0.1 ml/25 g B.W. i.p.) tended to slightly prolong barbiturate sleeping time but no statistical significance was noted between the effects of NSS and PEG 400. NTQ significantly prolonged the barbiturate sleeping time only when higher dose (20.0 mg/kg B.W.i.p.) was given whereas lower dose of NTQ (10.0 mg/kg B.W. i.p.) is devoid of this effect. In contrast, the barbiturate sleeping time was significantly prolonged by both low and high doses of ameltolide (1.0 and 10.0 mg/kg B.W.i.p., respectively).

3. Effects of test compounds on NMDA receptors expressed on *Xenopus laevis* oocytes

From 1-4 days after mRNA injection into the oocytes, currents evoked by application of different concentrations of the selective glutamate agonist, NMDA, plus 10 μM of glycine are shown in Figure 16. Without glycine, NMDA elicited no detectable current and non-injected oocytes did not respond to NMDA (data not shown). At a holding potential of –70 mV, log concentration-response curves of NMDA were constructed and EC_{50} value of 18.94 μM was determined.

To examine the inhibitory effects of NTQ on NMDA-induced currents, various concentrations of this compound (0.1-1,000 μM) were coapplied with 30 μM NMDA plus 10 μM glycine to the oocytes injected with mRNA and compared with that of ameltolide (0.1-1,000 μM). Ameltolide and NTQ by themselves did not induce currents or alter membrane resistance (data not shown). Ameltolide inhibited NMDA responses by 97.59+1.29 % in bell shaped manner and its IC_{50} value was 0.12 μM. NTQ markedly and concentration-dependently reduced the NMDA responses, however, NTQ seemed to potentiate the NMDA responses in high doses (100 and 1,000 μM). IC_{50} value of NTQ on the NR1a/NR2B receptors was 0.10 μM and inhibited the NMDA responses by 94.54+2.03 % (Figure 17 and 18).

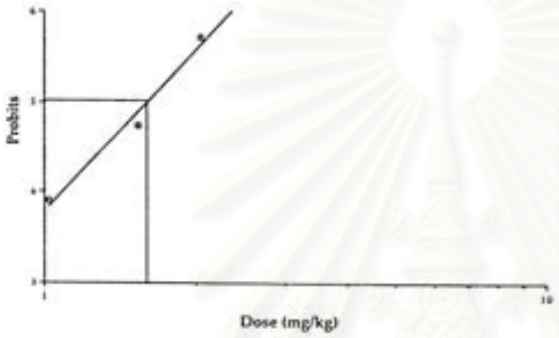


Ameltolide 15 min.

Probits = 5.0317+1.9101 Log (dose)

R^2 = 0.991

ED_{50} = 0.96 (0.42-2.20) mg/kg

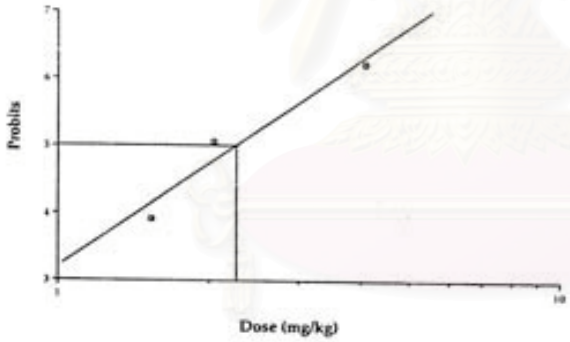


Ameltolide 30 min

Probits = 3.7857+5.9585 Log (dose)

R^2 = 0.978

ED_{50} = 1.60 (1.23-2.08) mg/kg



Ameltolide 60 min

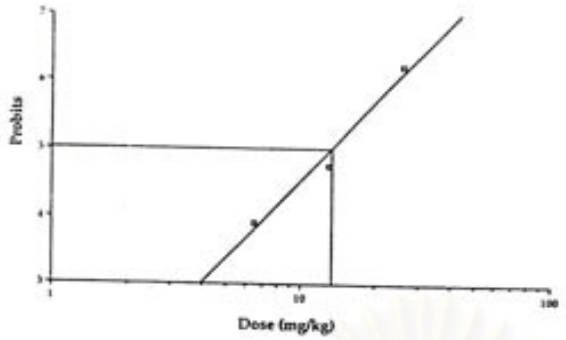
Probits = 3.1623+5.1085 Log (dose)

R^2 = 0.946

ED_{50} = 2.29 (1.78-2.95) mg/kg

Figure 6. Log dose response curves of ameltolide (i.p.) against MES at 15, 30 and 60 pretreated times.

(Number in parentheses represent 95% confidence interval)



NTQ 15 min

Probits = 0.70290+3.202 Log (dose)

$R^2 = 0.975$

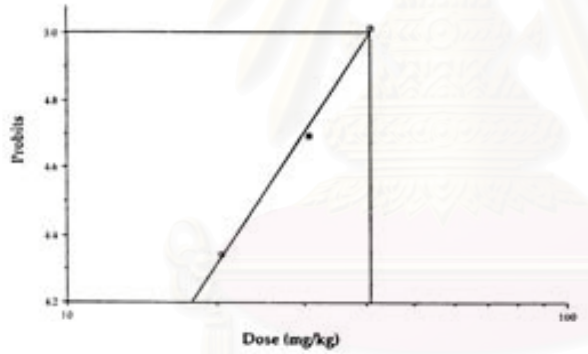
ED_{50} = 13.33 (7.41-23.99) mg/kg

NTQ 30 min

Probits = -1.5049+4.5282 Log (dose)

$R^2 = 0.971$

ED_{50} = 27.32 (19.24-38.79) mg/kg



NTQ 60 min

Probits = 1.4431+2.2101 Log (dose)

$R^2 = 0.995$

ED_{50} = 40.68 (22.48-73.63) mg/kg

Figure 7. Log dose response curves of NTQ (i.p.) against MES at 15, 30 and 60 min pretreated times.

(Number in parentheses represent 95% confidence interval)

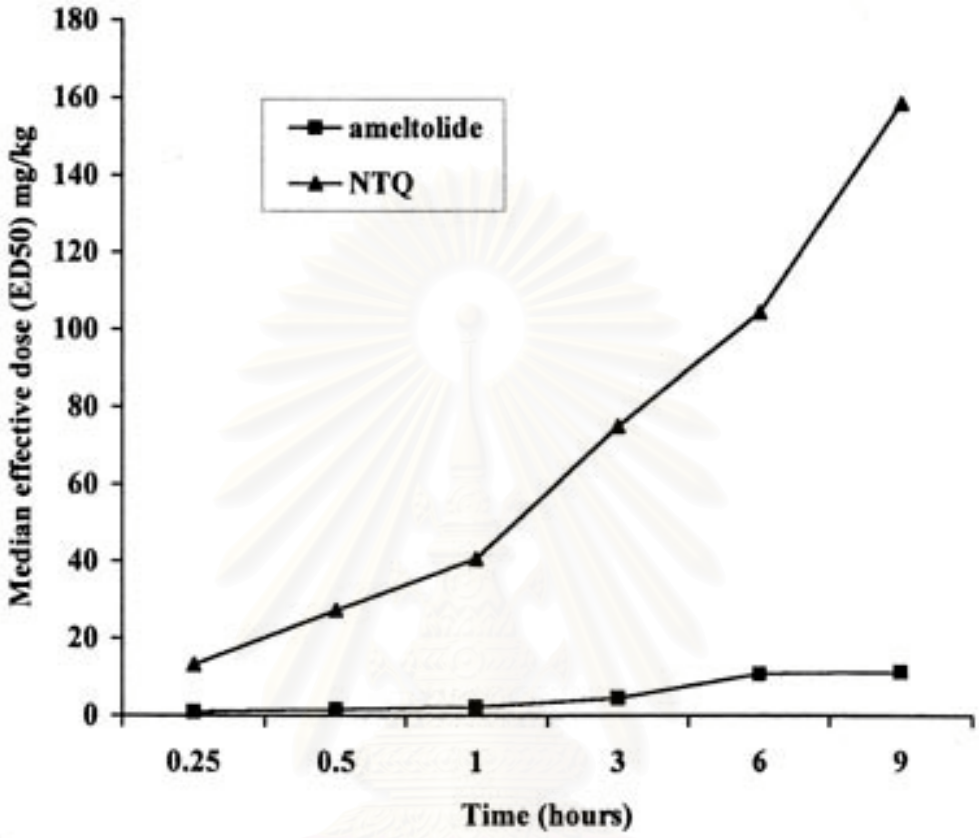


Figure 8. Protection against MES exhibited by ameltolide and NTQ at various pretreated times in mice.

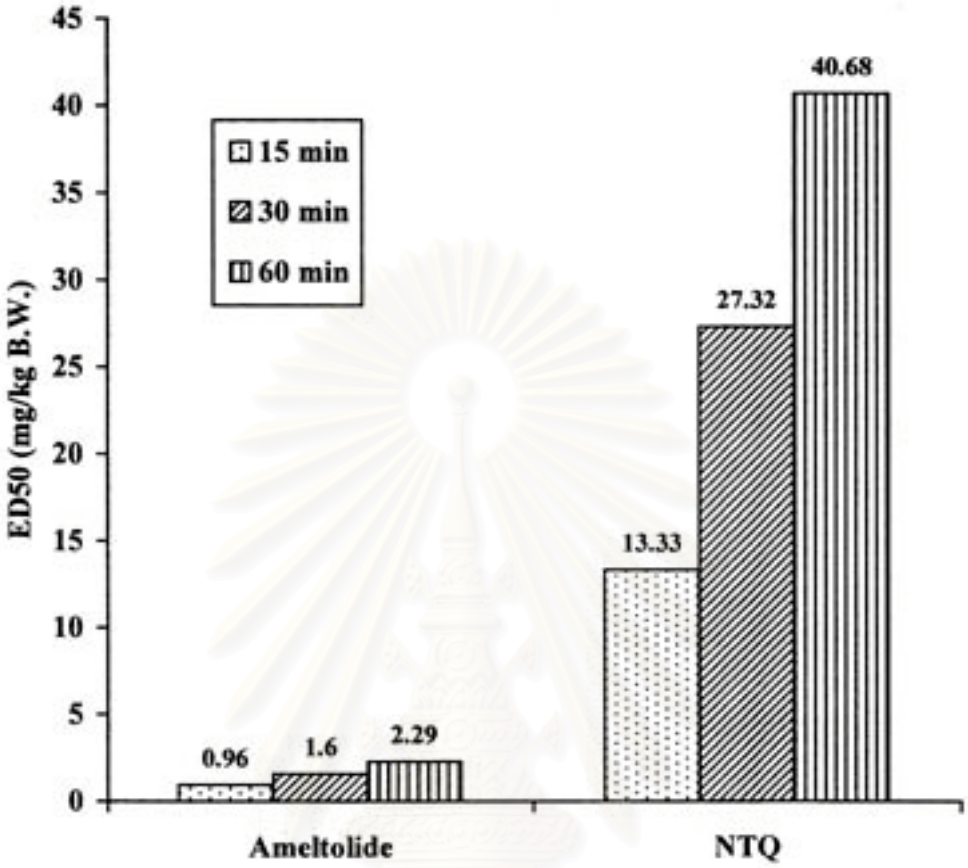


Figure 9. Comparison of ED_{50} at various pretreated times of intraperitoneally given ameltolide and NTQ against MES in mice.

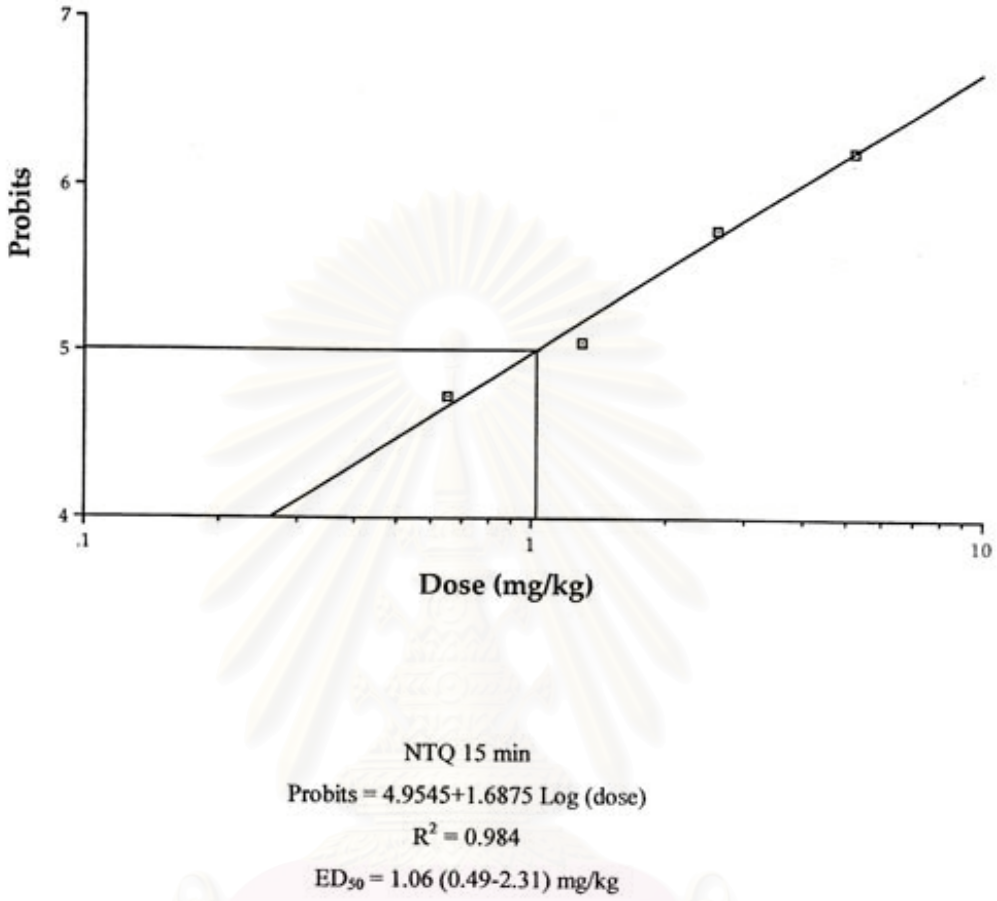
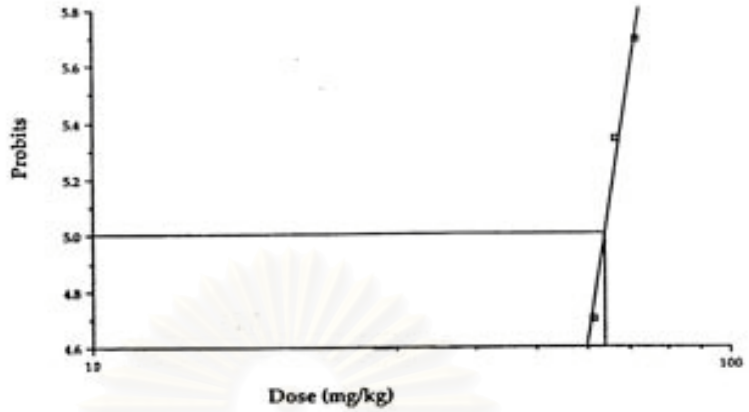


Figure 10. Log dose response curves of NTQ (i.p.) against PTZ at 15 min pretreated time.
(Number in parentheses represent 95% confidence interval)

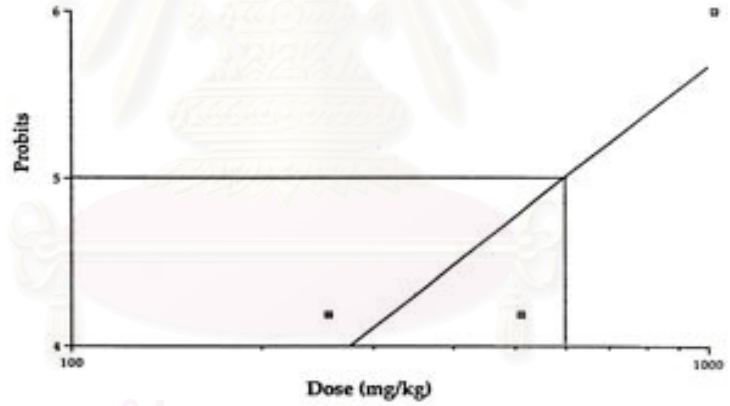


Ameltolide

Probits = -21.661+14.836 Log (dose)

$R^2 = 0.979$

LD_{50} = 62.67 (57.50-68.31) mg/kg



NTQ

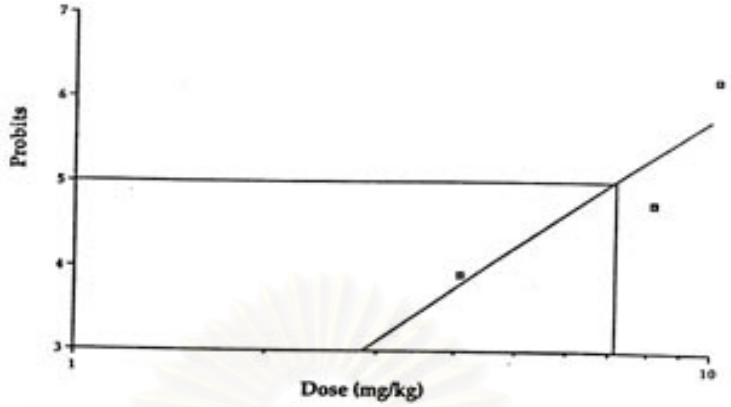
Probits = -3.3507+3.0063 Log (dose)

$R^2 = 0.750$

LD_{50} = 599.42(389.23-923.11) mg/kg

Figure 11. Log dose response curves of acute toxicity (lethality) of ameltolide and NTQ (i.p.) in mice.

(Number in parentheses represent 95% confidence interval)

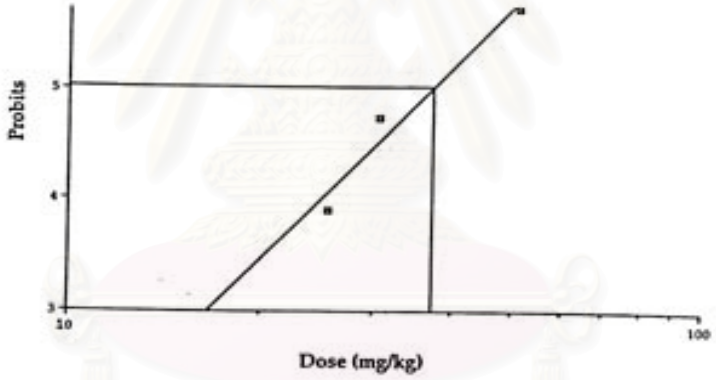


Ameltolide

Probits = 0.66721+5.0609 Log (dose)

R^2 =0.813

TD_{50} = 7.18(4.60-11.20) mg/kg



NTQ

Probits = -3.9617+5.7029 Log (dose)

R^2 = 0.954

TD_{50} = 37.28(28.24-49.21) mg/kg

Figure 12. Log dose response curves of neurotoxicity exhibited by ameltolide and NTQ (i.p.) in mice at optimal pretreated time.
(Number in parentheses represent 95% confidence interval)

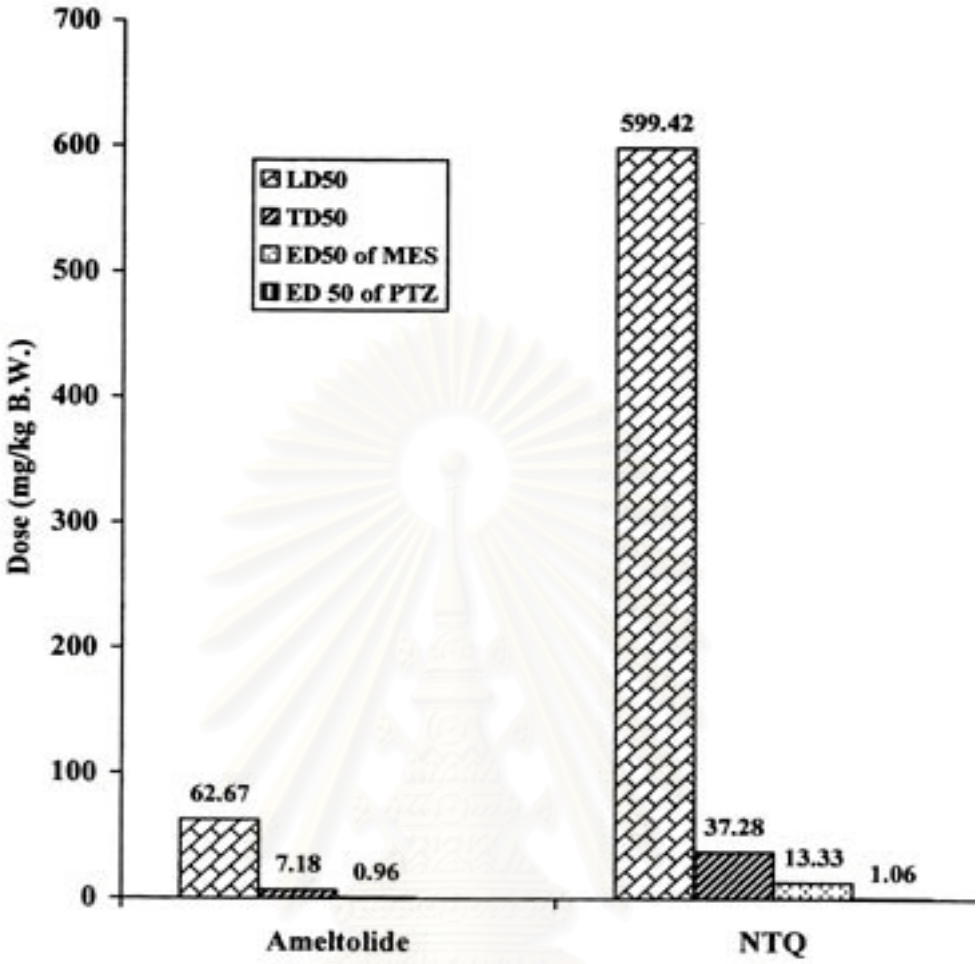


Figure 13. Illustration of the LD_{50}, TD_{50}, and ED_{50} elicited by an intraperitoneal administration of ameltolide and NTQ in MES and PTZ models.

Table 6. ED_{50} ,TD_{50} ,LD_{50} ,PI (TD_{50} /ED_{50}) and relative safety margin (LD_{50} /ED_{50}) of an intraperitoneal administration of ameltolide and NTQ in MES and PTZ models.

Parameter	Model	Ameltolide (mg/kg)	NTQ (mg/kg)
ED_{50}	MES	0.96	13.33
	PTZ	>10.00	1.06
TD_{50}	Rotorod	7.18	37.28
LD_{50}	-	62.67	599.42
PI (TD_{50}/ED_{50})	MES	7.48	2.80
	PTZ	-	35.17
Relative Safety Margin (LD_{50}/ED_{50})	MES	65.28	44.97
	PTZ	-	565.49

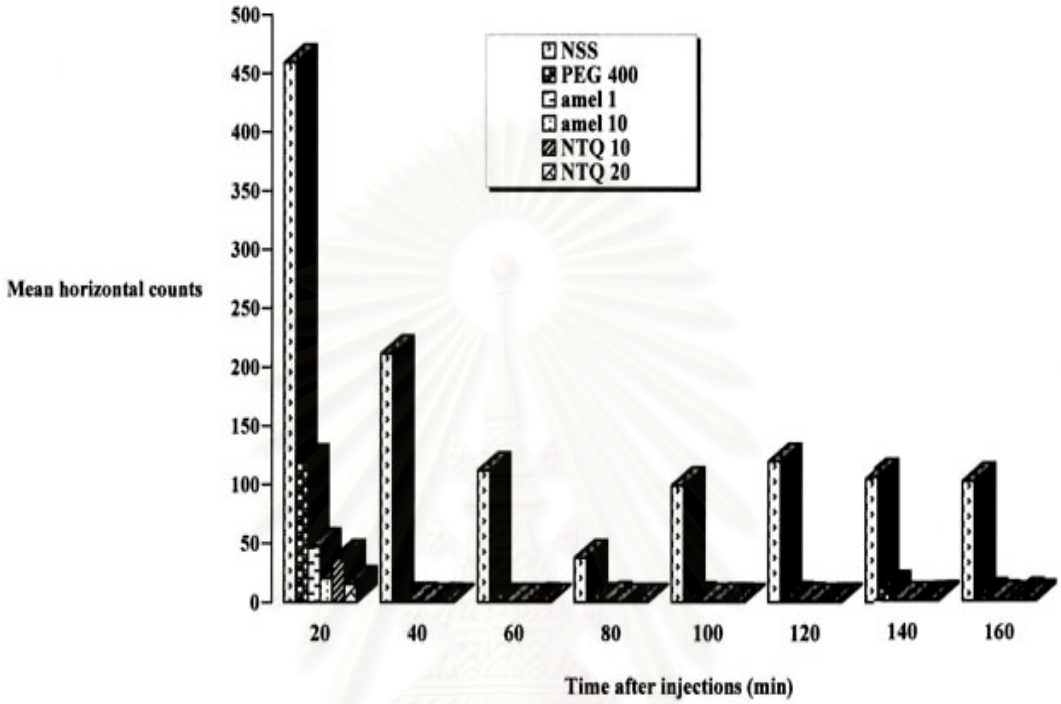
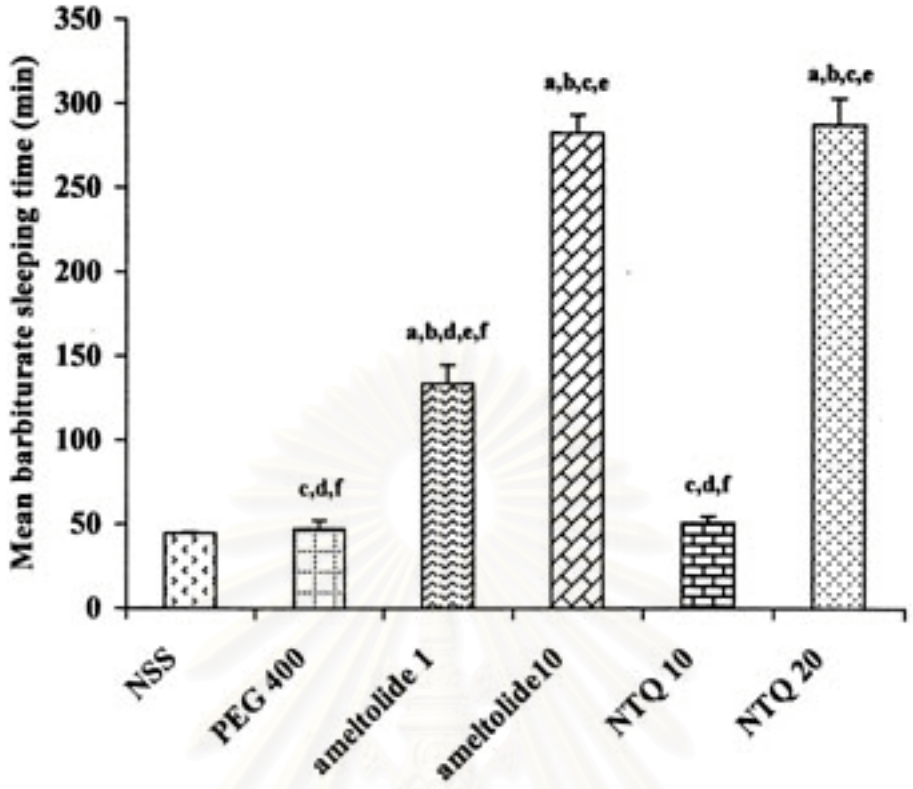


Figure 14. Effects of intraperitoneally administered of ameltolide or NTQ on horizontal counts of locomotor activity in mice at various times. Results are expressed as mean values (S.E.M. is not shown).



[a] p<0.05 denotes statistically significant difference from NSS

[b] p<0.05 denotes statistically significant difference from PEG 400

[c] p<0.05 denotes statistically significant difference from ameltolide 1 mg/kg

[d] p<0.05 denotes statistically significant difference from ameltolide 10 mg/kg

[e] p<0.05 denotes statistically significant difference from NTQ 10 mg/kg

[f] p<0.05 denotes statistically significant difference from NTQ 20 mg/kg

Figure 15. Effects of intraperitoneally administered ameltolide or NTQ on barbiturate sleeping time (mean+S.E.M) in mice.

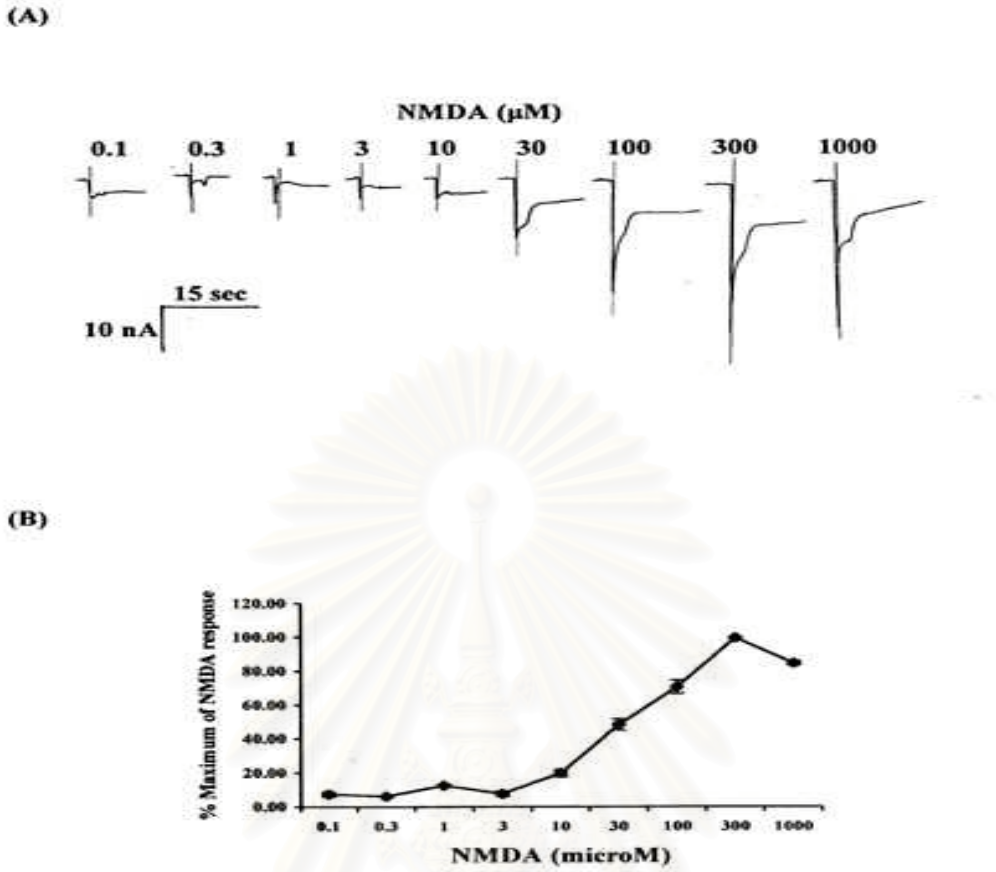


Figure 16. Pharmacological properties of the NR1a/NR2B receptors expressed in *Xenopus* oocytes injected with mRNA (A): Different concentrations of NMDA plus 10 μM of glycine applied to an oocyte induced inward currents denoted by downward deflection of the trace. (B): Log concentration-response curves of NMDA. Each point represents the mean+S.E.M of 4 oocytes. The EC_{50} value was 18.94 μM. Oocytes were voltage clamped at –70 mV.

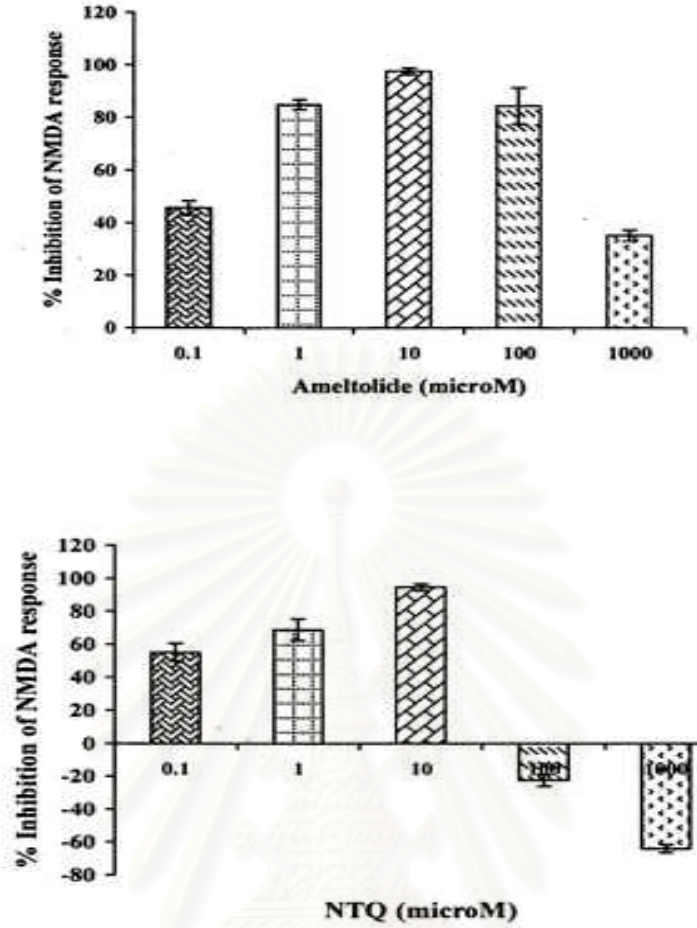


Figure 17. Inhibition of 30 μM NMDA induced currents by ameltolide and NTQ. Inhibitory effects of ameltolide and NTQ on 30 μM NMDA induced currents were 97.59+1.29 % and 94.54+2.03 %, respectively. Oocytes were voltage clamped at –70 mV. Each point represents the mean+S.E.M. of 3 oocytes.

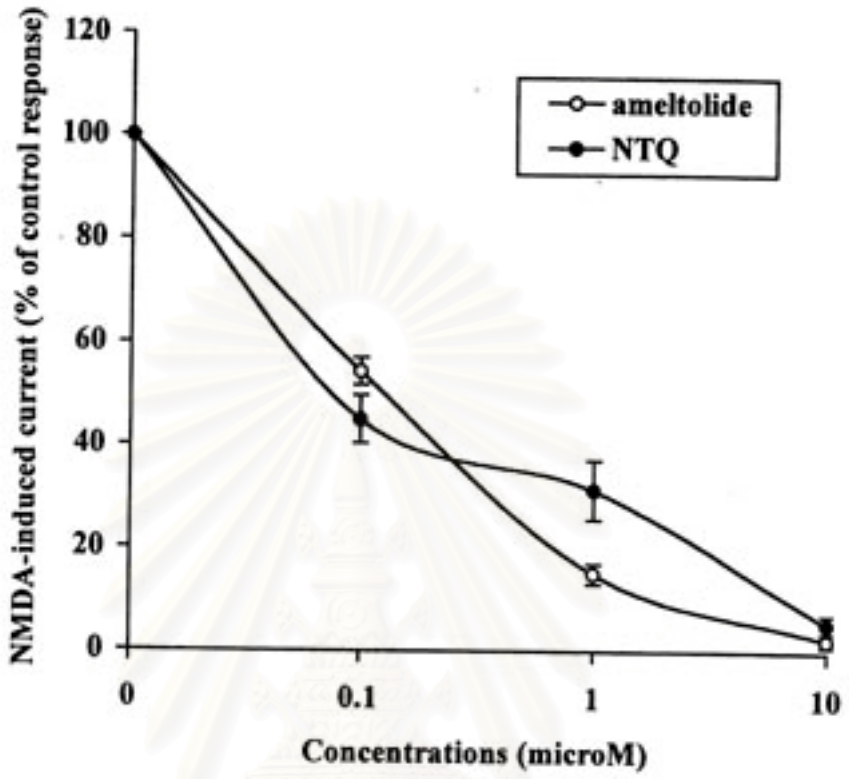


Figure 18. Concentration-inhibition curves for ameltolide and NTQ in *Xenopus* oocytes expressing heteromeric NR1a/NR2B receptor. Currents induced by NMDA (30 μM; with 10 μM glycine) were measured in the presence of various concentrations of ameltolide and NTQ. Data are expressed as a percentage of control NMDA currents. IC_{50} values of ameltolide and NTQ were 0.12 and 0.10 μM. Values are mean+S.E.M. from 3 oocytes at each concentration.

CHAPTER IV

DISCUSSION AND CONCLUSION

The MES and PTZ tests were used in an initial screening for anticonvulsant activity of an ameltolide analog, NTQ, in comparison to its parent compound, ameltolide. Both models were selected on the ground that they are most commonly used models and are highly reproducible. Furthermore, results in these two models provide clues to clinical efficacy as well as possible mechanism of the test compound (Rogawski and Porter, 1990). The MES test is an excellent animal model for the identification of new AEDs that block seizures spread and as such are likely to be effective for the management of generalized tonic-clonic seizures in humans. This particular test has been used extensively in the search for new AEDs since Putnam and Merritt successfully identified phenytoin in a systematic screening program. Their demonstration that it was feasible to discover a clinically effective AED using an experimental animal model provided the rationale for the introduction of the MES test into the routine screening protocol of numerous laboratories (White et al., 1995 and Putnam and Merritt, 1937). On the other hands, the PTZ test is an effective animal model that identifies those compounds that raise seizure threshold. This test would be useful for identifying drugs effective in the treatment of absence seizures (White et al., 1995).

The fact that ameltolide is very potent, through with narrow spectrum, attracted many investigators to further modify its structure searching for ameltolide analogs with improving pharmacological profiles (Clark, 1988; Potts et al., 1989; and Robertson et al., 1991). Many of them have been found to be ineffective while some of them became a board spectrum anticonvulsant exhibiting protection in both MES and PTZ models (Diouf et al., 1997; and Vamecq et al., 1998). NTQ is one among the latter. The results of the present studies demonstrated that intraperitoneally given NTQ demonstrate a board spectrum anticonvulsant activity in MES and PTZ tests (Figure 7, 10; Table 6). In accordance to previous work (Clark, 1988), ameltolide exclusively exerts its anticonvulsant activity in MES but not the PTZ model (Figure 6,

Table 6). NTQ was less potent than ameltolide, exhibiting the ED_{50} values of 13.33 mg/kg B.W. whereas the corresponding value for ameltolide was 0.96 mg/kg B.W.

Regarding to duration of anticonvulsant activity, it is apparent that intraperitoneal administration of either ameltolide or NTQ exhibited protection against MES at least until 9 hours after dosing, however, with an increment of the ED_{50} values (Figure 10). The ED_{50} values of NTQ were always higher than those of ameltolide at any given time, suggesting that the lower potency of NTQ than ameltolide throughout the observation period.

As shown in Table 6, NTQ and ameltolide demonstrated the LD_{50} of 599.42 and 62.57 mg/kg B.W., respectively. However in terms of the relative safety margin indicating discrepancy between the effective anticonvulsive dose and lethality, ameltolide seems to be safer than NTQ exhibiting the ratio between LD_{50} and ED_{50} at 65.28 whereas the corresponding value for NTQ is 44.97.

At therapeutic doses, an ideal AED should have low unwanted effects such as sedation, impairment of motor function or other adverse effects. The rotorod test of Dunham and Miya (1957) is the most commonly used screening test to estimate the minimal neurological deficit in experimental animals. As illustrated in Table 6, the TD_{50} of intraperitoneally given ameltolide and NTQ were 7.18 and 37.28 mg/kg B.W., respectively, resulting in neuroprotective indices ($PI=TD_{50}/ED_{50}$) of 7.48 for ameltolide and 2.80 for NTQ. Since it has been previously proposed that compounds with an estimated PI of at least 2 in the MES model should be further evaluated (Loscher and Nolting, 1990), the present finding renders such an opportunity for both compounds.

Determination of motor activity is considered to be simplest method for detecting CNS sedation effects (Thompson, 1990). Ameltolide (1 and 10 mg/kg B.W.) and NTQ (10 and 20 mg/kg B.W.) depressed locomotor activity to the same extent as did PEG 400 but not NSS, which demonstrated a significant lower degree of depression (Figure 14). Therefore, ameltolide and NTQ in the dose range tested seem to be devoid of a significant depressant effect on locomotor activity. Regarding the effect on barbiturate sleeping time, no statistical significance was noted between the

effects of NSS and PEG 400 (Figure 15). The barbiturate sleeping time was significant prolonged by both low and high doses of ameltolide (1 and 10 mg/kg B.W.) whereas NTQ significantly prolonged the barbiturate sleeping time only when high dose (20 mg/kg B.W.) was given. This finding demonstrated a lower degree of CNS depression of NTQ than ameltolide. Taking together with the results in locomotor activity test, rather low degree of CNS depression is expected from therapeutic dose of NTQ.

Most AEDs exert their antiepileptic properties through a few neurochemical mechanisms that are meanwhile basic pathophysiological mechanisms thought to cause seizures. The activity of numerous drugs is associated to increase in GABAergic activity. An another group of drugs decreases excitatory mechanisms, through the inhibition of ionic channels, or through a decrease in the activity of excitatory neurotransmitters. This understanding may make it possible to develop a group of agonists or antagonists with intended antiepileptogenic actions (Engelborghs et al., 2000; Moshe, 2000; Graeme and Martin, 2001; and Saidon, 2003). For example, excessive NMDA receptor activation is thought to be critical to a wide variety of pathological conditions, such as seizure activity. Subsequently, pharmacological agents that can selectively block subpopulations of NMDA receptors may have important therapeutic applications activity (Monaghan and Larsen, 1996).

Many methods are available for the study of mechanisms underlying membrane transport and signalling. The *Xenopus laevis* oocyte is now established as a transient expression system for voltage- and ligand- gated ion channels, because of their capability to translate exogenous mRNA and expressing functional proteins such as ion channels or receptors on their plasma protein (Sekiguchi et al., 1990; Weber, 1999). Thus, in the present study, it was used to probe the effect of NTQ and ameltolide on NMDA receptors comprising of NR1a and NR2B subunits. Generally the NR1 subunit can form functional homomeric NMDA receptors when expressed in *Xenopus* oocyte but whole cell currents activated by these receptors are very small. Much larger NMDA-evoked currents are seen after coexpression of NR1 and NR2 subunits. Therefore in the present experiment, mRNA of NR1a and NR2B were injected in a vegetal pole in a ratio of 1:4 to minimize the formation of homomeric NR1 receptors (Williams, 1993; Green, 2002).

Electrophysical recording of NMDA-evoked current, in the presence of glycine, demonstrated a linear relationship between current observed and concentration of NMDA 0.1-1,000 μM (Figure 16). The median effective concentration (EC_{50}) of NMDA was found to be approximately 30 μM, which was further used to analyze the effect of NTQ and ameltolide on NMDA receptors. While NTQ and ameltolide did not induce neither current nor a shift in membrane potential of oocyte, NTQ (0.1-10 μM) and ameltolide (0.1-10 μM) exhibited inhibition of NMDA-induced current at NR1a/NR2B receptors with high affinity with the IC_{50} = 0.10 μM and 0.12 μM for NTQ and ameltolide, respectively. Therefore it is suggestive that anticonvulsant activity exert by NTQ and ameltolide in animal model could be, at least, partly, accounted by their inhibiting effect on NMDA receptor.

As shown in Figure17, inhibitory effect of NTQ on NMDA-induced current was evidenced in the concentration between 0.1-10 μM whereas a potentiation of NMDA response was observed in high concentration (100-1000 μM). Comparatively, ameltolide (0.1-1,000 μM) exerted a bell-shaped inhibition of NMDA-induced current. Based on the different profile observed it is likely that NTQ and ameltolide may allosterically modulate activity of NMDA-induced current by different binding sites, which remain to be further elucidated.

In conclusion, the present study identified NTQ as a board spectrum anticonvulsant agent with lower safety margin and lower protective index than those exerted by ameltolide. However in its effective dose, NTQ is expected to produce less unwanted effect regarding CNS depression. Inhibition of excitation of NMDA receptor (NR1a/NR2B) may explain anticonvulsant effect exhibited by ameltolide and NTQ in animal models. Further structural modification of NTQ to improve its safety profile while preserving board spectrum property may lead to a discovery of new ameltolide analogs with favorable pharmacological and toxicological properties.

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APPENDICES

Horizontal counts

Group	Time after injection (min)	N	Mean	S.E.M.
NSS	20	8	459.00	13.37
	40		211.25	6.15
	60		112.00	8.82
	80		37.5	6.40
	100		99.00	4.57
	120		119.38	5.75
	140		104.63	5.98
	160		101.38	14.43
PEG 400	20	8	118.63	21.31
	40		0.88	0.52
	60		0.25	0.16
	80		0.38	0.26
	100		1.25	0.90
	120		1.88	1.04
	140		9.5	5.52
	160		3.25	1.36
Ameltolide 1	20	8	47.13	20.15
	40		1.13	2.48
	60		0.00	0.00
	80		1.63	3.81
	100		0.00	0.00
	120		0.88	1.64
	140		0.13	0.35
	160		1.25	1.49

Group	Time after injection (min)	N	Mean	S.E.M.
Ameltolide 10	20	8	20.13	6.73
	40		0.00	0.00
	60		0.13	0.35
	80		0.25	0.71
	100		0.63	1.06
	120		0.25	0.46
	140		0.25	0.46
	160		0.75	1.17
NTQ 10	20	8	37.38	3.19
	40		0.38	0.26
	60		0.38	0.26
	80		0.25	0.16
	100		0.38	0.38
	120		0.63	0.63
	140		0.75	0.41
	160		3.63	1.43
NTQ 20	20	8	15.25	1.42
	40		0.00	0.00
	60		0.00	0.00
	80		0.13	0.13
	100		0.13	0.13
	120		0.50	0.50
	140		1.00	0.57
	160		2.25	0.82

Barbiturate sleeping time

Group	Count	Mean	S.D.	S.E.M
NSS	8	44.63	1.60	0.57
PEG 400	8	47.00	10.23	5.12
Ameltolide 1	8	133.50	31.35	11.08
Ameltolide 10	8	282.63	29.85	10.55
NTQ 10	8	50.63	11.69	4.13
NTQ 20	8	287.25	45.70	16.16

[a] P<0.05 denotes statistically significant difference from NSS

[b] P<0.05 denotes statistically significant difference from PEG 400

[c] P<0.05 denotes statistically significant difference from ameltolide 1 mg/kg

[d] P<0.05 denotes statistically significant difference from ameltolide 10 mg/kg

[e] P<0.05 denotes statistically significant difference from NTQ 1 mg/kg

[f] P<0.05 denotes statistically significant difference from NTQ 10 mg/kg

[g] P<0.05 denotes statistically significant difference from NTQ 20 mg/kg

%Responses of NMDA at various concentrations after the injection of NR1a/NR2B mRNA in *Xenopus laevis* ooytes

NMDA (µM)	Count	Mean	S.D.	S.E.M
0.1	4	7.37	2.69	1.35
0.3	4	6.14	0.81	0.41
1.0	4	12.46	1.03	0.52
3.0	4	7.60	2.82	1.41
10.0	4	19.83	4.11	2.01
30.0	4	48.34	6.81	3.41
100.0	4	70.60	7.75	3.88
300.0	4	99.47	0.00	0.00
1,000.0	4	84.59	2.41	1.21

%Inhibition of 30 µM NMDA-induced currents by ameltolide

Ameltolide (µM)	Count	Mean	S.D.	S.E.M
0.1	3	45.67	4.65	2.68
1.0	3	84.95	3.26	1.88
10.0	3	97.59	2.23	1.29
100.0	3	84.39	12.12	7.00
1,000.0	3	35.12	3.59	2.07

%Inhibition of 30 µM NMDA-induced currents by NTQ

NTQ (µM)	Count	Mean	S.D.	S.E.M.
0.1	3	55.01	9.38	4.69
1.0	3	68.71	11.49	5.75
10.0	3	94.54	3.51	1.76
100.0	3	-22.53	5.87	2.94
1000.0	3	-64.05	4.23	2.12

VITAE

Miss Nuchjareeya Bhuthabthim was born on 3rd May 1977, in Lob Buri, Thailand. She had graduated with Bachelor Degree in Pharmacy in 1999 from Faculty of Pharmaceutical Sciences, Rangsit University, Pathumthani, Thailand. After graduation, she continues her studies in the Master's Degree program in Pharmaceutical Sciences at Chulalongkorn University.