KU Leuven
Biomedical Sciences Group
Faculty of Pharmaceutical Sciences
Department of Pharmaceutical and
Pharmacological Sciences

Discovery of antiseizure compounds in zebrafish models for the treatment of drug-resistant epilepsy

Jing Li

Jury:

Promoter:	Prof. Dr. Peter A. M. de Witte
Co-promoter:	Prof. Dr. Walter Luyten
Chair:	Prof. Dr. Myriam Baes
	Prof. Dr. dr. Lieven Lagae
	Prof. Dr. Joris Vriens
	Prof. Dr. Luc Pieters
	Prof. Dr. Ilse Smolders

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LIST OF ABBREVIATIONS

ACSF	Artificial cerebrospinal fluid
ADD	Antiepileptic Drug Development
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASD	Antiseizure drug
BBB	Blood/brain barrier
BNFCs	Benign familial nocturnal convulsions
CB	Cannabinoid
CBD	Cannabidiol
CBZ	Carbamazepine
CT	Computed tomography
DA	Dopamine
DBS	Deep brain stimulation
didy	*double indemnity*
dpf	days post fertilization
DMSO	Dimethyl sulfoxide
DS	Dravet syndrome
EEG	Electroencephalography
EKP	Ethylketopentenoate
EMA	European Medicines Agency
FDA	Food and Drug Administration
FFA	Fenfluramine
5-HT	5-hydroxytryptamine
GABA	γ-aminobutyric acid
GAD	Glutamic acid decarboxylase
GAT1	GABA transporter 1
GECIs	Genetically-encoded calcium indicators
GLUT1	Glucose transporter 1 deficiency
GPR55	G protein-coupled receptor 55
ICA	Independent component analysis
ILAE	International League Against Epilepsy
iZAP	Integrated zebrafish analysis platform
KD	Ketogenic diet
LEV	Levetiracetam
LFP	Local field potential
LTG	Lamotrigine
MES	Maximum electroshock

MO	Morpholino
M. officinalis	*Magnolia officinalis*
MRI	Magnetic resonance imaging
MS	Mass Spectrometer
MTC	Maximum tolerated concentration
NE	Noradrenaline
NMDA	N-methyl-D-aspartate
norFFA	Norfenfluramine
PEG200	Poly (ethylene glycol) M.W. 200
PHT	Phenytoin
PPAR	Peroxisome proliferator-activated receptor
PSD	Power spectral density
PTZ	Pentylenetetrazole
QOF	Quality of life
RNS	Responsive neurostimulation
RT	Room temperature
RT-qPCR	Real-time quantitative PCR
scPTZ	Subcutaneous pentylenetetrazole
SMEI	Severe myoclonic epilepsy of infancy
STP	Stiripentol
SUDEP	Sudden unexpected death in epilepsy
SV2A	Vesicle protein 2A
TCM	Traditional Chinese Medicine
TPM	Topiramate
TRPV1	Transient receptor potential of vanilloid type 1
VGCC	Calcium channels
VGPC	Potassium channels
VGSC	Sodium channels
VHC	Vehicle control
VNS	Vagus nerve stimulator
VPA	Valproate
WISH	Whole-mount in situ hybridization
ZFIN	Zebrafish Information Network

CHAPTER I

General Introduction

1. Epilepsy and epileptic seizure

1.1 Epilepsy

Epilepsy, derived from the Greek word *epilambanein*, meaning "to seize" or "to attack", refers to the common clinic characteristic of the disease.[1] In 2005, epilepsy was conceptually defined as a brain disorder characterized by an enduring predisposition to generate epileptic seizures.[2] Later in 2014, in order to emphasize the severity and impact of epilepsy, the International League Against Epilepsy (ILAE) defined epilepsy as a brain disease, rather than a brain disorder.[3] Epilepsy is clinically diagnosed when one of the following conditions are presented: (i) at least two unprovoked (or reflex) seizures occurring more than 24 hours apart; (ii) one unprovoked (or reflex) seizure and a probability of further seizures similar to the general recurrence risk after two unprovoked seizures (at least 60%) occurring over the next 10 years; and (iii) diagnosis of an epilepsy syndrome.[4] Until now, over 70 million people are affected by epilepsy,[5] with an annual incidence estimated in the ranges 40-70/100,000 for adults and 41-187/100,000 for children,[6] causing it to be one of the most common neurological diseases globally. In addition, epilepsy is always accompanied with neurological, psychiatric and cognitive comorbidities, that seriously affect quality of life (QOF) and are a heavy burden for patients.[7]

1.2 Epileptic seizure

The ILAE gives an up-to-date definition of epileptic seizure as the "transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain."[8] Therefore, abnormal excessive neuronal activity, or the imbalance of excitatory and inhibitory processes of the synchronous neuronal activities in the brain, are regarded as causing epileptic seizures.[7] Seizure can be provoked (resulted from acute injury, toxin, alcohol withdrawal or due to metabolic abnormalities like hypoglycemia) or unprovoked.[9] Unprovoked seizure may be a consequence of potential epilepsy.[9] Among patients with epilepsy, 20-30% experience seizure more than once a month, 12% once a week, and 8% undergo daily seizures.[10] The symptoms of seizure vary from objective signs to subjective symptoms, including body shaking, uncontrollable twitching, stiffening, difficulty responding and even unconsciousness.[7,11]

1.3 The classification of seizure and epilepsy

The classification framework for epilepsy and seizure has an important value in guiding clinic diagnosis and treatment, benefitting epilepsy research and promoting novel therapies. In 2017, the ILAE issued a revised operational classification of seizure and epilepsy, with a system defining three levels: seizure type, epilepsy type and/or epilepsy syndrome.[12] Possible etiology and comorbidities were incorporated in each level of the new classification (Figure I-1).[13]

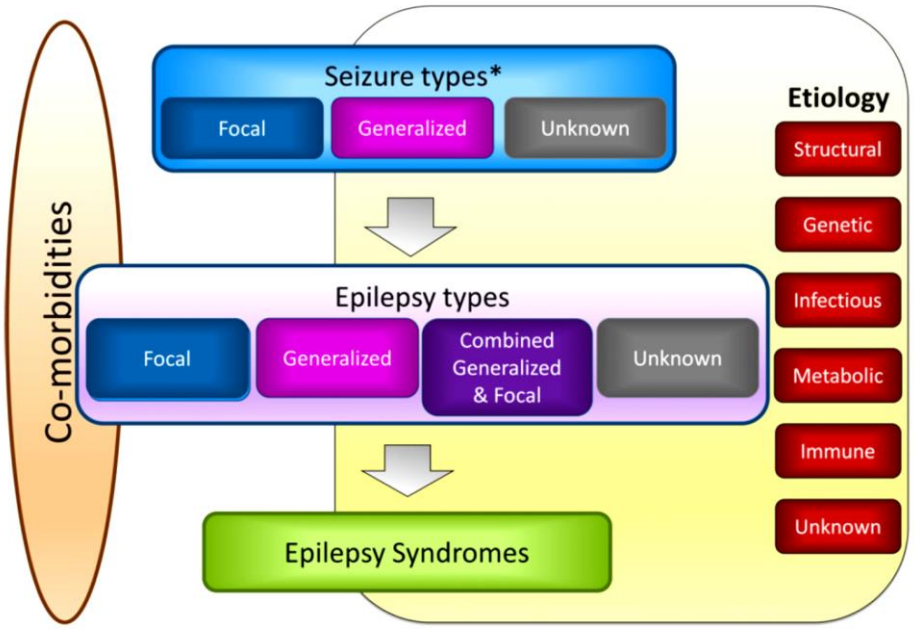

Figure I-1. The classification of the epilepsies published by ILAE in 2017. (from Scheffer *et al*., 2017)[13]

1.3.1 *Seizure type*

Based on the initial manifestations and whether the origin of seizure in the brain is focal or generalized, seizure type is classified into a) focal onset, b) generalized onset, and c) unknown onset (Figure I-2). Focal-onset seizure refers to the seizure "originating within networks limited to one hemisphere", whereas generalized-onset seizure is conceptually defined as "originating at some point within, and rapidly engaging, bilaterally distributed networks (in both hemispheres)".[12] Unknown onset means the seizure onset is not clearly located, but certain characteristic manifestations of epilepsy are still in evidence.[14] The focal-onset seizure can be further categorized based on the awareness level. Focal aware indicates that awareness is retained, otherwise it will be defined as impaired awareness. The next optional classifiers in focal onset are motor and nonmotor seizures, followed by some specific types, such as the myoclonic (irregular, brief focal jerking) activities of motor onset behaviors, and the autonomic

(gastrointestinal sensations) activities of nonmotor onset behaviors. In the case of generalized-onset seizures, they are always accompanied with impaired awareness, so that the main subdivision is directly into motor and nonmotor/absence seizure types. For unknown-onset seizures, excluding motor and nonmotor subcategories, if insufficient information is available to classify the seizure, it may be grouped into the unclassified type (Figure I-2). However, if more information is obtained later, the unknown onset seizure can be re-classified.[15,12]

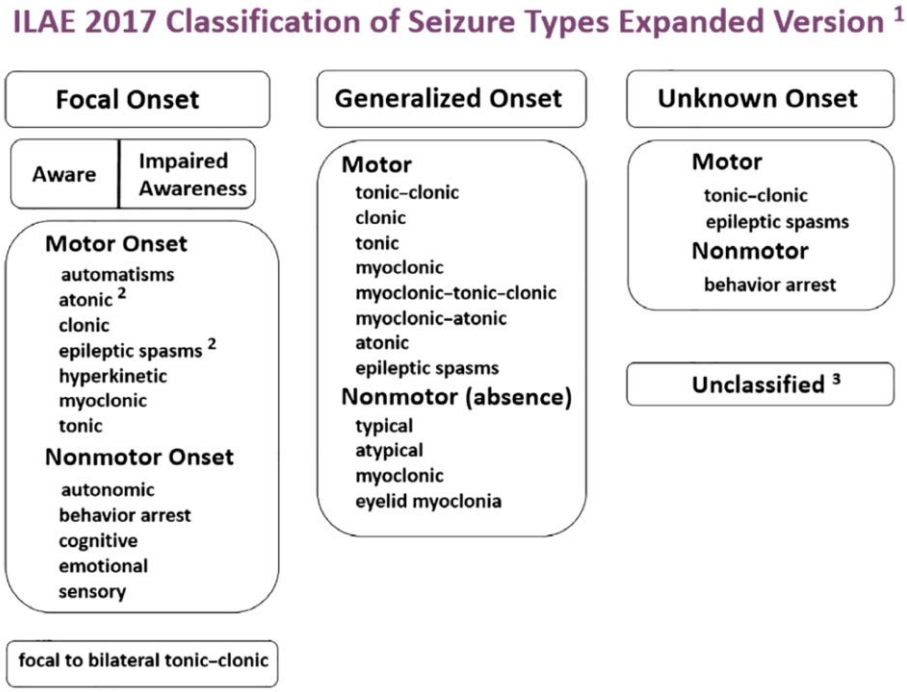

Figure I-2. The classification of seizure types published by ILAE in 2017. (from Fisher *et al*., 2017)[12]

1.3.2 *Epilepsy type*

The epilepsy type should be identified after the confirmation of the seizure type.[14] On one hand, similar to seizure type, the epilepsy types are a) focal, b) generalized, c) unknown, and d) combined generalized and focal type (Figure I-1). On the other hand, the classification of the epilepsy type covers a broader scope than that of seizure types. For example, multiple seizure types should be taken into consideration. Moreover, the classification of epilepsy type relies more on electroencephalography (EEG) findings and neuroimaging (e.g. magnetic resonance imaging (MRI)) features than classification of seizure, and also additional information from the syndrome and etiology.[14] For example, while the diagnosis of focal epilepsy is made on clinical grounds, this diagnosis is supported by a focal epileptic discharge typically shown in EEG signals, associated with both the unifocal and multifocal seizures limited to one hemisphere.[13] Patients with generalized epilepsy show spike-wave activity on EEG, which may

represent multiple generalized seizure types.[3,13] Combined generalized and focal epilepsy, a newly defined epilepsy type, is used for patients having both seizures types. Otherwise, if the available evidence is not fitting any of the above types, an unknown epilepsy type can be assigned.[3]

1.3.3 *Epilepsy syndrome*

As the third level, the diagnosis of an epilepsy syndrome needs more detailed information. In the clinic, the physician needs to consider a combining cluster of features, such as EEG findings, neuroimaging studies, age-dependent features, seizure triggers, diurnal variation, and also related comorbidities (e.g. intellectual and psychiatric problems).[3,16] Then a specific accepted epilepsy syndrome will be proposed. However, a formal full-list ILAE recognized epilepsy syndromes is not yet available.

Considering its critical impact on the management and therapeutic options, the underlying epilepsy etiology is addressed by the updated classification during the entire diagnostic pathway. Six etiologic categories have now been defined: a) structural, b) genetic, c) metabolic, d) abnormal immune, e) infectious, and f) unknown etiology (Figure I-1).[13] A structural etiology is assigned in the case of structural abnormality, which may be detected by EEG and neuroimaging.[13] The cause of a structural abnormality could be acquired (e.g. from stroke and brain tumors) or genetic (e.g. malformations of cortical development).[13] In the case of a genetic etiology, a presumed or known genetic variant is the cause of the epilepsy.[14] This could be inherited or caused by a *de novo* gene variant in the patients.[13,17] A notable example is *SCN1A* (encoding the voltage-gated sodium channel), the mutant of which is regarded as the cause for Dravet syndrome and genetic epilepsy with febrile seizure plus.[13] In some cases the origin of epilepsy and seizure is a metabolic disorder (e.g. glucose transporter 1 (GLUT1) deficiency). A genetic defect is present for a majority of the epilepsy-related metabolic disorders. Infectious etiology is the most common etiology of epilepsy, which could be acquired by viral, bacterial, or, occasionally, parasitic brain infections. The category of immune etiology refers to epilepsy which is due to an immune disorder (e.g. autoimmune encephalitis). Finally, an unknown etiology can be assigned if any classified cause is absent.[13] For a patient with epilepsy and seizures, it is possible that one or more etiological categories are present at the same time.[16]

Furthermore, attention to the presence of comorbidities at each level of the classification is essential (Figure I-1), since that it aids to early identification, diagnosis, and appropriate management.[13] As a matter of fact, more than 50% epileptic patients have at least one comorbid

disorder, such as learning, behavioral, psychiatric (e.g. depression, anxiety) and even somatic (e.g. type 1 diabetes) problems.[5] Moreover, epilepsy patients are at higher risk of developing several other diseases like dementia, migraine, heart disease, peptic ulcers, and arthritis.[18]

1.4 The diagnosis of epilepsy and epileptic seizure

Due to the complexity of epilepsies, an accessible standard diagnostic manual is not yet ready. In the clinic, a myriad of epilepsy mimics (e.g. psychogenic non-epileptic attacks) and seizure mimics (e.g. from convulsive syncope) mean there is a high chance of misdiagnosis. Therefore, differential diagnosis must be taken into account. A detailed history (family and personal history, medical history, and even psychosocial history), together with a reliable eyewitness account (e.g. tongue biting and postictal confusion), and home videos of events are crucial to diagnosis.[5,19,20] Also the patients should undergo a thorough clinical examination using best available diagnostic tools, for instance, the standard EEG, long-term video-EEG, and neuroimaging (e.g. MRI and computed tomography (CT)).[5] Laboratory investigations, such as blood glucose, blood counts, electrolyte panels (particularly sodium), lumbar puncture, toxicity screening (e.g. urine toxicity), and measuring serum neurological autoantibodies, can also provide extra information and help greatly in identifying specific causes. Recently, advances in genetic tests (including array comparative genomic hybridization, candidate epilepsy gene panels, and whole-exome sequencing) have dramatically benefited the diagnosis of genetic variants-related epilepsies.[7]

2. Management of epilepsy

The ultimate aim of the management of epilepsy is to control seizures (seizure free or at least reduce seizure), minimize adverse effects of treatment, and preserve good quality of life (QOL) in patients.[7] For most patients with epilepsy, pharmacological intervention is the first-line treatment. The physician will generally suggest individualized pharmacological treatment for a patient, considering the epilepsy and seizure type, the specific circumstances (e.g. age) of the patients, as well as the antiseizure drug (ASDs) efficacy and its pharmacokinetic and tolerability profile.[7] Around 70% of patients can achieve seizure control after commencing ASDs treatment. For those who fail to control their seizures by use of ASDs, non-pharmacological treatments options (surgery, neurostimulation and dietary therapies) should be considered.

2.1 Antiseizure drugs (ASDs)

2.1.1 *The three generations of ASDs*

Until now, around 30 drugs have been approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA).[7,21] They can be classified into three generations (Figure I-3). The first generation ASDs marketed from 1857 to 1958, including for example phenobarbital (1912), are regarded as the start of modern ASDs development. However, most of them are modifications of the barbiturate structure, and have quite low efficacy-to-tolerability characteristics.[7,22] From 1960s, the second generation ASDs represented by carbamazepine, diazepam and valproate were introduced, with a better tolerability.[23] Then, during the 1970-1988, the first Antiepileptic Drug Development (ADD) Program started.[23] Meanwhile, advances in clinical trial methodology (e.g. the application the randomized controlled trials) also led to a better understanding of the properties of available drugs and faster novel therapeutic discovery.[22] All of these advances contributed to the development of the third generation ASDs, and more than 16 new ASDs have been approved and marketed since the 1990s.[24] This new generation has many improvements in terms of pharmacokinetic properties and tolerability, and exert fewer drug interactions and adverse effects.[25] However, they are no more effective for seizure control than the 1st and 2nd generation ASDs, and are as prone to drug-resistance.[7]

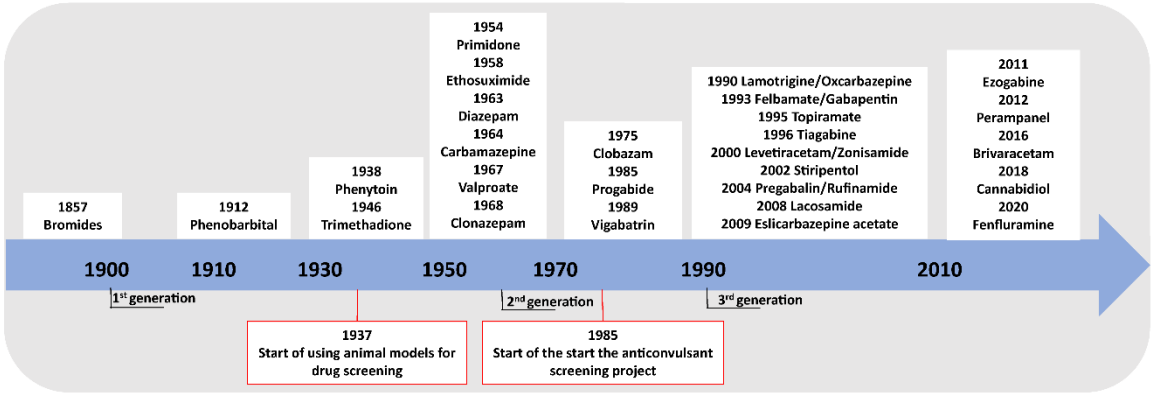

Figure I-3. The year of approval of ASDs from 1853 to 2020. (adapted from Löscher *et al.*, 2013 and Perucca *et al.*, 2019).[22,26]

2.1.2 *Mechanism of action of ASDs*

Current ASDs exploit diverse antiseizure mechanisms by modulating various molecular targets (Figure I-4). These targets can be classified into four broad types: a) voltage-gated ion channels, including sodium channel (VGSC), calcium channel (VGCC), and potassium channels (VGPC). b) inhibitory neurotransmission, namely γ-aminobutyric acid (GABA) transmission, where ASDs could enhance GABAergic inhibition through acting on the $GABA_A$ receptors, the GABA transporter 1 (GAT1) or GABA transaminase; c) excitatory neurotransmission, namely glutamate transmission, involving three types of ionotropic glutamate receptors which can mediate the attenuation of synaptic excitation: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxa-zole-propionate (AMPA) and kainate receptors;[27] and d) other molecular targets, mainly the synaptic vesicle protein 2A (SV2A) which functions in synaptic modulation,[27] and new emerging targets, such as serotonin (5-HT)[28] receptor. The current ASDs work on modulating one (e.g. pregabalin) or more (e.g. valproate) of the above molecular targets to exert their therapeutic effects.

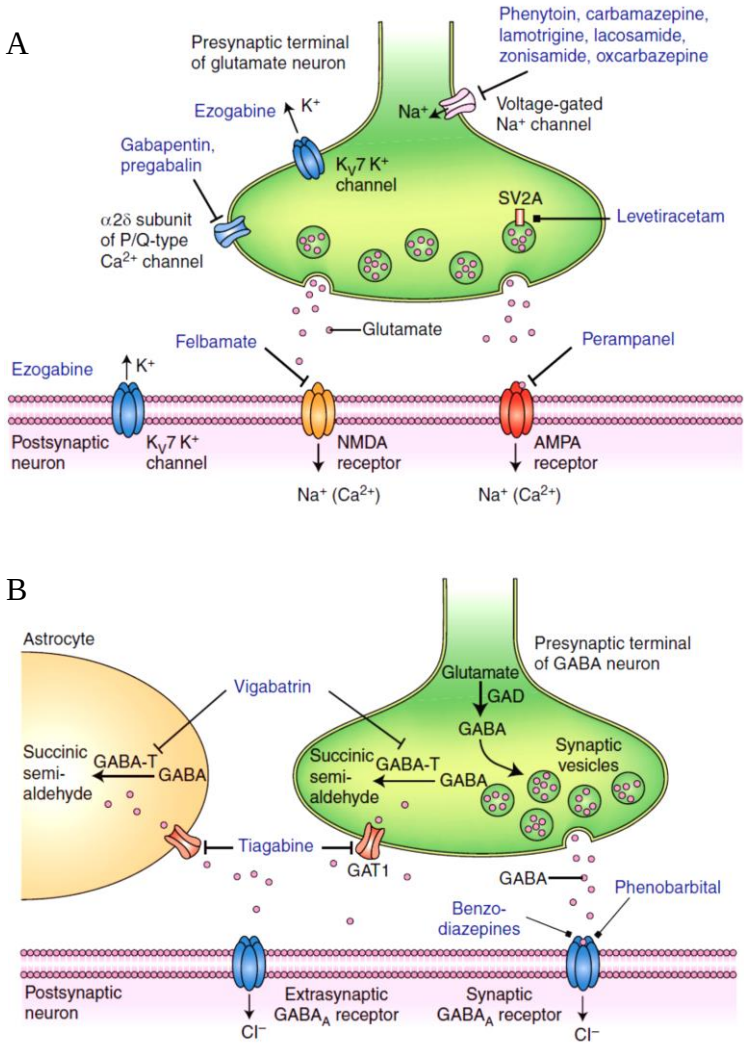

Figure I-4. Diverse molecular targets for antiseizure drugs (ASDs) at excitatory glutamatergic synapses (A) and at γ-aminobutyric acid (GABA)ergic synapses (B). AMPA: α-amino-3-hydroxy-5-methyl-4-isoxa-zole-propionate; GABA-T: GABA transaminase; GAT1: GABA transporter 1; GAD: glutamic acid decarboxylase; NMDA: N-methyl-D-aspartate; SV2A: synaptic vesicle protein 2A. (from Rogawski *et al*., 2016)[27]

2.1.2.1 Voltage-gated ion channels

1) Voltage-gated sodium channels (VGSC)

The VGSC are heteromeric protein complexes, comprising of a large α subunit and several smaller β subunits. Each α subunit has four subunit-like homologous domains (DI-IV) and each domain includes six transmembrane segments (S1-S6). VGSC are crucial for the initiation and propagation of action potential in excitable cells (e.g. neurons, myocytes) by modulating sodium ion influx, leading to the successful exploitation of VGSC blockers as ASDs, such as phenytoin, carbamazepine, oxcarbazepine, lacosamide, lamotrigine, and eslicarbazepine acetate.[29–31] The VGSC binding site of these ASDs is typically within the ion-conducting pore

that is mainly formed by the S5 and S6 segments.[32] Normally, the VGSC have three main conformational states: closed, open and inactivated. These conformational states occur consecutively when neurons are undergoing the depolarization process. The classical VGSC-blocking ASDs could trap and stabilize the VGSC in the inactivated state, thereby impeding the ongoing conformational states cycle, resulting in the blockade of the high-frequency repetitive spike firing of the neurons and reduction of the seizure activity.[27,33]

2) Voltage-gated calcium channels (VGCC)

VGCC are expressed in the neuronal cell membrane, conducting an inward calcium ion in response to depolarization. Calcium ions do not only alter the action potential, but also act as important signaling messengers in the neuronal cells. Several ASD (e.g. ethosuximide, pregabalin and gabapentin) can target VGCC channel to unleash their pharmacological activities.[27]

VGCC can be roughly divided into two major categories: low (LVA) and high voltage activated (HVA) channels, based on the membrane voltage range needed for their opening. Both the HVA and LVA channels contain the α1 subunit which forms the ion conducting pore and determines the subtype of the VGCC (Cav1-3). However, the ancillary subunits, namely β, $\alpha_2\delta$ and γ subunits, only exist in HVA channels.[27] The $\alpha_2\delta$1-3 subunits of the HVA channel are widely expressed in the different neurons (e.g. excitatory and GABAergic neurons) of the brain. The $\alpha_2\delta$-2 subunit is particularly involved in epilepsy, as reported by Dolphin *et al*., who showed that $\alpha_2\delta$-2 knockout and mutant mice display a spike-wave epilepsy and/or tonic-clonic seizures.[34] The pharmacological activities of gabapentin and pregabalin have been associated with a reduction in the trafficking of the $\alpha_2\delta$ subunits in the plasma membrane, which inhibits synaptic transmission.[34] There are three T-type VGCC that belong to the LVA and can be individually represented by three types of Cav3 channels, namely Cav3.1, Cav3.2, and Cav3.3. They are highly but differently expressed in the thalamocortical circuit, and might contribute to the spike-wave discharges of generalized absence seizures.[35] The high efficacy of ethosuximide for controlling generalized absence seizures is considered due to its inhibitory effect on the T-type VGCC.[27]

3) Voltage-gated potassium channels (VGPC)

VGPC play a key role in regulating the excitability of neurons through modulating the outflow of potassium ions to restore and maintain the resting membrane potential. For instance, opening of VGPC leads to the efflux of potassium ions, then the consequent hyperpolarization state

results in the stabilization of membrane potential and the reduction of cell excitability.[36] Loss-of-function mutations in certain VGPC could increase abnormal action potential firing, inducing the hyperexcitability of the neurons, and resulting in neurological disorders, like epilepsy.[37] Among VGPC, the Kv7 channel family is believed to be a particular epileptic burst firing "brake",[37] since they can mediate the M current. M current is a type of slowly activating and non-inactivating potassium current in the resting state and initial depolarization, which can raise the threshold for firing an action potential and thus reduce the neuronal excitability.[38] The brain Kv7.2 and Kv7.3 channel are encoded by KCNQ2 and KCNQ3 genes, the genetic defects of which were linked to benign familial nocturnal convulsions (BNFCs).[27,37] Ezogabine (retigabine) was a promising new ASD acting on focal seizures. Several experimental studies provide evidence that its antiseizure activity is highly related to its modulation in the Kv7 channel family, and in particular to its action on the M current.[27] However, long-term use of ezogabine (retigabine) was associated with bluish pigmentation of the skin and nails, and it was withdrawn from the market in 2017.[39]

2.1.2.2 γ-aminobutyric acid (GABA) transmission

GABA is the most important and abundant inhibitory neurotransmitter of brain interneurons, exerting a particularly important function of counterbalancing neuronal excitation.

GABA is formed within the cytoplasm of GABAergic axon terminals from glutamic acid by glutamate decarboxylase (GAD), after which the compound is loaded into synaptic vesicles by the vesicular GABA transporter (VGAT).[40,41] GABA unleashes its action through effects on $GABA_A$ and $GABA_B$ receptors.[41] $GABA_A$ receptors, which are ligand-gated chloride channels, represent an important target for ASDs and will be discussed below. $GABA_B$ receptors, which are G-protein-coupled receptors, display a different structure and function from $GABA_A$ receptors and are not a main target of the current ASDs.[27]

Synaptic GABA can be taken back up by GABA transporters and catabolized by GABA transaminase in the glia and presynaptic nerve terminals. An abnormal GABAergic inhibition is related to several acquired and genetic epilepsies. The enhancement of GABAergic inhibition is an important strategy for suppressing seizures, and is a critical mechanism of many current ASDs (Figure I-4).

1) The $GABA_A$ receptors

$GABA_A$ receptor are located in the postsynaptic membrane of the inhibitory synapse. Upon activation, they causes the influx of the chloride ion, resulting in the hyperpolarization of the

neurons.[42] Convulsants (e.g. pentylenetetrazol) often block $GABA_A$ receptors to induce the seizure activity. Conversely, the enhancement of $GABA_A$ receptors-mediated inhibition can exert an antiseizure effect. Therefore, $GABA_A$ receptors are an important molecular target for ASDs such as benzodiazepines (e.g. diazepam, clonazepam) and barbiturates (e.g. phenobarbital).[33] Both the benzodiazepines and barbiturates allosterically modulate $GABA_A$ receptors to exert their antiseizure action. However, their modulation modes are different. In case of benzodiazepines, they act on the γ_2 subunit of $GABA_A$ receptors (e.g. $(\alpha_1)_2(\beta_2)_2\gamma_2$, $\alpha_2\beta\gamma_2$, $\alpha_3\beta\gamma_2$ and $\alpha_5\beta\gamma_2$) to increase the frequency of the chloride channel opening, resulting in enhanced synaptic inhibition. Barbiturates are reported to exert their actions by extending the channel opening time.[27]

2) The $GABA_A$ transporters

There are four types of $GABA_A$ transporters (GAT), namely GAT1, BGT1, GAT2, and GAT3, whose functions are to transport GABA to the neurons and glial cells, which can terminate the action of GABA. Therefore, the GAT blockers can enhance the synaptic availability of GABA, raising its extracellular level, thereby prolonging GABAergic inhibition in the synaptic response. For instance, tiagabine is a highly selective inhibitor of GAT1, and presents a good antiseizure profile in the treatment of focal seizures.[27,43]

3) The GABA transaminase (GABA-T)

After uptake into the neurons and glial cells, GABA is catalysed into succinic semialdehyde and glutamate by a GABA-degrading enzyme, namely GABA-T, in the presence of 2-oxoglutarate.[27] Therefore, the inhibitors of the GABA-T may cause a widespread enhancement of CNS GABA levels.[27,44] ASDs like vigabatrin, designed to selectively and irreversibly inhibit GABA-T, can increase the available level of GABA in the synaptic cleft, resulting in enhanced GABAergic transmission.[44] The GABA-increasing effect of vigabatrin makes it effective in the treatment of refractory complex partial seizures (focal seizures) and infantile spasms.[44]

2.1.2.3 The glutamate receptors

Glutamate is the predominant excitatory neurotransmitter of brain; its level rises during seizures. Glutamate is released from the presynaptic neurons and exerts its excitatory action mainly through several ionotropic receptors, including AMPA, NMDA and kainate receptors. It has been documented that ionotropic glutamate receptors mediate the bulk of fast excitatory neurotransmission in the CNS.[33] Their expression was found altered in the neurons and glial cells after chronic seizures, which contributed to epileptogenesis.[45] Moreover, the participation

of AMPA receptors has been confirmed in both focal epilepsy and secondarily tonic-clonic seizures, so blocking AMPA receptors could impede epileptic synchronization.[27] In addition, the inhibitors of NMDA receptor subtypes have been proven to protect against seizures in *in vitro* and *in vivo* models.[33] Therefore, antagonists of ionotropic glutamate receptors have become a pivotal group of new ASDs.

Many ASDs, such as felbamate, valproate, and topiramate, can target at least one of the above three ionotropic glutamate receptors to exert their action. Of note, differently from the other receptor antagonists, AMPA receptor antagonists exert fewer effects on neuroplasticity and display lower potential for psychosis. So far, perampanel is the only known ASD identified for its selective targeting of the AMPA receptor. It not only exerts a pronounced clinical antiseizure action, but also display less adverse central nervous system effects at its lower doses.[27]

2.1.2.4 The other molecular targets

1) Synaptic vesicle protein 2A (SV2A)

SV2A, a transmembrane glycoprotein, is found in the synaptic vesicles of neurons, particularly in both the GABAergic and glutamatergic neurons of the brain. It acts as a key regulator of neurotransmitter release, involving multiple steps of the synaptic cyclical process, for example, the uptake of neurotransmitter into the vesicle, the transportation of vesicle between the cytoplasm and the presynaptic membrane, synaptic vesicle priming, calcium-dependent exocytosis, neurotransmitter release.[46] Several reports indicate that knocking out SV2A leads to a severe seizure phenotype in animal models. Moreover, SV2A mutations in humans have been associated with intractable temporal lobe epilepsy.[47] Currently, levetiracetam and its analogue brivaracetam display high binding affinities to SV2A protein. Also, they display reduced activity in $SV2A^{+/-}$ seizure mice models, which underlines their molecular target as being the SV2A protein.[46] Although the exact mechanism of current ASDs on SV2A are still obscure, abundant evidence shows that SV2A is a promising target for novel ASDs.[27]

2) Serotonin (5-HT) receptors

5-HT receptors are expressed in several different types of neurons in the brain. They are activated by the neurotransmitter serotonin, and drive a wide range of biological processes, such as appetite, sleep, memory and learning, mood, and epilepsy.[28,48] Recently, an increasing number of reports indicated that 5-HT receptors, especially 5-HT_{1A}, 5-HT_{2C}, 5-HT_{3}, 5-HT_{4} and 5-HT_{7}, are involved in epileptogenesis and/or seizure propagation. The 5-HT_{1A} receptor gene knockout mouse display a lower seizure threshold and higher seizure potency. In addition, 5-

HT receptor agonists have been shown to inhibit both focal and generalized seizures in animal models.[48–50] The assumed mechanism is that subtypes of the 5-HT receptors might directly or indirectly hyperpolarize the glutamatergic neurons, and depolarize the GABAergic neurons through effects on ionic conductance and/or concentrations within cells.[48] These findings all point to the potential of 5-HT receptors as targets for ASDs. New emerging ASDs, such as cannabidiol and fenfluramine, were reported to exert their antiseizure action in part via modulation of 5-HT receptors.[50,51]

2.1.3 *Monotherapy and polytherapy approaches*

It is preferable that patients with epilepsy achieve seizure freedom with single ASDs, as a monotherapy. However, more than 50% of patients fail with the first monotherapy owing to low tolerability or efficacy. Subsequently, an alternative monotherapy, or polytherapy could be considered as options (Table I-1).[39,52] Polytherapy is normally introduced if monotherapy is well tolerated, but only partially effective. Recently, the diverse antiseizure mechanisms, better pharmacokinetic and tolerability profiles of the new generation ASDs result in increased drug compliance, which further promotes the option of polytherapy, especially in the treatment of intractable epilepsy.[53] For example, in the treatment of Dravet syndrome, first line agents such as valproate are usually unable to reach an adequate seizure control in the patients. Therefore, second line ASDs, like stiripentol, and/or clobazam can be added, which can lead to a successful clinical outcome in the patients.[54]

In the use of the polytherapy, several principles should be adhered to: a) avoid negative pharmacokinetic interactions with other concomitants, b) prefer combination of drugs with different mechanisms of action,[39] c) prefer the combinations producing beneficial synergistic efficacy,[39,52] for example the combination of lacosamide and levetiracetam.[55] Otherwise, it might reduce efficacy or aggravate the adverse effects of the ASDs. For example, using two sodium channel blocking-predominated ASDs can lead to neurotoxic side effects owing to their pharmacodynamic interactions.[39]

2.1.4 *The limitation of the ASDs*

2.1.4.1 The adverse effects of ASDs

Eighty percent of patients will suffer adverse effects during their initial treatment with ASDs. In addition, 30-40% of patients will experience undesirable side effects which seriously impair their QOL and even lead to treatment failure. The assumed reason for the adverse effects of

current ASDs is that they utilize molecular targets to balance the hyper- or hypo-excitability of neurons, but this may interfere with normal neurotransmission to a significant extent.[7,26]

The adverse effects of ASDs are highly variable in the patients, ranging from moderate to serious, including weight gain/loss, hypersensitivity reactions, psychiatric and behavioral side effects, and neurological problems (Table I-1).[7] For example, several ASDs, such as valproate, carbamazepine, and perampanel can lead to obesity problems, while weight loss is conversely reported in certain ASDs, such as topiramate, zonisamide, felbamate, stiripentol and rufinamide. In addition, hypersensitivity reactions such as rash, malaise, fever, and even hyponatremia also occur, and most of these are immune-mediated. As the most common adverse effects, psychiatric and behavioral side effects include depressive mood, aggressive behavior and hyperactivity. The primarily neurological side effects, include sedation, dizziness, blurred vision, tremor and even cognitive deficits.[7]

2.1.4.2 The drug interaction in the ASDs

Drug interactions of ASDs can greatly attenuate their efficacy. Commonly used ASDs phenytoin, primidone, carbamazepine, lamotrigine, oxcarbazepine and eslicarbazepine acetate are enzyme inducers (Table I-1) able to stimulate synthesis of a broad range of enzymes, so affecting pharmacokinetic interactions, and consequently reducing the duration and action of other drugs, lowering their efficacy.[26] Therefore, in polytherapy, the use of an enzyme inducer should be avoided.[39,56] In addition, enzyme-inducing ASDs may associate to some comorbidities (e.g. osteoporosis, hyperlipidemia and vascular disease) since they are involved in endogenous metabolic pathways. Therefore, benefits and risks of the use of the enzyme-inducing ASDs should be evaluated.[56]

Table I-1. Characteristics of clinically approved ASDs

ASD	**Efficacy spectrum**	**Mono- or Polytherapy**	**Main limitation**	
			Drug interaction	Common adverse effects
Phenytoin	Focal seizure and generalized tonic-clonic seizure	Mono	Enzyme inducer	Cognitive problem, ataxia, incoordination, dysarthria, nystagmus, diplopia, and hypersensitivity syndrome
Trimethadione	Generalized seizure	-	-	Teratogenicity
Primidone	Focal seizure and generalized tonic-clonic seizure	Mono	Enzyme inducer	Sedation, debilitating drowsiness, dizziness, ataxia, nausea, and vomiting
Ethosuximide	Generalized seizure	-	-	Nausea, abdominal discomfort, anorexia, vomiting, diarrhea, drowsiness, insomnia, nervousness, dizziness, fatigue, ataxia, and behavior changes
Diazepam	Focal and generalized seizure	Poly	-	Sedation, dizziness, depression, fatigue, motor and cognitive impairment, dependence, tolerance (loss of efficacy)

Carbamazepine	Focal seizure and generalized tonic-clonic seizure	Mono	Enzyme inducer	Nausea, headache, dizziness, sedation, tiredness, cognitive impairment, blurred vision, diplopia, nystagmus, unsteadiness, incoordination, tremor and hyponatremia
Valproate	Most seizure types	Mono, Poly	Enzyme inhibitor	Gastric irritation with nausea, vomiting, and anorexia, diarrhea, fatigue, drowsiness, tremor, weight gain, hair loss, peripheral edema, and substantial teratogenicity
Clonazepam	Lennox-Gastaut syndrome and myoclonic seizure	Poly	-	Sedation, drowsiness, nystagmus, incoordination, unsteadiness, dysarthria, tolerance (loss of efficacy)
Clobazam	Lennox-Gastaut syndrome	Poly	-	Sedation, tolerance (loss of efficacy)
Progabide	Focal and generalized seizure, Lennox-Gastaut syndrome and myoclonic seizure	-	-	Clinical hepatotoxicity, not in wide use anymore
Vigabatrin	Focal seizure and infantile spasms	Poly	-	Sedation, fatigue, dizziness, ataxia, bilateral concentric visual field constriction
Lamotrigine	Most seizure types	Mono, Poly	Enzyme inducer	Dizziness, blurred vision, diplopia, unsteadiness, nausea, vomiting, headache, tremor, and rash
Oxcarbazepine	Focal seizure	Mono, Poly	Enzyme inducer	Drowsiness, headache, fatigue, dizziness, blurred vision, diplopia, nausea, vomiting, and ataxia
Felbamate	Focal seizure and drop seizure associated with Lennox-Gastaut syndrome	Mono, Poly	-	Gastrointestinal irritation with anorexia, nausea, vomiting, insomnia, irritability, headache, weight loss, lethal aplastic anemia, hepatic failure
Gabapentin	Focal seizure	Poly	-	Drowsiness, dizziness, ataxia, tiredness, weight gain, cognitive slowing problem and emotional lability
Topiramate	Most seizure types and drop seizure associated with Lennox-Gastaut syndrome	Mono, Poly	-	Cognitive adverse effects, sedation, fatigue, dizziness, ataxia, and depression
Tiagabine	Focal seizure	Poly	-	Dizziness, asthenia, nervousness, tremor, depression, and emotional lability
Levetiracetam	Most seizure types	Mono, Poly	-	Somnolence, dizziness, asthenia, and behavioral effects (e.g. hostility, nervousness, and aggression)
Zonisamide	Focal seizure	Mono, Poly	-	Sedation, ataxia, dizziness, nausea, fatigue, agitation/irritability, and anorexia
Stiripentol	Tonic-clonic seizure associated with Dravet syndrome	Poly	-	Loss of appetite, weight loss, insomnia, drowsiness, ataxia, hypotonia, and dystonia
Pregabalin	Focal seizure	Poly	-	Dizziness, somnolence, increased appetite, weight gain, peripheral edema
Rufinamide	Drop seizure associated with Lennox-Gastaut syndrome	Poly	-	Dizziness, fatigue, somnolence, and headache
Lacosamide	Focal seizure	Mono, Poly	-	Dizziness, headache, nausea, vomiting, diplopia, fatigue, and sedation
Eslicarbazepine acetate	Focal seizure	Poly	Enzyme inducer	Dizziness, somnolence, headache, diplopia, nausea, vomiting, fatigue, and ataxia
Perampanel	Focal seizure and tonic-clonic seizure	Mono, Poly	-	Dizziness, somnolence, headache, fatigue, ataxia, and blurred vision
Bivaracetam	Focal seizure	Mono, Poly	-	Somnolence, dizziness, and fatigue
Cannabidiol	Motor seizure associated with Dravet syndrome	Poly	-	Sedation, fatigue, decreased appetite, and diarrhea

	and Lennox-Gastaut syndrome			
Fenfluramine	Seizure associated with Dravet syndrome	Poly	-	Decreased appetite, diarrhoea, lethargy, and somnolence

Phenytoin, primidone, trimethadione, progabide are not commonly used nowadays. (the table is adapted from Löscher et *al*., 2013, Vossler *et al*., 2018, Devinsky *et al*., 2018, and Abou-Khalil *et al*., 2019, and Lagae *et al*., 2019)[7,26,39,57,58]

2.2 Non-pharmacological treatments

2.2.1 *Surgery treatment*

Surgery treatment is the only potential curative non-pharmacological treatment, offering a high chance in achieving seizure freedom in patients with medically refractory epilepsy (e.g. mesial temporal lobe epilepsy).[5] What's more, successful surgery can improve QOL and long-term psychiatric outcomes, reducing risk of sudden unexpected death in epilepsy (SUDEP).[59] Prior to surgery, a comprehensive pre-surgical evaluation (e.g. high-quality MRI imaging, prolonged video-EEG recordings, neuropsychological evaluation, and psychiatric assessments) is advised to help determine the epileptogenic zone, estimate risks of postsurgical deficits and predict outcomes.[5] The most suitable candidates for epilepsy surgery are patients with refractory focal epilepsy,[59] especially those with an MRI lesion.[7] Patients with these lesions can be completely resected, and typically above 80% remain seizure free over one year.[7] However, the risks of the surgery should also be noted regarding perioperative mortality and morbidity, post-surgery neurological and cognitive deficits.[59] Until now, only a small proportion of patients can be considered as surgical candidates, and the outcome of the surgery may be partial instead of full seizure freedom in many of those patients.[60]

2.2.2 *Neurostimulation*

Patients with drug-resistant epilepsy, who are not eligible for surgical treatment, may be offered neurostimulation as a palliative option. The vagus nerve stimulator (VNS) is the first approved neurostimulation device, comprising a stimulator (or 'pulse generator') which is placed in the chest and connected to the left vagus nerve in the neck through a stimulating wire. It can reduce the potential seizure generation or propagation by sending regular, mild electrical stimulations to the nerve.[9] Currently, the VNS is specifically used for treatment-resistant focal epilepsy.[61] In contrast with the VNS, the two electrodes used for deep brain stimulation (DBS) are placed within the brain (thalamic anterior nucleus). In a long term efficacy of DBS study for five years, around 50% of responders achieved more than 50% seizure reduction.[5,62] Recently, responsive

neurostimulation (RNS) has been approved. It is a closed-loop system, with the electrodes directly implanted at the sites of seizure onset in the brain (e.g. cortical or subcortical). Using RNS, the onset of seizure activity can be detected, then the electrical stimulation is delivered by the neurostimulator to the target seizure-onset zone, preventing progress of the seizure.[9,63] Though neurostimulation provides a new avenue to the treatment of drug-resistant epilepsy, only limited numbers of patients could reach seizure free status so far. In addition, several stimulation-related adverse events have been reported, such as cough, dyspnea, implant site pain and infection.[63,64]

2.2.3 *Ketogenic diet (KD)*

KD is a special high-fat, low-carbohydrate, and low-protein diet, initially used in the treatment of severe epilepsy in children. Recent decades have seen the effective use of KD to treat a range of intractable epilepsies (e.g. glucose transporter protein 1 deficiency syndrome),[7,51] as well as in the inhibition of epileptogenesis.[11] In the clinical use of KD, the John Hopkins Hospital protocol is usually advised, which is a 4:1 ratio of fat (i.e. long-chain triglycerides of 16-20 carbon atoms) to combined proteins and carbohydrates.[65,66,67] Recently, some modified KDs have been developed, including the long-chain triglycerides diet,[68] the Atkins diet or the low-glycemic diet.[7]

These diets can alter the metabolism by using fats as a main energy source, inducing to the liver to catabolize the fatty acids, leading to an increased ketone bodies concentration in the blood, known as a state of ketosis.[69] Ketosis has been reported to promote a reduction in the frequency of epileptic seizure both in animal models and in patients.[66,67,70,71] For example, over 50% of patients treated with KD were found to achieve seizure reduction, and 10% could reach seizure free status.[7,63] The underlying mechanism of KD is still obscure. The mechanism was assumed to relate to its effects on AMPA receptors, potassium channels, GAD enzyme, peroxisome proliferator-activated receptor (PPAR), and mitochondrial biosynthesis.[27,68] However, diverse adverse effects from long-term complications of KD were reported, including gastrointestinal symptoms, nutritional deficiency, metabolic abnormalities, kidney stones, and cardiac abnormalities. Therefore, rigorous clinical trials of efficacy and safety of KD are required in the future.[7,63]

3. Drug-resistant epilepsy

Drug-resistant epilepsy, also termed as pharmacoresistant epilepsy, is defined as the failure of trials of two tolerated, appropriately chosen and used ASDs schedules (whether as monotherapy or in polytherapy) to achieve sustained seizure freedom.[72] It is the major challenge for the pharmacotherapy of epilepsy and affects one third of epilepsy patients. Though considerable advances have been made in the understanding of epilepsy and in developing novel ASDs with diverse mechanisms of action, the percentage of patients with drug-resistant epilepsy is unchanged.[73] Besides, drug-resistant epilepsy is always associated with a heavy burden and adverse side effects of the medication, serious psychiatric and neurocognitive comorbidities, a high risk of SUDEP, and reduced QOL in patients.[7]

3.1 Mechanism of drug-resistant epilepsy

The lack of significant progress in the treatment of drug-resistant epilepsy is thought to be due to the limited comprehension of its underlying mechanism. Recently, several hypotheses have been proposed to explain the neurobiological basis of drug resistance in epilepsy, mainly the pharmacokinetic hypothesis, neural network hypothesis, intrinsic severity hypothesis, gene variant hypothesis, target hypothesis, and transporter hypothesis (Figure I-5).[74]

1) Pharmacokinetic hypothesis

The pharmacokinetic hypothesis supposes that changes in the metabolism and elimination of ASDs (e.g. by abnormally expressed CYPs and drug transporters) can decrease the level of drug in the plasma, so that only limited amounts of ASD pass the blood-brain barrier (BBB) and can act in the brain epileptic focus. For instance, an increased expression of an efflux transporter, like p-glycoprotein, in the peripheral organs (e.g. liver) is reported to coincide with low plasma levels of ASDs in drug-resistant epilepsy patients.[73,74]

2) Neural network hypothesis

The neural network hypothesis proposes that cellular alterations of the neurons that accompany recurrent epileptic seizures, such as seizure-induced neural degeneration, but also synaptic reorganization, could aberrantly remodel the neural network and produce hyperexcitable circuits. This may restrain the endogenous antiseizure system and impede entry of ASDs to neuronal targets, eventually leading to the drug-resistant problem.[73–75]

3) Intrinsic severity hypothesis

The intrinsic severity hypothesis states that the common neurobiological factors which contribute the severity of the disease can also induce drug resistance. In this regard, several clinical conditions, especially where there is increased occurrence of epileptic seizures, might be considered as predictive factors and biomarkers for refractory epilepsy.[74]

4) Gene variant and target hypothesis

The gene mutation hypothesis emphasizes that mutations and polymorphisms of genes, especially those that encode for relevant functional proteins, such as the targeting sites (e.g. neurotransmitter receptors and voltage-gated ion channels) may contribute to a decrease of the sensitivity towards ASDs, leading to inherent drug-resistant epilepsy.[73,74,76]

5) Transporter hypothesis

The transporter hypothesis assumes that increased expression of drug efflux transporter in the the blood-brain barrier and blood-cerebrospinal fluid barrier leads to an overall low brain concentration of ASDs, and consequently to a decrease in their effectiveness.[73,76] The transporter hypothesis is an emerging hypothesis explaining especially drug-resistance against a broad range of ASDs with different therapeutic targets.[76]

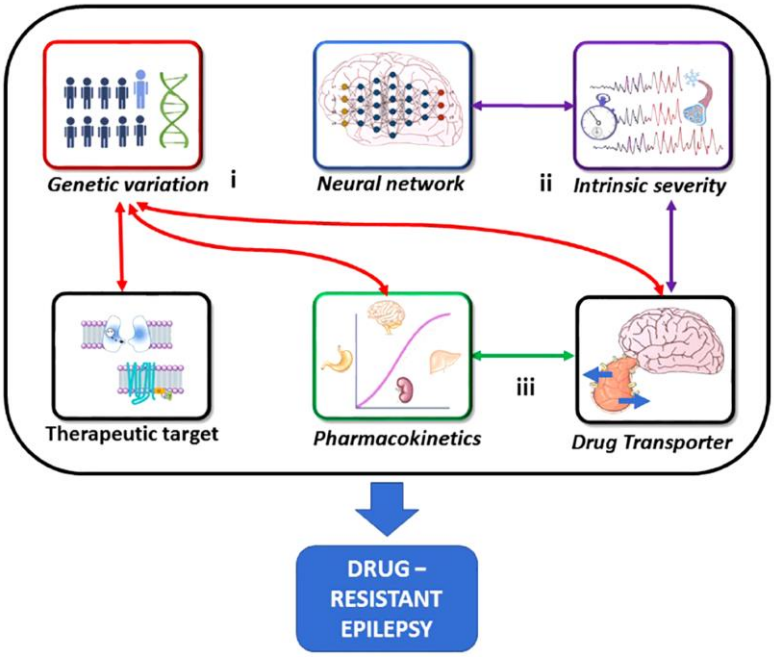

Figure I-5. Diagram showing the classical hypotheses of drug resistance in epilepsy and the connections between the different accepted hypotheses. (from Pérez-Pérez *et al.*, 2019)[73]

Each hypothesis can explain the underlying mechanism of drug-resistant epilepsy to some extent, but drug resistance is most likely multifactorial. In addition, a close relationship exists

between different hypotheses (Figure I-5), for example, gene variations can lead to the abnormal expression of the functional protein in therapeutic targets and/or pharmacokinetics of the ASDs. However, further clinical trials are still needed to validate the above hypotheses.

3.2 Dravet syndrome (DS)

DS was initially described in 1978 as severe myoclonic epilepsy of infancy (SMEI) by Charlotte Dravet and later renamed to DS by ILAE in 1989.[58,77] It is a rare (incidence of 1 in 15,700 to 41,000 live births), but highly treatment-resistant, developmental epileptic encephalopathy initiated in the first year of life.

The presentation of seizures in DS evolves with age.[78] Seizure onset is during infancy (before age of 12 months). The first seizure is usually hemiclonic (affecting only one side of the body), or generalized (affecting both sides of the body simultaneously) clonic or tonic-clonic activity, and is characterized by a long-lasting duration and triggered by fever.[79] However, cerebral imaging (e.g. MRI) and EEG recordings of the brain are usually normal at this stage.[80] In the following stage, additional multiple seizure types, such as convulsive, focal, atypical absences, and myoclonic seizures, are presented.[77,80] In addition, developmental and psychomotor delays are observable from the second year of life, such as ataxic and speech problems.[81] EEG evidence of epileptic activity with spike and wave or polyspike discharges are typically shown.[80] Later, the clonic seizures are more frequent and persistent, and subsequently motor dysfunction (e.g. ataxia, crouch gait and incoordination), behavioral disorder (e.g. hyperactivity and oppositional disorder), and cognitive impairment (e.g. executive dysfunctions and language deficit) are apparent in the patients.[79] Moreover, DS related comorbidities including sleep issues, frequent infections, and psychiatric disturbances are pronounced.[82] Besides, an increased incidence of mortality, especially a higher risk of SUDEP (9.32 per 1000 person-years), is reported in DS.[83]

3.2.1 *The etiology of DS*

Around 80% patients with DS have mutations in the *SCN1A* gene which codes an α subunit of the brain voltage gated sodium channel (VGSC) type-1 ($Na_V1.1$).[84,85] Most loss-of-function mutations of the *SCN1A* gene arise *de novo*, but 5-10% of patients inherit mosaic mutations from a non-affected transmitting parent.[79,86] The majority are truncating mutations and missense mutations, while exons deletions or chromosomal rearrangements of the *SCN1A* gene also exist in DS.[84] According to a recent study, if combining additional intronic alterations,

copy number variants and mosaicism, *SCN1A* abnormalities are demonstrable in almost all patients with DS.[87]

The VGSC family plays crucial roles in the initiation and propagation of the action potentials. Normally, haploinsufficiency of a VGSC channel can reduce the sodium-dependent action potentials and lower the excitability of neurons, therefore leading to a reduction of the seizure activities (e.g. the mechanism for VGSC blocking ASDs). However, the type I sodium channel, namely $Na_V1.1$, is specifically located in the initial segments of GABAergic inhibitory interneurons cell bodies in the brain.[31,29] In the DS mouse model, haploinsufficiency of the $Na_V1.1$ caused a dramatic reduction of sodium current density of the hippocampal GABAergic interneurons. This reduction does not occur in the excitatory neurons, which resulted in hyperactivity and spontaneous seizures in the *scn1a* mutant mice.[88,89] In addition, the loss of $Na_V1.1$function was reported to be associated with multiple neurological disorders in the animal models, like cognitive impairment, and abnormal social behaviors of the mice.[89,90] Recently, Richards *et al*., used the venom peptide Hm1a to selectively potentiate $Na_V1.1$ channels, and it restored the function of GABAergic inhibitory interneurons without affecting excitatory neurons, leading to significantly reduced seizures and mortality of the DS mice.[91] These animal experiments are strong evidence that the *SCN1A* loss-of-function mutation affecting the $Na_V1.1$ channel could be the main pathogenesis of DS.

Other than *SCN1A*, also mutations in additional genes, such as *SCN2A*, *SCN8A*, *CHD2*, *GABRA1*, *STXBP1* and *PCDH19*, have been identified to be related to DS-like phenotypes that exist in a small percentage of cases.[84,86,92,93] However, the main syndrome spectrum remains to be more likely related to the mutation in *SCN1A* rather than any other gene.[87]

3.2.2 *The management of DS*

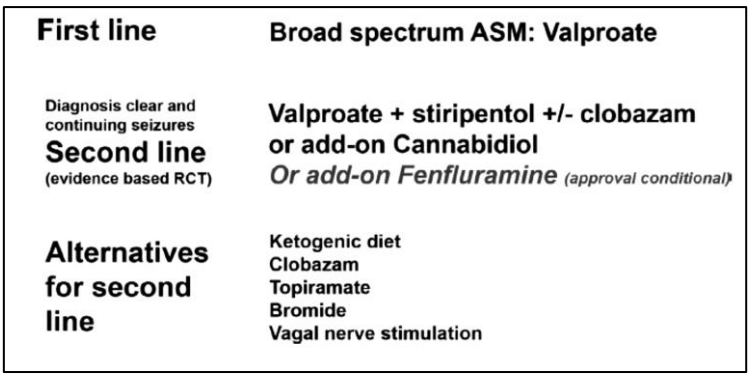

Figure I-6. The updated treatment algorithm for Dravet Syndrome (DS), the experts opinions of Europe. ASM: antiseizure medication; RCT: randomized clinical trial. (from Cross *et al*., 2019)[54]

The main therapeutic goals for the management of DS include reaching optimal seizure control, optimizing neurodevelopmental outcome, and improving QOL for patients. However, current therapies only have effect on seizures. To date, several therapeutic options are available, which can be classified into ASDs treatment and non-pharmacological treatments (Figure I-6). Although non-pharmacological treatments are increasingly used today, ASDs are still the main basic treatment.

3.2.2.1 ASDs treatment

Since DS is highly drug-resistant epilepsy, complete seizure remission is typically not achievable with current ASDs. Polytherapy, namely a combination of two or three ASDs, is usually required for patients to achieve a significant reduction in seizure burden. However, a clinical "standard of care" recommending ASDs treatment for most patients has not been published yet from FDA or EMA.[54,94–96] Moreover, the efficacy and tolerability of ASDs should be considered when commencing polytherapy. In particular, VGSC-blocking ASDs, such as carbamazepine, oxcarbazepine, phenytoin and lamotrigine, should be avoided since they can aggravate seizures in DS patients.[78,97]

Valproate is a broad spectrum ASD with diverse antiseizure mechanisms (e.g. enhance GABA transmission and inhibit T-type Ca^{2+} channels) and a wide therapeutic range in different seizure types.[7,26,39,57] Both retrospective studies and the newly updated treatment algorithm suggest valproate as the first-line ASDs treatment,[54,77,78,98] and it is indeed the most used ASD in DS patients at present.[95,99] Unfortunately, it rarely provides adequate seizure control, which requires the addition of second-, alternative second or third-line therapies. The add-on ASDs used clinically in DS vary between different reports, with clobazam, stiripentol, and topiramate the most used and showing clear benefits.[55,79,96,99] Recently, stiripentol has been approved by EMA as an adjunctive therapy (in combination with clobazam and valproate) for DS. It is an ASD targeting the GABAergic neurotransmission system,[100] which can increase the efficacy of its co-administered ASDs (e.g. clobazam) due to its inhibitory effects on certain cytochrome P450 enzymes.[101,102] A good response (≥50% seizure reduction) in approximately 55-70% of DS patients (a high responder rate) was reported with the add-on stiripentol therapy.[103] Another example is topiramate, also a broad spectrum ASD with multiple antiseizure mechanisms. It has been recommended as second-line medication for DS by a North American Consensus Panel, along with stiripentol.[98] However, despite multiple polytherapy regimens being proposed, there are still ~ 45% of patients experiencing more than three tonic-clonic seizures

monthly.[95] Recently, emerging antiseizure agents, such as fenfluramine and cannabidiol (both approved by FDA), have been demonstrated for their specific efficacy in DS and incorporated as second line ASDs in the newly published treatment algorithm (Figure I-6).[54]

1)Fenfluramine (FFA)

FFA has been approved by FDA for treatment of seizures associated with DS in 2020. It was initially used as an anorectic in polytherapy with phentermine, but was withdrawn from the market owing to the cardiopulmonary side effects at high dosages, in 1997.[54] Recently, the successful application of low dosage FFA as the add-on therapy for the treatment of DS was reported by Ceulemans *et al*., including the achievement of seizure free cases.[104,105] Later clinical trials have further confirmed the efficacy and safety of FFA in DS. Importantly, no cardiovascular adverse effects were observed in these trials.[58,106] For example, in a recent randomized and double-blind clinical trial with placebo control, add-on FFA at 0.7 and 0.2 mg/kg per day was able to reduce seizure frequency in patients by 74.9% and 42.3%, respectively. Moreover, FFA at 0.7 and 0.2 mg/kg per day was generally well tolerated, and no cardiopulmonary cases were reported.[58] The antiseizure mechanism of FFA is assumed to be due to its action partly on the 5-HT receptors which are emerging molecular targets of ASDs. FFA is not only a 5-HT releaser but also a serotonin reuptake inhibitor, so that it can lead to an increased 5-HT level in the brain.[107,108] Moreover, it can also target the sigma-1 receptors in the CNS.[109] Recently, based on the 5-HT mechanism of FFA, Sourbron *et al*. tested selective 5-HT receptor agonists using a DS zebrafish model (*scn1Lab*$^{-/-}$ mutant model), and found FFA could mediate the 5-HT_{1D}, 5-HT_{2C}, sigma-1, and possibly also the 5-HT_{2A} receptors to perform its anti-seizure activity.[50,110] Interestingly, the 5-HT_{2B} receptor, which was thought to be responsible for this FFA-induced cardiopulmonary problem, was not involved in the antiseizure action.[50,110,111]

2) Cannabidiol (CBD)

One of the active compounds of *Cannabis sativa*, CBD, has been approved for treatment of children with DS, by the FDA in 2018 and EMA in 2019.[112] It was reported as displaying a prominent efficacy in reducing seizure frequency in clinical trials.[113,114] In Devinsky *et al*'s study, a double-blind and placebo-controlled trial was conducted in 120 children and adults with DS. Forty percent of the patients treated with add-on CBD at 20 mg/kg per day had at least 50% reduction in convulsive seizure frequency (placebo: 27%). Among the patients who

became seizure-free, 5% were treated with add-on CBD, and 0% was with placebo.[115] In addition, the effects of CBD on seizures are not limited to DS. CBD appears to exert a more general action as it also displayed antiseizure activity against the Lennox-Gastaut syndrome[114,116] and a range of animal seizure and epilepsy models.[117–119] Recent preclinical experiments indicated CBD to be a multitarget agent, it can block the G protein-coupled receptor 55 (GPR55), and desensitize the transient receptor potential of vanilloid type 1 (TRPV1) channels, therefore leading to reduced calcium levels and cell excitability.[119–121] Also, it can inhibit the adenosine reuptake, resulting in the enhanced extracellular adenosine levels and abnormal synaptic effects.[122] In addition, it has effects on 5-HT receptors.[51,122] Of note, CBD was not able to activate cannabinoid type 1 (CB1) and type 2 (CB2) receptors at its physiologically achievable concentrations, thereby lacking psychotropic activity.[96,121] However, the precise antiseizure mechanism of CBD remains unknown.

3.2.2.2 Non-pharmacological treatments

There are two main options in the non-pharmacological treatment for DS, KD and VNS. KD is usually selected after the failure of three or four ASDs.[54] This treatment not only benefits the seizure control but also improves cognition and behavior in most DS patients.[98] VNS involves a surgical implantation of the device and could be considered after the failure of ASDs treatment and KD. It has a long-term efficacy in achieving minimal to moderate seizure control.[78,98] However, both KD and VNS only have been evaluated in small case series,[51] and they are suggested as alternative second-line treatments in the newly published treatment algorithm for DS (Figure I-6).[54,78]

4. ASDs discovery in animal models

Until now, the discovery and development of ASD candidates has mainly relied on the use of preclinical epilepsy and epileptic seizure models. Among these models, the use of *in vitro* models is limited since they lack the complexity of living organisms and are therefore unsuited to a systematic use in ASD discovery projects.[123] Since the discovery of the antiseizure properties of phenytoin using the electroshock seizure model in 1930s, whole-animal models, especially rodent models, are commonly used in ASDs discovery projects, and they have successfully identified numerous clinically effective ASDs.[23] In addition, over the last decade, zebrafish has rapidly emerged as a promising model for ASDs discovery on account of their ideal features for high-throughput screening.[124]

4.1 The rodent seizure and epilepsy models

In general, preclinical rodent seizure and epilepsy models fall into at least four groups, namely chemical models (e.g. subcutaneous pentylenetetrazole (scPTZ) seizure model), electrical models (e.g. 6 Hertz (6 Hz) psychomotor seizure model), lesion models (e.g. traumatic brain injury model), and genetic models (*Scn1a*$^{+/-}$ mutant model).[23,125,126] Of note, the chemical, electrical and lesion seizure models can also result into epilepsy models as spontaneous seizures can be present in the chronic phase after the insult. For instance, the systematic or intracerebral injection of chemicals (e.g. kainic acid) into the rodents can induce status epilepticus, and afterwards trigger spontaneous recurrent seizures.[23,127] Moreover, those models have similar pharmacological responses to ASDs as humans, which facilitate their intensive use in the discovery and development of ASD candidates.[123]

4.1.1 *The gatekeeper models*

Due to advantages in saving labor, time and cost, and predictability of clinical activity, the optimized maximum electroshock (MES) rodent model and the scPTZ rodent model have been employed as gatekeepers and are widely used in multiple ASD screening projects.[23,123,125,128] MES and scPTZ models are respectively electrically- and chemically-induced acute seizure models, each using normal (non-epileptic) mice or rats.[123] For the typical MES test, a suprathreshold (50-60 Hz) electrical stimulus (50 mA in mouse and 150 mA in rats) is administered to the mouse or rat by the transcorneal or, less often, transauricular electrodes for a short duration (0.2 s). The animal will then experience a severe tonic seizure period, a following clonic seizure phase, and finally a tonic hindlimb extension.[123,125] This test has been regarded as a measure of human generalized tonic-clonic seizures, since most clinically proven ASDs used for treating generalized tonic-clonic seizures are effective in this model.[128,129] In contrast, the scPTZ model is generally regarded as predictive of antiseizure drug activity against nonconvulsive generalized (myoclonic, absence) seizures.[125] Typically, PTZ is administrated to the rodents through subcutaneous administration at a fixed dose, evoking clonic seizures (e.g. vibrissae and/or forelimbs) for at least 5s.[129] Remarkably, these two simple models have helped to discover many of the ASDs so far approved by FDA and EMA.[23,125]

However, it has been argued that the conventional models (i.e. MES and scPTZ) can only identify “me too” drugs and fail to pick up compounds that have novel antiseizure mechanisms and utility, which may have potential efficacy for refractory epilepsies.[125] For example, levetiracetam, which is broad spectrum ASD with an antiseizure mechanism related to the

SVA2, is not effective in the conventional models. Therefore, a variety of novel drug-resistant models have been further explored and developed in recent years.

4.1.2 *Drug-resistant epilepsy models*

In line with the operational definition of ASDs resistance in patients with epilepsy, "drug resistance in animal models" is defined where animals do not respond or respond poorly to at least two current ASDs in monotherapy, at the maximum tolerated doses.[125,130] Accordingly, a 6 Hz psychomotor seizure model, as an acute electrically-induced drug resistant seizure model, has been introduced into the routine workflow of ASDs screening projects.[129] The psychomotor seizures are electrically induced (6 Hz, 0.2 ms rectangular pulse width, 3 s duration, 44 mA) in mice through the cornea, and are characterized by stun, clonus, twitching of the vibrissae, and straub tail. Increased levels of molecular biomarkers, especially early proto-oncogenes such as *c-fos*, have been found in some specific areas of the brain (e.g. dentate gyrus).[131] This model has been employed to screen novel antiseizure compounds and to distinguish their efficacy. Only a few ASDs (e.g. levetiracetam and valproate) completely protect the animals from the seizures.[123,131,132] However, considering this model cannot reflect the pathophysiology of drug-resistant epilepsy, it is widely accepted that 6 Hz model is only suitable for early *in vivo* ASDs screening.[123]

In order to identify differentiated therapies, emphasizing the activity spectrum of candidate compounds, several "disease-specific" chronic rodent epilepsy models have been proposed for use in ASDs screening projects.[123,133] From 2015, the Epilepsy Therapy Screening Program (ETSP) (previously known as the Anticonvulsant Screening Program), a preclinical screening program that contributed to the approval of most of the clinically-available ASDs, extended its screening workflow with an "Identification" phase and a "Differentiation" phase (Figure I-7). In addition to the MES and 6 Hz models, several models with chronic seizure activity were incorporated, including kindling models induced by repeated application of electrical or chemical stimuli (e.g. 60 Hz corneal kindled seizure model and lamotrigine-resistant amygdala kindled model) and the post status epilepticus (SE) models induced by electrical stimulation or administration of large doses of convulsant and characterized by development of spontaneous recurrent seizure after the initial insult (limbic SE) (e.g. intrahippocampal kainate mouse model of mesial temporal lobe epilepsy). Also, considering epilepsy could develop from acute inflammation of the CNS, Theiler's virus model of acute seizure was implemented in the differentiation phase of the ETSP.[23,133] Although these models are able to identify potential

antiseizure compounds acting on specific populations, they are always complicated, extremely time-consuming and laborious in providing useful differentiated data.

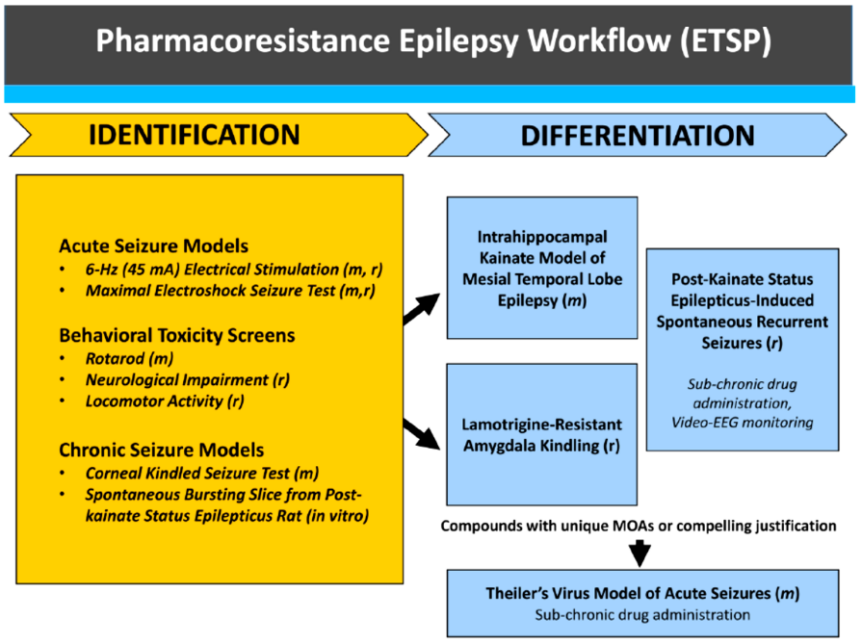

Figure. I-7 Pharmacoresistance epilepsy workflow for the Epilepsy Therapy Screening Program (ETSP). m: mice; r: rat; MOA: mechanism of action. (from Löscher *et al*., 2017)[23]

4.1.3 *Readout assays in rodent epilepsy and seizure models*

Video and EEG recordings are two commonly used technical approaches to show evidence of seizures in rodent models. Behavioral signs of seizure activity in rodents vary depending on the type of seizure modeled, and may be similar to the features of the clinical epilepsy phenotype in humans.[134] Typical ways to quantify seizure-activity include seizure duration, threshold, severity, and incidence.[134] It is also possible to detect and score rodent seizure behavior as locomotion and social interaction in the home cage.[135] Moreover, with the recent advances in video tracking technologies, some automated behavioral detection platforms (e.g. behavioral spectrometer) have been developed, which can collect complicated behavioral information and characterize hyperactivity.[136]

EEG recording of burst electrical discharges on the scalp or surface of the brain is the golden standard to clinically diagnose epilepsy, and is often used in epilepsy animal models too. If the seizures in rodents are behaviorally subtle, or if it is unclear whether a particular alteration in behavior is a seizure, EEG recordings are necessary.[134] Typically, EEG recordings are conducted in freely moving rodents, after single or multiple recording electrodes were intracranially placed.[127] EEG signals can be acquired by multiple approaches, such as the

adapted clinical video EEG systems, the commercial systems for wireless radiotelemetry (e.g., Epitel, DSI, TSE, Indus Industries, or Millar), or the laboratory amplifiers that have associated recording software.[134] In addition, fast and automated tools for the detection of electrographic seizures have also been developed.[137,138]

4.2 The zebrafish models

Zebrafish (*Danio rerio*) is a tropical freshwater fish originated from the Ganges region (South and South-East Asia). The initial 'modern' zebrafish research was performed by George Streisinger in the 1960s. Since then, zebrafish models have been favorably used in several areas of biomedical research, such as for genetic research and disease modelling, as well as medium-to-high throughput pharmacological and genetic screening programs.[134,139,140] Hereafter, the advantages (Figure I-8) and limitations of the zebrafish models, and their application in epilepsy-related studies will be discussed.

4.2.1 *Advantages and limitations of the zebrafish models*

Advantages of zebrafish models

1) Zebrafish are vertebrate models with genetic, physiological and CNS features that are highly conserved across vertebrates, including humans.[141] For example, the zebrafish model genome has been well-characterized, and 70% of human genes (and 82% of disease-related human proteins) have at least one zebrafish orthologue.[142] In particular, homologous functions of the major neurotransmitters, brain-derived neurotrophic factors, as well as several key brain areas have been described in zebrafish.[143–145]

2) Zebrafish can be maintained at a low cost and effort level thanks to their high fertility (50-500 eggs per mating), fast embryonic development (major organ systems are fully functional by 5 days postfertilization (dpf) and long lifespan (5 years).[133] Additionally, the use of zebrafish larvae (before 6 dpf) implements the 3R principles (Replacement, Reduction, Refinement) of humane animal research.

3) In case of genetic modification of zebrafish, diverse genetic tools, such as morpholino (MO)-based gene knockdown and CRISPR/Cas9-based genome editing can be used.[134,146] Also, the availability of the Zebrafish Information Network (ZFIN), a genome informatics database containing accumulating genetic and phenotypic data, aids and accelerates the searching of zebrafish genetic, genomic and phenotype data.

4) Regarding the compound administration, chemicals can simply be added to the fish medium, then absorbed through the gastrointestinal tract, skin and gills of zebrafish.[134] Zebrafish-absorbed compounds typically fulfil certain criteria: (a) molecular weight ≤ 500, (b) clog P ≤ 5.3, (c) HBD ≤ 3, (d) HBA ≤ 7, (d) PSA ≤ 124 A°, and (e) rotatable bonds ≤ 9.46.[147] Moreover, due to their small size (3.5-4.5 mm at 3-7 dpf) zebrafish larvae can be arrayed in microtiter plates. All these features facilitate their use in phenotype-based, medium-to-high throughput screening research.

5) Concerning compound distribution and metabolism, the existence in zebrafish of the blood/brain barrier (BBB) and tissue-specific transporters have been strongly evidenced.[141,148] Moreover, most zebrafish liver cell types and their functions appear similar to mammals.[149] Importantly, a good translation of the pharmacological profiles of compounds between zebrafish and rodent models has been documented.[141]

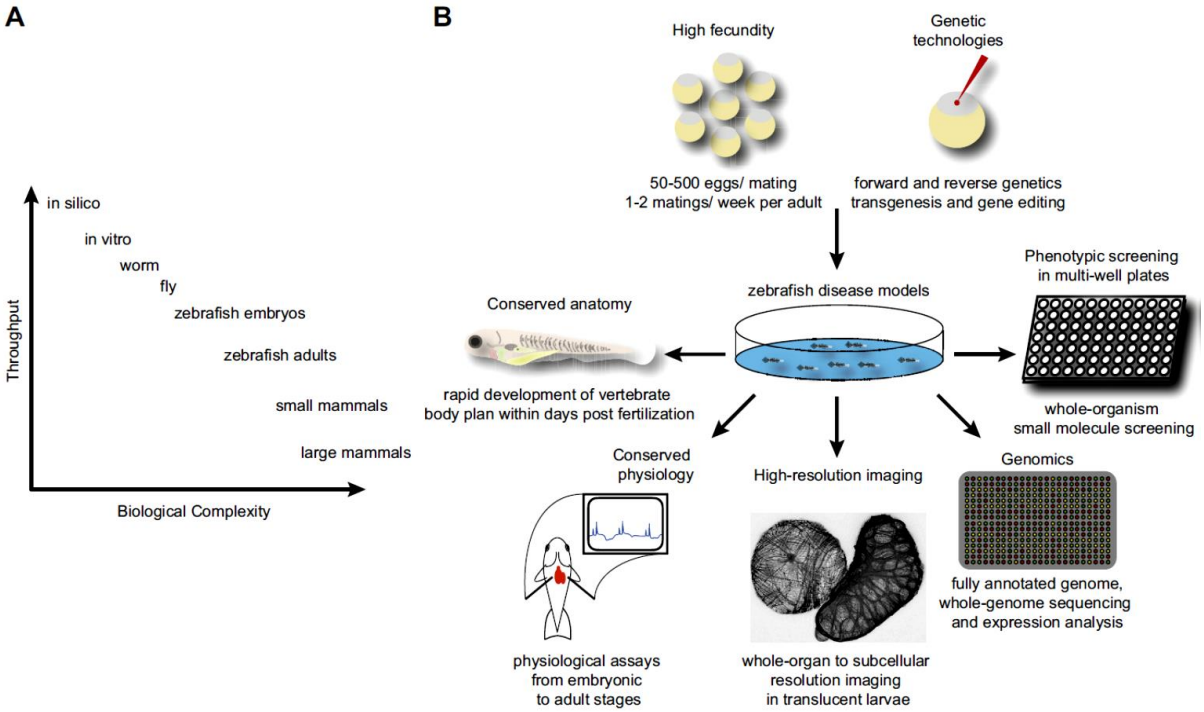

Figure I-8. Advantages of zebrafish for biomedical research. A: zebrafish occupy a unique position in biomedical research as a vertebrate organism suited to large-scale genetic and chemical screening. B: zebrafish are amenable to a broad spectrum of genomic, physiological, imaging, and small-molecule screening approaches. (From Gut *et al*., 2017)[146]

Limitations of zebrafish models

1) As a non-mammalian model, zebrafish are more evolutionarily distinct from humans compared with the rodent models. Comparing with other mammalian models, they possess

some different organ systems (e.g. a swim bladder present instead of lungs) and lack certain mammalian organs completely (e.g. layered cerebral cortex in the brain). [134]

2) For high-throughput screening, adult fishes are always excluded due to their "big size". In addition, false negatives are likely to occur since the absorption of the compounds can be influenced by certain variables, such as the solubility of the compound.[147] Also, the methods available for administration of insoluble compounds, i.e. by injection (into the yolk sac of the embryonal stage) and oral (with microgavage), are limited by capacity and considered to be technical challenging.[140]

3) Finally, the additional duplication of the whole zebrafish genome results in a mixture of paralog genes (new function), ortholog genes (same function retained for both) and even loss of redundant gene duplicates,[139] impeding the modelling of human diseases in zebrafish.

4.2.2 *Zebrafish models for epilepsy and seizure*

The first zebrafish epilepsy model was introduced in 2005.[150] From then on, many zebrafish models have been developed and employed in the epilepsy studies. Seizure-like behavioral and physiological responses (e.g. alter brain electrical activity) are displayed in zebrafish by exposure to various convulsant or genetic manipulations. Zebrafish models are classified into chemically-induced epileptic seizure models and genetic epilepsy models.[134]

4.2.2.1 Zebrafish epileptic seizure models

Zebrafish epileptic seizure models are mainly chemically induced. Commonly used convulsants, mainly pentylenetetrazole (PTZ),[150,151] picrotoxin,[152] pilocarpine,[153] kainate[154,155], caffeine[151] and strychnine[124], can be administrated to larval and adult zebrafish by immersion or injection to evoke robust seizure-like responses.[124] The seizure-like behaviors are varied among the different models, but generally include the circling, hyperactivity, spasms, ataxia, clonus-like head-shaking convulsions, twitching, jerking and tremor.[124,134] Among convulsant chemicals, PTZ as a GABA antagonist, was the first convulsant introduced to the zebrafish model.[150] Typically, after exposure to PTZ for a few minutes, vigorous locomotor convulsions are presented, and are characterized by three clear stages. The first stage involves significantly increased swim activity, followed a rapid "whirlpool-like" circling swim behavior at the second stage, and subsequently ending in a series of brief clonus-like convulsions at the third stage, leading to a loss of posture.[150,154,156] Moreover, epileptiform brain activity and up-regulation of *c-fos* expression are displayed.[150] This model was validated by testing known ASDs, and a good correlation with results from the rodent PTZ seizure model was confirmed in the overall

data. For example, among the 13 tested ASDs, diazepam, ethosuximide, valproate and tiagabine are effective in counteracting PTZ-evoked seizures in zebrafish, consistent with published rodent data.[157] Recently, the PTZ zebrafish seizure model has been accepted as a standard test for the discovery of antiseizure compounds.[157,158]

Moreover, chemically induced (e.g. pilocarpine and PTZ) chronic seizure models have been documented, using adult zebrafish. Other than seizure behaviors, they can display epilepsy-related molecular alterations (abnormal neurotransmitters levels) or comorbidities (e.g. cognitive dysfunction), demonstrating their potential for exploring novel therapies.[124,159,160]

In order to fulfil the demand for new medications to treat refractory seizures, new zebrafish models are being explored and specifically generated for the purpose of discovering antiseizure agents with novel targets and mechanisms.[156,161] This is the reason that the ethylketopentenoate (EKP)-induced zebrafish seizure model was developed. EKP is an inhibitor of rate-limiting enzyme glutamate decarboxylase (GAD), which can impede the synthesis of GABA from glutamate, and consequently reduce GABA levels and the functional loss of inhibitory interneurons.[156,162] The available preclinical and clinical data have demonstrated that a lower activity of GAD is associated with several epilepsies, such as drug-resistant temporal lobe epilepsy.[156,161,163,164] According to a recent study, seizures elicited in EKP-treated zebrafish demonstrate a high level of resistance against commercially available ASDs. Among the 13 tested ASDs, only perampanel, which is an AMPA receptor inhibitor, showed a clear inhibitory effect on both the EKP-triggered excessive behavior and brain activity. Conversely, GABAergic compounds like tiagabine (which prevents GABA reuptake) and valproate (which inhibits GABA degradation) were inactive. Thus, the EKP model is regarded as an interesting discovery platform to find mechanistically novel ASDs.[161]

Besides the chemically-induced model, a hyperthermia-induced (induced by increased bath temperature) zebrafish seizure model has been generated, which displays reproducible acute electrographic seizures. The basis of this mechanism is assumed to be the effect of enhanced brain temperature on TRPV receptors, increasing neuronal excitability.[165] Moreover, this model may be considered to replicate febrile seizure, which is normally triggered by the increasing of body temperature, to some extent.

4.2.2.2 Zebrafish genetic epilepsy models

The sequencing of the zebrafish genome was completed in 2013, confirming the high homology (70%) of zebrafish and human genes.[166] Given the key role of genetic factors that

play an important role in epilepsy, zebrafish genetic epilepsy models have been extensively investigated. Both the forward and reverse genetics approaches have been employed to generate the most common genetic manipulations in zebrafish. As forward genetics strategies, N-ethyl-N-nitrosourea (ENU) mutagenesis and retroviral insertional mutagenesis have been used to generate random mutant models, such as *scn1Lab* mutant zebrafish[167] and *mind bomb* mutant[168] zebrafish. In the case of reverse genetics technologies, the use of morpholino oligonucleotides (MOs) and CRISPR/Case9 for characterizing a specific gene in zebrafish through its overexpression/knockdown/knockout, has yielded several models, such as *kcnq3* morphant model,[169] *scn1Lab* morphant model,[170] *cpa6*[153] morphant model and *stxbp1b* mutant model.[171,172] Most genetically stable mutant and morphant zebrafish models can appropriately recapitulate key features and even the pathophysiology of human epilepsy patients,[134] which not only benefits the understanding of disease mechanisms but also helps with identifying personalized therapeutics for specific epilepsies.[173]

Recently, both the *scn1Lab* morphant and mutant zebrafish model were developed to recapitulate DS, based on the fact that the zebrafish *scn1Lab* gene is evolutionarily related to the mammalian *SCN1A* gene, and *SCN1A* mutation is the main etiology of the DS.[161,170,168,167] The homozygous zebrafish larvae of these models display spontaneously occurring seizures as well as electrographic seizure from 3-4 dpf. These mutants have an abnormal pigmentation pattern and early fatality in 14 dpf. The *scn1Lab* morphant model is a transient model generated by using morpholino antisense oligomers targeting *scn1Lab*. Zheng *et al*. conducted a pharmacological evaluation on this model using commercially available ASDs. They found that the *scn1Lab* morphant model is highly resistant to ASDs, with only valproate and FFA that showed significant counteraction of the epileptiform discharges.[170] Interestingly, the *scn1Lab double indemnity* (*didy*) mutant model was initially discovered from a random forward genetic screen of point mutations induced by the ENU (a highly potent mutagen), identified due to its inability to sustain saccadic eye movements during the optokinetic response.[167,175] In the heterozygous mutant zebrafish, a point mutation transforms a thymine ($AT^{3632}G$) to a guanine ($AG^{3632}G$) in the *scn1Lab* gene, leading to a malfunction of the corresponding protein.[50] Recently, a phenotype-based high-throughput compound screening project was carried out using those mutants, and finally identified clemizole, a FDA-approved histamine antagonist, to be a potent antiseizure drug for DS, thus providing a proof-of-concept for the use of zebrafish genetic epilepsy models in the high-throughput ASD discovery.[167]

4.2.2.3 The readout assays in zebrafish epilepsy and seizure models

The typical phenotypes in the zebrafish epilepsy and epileptic seizure models include seizure like behaviors, brain epileptiform discharges and upregulated expression of molecular biomarkers. In order to trace and quantify these features in zebrafish, several readout assays have now been developed and employed.

The availability of commercial video tracking systems (e.g. ZebraLab or Ethovision) has dramatically enhanced neurobehavioral analyses in zebrafish, and is now frequently used in zebrafish epilepsy and seizure models. Typically, these tracking systems are compatible for studies in a 96-well plate format, detecting total larval movement, freezing, and burst activities of the zebrafish by a top-view camera.[176] Such assays can monitor and quantify the behavioral effects of genetic modification and administered compounds (proseizure and antiseizure) on the zebrafish, and have been employed as a rapid, and high-throughput approach in screening projects.[134]

To record the epileptiform brain activity in the zebrafish model, local field potential (LFP) recordings were developed, which mimic human EEG.[134] During the recording, zebrafish are immobilized in agarose, and the electrode positioned in or on larva's middle brain or forebrain structures to capture the hyper-synchronized neuronal activities, which are present as the interictal- and ictal-like epileptiform discharges.[134,177] In addition, the epilepsy parameters of the recording, including duration, clustering, frequency, and amplitude can be used to quantify the effects of genetic modification and administered compounds on the zebrafish.[134,150]

Molecular biomarkers can be used to examine and further confirm the seizure-like behavior of the zebrafish and reduce false positive results from the above assays. Changes in expression of molecular biomarkers can be readily detected by whole-mount in situ hybridization (WISH) or real-time quantitative PCR (RT-qPCR).[134,173] Early proto-oncogene *c-fos* is the most common molecular biomarker of epilepsy, believed to be related to the neuronal activation and brain hyperactivity of zebrafish, and its increased expression has been found in diverse zebrafish epilepsy and seizure models.[150,178] Others, like neuronal domain-containing protein 4 (*npas4*) and sestrin 3 (s*esn3*), and proflammatory cytokines (e.g. *IL-1*, *IL-6*, *TNF-α*), which are implicated in human and rodent epilepsy, are also now regularly examined as molecular biomarkers in zebrafish epilepsy and seizure models.[124,173,178,179]

5. Natural products in ASDs discovery

Natural products are small molecules mainly originating from plants, bacteria, and fungi, as well as marine flora and fauna.[180] Historically, natural products and their derivatives played a significant role in medicine. With the recent developments in the isolation and purification technologies, and the advances in the combinatorial chemistry approaches, numerous biologically active natural products and their derivatives have been identified and synthesized.[181] More recently, a variety of high-throughput screening systems are being developed to facilitate the use of natural products in drug discovery campaigns.[182] For instance, a public-accessible library of natural product fractions (original from more than 125,000 crude extracts) for high-throughput screening has been established by the US National Cancer Institute (NCI), with the aim of accelerating natural product-based drug discovery.[183] Therefore, natural products and their derivatives have become a productive and excellent source for drugs and drug leads.[182,184] According to an updated survey from Newman *et al*., among the 1328 new chemical entities approved as drugs between 1981 and 2016, around 40% (549) of them have a natural origin or are related to natural compounds (e.g. semi-synthesis products that have similar structure as known natural products).[185,186]

In ASDs discovery, three main strategies typically employed are: a) the random and phenotypic screening of newly synthesized potential antiseizure chemicals; b) the structural modification of known ASDs; and c) target-based and mechanism-oriented novel drug design. Though numerous ASDs have been found according these three strategies, their efficacy for treating drug-resistant epilepsy is low.[26] Therefore, the innovative strategies and new avenues in ASDs discovery are urgently sought. As there are centuries-old traditions of using natural products for the treatment and control of epilepsy and related syndromes, they have been regarded as a promising source for the discovery of new ASDs. To date, a variety of antiseizure natural products have been identified which showed efficacy against drug-resistant epilepsy.[184,187–189] This can be illustrated by CBD, a constituent of *Cannabis sativa*, which was approved by FDA in 2018 and by EMA in 2019 for the treatment of children with DS.

5.1 Traditional Chinese medicine (TCM) in ASDs discovery

Natural product-based drug discovery can be conducted by a random screening, or guided by a molecule category known to provide a given activity (chemotaxonomic targeting) or especially, by a plant's ethnopharmacological use, such as folk medicine or ethnomedical medicine.[180] TCM is one of the most widely practiced forms of botanical therapy in the world

that has been in use for more than 2500 years. In 2015, the Nobel Prize for Physiology or Medicine was awarded to Prof. Tu for her discovery and development of artemisinin, an antimalarial agent isolated from the TCM *Artemisia annua*. This success shows the potential of TCM for drug discovery, especially the possibility of translating TCM to novel drug development.[190]

TCM includes multiple medicinal plants against epilepsy and seizures, and the antiseizure activities of some have been examined in *in vitro* and *in vivo* epilepsy and seizure models. An overview of antiseizure medicinal plants and their main active components are summarized in Table I-2. The main active ingredients include alkaloids, flavonoids, terpenoids, saponins, and coumarins, exerting effects on multiple antiseizure molecular targets, such as $GABA_A$ receptor and ion channels (e.g. VGSC, VGCC, VGPC and TRP).[187,191,192]

Moreover, TCM also has been employed as a complementary and alternative medicine (CAM) to control epilepsy-related comorbidities, and to increase the curative effects and attenuate side effects of ASDs.[193–195] Taken together, the investigation of TCM might provide us new insights for the discovery and development of ASDs.

Table I-2. The medicinal plants in TCM used in epilepsy therapy

Medicinal plant	Main active components	Reference
***Acorus tatarinowii* Schott**	Asarone	Liu *et al*., 2015; Xiao *et al*., 2015.[196,197]
***Anemarrhena asphodeloides* Bge.**	Anemarsaponin	Oh *et al*., 2007; Xiao *et al*., 2015. [196,198]
***Astragalus membranaceus* (Fisch.) Bge.var.mongholicus (Bge.) Hsiao**	Saponin	Xiao *et al*., 2015.[196]
***Bupleurum chinense* DC.**	Saikosaponin A	Xie *et al*., 2013. Yu *et al*., 2012.[199,200]
***Coptis chinensis* Franch.**	Berberine	Xiao *et al*., 2015; Bhutada *et al*., 2010.[196,201]
***Crocus sativus* L.**	Safranal	Rajabian *et al*.,2019.[202]
***Curcuma longa* L.**	Curcumin	Kaur *et al*., 2015; Dhir *et al*., 2018.[203,204]
***Cynanchum otophyllum* C.K.Schneid.**	Steroidal saponins	Li *et al*., 2014.[205]
***Gastrodia elata* Blume.**	Vanillin	Yip *et al*., 2020; Xiao *et al*., 2015.[196,206]
***Ginkgo biloba* L.**	Bilobalide	Mazumder *et al*., 2017; Xiao *et al*., 2015.[196,207]
***Glycyrrhiza glabra* L.**	Licorice flavonoid, saponins	Luo *et al*., 2014; Singh *et al*., 2015.[208,209]
***Magnolia officinalis* Rehder & E.H.Wilson**	Magnolol, honokiol	Vega-García *et al*., 2019; Chen *et al*., 2011.[210,211]
***Panax ginseng* C.A.Mey.**	Ginsenoside	Zheng *et al*., 2018; Xiao *et al*., 2015.[196,212]
***Pinellia ternata* (Thunb.) Breit.**	Pinelliae alkaloids	Deng *et al*.,2019.[213]
***Poria cocos* (Schw.) Wolf.**	Triterpenes	Gao *et al*., 2016.[214]
***Salvia miltiorrhiza* Bge.**	Tanshinone	Buenafe *et al*., 2013.[215]
***Scutellaria baicalensis* Georgi.**	Baicalin	Liu *et al*., 2012.[216]
***Semen Pharbitidis* (Linn.) Choisy**	Pharbitin	Liu *et al*., 2019.[217]
***Uncaria rhynchophylla* (Miq.) Miq.ex Havil.**	Rhynchophylline, isorhynchophylline	Ho *et al*., 2014; Xiao *et al*., 2015.[196,218]

CHAPTER II

Research Objectives

Objective 1

Epilepsy is a brain disease characterized by recurrent seizures due to abnormal synchronous neuronal activities in the brain. To date, drug-resistance is the major challenge for the pharmacotherapy of epilepsy, affecting about one third of epilepsy patients. Therefore, innovative *in vitro* and *in vivo* experimental models are being developed and used to find novel ASDs to treat therapy-resistant epilepsies.[76] In the recent past, our research group has been using a pentylenetrazole (PTZ)-induced zebrafish seizure model that displays generalized tonic-clonic seizures and epileptiform brain activity, as a standard assay for the discovery of ASDs. Moreover, we developed and validated a chemically-induced EKP zebrafish seizure model that proved to exhibit a high pharmacoresistant profile.[161] Hence, the latter model is well suited for the discovery of new hits and the identification of compounds of interest that could be used in the fight against pharmacoresistant epilepsy.

Comparing with random screening of compounds, evidence suggest that medicinal plant-based drug discovery approach results in a faster and cheaper identification of active ingredients, as the candidate plants have been preselected and used by ethnomedical practitioners.[219–221] Moreover, since natural products from medicine plants are typically characterized by a large chemical diversity, they have been regarded as a promising source for the finding of novel ASDs, and several natural products (e.g. cannabidiol) have already been identified as promising against drug-resistant epilepsy.[112,184,187–189] Considering that TCM is rich of medicinal plants against epilepsy and seizures, in this project, neuroprotective and antiseizure medicinal plants from TCM were collected, and used to explore the possibility to discover new antiseizure lead compounds.

The specific aims of this part of the thesis therefore include:

1) to screen extracts of single plants employed in Traditional Chinese Medicine (TCM) against epilepsy in the PTZ zebrafish model, and after selection also the EKP zebrafish model,

2) to assess the antiseizure activity of major constituents of plant(s) of interest as novel lead compounds in the PTZ and EKP zebrafish models, and

3) to translate the findings from the zebrafish platform to the 6 Hz (44 mA) mouse model which represents a high potential platform to identify ASDs with a novel mechanism-of-action.

Objective 2

Dravet syndrome (DS) is a rare but severe developmental epileptic encephalopathy that is highly drug-resistant to commonly used ASDs.[58,222] A *de novo* mutation in the gene *SCN1A* which encodes for an α-subunit of the brain voltage gated sodium channel type-1 (NaV1.1), has been regarded as the genetic cause of the disease for a large majority of patients.[223]

In 2020, FDA approved fenfluramine (FFA) for treatment of seizures associated with DS. Chemically, FFA used in the clinic is a racemic mixture (including (-)-FFA and (+)-FFA enantiomers). In addition, pharmacokinetic investigations have shown that FFA is substantially metabolized to norfenfluramine (norFFA).[107,224] Moreover, circulating norFFA also consists of a racemic mixture of (-)-norFFA and (+)-norFFA. Although clinically used it is therefore presently not known whether the efficacy of racemic FFA is due to a single enantiomer of FFA, or to both, and whether the norFFA enantiomers also contribute significantly.

A zebrafish *scn1Lab double indemnity* (*didy*^{552}) mutant model that reflects well the genetic basis and characteristics of DS, has been successfully used for the discovery of "precision drugs" against DS.[167] Homozygous *scn1Lab* mutants display spontaneously occurring seizures and brain epileptic discharges.[167] In our previous work, we investigated the potential action mechanism of FFA with the *scn1Lab*$^{-/-}$ mutant model, and elucidated that FFA could modulate 5-HT$_{1D}$, 5-HT$_{2C}$, sigma-1, and possibly 5-HT$_{2A}$ receptors.[50,110]

The specific aims of this part of the thesis therefore include:

1) to pharmacologically validate the *scn1Lab*$^{-/-}$ mutant Dravet syndrome (DS) model and the experimental conditions used, with a set of ASDs typically deployed in the fight against DS, including combination therapy,

2) to explore the antiseizure activity of enantiomers of FFA and norFFA in the *scn1Lab*$^{-/-}$ mutant Dravet syndrome (DS) model, and

3) to examine the uptake of all four compounds in the larval zebrafish heads as a function of incubation time using LC-MS.

CHAPTER III

Zebrafish-Based Screening of Antiseizure Plants Used in Traditional Chinese Medicine: *Magnolia officinalis* Extract and Its Constituents Magnolol and Honokiol Exhibit Potent Antiseizure Activity in a Therapy-Resistant Epilepsy Model

Zebrafish-based screening of antiseizure plants used in Traditional Chinese Medicine: *Magnolia officinalis* extract and its constituents magnolol and honokiol exhibit potent antiseizure activity in a therapy-resistant seizure model

Jing Li[1], Daniëlle Copmans[1], Michèle Partoens[1], Borbála Hunyadi[2], Walter Luyten[3] and Peter A. M. de Witte[1]

[1] Laboratory for Molecular Biodiscovery, Department of Pharmaceutical and Pharmacological Sciences, KU Leuven, 3000 Leuven, Belgium

[2] STADIUS Center for Dynamical Systems, Signal Processing and Data Analytics, Department of Electrical Engineering (ESAT), KU Leuven, 3001 Leuven, Belgium

[3]Department of Biology, KU Leuven, 3000 Leuven, Belgium

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1. Abstract

With the aim to discover interesting lead compounds that could be further developed into compounds active against pharmacoresistant epilepsies, we first collected fourteen medicinal plants used in Traditional Chinese Medicine (TCM) against epilepsy. Of the six extracts that tested positive in a pentylenetetrazole (PTZ) behavioral zebrafish model, only the ethanol and acetone extracts from *Magnolia officinalis* (*M. officinalis*) also showed effective antiseizure activity in the ethylketopentenoate (EKP) zebrafish model. The EKP model is regarded as an interesting discovery platform to find mechanistically novel antiseizure drugs, as it responds poorly to a large number of marketed antiseizure drugs.

We then demonstrated that magnolol and honokiol, two major constituents of *M. officinalis*, displayed an effective behavioral and electrophysiological antiseizure activity in both the PTZ and the EKP models. Out of six structural analogs tested, only 4-O-methylhonokiol was active, and to a lesser extent tetrahydromagnolol, whereas the other analogs (3,3'-dimethylbiphenyl, 2,2'-biphenol, 2-phenylphenol, 3,3',5,5'-tetra-tert-butyl-[1,1'-biphenyl]-2,2'-diol) were not consistently active in the aforementioned assays.

Finally, magnolol was also active in the 6 Hz psychomotor mouse model, an acute therapy-resistant rodent model, thereby confirming the translation of findings from zebrafish larvae to mice in the field of epilepsy.

We also developed a fast and automated power spectral density (PSD) analysis of local field potential (LFP) recordings. The PSD results are in agreement with the visual analysis of LFP recordings using Clampfit software and manually counting the epileptiform events.

Taken together, screening extracts of single plants employed in TCM using a combination of zebrafish- and mouse-based assays, allowed us to identify allyl biphenol as a chemical scaffold for the future development of compounds with potential activity against therapy-resistant epilepsies.

KEYWORDS: zebrafish, antiseizure drug discovery, magnolol, honokiol, pharmacoresistant model, antiseizure activity

2. Introduction

Epilepsy is a chronic and devastating brain disease characterized by an imbalance of excitatory and inhibitory processes, resulting in unpredictable, unprovoked, recurrent seizures.[1] So far, pharmacological intervention is the main treatment, since only few people are considered suitable for a ketogenic diet or surgical intervention.[2,3] Unfortunately, the marketed antiseizure drugs (ASDs) control seizures in only about 70% of the patients,[4] Moreover, a considerable number of patients also experiences mild to moderately severe side effects.[5,6]

Before the advent of chemically synthesized compounds, medicinal plants have been used for centuries to treat epilepsy, and control related syndromes in many countries around the world.[7] Consequently, plant extracts have been regarded as a reliable source for the discovery of new ASDs.[8] This is illustrated by the recent finding that cannabidiol (CBD), a constituent of *Cannabis sativa*, demonstrated potent antiseizure activity in several animal models, and also in clinical practice.[8,9]

Traditional Chinese Medicine (TCM) is one of the most widely practiced forms of botanical therapies in the world; it includes multiple recipes of medicinal plants against epilepsy and seizures.[10] Moreover, relying on the robust development of chromatographic and spectroscopic methods,[11] thousands of pure compounds have been purified and identified from TCM, facilitating the identification of active substances using a variety of *in vivo* and *in vitro* assays.

Relevant epilepsy and epileptic seizure models are crucial for the discovery of new ASDs. There are over a hundred models developed to date,[12] mainly grouped into seizure-induced and genetic models.[13] Rodents and zebrafish are the most frequently used animals in these models. Compared with rodent models, zebrafish models have advantages for large-scale drug screenings thanks to high fertility, fast embryonic development, small size, ease of maintenance and of drug administration.[14] Furthermore, zebrafish show high cell- and organ homologies to humans, and approximately 85% of human disease-related genes can be correlated to at least one zebrafish orthologue.[15]

To discover interesting lead compounds that could be further developed into compounds active against pharmacoresistant epilepsies, in this work first neuroprotective and antiseizure medicinal plants were identified based on the Chinese Pharmacopoeia and data published in literature using search terms like “epilepsy”, “seizure”, “TCM”, and “traditional Chinese medicine”.[10,16-19] Then, extracts of the selected plant were tested for their antiseizure activity using a pentylenetetrazole (PTZ)-induced zebrafish locomotor model. PTZ-based animals

models display generalized tonic-clonic seizures and epileptiform brain activity, and have been used extensively as a standard assay for the discovery of ASDs.[20,21] Active extracts were then further examined for their locomotor effect in an ethylketopentenoate (EKP)-induced zebrafish seizure model. Seizures elicited in zebrafish treated with EKP demonstrate a high level of resistance against commercially available ASD, hence the EKP model is regarded as an interesting discovery platform to find mechanistically novel ASDs.[22]

We show that extracts of *Magnolia officinalis*, its main constituents magnolol and honokiol, as well as the structurally related allyl biphenolic methylhonokiol, exerted a potent and effective inhibition of PTZ- and EKP-induced locomotor and brain hyperactivity in zebrafish larvae. Moreover, magnolol was also active in the 6 Hz psychomotor mouse model, an acute therapy-resistant rodent seizure model.[23,24]

3. Results and discussion

3.1 Zebrafish-based screen of antiseizure medicinal plant extracts

Fourteen medicinal plants (Table III-1) used in TCM to treat seizures, were collected. Their medicinal parts were ground into a powder and extracted in parallel with three different solvents of increasing polarity (acetone, ethanol or water). A PTZ-induced locomotor assay in zebrafish was used as a first test for the antiseizure activity of all 42 extracts, administered to the medium. Extracts were prepared at concentrations ranging from 50 to 6.25 μg/mL, following a 2-fold serial dilution. An extract was considered effective if it reduced not less than 40% of PTZ-induced seizure movement, compared with the PTZ control group at its maximum tolerated concentration (MTC).

Based on this criterion, six plant extracts from four medicinal plants, i.e. *Anemarrhena asphodeloides* (*A. asphodeloides*), *Bupleurum chinensis* (*B. chinesis*), *M. officinalis* (see Fig. 1 A-D) and *Scutellaria baicalensis* (*S. baicalensis*), were identified as effective (Table III-1).

It is notable that most of these plants have been previously described to have neuroactivity and/or therapeutic effects. For instance, an extract of *A. asphodeloides* had a favorable effect in ischemia-induced brain injury in rats,[25] likely due to sarsasapogenin and possibly related compounds that possess neuroprotective activity.[26] Saikosaponin was shown to be responsible for the antiseizure activity of *B. chinensis* in a variety of epilepsy models, as a result of an inhibition of the NMDA receptor current and the persistent sodium current (INaP).[27] Moreover,

saikosaponin A also counteracted the inflammatory response and displayed the neuroprotective effects in traumatic brain injury rats.[28] Of interest, though the blood/brain barrier (BBB) permeability of saikosaponin might be restricted due to its hydrophilicity and its large molecular mass, several *in vitro* and *in vivo* studies demonstrated that saikosaponin can be converted into its lipophilic aglycon (i.e. saikogenin) by means of gastric fluid and the intestinal flora.[29,30]

Table III-1. Antiseizure activity of medicinal plant extracts using zebrafish-based seizure models

Medicinal plant	Plant part	PTZ model			EKP model		
		Water extract (a)	Ethanol extract (b)	Acetone Extract (c)	Water extract (a)	Ethanol extract (b)	Acetone extract (c)
***Anemarrhena asphodeloides* Bunge**	rhizome	×	√	√	–	×	×
***Angelica pubescens* Maxim.**	root	×	×	×	–	–	–
***Asarum heterotropoides* f. mandshuricum (Maxim.) Kitag.**	rhizome and root	×	×	×	–	–	–
***Astragalus membranaceus* var. mongholicus (Bunge) P.K.Hsiao**	root	×	×	×	–	–	–
***Bupleurum chinensis* DC.**	root	×	×	√	–	–	×
***Coptis chinensis* Franch.**	rhizome	×	×	×	–	–	–
***Gastrodia elata* Blume**	tuber	×	×	×	–	–	–
***Magnolia officinalis* Rehder & E.H.Wilson**	bark	×	√	√	–	√	√
***Notopterygium incisum* K.C.Ting ex H.T. Chang**	rhizome and root	×	×	×	–	–	–
***Paris polyphylla* var. yunnanensis (Franch.) Hand.-Mazz.**	rhizome	×	×	×	–	–	–
***Poria cocos* (Schw.) Wolf**	sclerotium	×	×	×	–	–	–
***Rehmannia glutinosa* (Gaertn.) DC.**	tuberous root	×	×	×	–	–	–
***Scutellaria baicalensis* Georgi**	root	×	√	×	–	×	–
***Nelumbo nucifera* Gaertn.**	plumule	×	×	×	–	–	–

× Toxic effect or < 40% reduction of seizure movement in comparison to PTZ/EKP control. √ ≥ 40% reduction of seizure movement in comparison to PTZ/EKP control. – Not tested in this model.

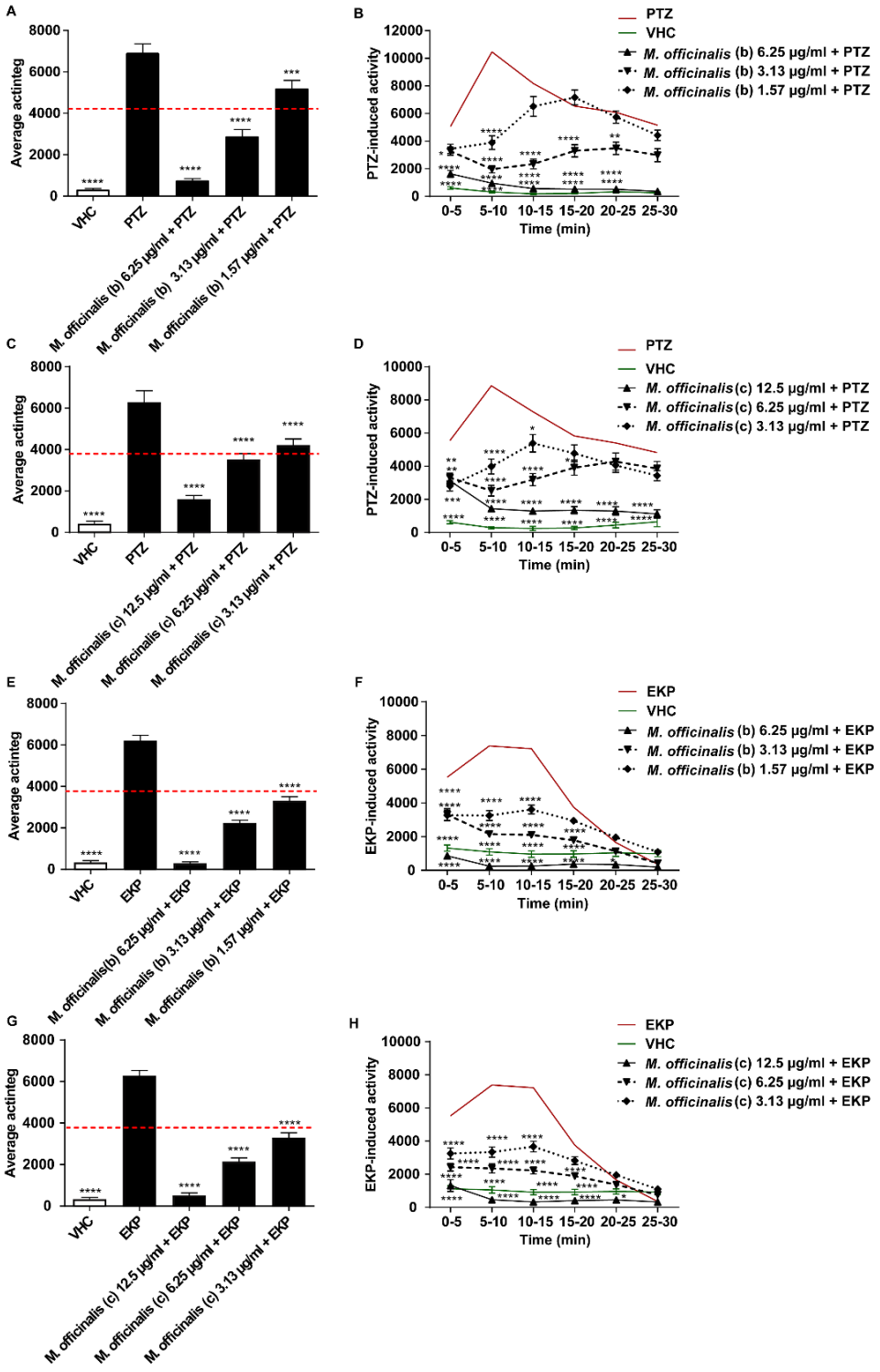


Figure III-1. Behavioral antiseizure activity of *M. officinalis* ethanol and acetone extract in the PTZ/EKP zebrafish models. Behavioral antiseizure activity of *M. officinalis* ethanol extract (b) and acetone extract (c) in the zebrafish pentylenetetrazole (PTZ) seizure model (A-D) and ethylketopentenoate (EKP) seizure model (E-H), respectively, after 2-hour incubation. PTZ-induced seizure-like behavior is expressed as mean actinteg units/5 min (± SEM) during the 30-min recording period (A, C) and over 5-min time intervals (B, D). EKP-induced seizure-like behavior is expressed as mean actinteg units/5 min (± SEM) during the 20-min recording period (E, G) and over 5-min time intervals (F, H). The red dashed line represents 60% of seizure-related movement of the PTZ/EKP control (A, C, E, G). Results were pooled from 3 independent experiments with 10 larvae per experiment. Statistical analysis: one-way ANOVA with Dunnett's multiple comparison test (A, C, E, G), two-way ANOVA with Bonferroni posttests (B, D, F, H). Significance levels: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. Abbreviation: VHC, vehicle.

For *M. officinalis* and other Magnolia species, a broad range of therapeutic effects have been described, including anxiolytic, central nervous system (CNS) depressant, anti-inflammatory, anti-tumoral and anti-epileptic.[31,32] The major constituents of this species are phenolic compounds, notably magnolol and honokiol, that make up to about 3-10% of their dry weight, and were shown to be non-toxic.[33,34] Finally, in case of *S. baicalensis*, a flavone glucuronide called baicalin has been isolated that exhibited antiseizure and neuroprotective effects in a pilocarpine-induced epilepsy model in rats.[35] Interestingly, Zhang *et al.*[36] investigated the distribution of baicalin metabolites in various tissues of rats, and found five metabolites in the brain, including methylbaicalein, a deglucuronidated and methylated aglycon. To what extent each of the metabolites contributes to the *in vivo* activity however is not known.

To find interesting hit compounds starting from TCM plant extracts that could be used against drug-resistant epilepsies, we then further tested the six effective plant extracts in the zebrafish EKP seizure model. In this case, only extracts of *M. officinalis* significantly inhibited EKP-triggered seizure behavior (see Table III-1 and Figure III-1 E-H) in an effective way (> 40% reduction as compared to the EKP control group). Moreover, magnolol and honokiol that are the biologically active constituents present in *M. officinalis* extracts were chosen for further investigations.[37]

3.2 Activity of magnolol and honokiol and their analogs in zebrafish PTZ- and EKP-seizure models

The antiseizure activity of magnolol and honokiol were tested in the PTZ and EKP model using a locomotor assay. A compound was considered effective if it reduced not less than 40% of PTZ/EKP-induced seizure movement, compared with the PTZ/EKP control group at its maximum tolerated concentration (MTC). Both magnolol and honokiol significantly and effectively (> 40% reduction) counteracted PTZ-provoked locomotor activity of larvae after a 2-hour incubation, but only at their MTC (Figure III-2A-D). Lower concentrations were not active. Conversely, when tested in the EKP model, both compounds showed significant activity in a clear concentration-dependent manner (Figure III-2E-H). Magnolol was more potent than honokiol, as it effectively inhibited the EKP-induced locomotor activity by more than 40% at its ½ MTC as well.

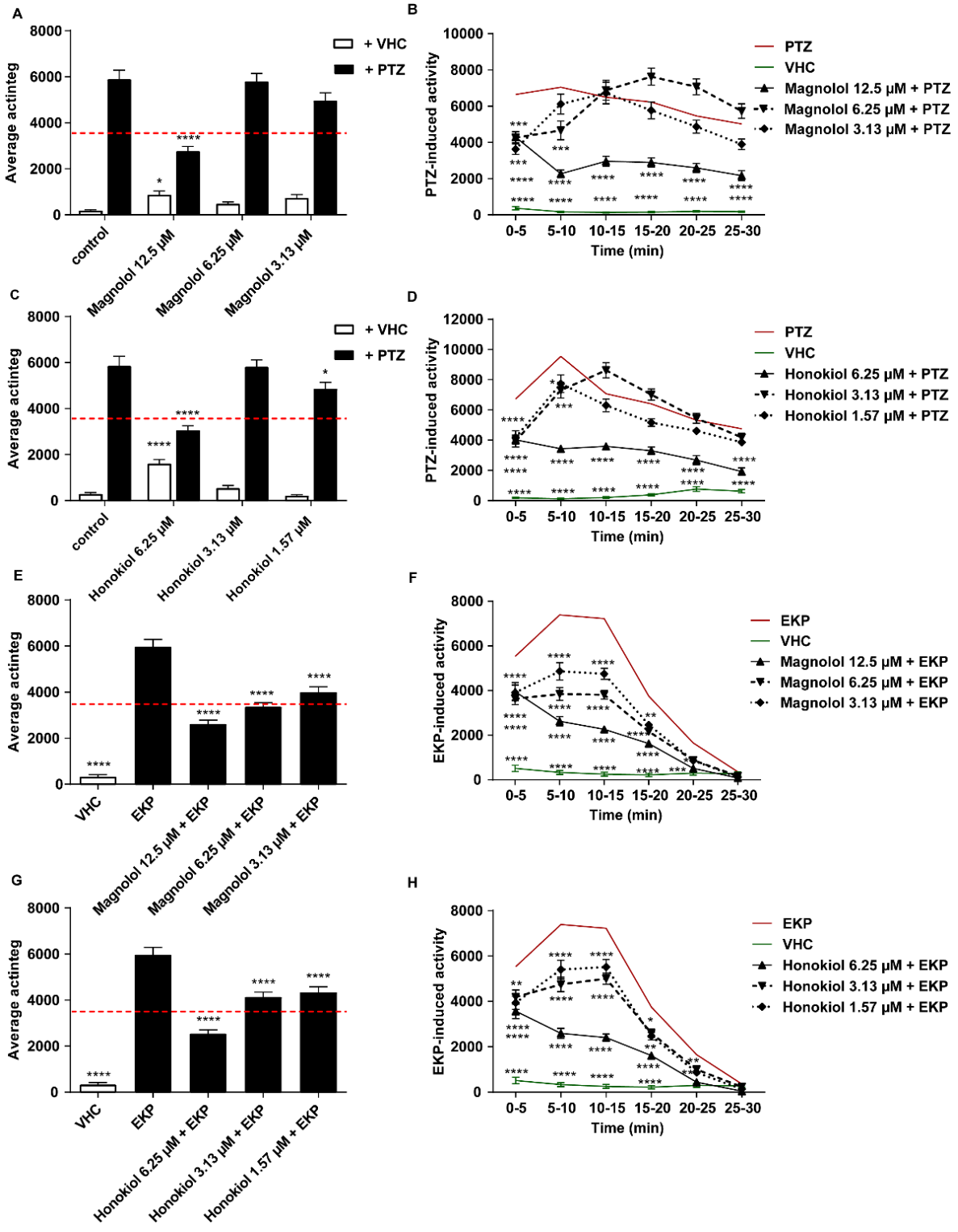


Figure III-2. Behavioral antiseizure activity of magnolol and honokiol in the PTZ/EKP zebrafish models. Behavioral antiseizure activity of magnolol and honokiol in the zebrafish pentylenetetrazole (PTZ) seizure model (A-D) and ethylketopentenoate (EKP) seizure model (E-H), respectively, after a 2-hour incubation. PTZ-induced seizure-like behavior is expressed as mean actinteg units/5 min (± SEM) during the 30-min recording period (A, C) and over 5-min time intervals (B, D). EKP-induced seizure-like behavior is expressed as mean actinteg units/5 min (± SEM) during the 20-min recording period (E, G) and over 5-min time intervals (F, H). The red dashed line represents 60% of seizure-related movement of the PTZ/EKP control (A, C, E, G). Results were pooled from 4 independent experiments with 10 larvae per experiment. Statistical analysis: one-way ANOVA with Dunnett's multiple comparison test (A, C, E, G), two-way ANOVA with Bonferroni posttests (B, D, F, H). Significance levels: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. Abbreviation: VHC, vehicle.

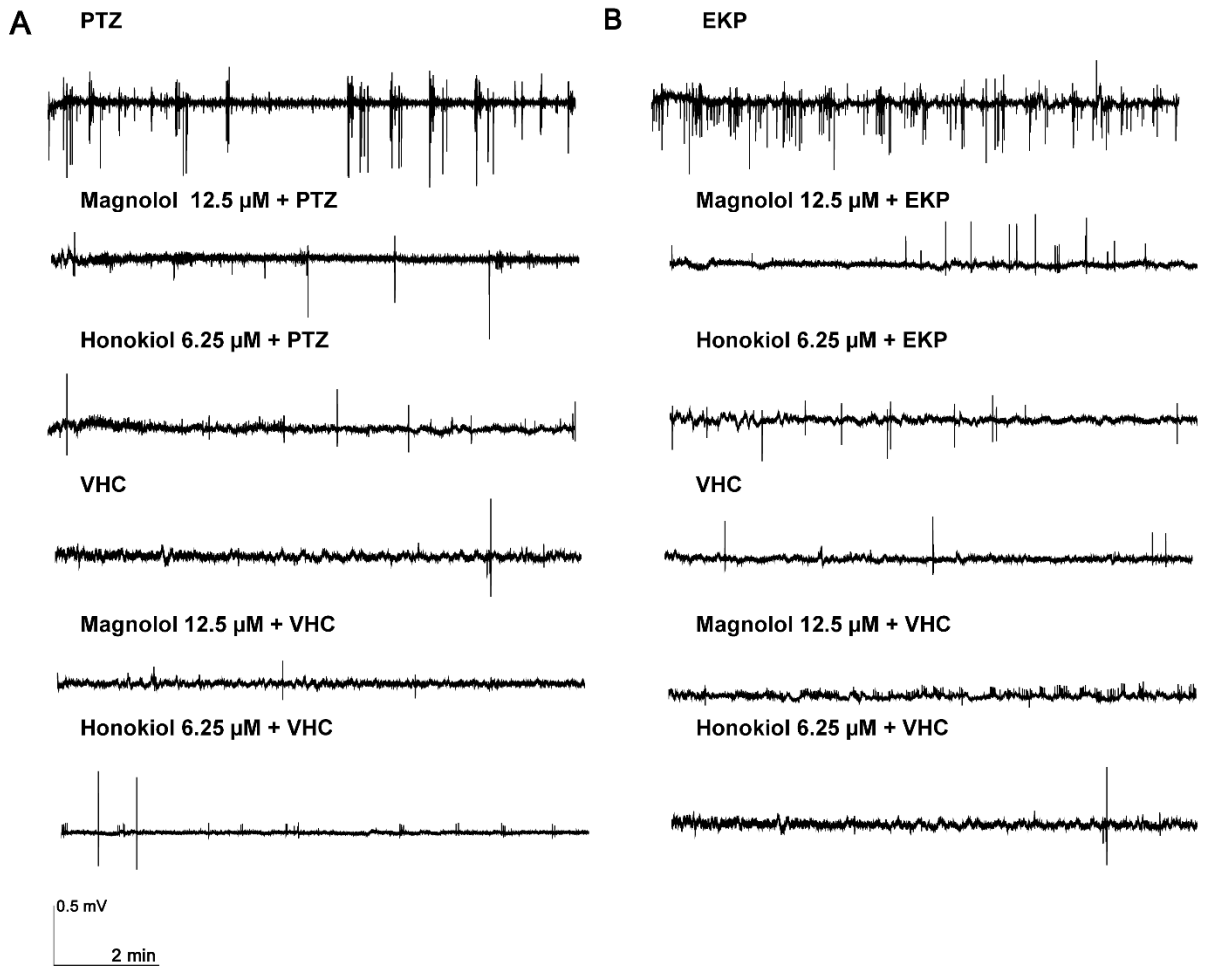


Figure III-3. Representative local field potential recordings. Ten-min noninvasive local field potential (LFP) recordings from the optic tectum of larvae pre-exposed to vehicle (VHC), pentylenetetrazole (PTZ), ethylketopentenoate (EKP), compound supplemented with PTZ/EKP, or compound supplemented with VHC. Larvae were incubated with 12.5 µM magnolol or 6.25 µM honokiol for 2 h.

To confirm these results, the effects of magnolol and honokiol were investigated at their respective MTCs on the epileptiform brain discharges induced by PTZ and EKP. Brain activity was monitored by non-invasive local field potential (LFP) measurements, using an electrode positioned on the skin above the optic tectum. The recordings were scored by visual inspection using Clampfit software by counting the epileptiform events present, as described before by our,[20,38] and other groups.[39,40] As shown in Figure III-3A and 4A-B, both compounds significantly reduced the frequency of epileptiform events, resulting in a decrease of cumulative duration compared to PTZ-treated controls. A similar effect was observed in the EKP model (Figure III-3B and 4C-D).

Somewhat surprisingly, we also found that magnolol and honokiol increased the locomotor activity in control conditions (i.e. in the absence of PTZ/EKP) (Figure III-2A and 2C). However, a similar hyperactivity in control conditions has been observed also for other antiseizure

compounds.[38,41] In addition, the electrophysiological analysis of brain activity in compound-treatment conditions did not reveal any abnormalities (Figure III-3 and 4). This outcome suggests that magnolol and honokiol do not affect normal brain activity significantly, and that the increased locomotor activity may be due to some undefined effect on the peripheral parts of the larval body.

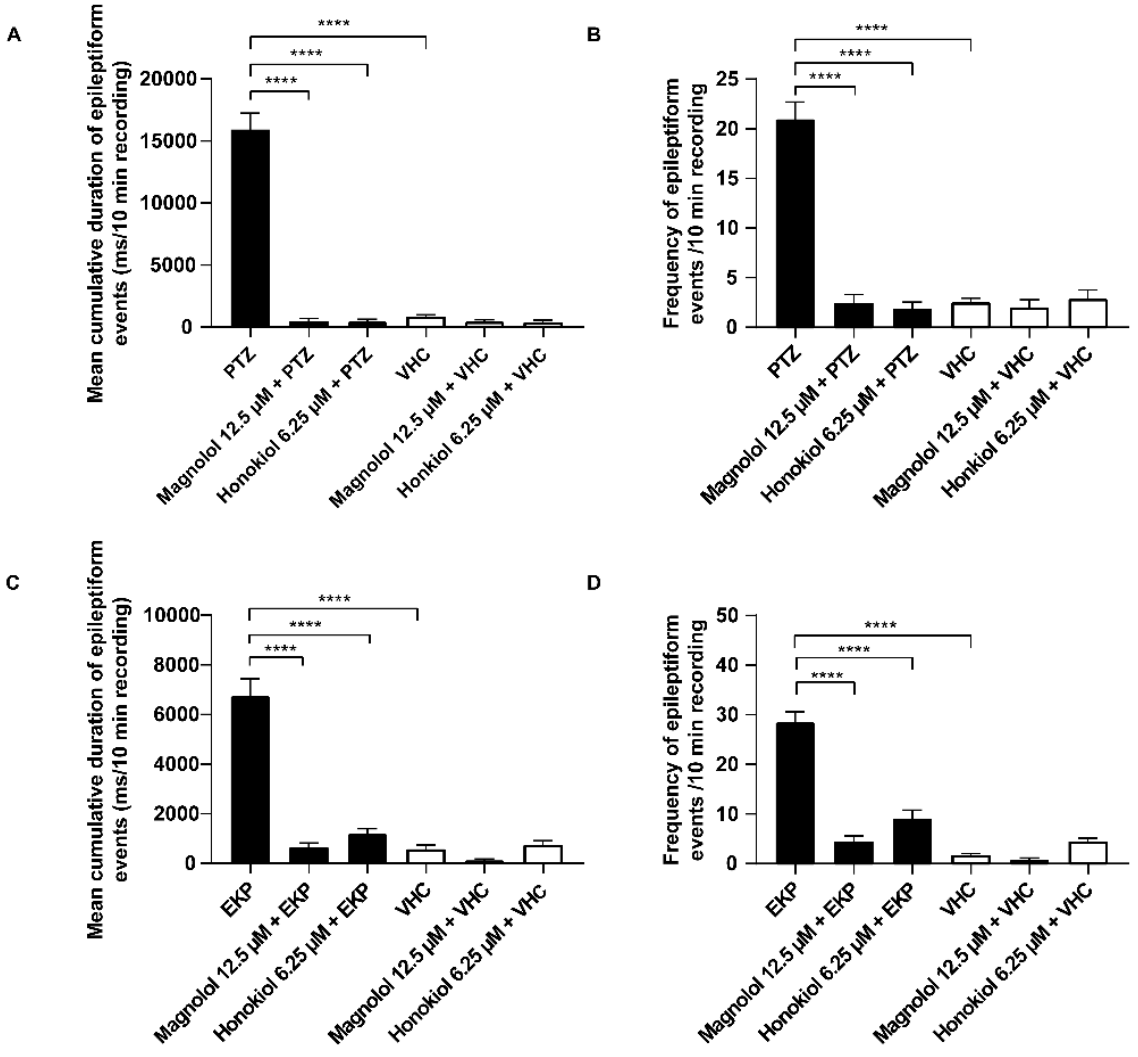


Figure III-4. Electrophysiological antiseizure activity of magnolol and honokiol (visual analysis) in the PTZ/EKP zebrafish models. Noninvasive local field potential (LFP) recordings from the optic tectum of larvae pre-exposed to vehicle (VHC), pentylenetetrazole (PTZ), ethylketopentenoate (EKP), compound supplemented with PTZ/EKP or compound supplemented with VHC. Larvae were incubated with 12.5 µM magnolol or 6.25 µM honokiol for 2 h. Epileptiform discharges are quantified by the cumulative duration (mean ± SEM) (A, C) and number (mean ± SEM) (B, D) of events per 10-min recording. Number of larvae per condition: n = 31-33 for the VHC/PTZ/EKP group, n=15-17 for the magnolol/honokiol+PTZ/EKP/VHC group. Statistical analysis: one-way ANOVA with Dunnett's multiple comparison test. Significance levels: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

Looking for a fast and reliable alternative to the visual analysis of LFP recordings, an automated method was developed that enabled us to quantify the increase in spectral power caused by epileptic activity of multiple LFP recordings simultaneously. Power spectral density (PSD) computation has been used successfully before to analyze electrophysiological signals of the brain in rodents and patients.[42-44]

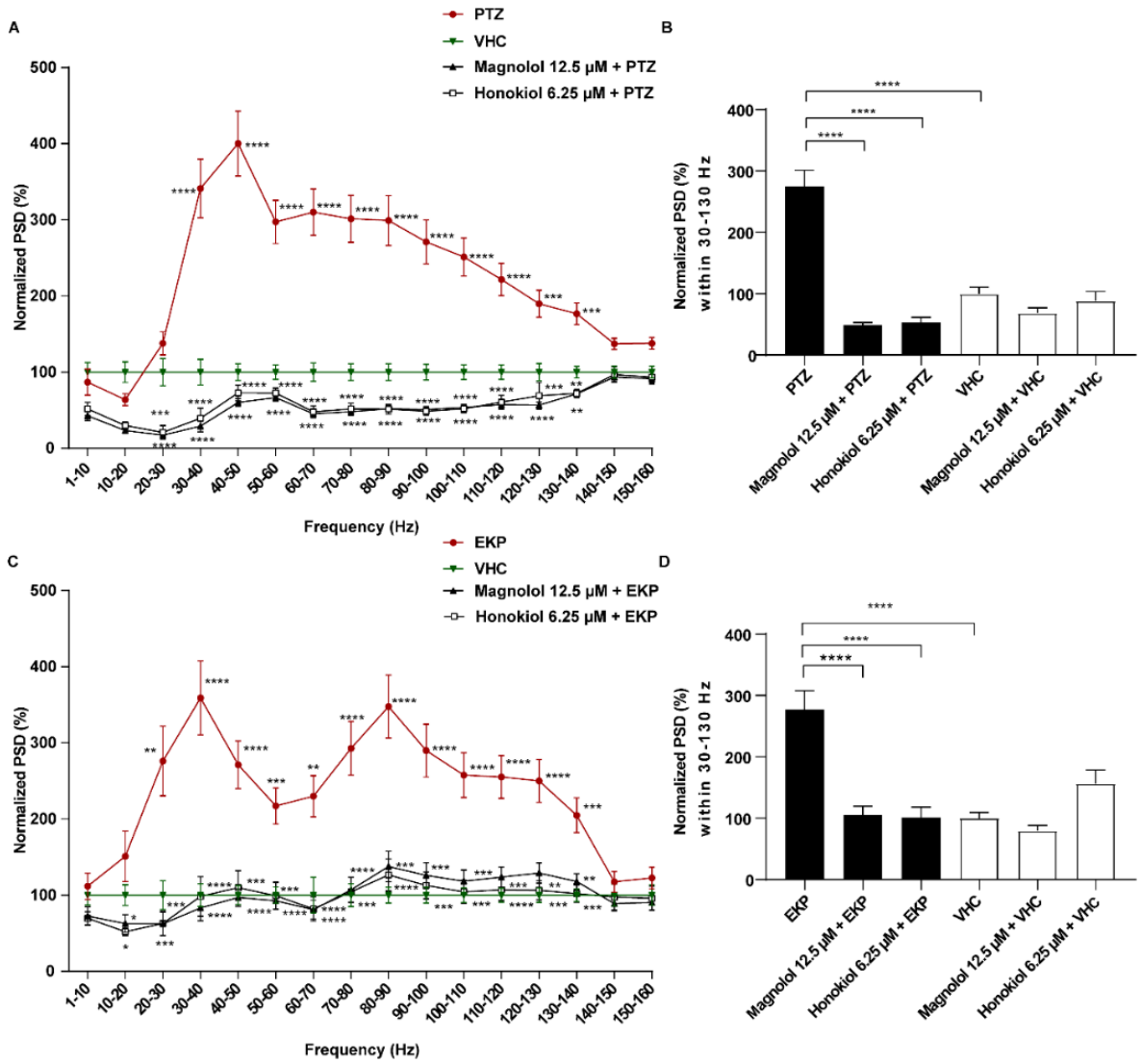


Figure III-5. Electrophysiological antiseizure activity of magnolol and honokiol (PSD analysis) in the PTZ/EKP zebrafish models. The power spectral density (PSD) ranging from 0-160 is normalized against the (vehicle) VHC control, and the data are plotted as mean (± SEM) PSD per 10Hz (A, C) and per condition over the 30-130 Hz region (B, D). For the sake of clarity, the data of magnolol/honokiol+VHC are not shown in Figure 5A and 5C. Number of larvae per condition: n = 31-33 for the VHC/PTZ/EKP group, n=15-17 for the magnolol/honokiol+PTZ/EKP/VHC group. Statistical analysis: two-way ANOVA with Bonferroni posttests, PTZ/EKP control in comparison to VHC group, magnolol/honokiol+PTZ/EKP group in comparison to PTZ/EKP control (A, C), one-way ANOVA with Dunnett's multiple comparison test (B, D). Outliers were identified via the Iterative Grubbs test (Alpha = 0.1) (A-D). Significance levels: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

As shown in Figure III-5A and 5C, the power spectral density estimates of signals in each LFP recording was determined per 10 Hz frequency band, ranging from 0-160 Hz using Welch's method. Next, the PSD was normalized against the VHC control and the data were plotted as mean (±SEM) PSD per 10Hz. PTZ and EKP treatment induced a significantly higher LFP power in the frequency range between 30-130 Hz. Therefore, the PSD were plotted as mean (±SEM) PSD per condition over the 30-130 Hz region (Figure III-5B and 5D). As shown in Figure III-5B and 5D, magnolol and honokiol had a major and statistically significant inhibitory effect on the PSD obtained after PTZ and EKP treatment, in line with the outcome of the visual analysis (Figure III-4). These results therefore validate PSD analysis as an accurate method to examine LFP recordings, increasing dramatically the data throughput possible.

Valproate and perampanel, used as positive controls in the PTZ and EKP models, respectively, exhibited a clear inhibitory effect on the behavioral (Figure III-7) and electrographical seizures (Figure III-8, supplementary Figure III-1A and 1D), as observed before.[20,22]

Overall, our results demonstrate that magnolol and honokiol are among the main antiseizure constituents present in *M. officinalis* extracts. To start understanding the structure-activity relationship of these biphenolic structures, six commercially available analogs were examined at their MTCs for their inhibitory activity in both the PTZ- and EKP-zebrafish assays (Figure III-6 and Table III-2). Compound 7 (Figure III-6 and Table III-2) precipitated in the VHC before reaching the MTC. In this case, the maximum soluble concentration (MSC) (6.25 µM) was used instead. Tetrahydromagnolol was tested at ½ MTC in case of the EKP assay, as this compound induced toxicity at its MTC when examined in the presence of EKP.

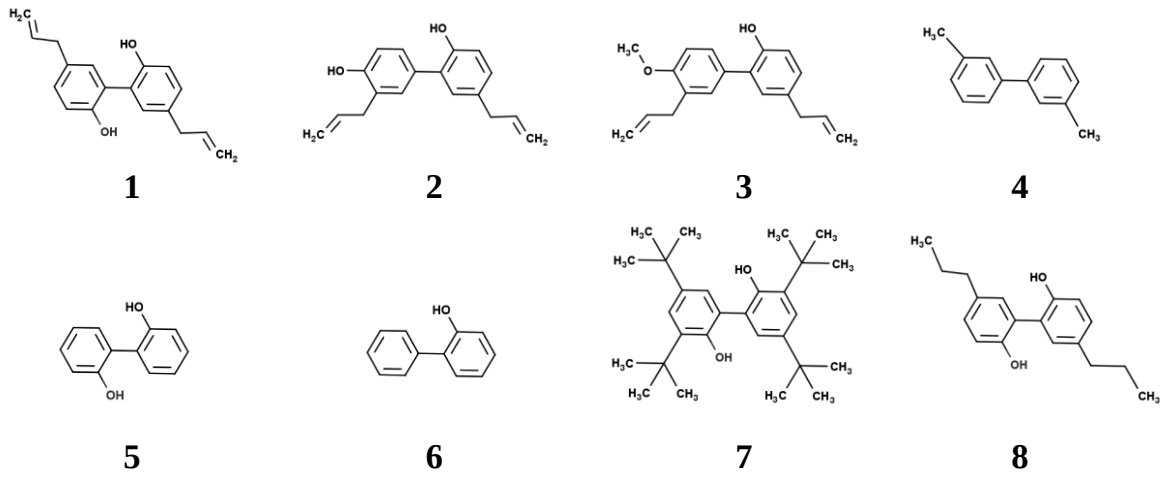


Figure III-6. Structures of magnolol, honokiol and their analogs.

Table III-2. Physicochemical properties and MTC of magnolol and honokiol and their analogs

Compound	MW	clog P	clog D (pH=7.6)	PSA (pH=7.6)	Rotor	HBD (pH=7.6)	HBA (pH=7.6)	MTC (μM)
Magnolol	266.34	5.25	5.17	40.46	5	2	2	12.5
Honokiol	266.34	5.25	5.17	40.46	5	2	2	6.25
4-O-Methylhonokiol	280.37	5.28	5.34	29.46	6	1	2	1.57
3,3′-Dimethylbiphenyl	182.27	4.67	4.65	0	1	0	0	50
2,2′-Biphenol	186.21	3.16	3.00	40.46	1	2	2	25
2-Phenylphenol	170.21	3.45	3.31	20.23	1	1	1	12.5
3,3',5,5'-Tetra-tert-butyl-[1,1'-biphenyl]-2,2'-diol	410.64	9.67	9.19	40.46	5	2	2	6.25
Tetrahydromagnolol	270.37	5.68	5.81	40.46	5	2	2	3.13

MW: molecular weight, clog P: calculated partition coefficient, clog D: calculated distribution coefficient, PSA: polar surface area, Rotor: rotatable bonds, HBD: number of hydrogen bond donors, HBA: number of hydrogen bond acceptors, all physicochemical properties of compounds were calculated using ChemAxon software imbedded in MarvinSketch 20.2.0.

Methylhonokiol (compound 3) and tetrahydromagnolol (compound 8) exhibited a significant inhibitory effect on the PTZ-increased locomotor activity (Figure III-7A), although the latter did not effectively (< 40% inhibition) reduce the PTZ-associated activity. In the EKP locomotor model, all analogs except for compound 4 (dimethylbiphenyl) and 7 (tetra-tert-butyl[biphenyl]diol), were significantly active, but only methylhonokiol (compound 3) inhibited the EKP-associated activity in an effective way (> 40% reduction) (Figure III-7B).

In general, this outcome is in agreement with the electrophysiological results (LFP recordings, PSD analysis). In the PTZ model, methylhonokiol (compound 3) was significantly active, but also tetrahydromagnolol (compound 8) exhibited similar inhibitory activity (Figure III-8A, and supplementary Figure III-1B-C). Conversely, in the EKP model, only methylhonokiol (compound 3) had an inhibitory effect on the epileptiform discharges. Tetrahydromagnolol (compound 8) exhibit some inhibitory (non-significant, p=0.15) activity (Figure III-8B, and supplementary Figure III-1E-F).

Taken together, only magnolol, honokiol, as well as its methylated analog (compound 3) had a substantial inhibitory effect on abnormal locomotor activity, and especially epileptiform brain discharges, induced by both PTZ and EKP. Tetrahydromagnolol was somewhat less active, whereas the other compounds were not consistently active.

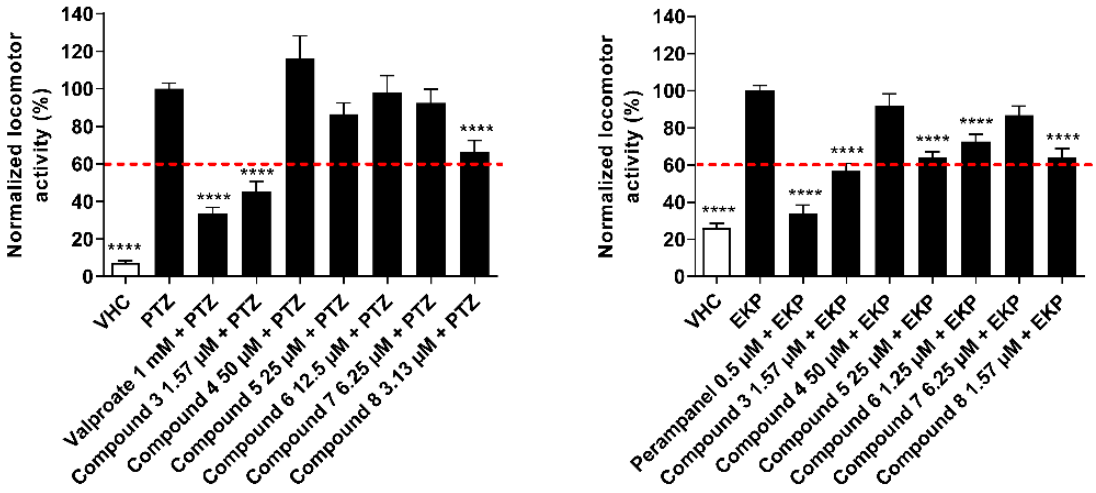


Figure III-7. Behavioral antiseizure activity of structural analogs in the PTZ/EKP zebrafish models. Antiseizure activity of structural analogs in the zebrafish pentylenetetrazole (PTZ) seizure model (A) and ethylketopentenoate (EKP) seizure model (B), respectively, after a 2-hour incubation. PTZ-induced seizure-like behavior within a 30-min recording normalized to PTZ control (mean ± SEM) (A). EKP-induced seizure-like behavior within a 20-min recording normalized to EKP control (mean ± SEM) (B). The red dashed line represents 60% of seizure-related movement of the PTZ/EKP control (A-B). Valproate and perampanel were used as positive controls for the PTZ and EKP model, respectively. Results were pooled from 3 independent experiments with 10 larvae per experiment. Statistical analysis: one-way ANOVA with Dunnett's multiple comparison test. Significance levels: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. Abbreviation: VHC, vehicle.

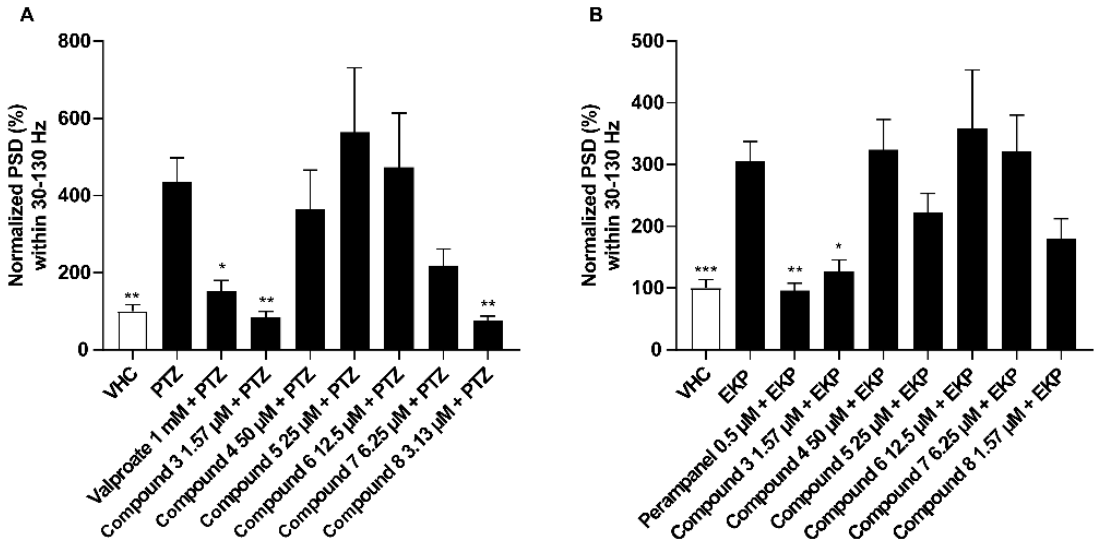


Figure III-8. Electrophysiological antiseizure activity (PSD analysis) of structural analogs in the PTZ/EKP zebrafish models. The power spectral density (PSD) ranging from 0-160 Hz is normalized against the (vehicle) VHC control and the data are plotted as mean (± SEM) PSD per condition over the 30-130 Hz region (A, B). Valproate and perampanel were used as positive controls. Number of larvae per condition: n = 16-19 for the VHC/PTZ/EKP group, n=12-17 for the valproate/perampanel+PTZ/EKP group, n=10 for analogs+PTZ/EKP. Statistical analysis: one-way ANOVA with Dunnett's multiple, outliers were identified via the Iterative Grubbs test (Alpha = 0.1). Significance levels: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

As the BBB (blood/brain barrier) is not fully matured in 7 dpf larvae,[45] we can assume that all absorbed compounds penetrated into the central nervous system (CNS). Conversely, the inactivity of some of the compounds tested could be due to a lack of body absorption during larval exposure or, after absorption, due to a limited affinity for the protein targets concerned. Interestingly, recently hundreds of compounds reported to be active in zebrafish assays were chemically described, and it was found that zebrafish-absorbed compounds typically fulfil certain criteria: (a) MW ≤ 500; (b) clog P ≤ 5.3; (c) HBD ≤ 3; (d) HBA ≤ 7; (d) PSA ≤ 124 A°; (e) rotatable bonds ≤ 9.[46] From Table III-2 it can be seen that all compounds used in this work comply with the requirements, except for compound 7 (log P: 9.67). So it is anticipated that compound 7 was not active in our *in vivo* assays because of a lack of absorption in larval zebrafish.

Magnolol and honokiol have demonstrated positive allosteric modulatory effects on γ-aminobutyric acid type A ($GABA_A$) receptors.[32,47] In particular, honokiol exhibits a positive effect on chloride current (I_{GABA}) through $GABA_A$ receptors of seven different subunits compositions, showing most activity on $\alpha_3\beta_2$, $\alpha_2\beta_2$, $\alpha_1\beta_2$ and $\alpha_1\beta_1$.[48] As compared to honokiol, its methyl-derivative (compound 3) was about twice as active at $\alpha_1\beta_2$ receptors as I_{GABA} modulator at 30 µM, whereas magnolol was equally active.[48]

According to the pharmacophore model established using the $\alpha_1\beta_2$ $GABA_A$ receptor, active honokiol derivatives exhibit hydrophobic regions (represented by the 2-propenyl substituents in honokiol), one acceptor (aromatic ring) and one donor region (hydroxy group).[48] Of interest, also Fuchs *et al.* found that an alkyl residual is essential for the action of biphenolics at $GABA_A$ receptors.[49] These data therefore suggest that compounds 4, 5 and 6 are not active in the PTZ assay as they lack at least one of the pharmacophore characteristics.[48] Moreover, replacing the 2-propenyl (i.e. allyl) by a propyl-substituent (as present in tetrahydromagnolol) modified the activity, indicating that also the type of alkyl group is of importance.

Furthermore, as PTZ prompts neuronal excitability predominantly by antagonizing GABAergic inhibition,[50] we conclude that potentiation of GABAergic transmission probably accounts for the antiseizure activity of the active allyl biphenolics observed in the PTZ model.

The EKP zebrafish model shows a poor response to several existing ASDs. For instance, out of thirteen clinically tested antiepileptics, only the (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) AMPA receptor inhibitor perampanel showed a clear inhibitory effect on both the EKP-triggered excessive behavior- and brain activity,[22] whereas GABAergic

compounds like tiagabine (which prevents GABA reuptake) and valproate (which inhibits GABA degradation) were inactive,[22] Thus, the positive allosteric modulatory effect on $GABA_A$ receptors exerted by these compounds is unlikely to account for their inhibitory effects observed in the EKP zebrafish model.

However, allyl biphenolics can interact with other molecular targets as well, which might explain the activity observed in the EKP model. For instance, magnolol and honokiol have potent effects on cannabinoid (CB) receptors at low micromolar concentrations,[51-53] and CB receptors are therapeutic targets for epilepsy.[54,55] Furthermore, it was found that magnolol can weaken both glutamate- and NMDA-induced neurotoxicity, prevent the increasing Ca^{2+} influx caused by glutamate,[56] and exhibits affinity for the AMPA receptor.[57]

3.3 Antiseizure analysis of magnolol in the mouse 6-Hz psychomotor seizure model

Finally, we tested whether the antiseizure activity of magnolol, as detected in zebrafish models, translates to the 6 Hz (44 mA) psychomotor mouse model, a standard rodent model able to detect compounds with novel antiseizure mechanisms, and with potential activity against drug-resistant seizures.[23,58] Seizures are characterized by stun, clonus, twitching of the vibrissae, and straub tail.

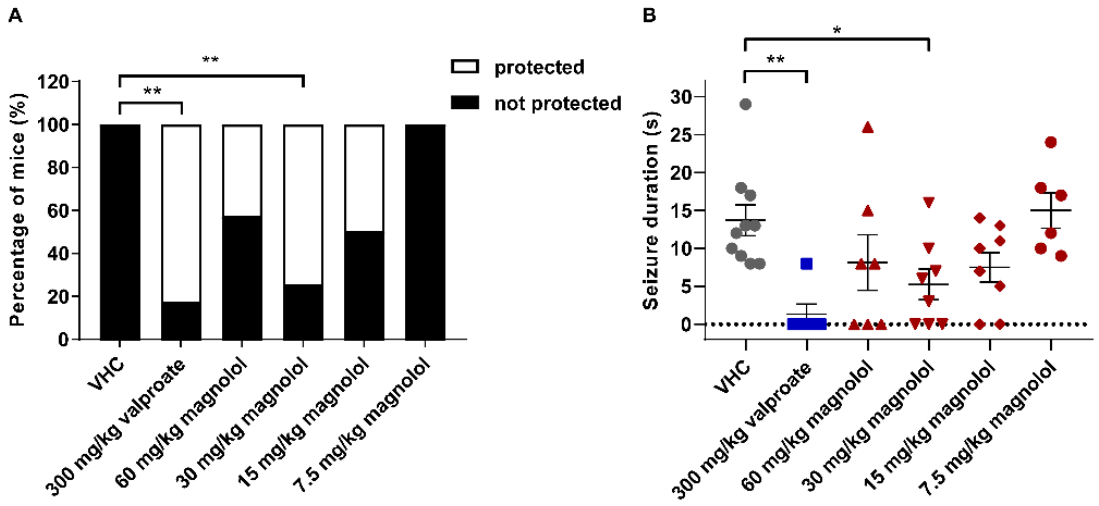


Figure III-9. Antiseizure activity of magnolol in the mouse 6-Hz psychomotor seizure model. Psychomotor seizures were electrically induced (6 Hz, 0.2 ms rectangular pulse width, 3 s duration, 44 mA) through cornea, 60 min after intraperitoneal injection of vehicle (VHC, n = 10), positive control valproate (n = 6), or test compound (n = 6-8 per dose). Number of mice protected against seizures are depicted (A) and defined by a seizure duration shorter than 8 s (B). Data are shown as the mean ± SEM. Statistical analysis: Fisher's exact test (A), one-way ANOVA with Dunnett's multiple comparison test (B), outliers were identified via the Iterative Grubbs test (alpha = 0.01) (A, B). Significance levels: *$p \leq 0.05$, **$p \leq 0.01$

As shown in Figure III-9A-B, VHC-injected mice showed a mean (± SEM) psychomotor seizure duration of 14 ± 2 s. Mice were regarded as protected when the seizure duration was shorter than 8 s.[59] Valproate-treated mice (positive control group) were nearly all protected (83%), and showed dramatically reduced seizure duration, in line with our previous results[247]. Magnolol at a dose of 30 mg/kg significantly decreased the mean seizure duration and protected 75% of the mice compared with the VHC group. Moreover, a dose-dependent relationship was found at doses between 30 mg/kg and 7.5 mg/kg magnolol (Figure III-9A-B).

4. Conclusion

Taken together, screening extracts of single plants employed in TCM using a combination of zebrafish- and mouse seizure models, allowed us to identify allyl biphenol as a chemical scaffold for future discovery of compounds possibly active in therapy-resistant epilepsies. The compounds are endowed with an interesting multi-target profile encompassing $GABA_A$, cannabinoid and AMPA receptors. Yet, whether these or other unknown molecular targets play a key role is presently unexplored, and warrant further investigation. Knowing the molecular target(s) is probably necessary for further lead optimization.

5. Methods

5.1 Animals and maintenance

Zebrafish. Adult zebrafish (*Danio rerio*) of the AB strain were kept under standard husbandry conditions (28 °C, pH 6.5-7.8, 14/10 h light/dark cycle). Eggs and embryos were obtained following natural spawning, then sorted and raised in embryo medium (0.3x Danieau's solution (DS): 1.5 mM HEPES, pH 7.6, 17.4 mM NaCl, 0.21 mM KCl, 0.12 mM $MgSO_4$, and 0.18 mM $Ca(NO_3)_2$) in a Peltier-cooled incubator (IPP 260, Memmert, Schwabach, Germany) at 28 °C until 5-7 days post fertilization (dpf).

Mice. Male NMRI mice (provided by Charles River Laboratories) used in the experiments were 9 weeks old and weighed 18-20 g. Animals were housed in groups of 4 or 5 per cage, and maintained under standard animal housing conditions at 22 °C with a 14/10 h light/dark cycle. Water and food were available *ad libitum*. The animals were fed a pellet diet and water *ad libitum*, and were allowed to acclimate for one week before experiments were conducted.

All animal experiments were approved by the Ethics Committee of the University of Leuven (approval numbers 027/2017 and 023/2017) and by the Belgian Federal Department of Public Health, Food Safety & Environment (approval number LA1210199).

5.2 Plant extract preparation

Dried medicinal plant material was purchased from Beijing Tongrentang Pharm. (BTP) Co. Ltd. (Beijing, China). The purchased plant materials were authenticated according to the Chinese Pharmacopeia (version 2010) by a local botanist. Vouchers of plant materials were deposited in our lab. The medicinal parts were ground, and the powder obtained was immersed in acetone, ethanol or water at 1 g/10 mL, after which the suspension was sonicated 5x for 15 min over a 24-h period, followed by a centrifugation at 3500 × g for 10 min. One mL supernatant of each extract was transferred into 1.5 mL Eppendorf tubes for further evaporation using a Savant SpeedVac Concentrator in case of aqueous and ethanol extracts. Acetone extracts were dried by air blowing. Residues were dissolved in Milli-Q water or dimethyl sulfoxide (DMSO) for aqueous and organic extracts, respectively, at a final concentration of 40 mg/mL.

5.3 Compounds preparation

Magnolol, honokiol, 3,3′-dimethylbiphenyl, 2,2′-biphenol, 2-phenylphenol, 3,3',5,5'-tetra-tert-butyl-[1,1'-biphenyl]-2,2'-diol, and valproate were purchased from Sigma-Aldrich (analytical standard). Other compounds used were: 4-O-methylhonokiol (Enzo), tetrahydromagnolol (Cayman Chemical Company) and perampanel (Eisai). Compounds were dissolved in 100% DMSO at a concentration of 200 mM, and stored at -20 C°. Stock solutions were diluted 100-fold in embryo medium before use (final DMSO concentration: 1% w/v).

All physicochemical properties of compounds (MW, clog P, clog D, PSA, Rotor, HBD, HBA) were calculated using the ChemAxon software imbedded in MarvinSketch 20.2.0 (ChemAxon, Hungary) requiring only 2D structural formula as input.

PTZ was purchased from Sigma-Aldrich (analytical standard). EKP was synthesized by the Laboratory of Organic Synthesis (Prof. Wim De Borggraeve, KU Leuven) according to the method described.[22] EKP was dissolved in 100% DMSO at a concentration of 800 mM, and stored at -80 C°. PTZ was dissolved in embryo medium at a concentration of 40 mM, and freshly prepared before use.

5.4 Toxicity evaluation

The maximum tolerated concentration (MTC) of extracts and compounds was determined by a method described before.[38] A dozen zebrafish larvae of 5 dpf were individually incubated in single wells of a 96-well plate, and were treated separately with extracts or compounds at concentrations ranging from 6.25 to 50 µg/mL, and from 3.13 to 200 µM, respectively, in 100 µL VHC (1% DMSO). After 18 h, larvae were individually examined for their touch response, posture, edema, signs of necrosis, morphology and swim bladder. The MTC was defined as the highest concentration at which an extract or compound did not exert any sign of toxicity in any of the larvae used. 3,3',5,5'-Tetra-tert-butyl-[1,1'-biphenyl]-2,2'-diol (compound 7, Figure III-6 and Table III-2) precipitated in the VHC before reaching the MTC. In this case, the maximum soluble concentration (MSC) (6.25 µM) was used for further testing of activity.

5.5 Locomotor activity evaluation

Zebrafish larvae of 5 dpf (n=10) were individually positioned in single wells of a 96-well plate, and incubated in 100 µL VHC or VHC supplemented with extract or compound for 2 h at 28 °C in the dark. Then, 100 µL of VHC, or VHC supplemented with PTZ (40 mM) or EKP (1 mM)

was added, followed by placing the plates immediately in an enclosed tracking device (ZebraBox Viewpoint, France). Locomotion activity was expressed in "actinteg" units, and plotted per 5 min by ZebraLab software (Software Viewpoint, France). Total locomotor activity accumulated over the total tracking period of 30 min (PTZ model) or 20 min (EKP model), respectively, was calculated as well.

5.6 Local field potential recordings

Epileptiform activities were measured by non-invasive local field potential (LFP) recording of the optic tectum (midbrain) of 7 dpf zebrafish larvae. Larvae were treated as described above. After incubation, 100 µL of VHC, or VHC supplemented with PTZ (40 mM) or EKP (1 mM) was added. After 15 min (PTZ model) or 8 min (EKP model), the larvae were immobilized in 2% low-melting-point agarose (Invitrogen) at room temperature (RT). A single glass electrode filled with artificial cerebrospinal fluid (ACSF) (124 mM NaCl, 2 mM KCl, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 1.25 mM KH_2PO_4, 26 mM $NaHCO_3$, and 10 mM glucose) was positioned on the skin above the optic tectum. Local field potential recordings were performed according to the method reported by Copmans *et al.*[38] and Zhang *et al*.[22]

Recordings were visually inspected using Clampfit 10.2 software (Molecular Devices Corporation, USA[60]). An electrical discharge was considered as epileptiform if it corresponded to a poly-spiking event comprising at least 3 spikes with a minimum amplitude of three times the baseline amplitude and a duration of at least 100 ms.

In addition, power spectral density (PSD) analysis of the recordings was performed using MatLab R2018 (MATrix LABoratory, USA) software.[61] In brief, the power spectral density of the signals were estimated using Welch's method of averaging modified periodograms with 512-point fast fourier transform of 80% overlapping 100 sample (100 ms) long segments and a Hamming window. Then, the PSD estimate of each LFP recording was summed over each 10 Hz frequency band, ranging from 0-160 Hz. This analysis assumes that the epileptiform activity manifests as a high power oscillation at certain frequencies. If epileptic activity occur often throughout the recording, that will lead to an increased PSD estimate in the corresponding frequency band. Note that such frequency-domain analysis, along with other time-domain signal characteristics were successfully used to automatically detect and count epileptic events in LFP recordings.[61] Next, the PSD estimates were normalized against the VHC control and the data were plotted as mean (±SEM) PSD per 10 Hz and per condition over the 30-130 Hz region. Outliers were identified via the Iterative Grubbs test (alpha = 0.1).

5.7 Mouse 6-Hz psychomotor seizure model

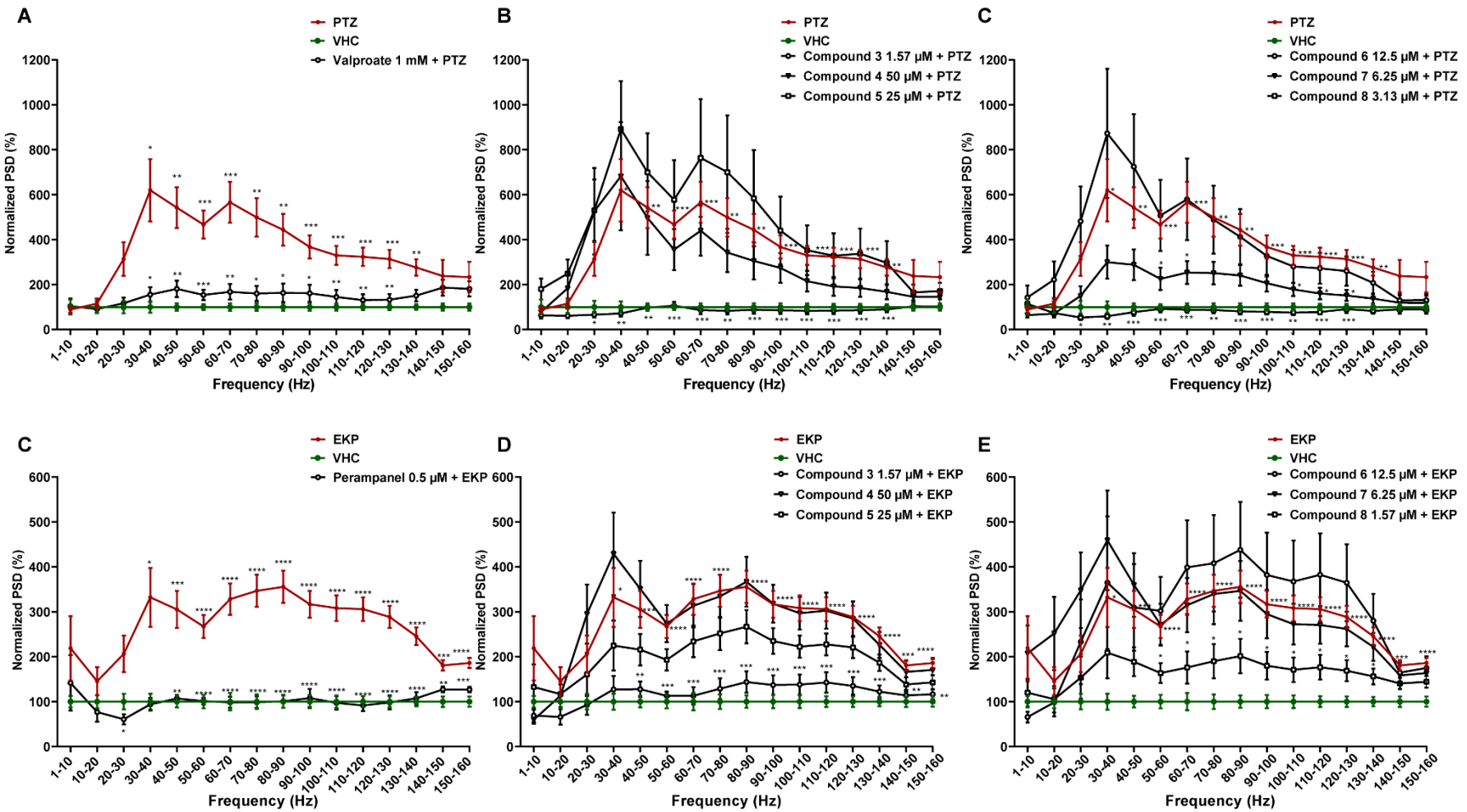
The antiseizure activity of compounds was investigated in the mouse 6-Hz (44 mA) psychomotor seizure model as described before.[38] NMRI mice (10 weeks old) weighing 27-31 g were randomly assigned to one of 6 groups, with every group consisting of at least 6 mice. Mice were injected i.p. with valproate (300 mg/kg), magnolol (doses ranging from 7.5 to 60 mg/kg) dissolved in 200 µL VHC (DMSO:PEG200:saline, 0.25:0.25:0.5, v/v/v), or 200 µL VHC (control). One hour after injection, an ECT Unit 5780 stimulator (Ugo Basile, Comerio, Italy) was used for inducing psychomotor seizures through corneal electrical stimulation (6 Hz, 0.2 ms rectangular pulse width, 3 s duration, 44 mA). An ocular anesthetic (lidocaine, 0.5%) was applied to the cornea before stimulation. Psychomotor seizures characterized by stun, clonus, twitching of the vibrissae, and straub tail were scored, and video-monitored. Seizure durations were determined by blinded video analysis to confirm or correct the initial observations. Mice were considered protected in case all four signs of psychomotor seizures were absent within 8 s after stimulus delivery.[59]

5.8 Statistical analysis

GraphPad Prism 8 software (GraphPad Software, Inc, USA) was used for statistical analyses. For all analyses, differences between a treatment group and the equivalent control groups were considered statistically significant if the p-value was below 0.05 ($p < 0.05$), indicated by an asterisk (*). P-values below 0.01, 0.001 and 0.0001 were marked by two (**), three (***) or four (****) asterisks.

6. Supporting information

Electrophysiological antiseizure activity (PSD analysis) of structural analogs in the PTZ/EKP zebrafish models, data are plotted as mean (± SEM) PSD per 10 Hz.



Supplementary Figure III-1. Electrophysiological antiseizure activity (PSD analysis) of structural analogues in the PTZ/EKP zebrafish models. The power spectral density (PSD) ranging from 0-160 Hz is normalized against the vehicle (VHC) control, and the data are plotted as mean (± SEM) PSD per 10 Hz (A-F). Number of larvae per condition: n = 16-19 for the VHC/PTZ/EKP group, n=12-17 for the valproate/perampanel+PTZ/EKP group, n=10 for analogues+PTZ/EKP. Statistical analysis: two-way ANOVA with Bonferroni posttests, PTZ/EKP control in comparison to VHC group, valproate/perampanel/analogues+PTZ/EKP group in comparison to PTZ/EKP control. Outliers were identified and removed based on the Iterative Grubbs (Alpha = 0.1). Significance levels: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

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CHAPTER IV

Antiseizure Activity of Enantiomers of Fenfluramine and Norfenfluramine in a Zebrafish Model of Dravet Syndrome

Antiseizure activity of enantiomers of fenfluramine and norfenfluramine in a zebrafish model of Dravet syndrome

Jing Li[1], Jo Sourbron[2], Maxim Nelis[3], Lieven Lagae[2], Deirdre Cabooter[3] and Peter A. M. de Witte[1]

[1] Molecular Biodiscovery, Department of Pharmaceutical and Pharmacological Sciences, University of Leuven (KU Leuven), B-3000 Leuven, Belgium

[2] Department of Pediatric Neurology, University Hospitals Leuven, B-3000, Leuven, Belgium

[3] Pharmaceutical Analysis, Department of Pharmaceutical and Pharmacological Sciences, University of Leuven (KU Leuven), B-3000, Leuven, Belgium

1. Abstract

Dravet syndrome (DS) is a rare genetic encephalopathy that is characterized by severe seizures and prominent comorbidities. Most DS patients are highly resistant to treatment with commonly used antiseizure drugs (ASDs), even when treated with polytherapy regimens. In 2020, FDA has approved fenfluramine (FFA) for treatment of seizures associated with DS. However, the clinically used FFA is a racemic mixture (i.e. (±)-FFA), that is substantially metabolized to norfenfluramine (norFFA), and it is presently not known whether the efficacy of FFA is due to a single enantiomer of FFA, or to both, and whether the norFFA enantiomers also contribute significantly.

In this study, the antiseizure activity of enantiomers of FFA (i.e. (+)-FFA and (-)-FFA) and norFFA (i.e. (+)-norFFA and (-)-norFFA) was explored using the zebrafish *scn1Lab*$^{-/-}$ mutant model of DS in behavioral and electrophysiological assays. To validate the experimental conditions used, we assessed the activity of a set of ASDs typically used in the fight against DS, including combination therapy. Overall, our results are highly consistent with the treatment algorithm proposed by the updated current practice in the clinical management of DS.

Our results show that (+)-FFA, (-)-FFA and (+)-norFFA displayed significant antiseizure effects in the preclinical model, and thus can be considered as compounds actively contributing to the clinical efficacy of FFA. In case of (-)-norFFA, the results were less conclusive.

We also investigate the uptake kinetics of the enantiomers of FFA and norFFA in larval zebrafish heads using Liquid Chromatography-Mass Spectrometry (LC-MS). The data show that the total uptake of each enantiomer of FFA and norFFA increased in a time-dependent fashion. A somewhat similar uptake was observed for the (+)-norFFA and (-)-norFFA enantiomers, implying that the levo/dextrotation of the structure did not dramatically affect the uptake. Significantly, when comparing (+)-FFA with the less lipophilic (+)-norFFA, the data clearly show that the nor-metabolite of FFA is taken up less than the parent compound.

KEYWORDS: Dravet syndrome, zebrafish, antiseizure activity, fenfluramine, norfenfluramine, enantiomers, serotonin.

2. Introduction

Dravet syndrome (DS) is a rare, but severe developmental epileptic encephalopathy that begins in infancy.[1,2] The first seizures are typically triggered by fever, and are characterized by long-lasting hemiclonic or generalized clonic or tonic-clonic convulsions. Later, the seizures evolve with age, and multiple seizure types may occur, such as focal, atypical absences, and myoclonic seizures. Furthermore, motor dysfunction, behavioural disorder, and cognitive impairment appear.[3,4] Also increased incidence of mortality is reported in DS patients, especially due to a higher risk of sudden unexpected death.[5,6] Regarding the genetic architecture of DS, a *de novo* mutation in the gene *SCN1A* which encodes for an α (pore-forming) subunit of the brain voltage gated sodium channel type-1 ($Na_V1.1$), occurs in a large majority of patients.[7]

Most DS patients are highly resistant to treatment with commonly used antiseizure drugs (ASDs). For instance around 45% of DS patients in Europe experienced on average more than four tonic-clonic seizures per month, even when treated with polytherapy regimens.[8] Algorithms for management of DS have been proposed by experts in North America[9] and Europe[10] to optimize the treatment outcome of DS patients with a minimal risk for toxicity. The most recent flowchart proposed by Cross and coworkers consists of valproate (VPA) as a first line treatment.[10] When a clear DS diagnosis is given and seizures continue, stiripentol (STP) (with or without clobazam (CLB)) or cannabidiol (CBD) or fenfluramine (FFA) can be added. As an alternative, the administration of topiramate (TPM) or a ketogenic diet may be considered [10].

In 2020, FDA approved fenfluramine for treatment of seizures associated with DS. As a potent releaser and reuptake inhibitor for 5-hydroxytryptamine (5-HT, serotonin), FFA was initially used as an anorectic in polytherapy with phentermine, but was withdrawn from the market in 1997 due to cardiopulmonary side effects at high dosages.[10] However, the successful use of low dosage FFA as an add-on therapy for the treatment of DS was reported by Ceulemans *et al.*,[11,12] including the achievement of seizure free cases. Later clinical trials have further confirmed the efficacy and safety of FFA in treatment of DS. Importantly, no cardiovascular adverse effects were observed in these trials.[1]

Chemically, fenfluramine used in the clinic is a racemic mixture, meaning that equal amounts of left-, and right-handed stereo-isomers (enantiomers) of the chiral molecule are present, i.e. (-)-FFA or levoFFA and (+)-FFA or dexFFA (Figure IV-1). In addition, pharmacokinetic investigations have shown that FFA is substantially metabolized to norfenfluramine (norFFA),

a *N*-dealkylated derivative of FFA resulting in circulating plasma levels that are similar to or greater than that of FFA itself, in human and animal models.[13,14] Notably, circulating norFFA also consists of a racemic mixture of (-)-norFFA and (+)-norFFA (Figure IV-1). As the various FFA and norFFA enantiomers are endowed with somewhat differing pharmacological profiles involving especially 5-HT, and type 1 sigma (σ1) receptors,[13,15] possibly resulting in a different antiseizure activity, it is presently not known whether the efficacy of racemic FFA in the treatment of DS is due to a single enantiomer of FFA, or to both, and whether the norFFA enantiomers also contribute significantly.

Zebrafish are vertebrate models with genetic, physiological and CNS features that are highly conserved across vertebrates, including humans. Moreover, diverse genetic tools, such as morpholino (MO)-based gene knockdown and CRISPR/Cas9-based genome editing can easily be used in zebrafish to model human genetic diseases.[16–18] In addition, the high-throughput screening capacity of zebrafish-based models offers great opportunities for screening and discovery of precision medicine drugs.[19,20] Since zebrafish *scn1Lab* is evolutionarily close to the mammalian *SCN1A* gene,[21] a zebrafish *scn1Lab double indemnity* (*didy*^{552}) mutant model has been used to find new medication to treat DS patients.[22] Homozygous *scn1Lab* mutants display spontaneously occurring seizures and brain epileptic discharges, facilitating their use in phenotype-based screening projects.[22] In our previous work, Sourbron *et al*.[23,24] investigated the potential action mechanism of FFA with the *scn1Lab*$^{-/-}$ mutant model, and elucidated that FFA could modulate 5-HT$_{1D}$, 5-HT$_{2C}$, sigma-1, and possibly 5-HT$_{2A}$ receptors to perform its antiseizure activity.

In this study, the antiseizure activity of the enantiomers of FFA and norFFA was explored using the zebrafish *scn1Lab* mutant model of DS in behavioral and electrophysiological assays. To validate the experimental conditions used, we explored the activity of a set of ASDs typically used in the fight against DS, including combination therapy. Finally, also the uptake of all four compounds in the larval zebrafish heads as a function of incubation time was assessed using Liquid Chromatography Mass Spectrometry (LC-MS). Our data show that (+)-FFA, (-)-FFA and (+)-norFFA can be considered as active compounds and metabolites contributing to the clinical activity of FFA. In case of (-)-norFFA the results were less conclusive.

3. Material and Methods

3.1 Zebrafish maintenance

Husbandry conditions of adult wild-type (AB-strain) and *scn1Lab* heterozygous mutant zebrafish (Danio rerio) were as described previously [23]. Fertilized eggs were collected following natural spawning. Then embryos and larvae were sorted and raised in embryo medium (0.3× Danieau's solution: 1.5 mM HEPES, pH 7.6, 17.4 mM NaCl, 0.21 mM KCl, 0.12 mM $MgSO_4$, 0.18 mM $Ca(NO_3)_2$) in a Peltier-cooled incubator (IPP 260, Memmert, Schwabach, Germany) at 28 °C, using a 14/10 light/dark cycle. At 6 days post-fertilization (6 dpf) *scn1Lab*$^{-/-}$ mutant larvae were selected by their darker appearance, lack of a swim bladder and slight curvature of the body, as performed before.[23]

All zebrafish experiments carried out were approved by the Ethics Committee of the University of Leuven (approval numbers 027/2017 and 023/ 2017) and by the Belgian Federal Department of Public Health, Food Safety, and Environment (approval number LA1210199).

3.2 Compound preparation

Valproate (VPA), topiramate (TPM), stiripentol (STP), cannabidiol (CBD), clobazam (CLB), levetiracetam (LEV), carbamazepine (CBZ), and lamotrigine (LTG) were purchased from Sigma-Aldrich. Phenytoin (PHT) was from Acros Organics. (±)-Fenfluramine ((±)-FFA) was a gift from Prof. Berten Ceulemans (University of Antwerp, Belgium). The enantiomers of FFA and norfenfluramine (norFFA) were provided by Zogenix International (Emeryville, USA). Compounds were dissolved in dimethylsulfoxide (DMSO), and diluted in embryo medium to achieve a final DMSO concentration of 0.1% w/v. Embryo medium with 0.1 % w/v DMSO served as a vehicle control (VHC).

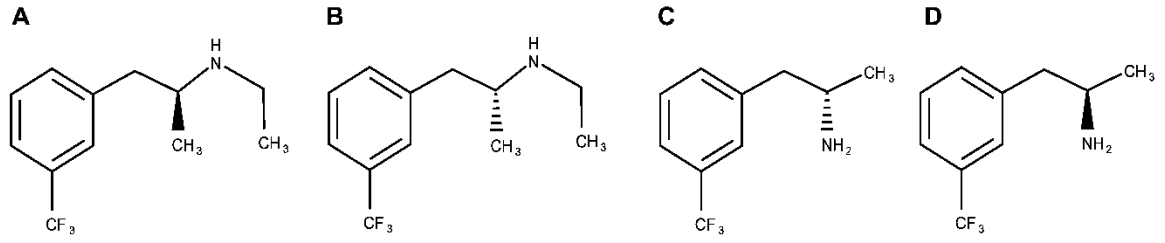

Figure IV-1. The structure of (+)-FFA (A), (-)-FFA (B), (+)-norFFA (C) and (-)-norFFA (D).

3.3 Toxicity evaluation

To evaluate the maximum tolerated concentration (MTC) of the individual compounds, a dozen of WT zebrafish larvae (6 dpf) were individually incubated in single wells of a 96-well plate, and treated with two-fold serial dilutions of the compounds. After 22 h incubation under standard conditions (28 °C, 14/10 light/dark cycle), larvae were individually examined for their touch response, posture, edema, signs of necrosis, morphology, heartbeat rate, and swim bladder condition under the microscope. The MTC was defined as the highest concentration at which a compound did not exert any sign of toxicity in any of the larvae used.

3.4 Locomotor activity measurement

WT and homozygous zebrafish larvae (*scn1Lab*$^{-/-}$ mutants) (6 dpf) were individually positioned in single wells of a 96-well plate, and incubated in 100 µL VHC or VHC supplemented with compound or combined compounds for 22 h at 28 °C on a 14/10 h light/dark cycle. Then, the plates were placed immediately in an enclosed tracking device (ZebraBox Viewpoint, France), followed by a 30 min chamber habituation and 10 min recording. Locomotion activity was quantified by the lardist parameter (total distance in large movements) with "cm" units, and plotted per 100 seconds by ZebraLab software (Software Viewpoint, France), as reported previously by our group.[23,24] The total locomotor activity accumulated over the total tracking period of 10 min. Final data were pooled from three or four independent experiments, with at least five larvae for each treatment and 20-30 larvae for each experimental condition. The compound-treated *scn1Lab*$^{-/-}$ mutant locomotor data were analyzed by normalizing against VHC-treated control group data, and displayed as percentage.

3.5 Local field potential recordings

WT and homozygous zebrafish larvae (*scn1Lab*$^{-/-}$ mutants) (6 dpf) were treated as described above. After incubation, larvae were immobilized in 2% low-melting-point agarose (Invitrogen) at room temperature (RT) and the epileptiform activities were measured by noninvasive local field potential (LFP) recording from the skin above the optic tectum (midbrain). The single glass electrode filled with artificial cerebrospinal fluid (ACSF) (124 mM NaCl, 2 mM KCl, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 1.25 mM KH_2PO_4, 26 mM $NaHCO_3$, and 10 mM glucose) was positioned on the skin above the optic tectum. Each recording lasted for 10 min. Epileptiform activity was quantified by using Clampfit 10.2 software (Molecular Devices Corporation, USA60), as reported previously by our group.[23,24]

3.6 Measurement of compound concentration in heads

3.6.1 *Extraction procedure*

WT and homozygous zebrafish larvae (*scn1Lab*$^{-/-}$ mutants) (6 dpf) were treated as described above. After incubation with compounds, larvae were washed and euthanized by exposure to cold Milli-Q water. Then, the heads of larvae were carefully separated under the microscope, and five heads were transferred collectively into one 1.5 mL Eppendorf tube with acid-washed glass beads (diameter: 710-1180 µm, Sigma Aldrich) and 275 µL extraction medium (HPLC grade methanol, Sigma Aldrich). Next, the samples were homogenized by 10 min of ultrasonication (Diagenode Bioruptor Plus, Belgium) at 4 °C. The overall ultrasonication process encompassed 10 cycles of 30 s with pauses of 30 s in-between with high energy input.[25] After the subsequent centrifugation (14500 g, 15 min), 200 µL of supernatant were collected from each tube, and stored in -80 °C for further LC-MS processing.

3.6.2 *HPLC instrumentation and quantification*

Analyses were performed using an Infinity 1200 LC system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler, binary pump, and a thermostated column oven compartment. An YMC-Triart C18 (50 x 2.0 mm; d_p = 1.9 µm) column was utilized for the chromatographic separations at 40 °C. Samples were injected in a volume of 1 µL and the flow rate was set to 0.2 mL/min. The mobile phase consisted of 10 mM ammonium acetate in H_2O: acetonitrile 75:25 (*v/v*) and separations were carried out isocratically. The elution time of (-)- and (+)-FFA was 4.1 min, while the elution time of (-)- and (+)-norFFA was 2.6 min. The LC instrument was hyphenated to a mass spectrometer (MS) with a triple quadrupole detector (API 3000, Applied Biosystems, Carlsbad, CA, USA) and equipped with an electrospray ionization source. The MS/MS analysis was conducted in multiple reaction mode (MRM) in positive mode. The MS settings were optimized by direct infusion of compound standards diluted in methanol. The optimal MS parameters are summarized in Table IV-1.

Quantification was performed through the use of a calibration curve created for each compound separately in blank matrix, which was derived from a pooled set of blank fish heads (n=40). Calibrators consisted of 90 µL of blank matrix spiked with 10 µL of the corresponding standard dissolved in MeOH to obtain a concentration range varying between 0.025 µM to 7.5 µM for (+)-FFA, 0.02 µM to 5 µM for (-)-FFA, 0.05 µM to 10 µM for (+)-norFFA, and 0.02 µM to 10 µM for (-)-norFFA. Ranges consisted of at least 5 concentrations, each of which was analyzed

in replicate (n=5). A weighed least squares (WLS) regression model with $1/x^2$ weighing was utilized such that back-calculated concentrations did not deviate more than 15% from their nominal value. The final values of the regression coefficients (R^2) were 0.993, 0.998, 0.992, and 0.990 for (+)-FFA, (-)-FFA, (+)-norFFA and (-)-norFFA, respectively.

Table IV-1. Optimized mass settings for FFA and norFFA

Compound	Transition (Da)	DP (V)	FP (V)	EP (V)	CE (eV)	CXP (V)	NEB (psi)	CUR (psi)	CAD (psi)	Heater gas (L/min)	TEM (°C)	IS (V)
FFA	232 > 159	40	180	10	29	10	8	6	4	7	300	5500
norFFA	204 > 159	33	120	10	29	8	9	9	6	4	300	5500

DP declustering potential, FP focusing potential, EP entrance potential, (CE) the collision energy, and (CXP) the collision cell exit potential, (NEB), the nebulization gas, (CUR) the curtain gas, (CAD) the collision gas, (TEM) the temperature and (IS) the ionspray voltage

The mean head weight (± S.D.) of a 6 dpf and 7 dpf zebrafish larva was 192 ± 10 µg and 196 ± 13 µg, respectively, as measured by weighing three batches of 50 fresh heads after removing excess water with filter paper. The final uptake was calculated according to the method reported by Copmans *et al*.,[26] and expressed as amount/head weight (µg/g).

3.7 Statistical analysis

For all results obtained, one-way ANOVA followed by Dunnett's multiple comparison tests were used, which were performed in GraphPad Prism 8 software (GraphPad Software, Inc, USA). In case of locomotor data and local field potential recordings (Figure IV-2, 3, 5 and 7), significance was calculated only when compound treatment decreased the seizure activity. Significance levels: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

4. Results and Discussion

4.1 Pharmacological evaluation of the zebrafish *scn1Lab*$^{-/-}$ mutant model

To validate the zebrafish *scn1Lab*$^{-/-}$ mutant model and the experimental conditions used in this study, we first tested a series of antiseizure drugs (ASDs) proposed by different treatment algorithms for DS,[8–10,27,28] including valproate (VPA), racemic fenfluramine ((±)-FFA), topiramate (TPM), stiripentol (STP), cannabidiol (CBD), clobazam (CLB) and levetiracetam (LEV). In addition, ASDs that should be avoided by DS patients, like carbamazepine (CBZ), phenytoin (PHT) and lamotrigine (LGT) were examined as they target the sodium channel resulting in seizure aggravation.[6,29]

Various concentrations of the ASDs were examined for their adverse effects, allowing a maximum tolerated concentration (MTC) to be determined. MTC is defined as the highest concentration at which the compound did not exert any sign of toxicity in any of the larvae tested. MTC values were determined as 1 mM for VPA, 50 µM for (±)-FFA, 200 µM for TPM, 50 µM for STP, 6.25 µM for CBD, 100 µM for CLB, 10 mM for LEV, 50 µM for CBZ, 100 µM for PHT, and 100 µM for LTG. By using these MTCs for all further investigations, we sought to reduce the risk of false positive results to a minimum, which is particularly critical for locomotor activity measurements.

Next, the effect of each of these ASDs as a single treatment on locomotor and brain activities was evaluated by behavioral and electrophysiological assays using the zebrafish *scn1Lab*$^{-/-}$ mutant model. As shown in Figure IV-2, VPA elicited a complete rescue of the epileptiform locomotor activity (Figure IV-2A, $P \leq 0.0001$) and epileptiform brain discharges of the mutant larvae, as monitored by measuring the cumulative duration (Figure IV-2A1, $P \leq 0.01$) and frequency (Figure IV-2A2, $P \leq 0.0001$).

Of interest, VPA rarely provides adequate seizure control in DS patients, so that it requires the addition of others ASDs as second-line therapies,[10] whereas in our hands VPA fully corrected the seizure phenotype of the *scn1Lab*$^{-/-}$mutants. In the clinic however VPA shows dose-limiting side-effects after prolonged use like fatigue, hair loss and hyperammonemia amongst others.[30] Possibly the relative short treatment of zebrafish used in this study allowed to use an immersion concentration that exceeds the corresponding clinical dose of VPA, resulting in an enhanced efficacy.

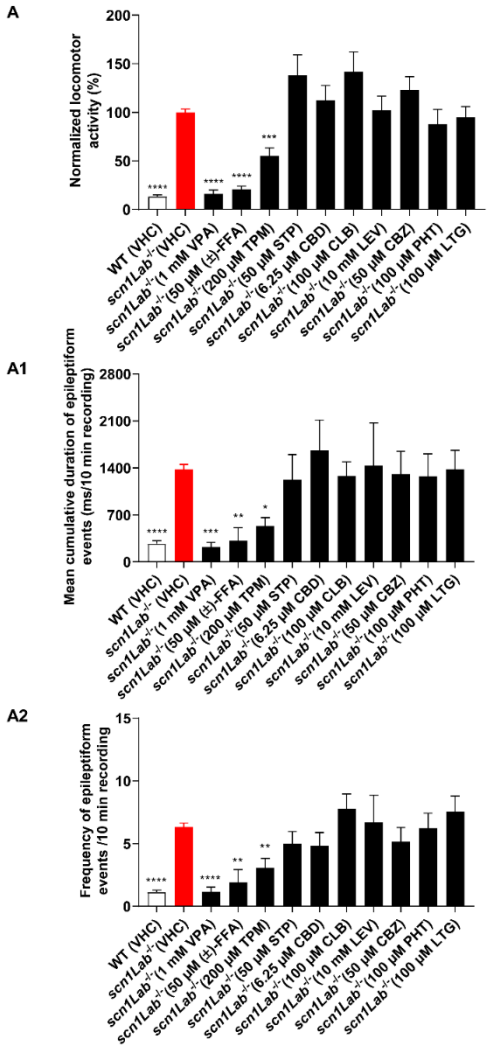

Figure IV-2. Behavioral (A) and electrophysiological (A1-2) antiseizure activity of valproate (VPA), (±) fenfluramine ((±)-FFA), topiramate (TPM), stiripentol (STP), cannabidiol (CBD), clobazam (CLB), levetiracetam (LEV), carbamazepine (CBZ), phenytoin (PHT), and lamotrigine (LTG) in the zebrafish *scn1Lab*$^{-/-}$ mutant model. (A) Locomotor activity of larvae pre-exposed to antiseizure drugs (ASDs) for 22 hours. Locomotor activity was normalized against VHC-treated *scn1Lab*$^{-/-}$ mutant larvae (colored in red) and displayed as a percentage (± SEM). Results were pooled from 3-4 independent experiments, with 207 larvae for the VHC-treated group, 22-31 larvae for each ASD-treated group. (A1-2) Noninvasive local field potential (LFP) recordings from the optic tectum of larvae pre-exposed to antiseizure drugs (ASDs) for 22 hours. Epileptiform discharges are quantified by the cumulative duration (mean ± SEM) (A1) and frequency (mean ± SEM) (A2) of events per 10-min recording. With 72 larvae for the VHC-treated group, 10-15 larvae for each ASD-treated group. Statistical analysis: one-way ANOVA with Dunnett's multiple comparison test. Significance levels: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. Abbreviation: WT, wide type, VHC, vehicle.

Similarly to VPA, (±)-FFA (Figure IV-2A, P ≤ 0.0001) and TPM (Figure IV-2A, P ≤ 0.001) not only significantly counteracted the increased locomotor activity observed in *scn1Lab*$^{-/-}$ mutants, but also dramatically attenuated the frequency of epileptiform events (Figure IV-2A1, P ≤ 0.01 for both), resulting in a decrease of cumulative duration (Figure IV-2A2, P ≤ 0.01 for (±)-FFA, P ≤ 0.5 for TPM).

By contrast, STP, CBD, CLB and LEV failed to rescue both the hyper-locomotor activity and brain epileptic discharges of *scn1Lab*$^{-/-}$ mutants (Figure IV-2). Also, as expected, no inhibitory effects could be observed with CBZ, PHT and LTG treatments (Figure IV-2).

As STP, CBD and (±)-FFA are typically combined in the clinic with VPA for second line treatment,[10] we further validated the *scn1Lab*$^{-/-}$ mutant model by exploring the activity of this combination treatment. Since VPA as a single treatment at 1 mM completely reduced the read-outs to the level exhibited by AB control larvae, we modified its concentration to 250 µM. This concentration exerted a limited and statistically non-significant effect on the locomotor activity and epileptiform brain discharges of the mutant larvae (Figure IV-3).

Significantly, the combination of VPA (250 µM) and STP (50 µM) turned out to effectively alter the locomotor activity and decrease the epileptiform discharges of the mutant larvae, whereas the single treatments were not active (Figure IV-3A).

Furthermore, in order to investigate the combination outcome of VPA (250 µM) and (±)-FFA, the concentration of the latter compound was reduced from 50 µM (MTC) to 3.13 µM. This concentration continued to induce a significant effect on the locomotor activity but not on the epileptiform brain discharges of the mutant larvae (Figure IV-3B, and 3B1-2). The combined compounds diminished the locomotor activity of mutant larvae treated with 3.13 µM (±)-FFA (single treatment) by more than half on average (i.e. (±)-FFA: 40 ± 8 % *vs* (±)-FFA+VPA: 15± 5 % (mean ± SEM)), although the difference observed was statistically not significant (Figure IV-3B). The LFP results further show that the combination of VPA and (±)-FFA was highly effective in reducing the epileptiform discharges of the mutant larvae, whereas the single treatments were not (Figure IV-3B1-2).

Finally, when VPA (250 µM) was combined with CBD (6.25 µM), the VPA+CBD-treatment showed a clear effect on the locomotor and LFP results obtained with the mutant larvae as compared to the single treatments that were not active (Figure IV-3C and 3C1-2).

As far as alternatives for second line treatment are concerned,[10] i.e. TPM and CLB, only the former exhibited a pronounced therapeutic activity in the *scn1Lab*$^{-/-}$ mutant model. Noticeably, CLB was previously proven to be ineffective in the DS zebrafish mutant model.[31] Although CLB has also been suggested as a first-line drug by the North American consensus panel, it typically only displays efficacy in DS patients when combined with VPA and STP.[9] Whether CLB therefore classifies as a true false negative in the *scn1Lab*$^{-/-}$ mutant model is yet to be

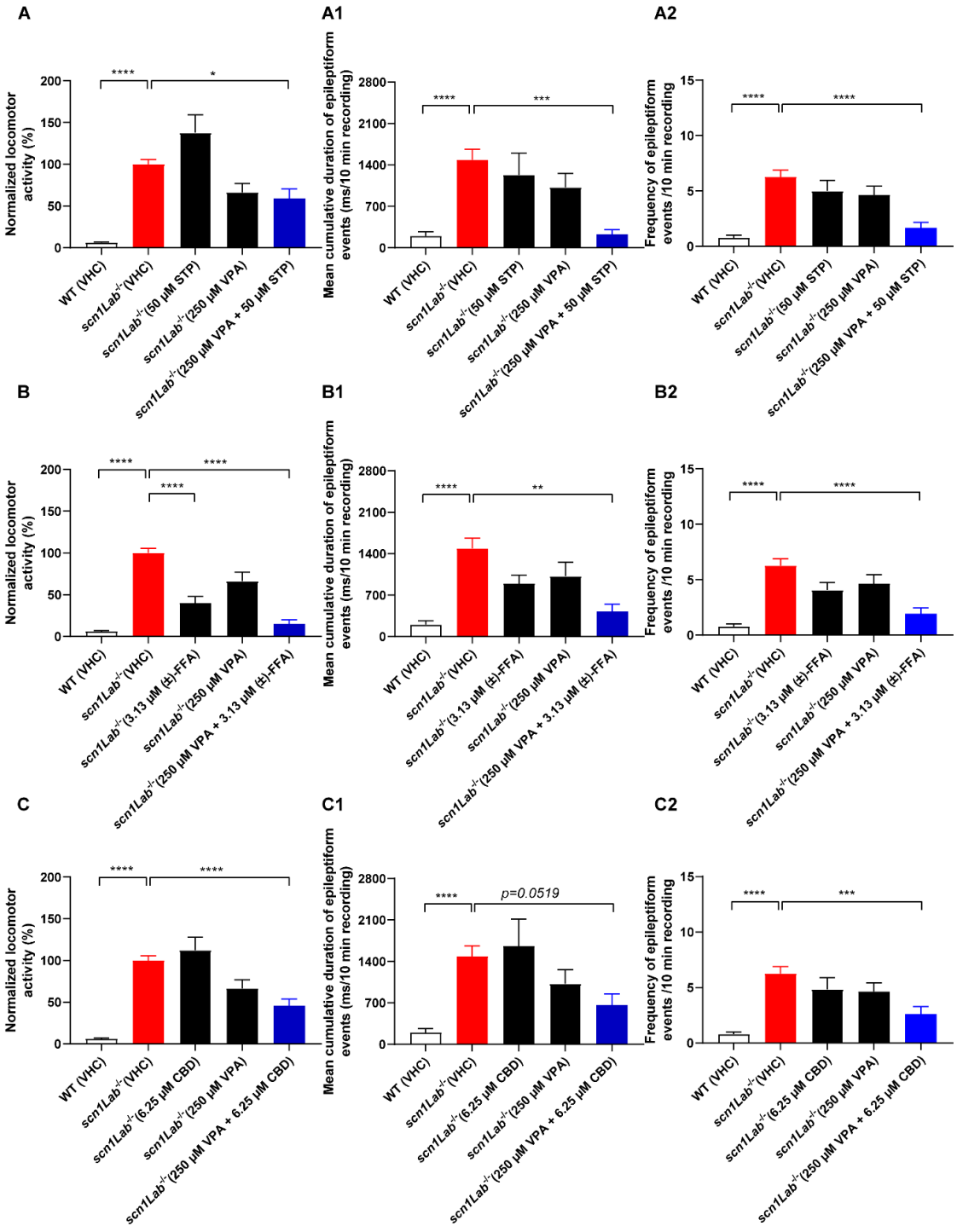

Figure IV-3. Behavioral (A, B, C) and electrophysiological (A1-2, B1-2, C1-2) antiseizure activity of combinatorial treatment (colored in blue) of valproate (VPA) with stiripentol (STP) (A, A1-2), (±) fenfluramine ((±)-FFA) (B, B1-2), and cannabidiol (CBD) (C, C1-2) in the zebrafish *scn1Lab*$^{-/-}$ mutant model. (A, B, C) Locomotor activity of larvae pre-exposed to antiseizure drugs (ASDs) for 22 hours. Locomotor activity was normalized against VHC-treated *scn1Lab*$^{-/-}$ mutant larvae (colored in red), and displayed as a percentage (± SEM). Results were pooled from 3-4 independent experiments, with 120 larvae for the VHC-treated group, 20-30 larvae for each ASD-treated group. (A1-2, B1-2, C1-2) Noninvasive local field potential (LFP) recordings from the optic tectum of larvae pre-exposed to antiseizure drugs (ASDs) for 22 hours. Epileptiform discharges are quantified by the cumulative duration (mean ± SEM) (A1, B1, C1) and frequency (mean ± SEM) (A2, B2, C2) of events per 10-min recording. With 23 larvae for the VHC-treated group, 12-14 larvae for each ASD-treated group. Statistical analysis: one-way ANOVA with Dunnett's multiple comparison test. Significance levels: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. Abbreviation: WT, wide type, VHC, vehicle.

investigated into more detail, for instance by exploring its additional activity in combination with VPA and STP, as suggested by Cross *et al.*[10]

LEV has been categorized as a third-line drug for DS by the North American consensus panel[9], but is not mentioned in the treatment options by Cross *et al.*[10] As a matter of fact, there is limited clinical evidence regarding its efficacy in the clinic, with retrospective studies demonstrating low responder rates in DS patients.[28] Significantly, the compound also failed to suppress the seizure-like activity of *scn1Lab*$^{-/-}$ mutants in this study, as shown before.[22]

Taken together, our results mirror well the order of multiple treatment options proposed by the updated current practice in management of DS.[10] Notably, a similar validation of the *scn1Lab*$^{-/-}$ mutant zebrafish DS model was previously performed, using a shorter incubation protocol than present in this study.[22,32] However, to our knowledge, this is the first study that shows the activity of combined ASD therapy, thereby further corroborating the zebrafish DS model.

4.2 Determination of the time-dependent concentration of enantiomers of FFA and norFFA in zebrafish head

In order to investigate the uptake kinetics of the enantiomers of FFA and norFFA in larval zebrafish heads, we immersed larvae in solutions of the individual compounds at their MTC for 30 min, 4 h and 22 h. Next, the compounds present in extracts of the heads were quantified by LC-MS analysis. The data depicted in Figure IV-4 show that the total uptake (amount of compound/head weight) of each enantiomer of FFA and norFFA increased in a time-dependent fashion.

A somewhat similar uptake was observed for the (+)-norFFA and (-)-norFFA enantiomers, implying that the levo/dextrotation of the structure did not dramatically affect the uptake. Conversely, a direct comparison between (+)-FFA and its (-)-enantiomer is hard to draw as different immersion concentrations were used due to the different MTCs of the respective compounds.

Surprisingly, a substantial increase in concentrations could still be observed in the 4 h-22 h time window. To the best of our knowledge, this study is the first to quantify the uptake of any drug compound in heads of zebrafish larvae as a function of time, and consequently it is not possible to conclude whether FFA and norFFA are actually unique in this respect. However, a certain parallel is apparent with the clinical condition, where accumulation of (+)-FFA and (+)-

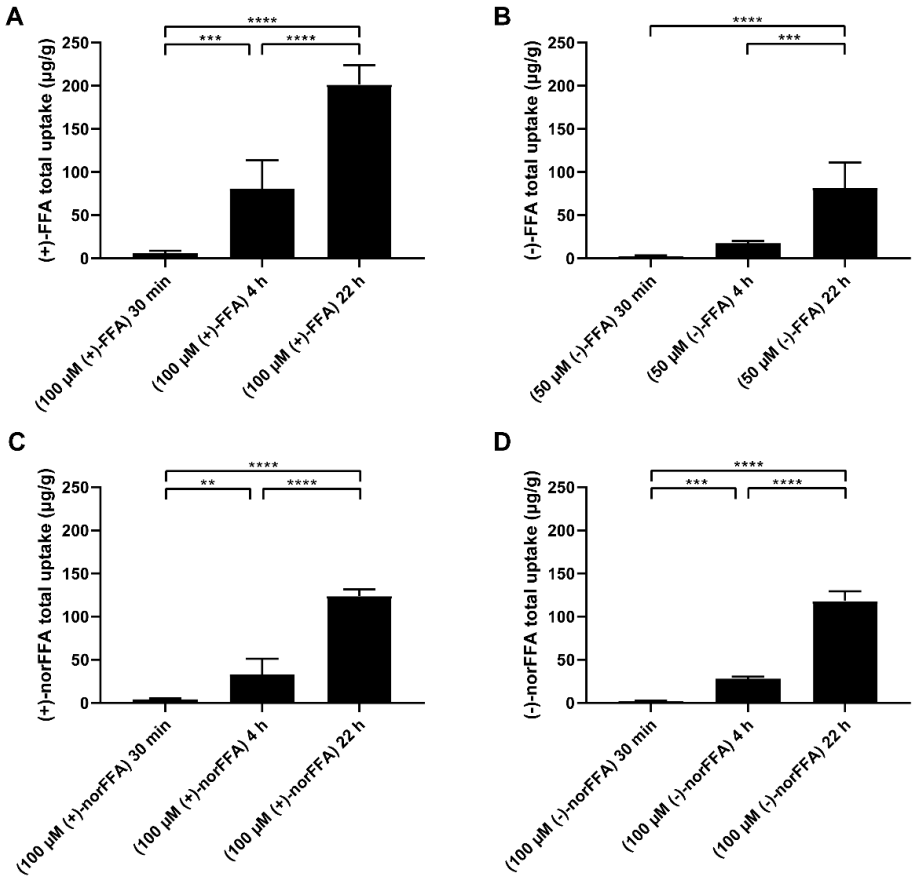

Figure IV-4. The total uptake of (+)-FFA (A), (-)-FFA (B), (+)-norFFA (C) and (-)-norFFA (D) in the larvae head after different exposure times (30 min, 4 h and 22 h). The head concentration of larvae pre-exposed to (+)-FFA (A), (-)-FFA (B), (+)-norFFA (C) and (-)-norFFA (D) at their respective MTCs for 30 min, 4 h and 22 h, separately, and total uptake of compound=compound concentration/head weight. With 5 larvae head for each sample, and 5 replicates for a total of 25 larvae heads per treatment. Statistical analysis: one-way ANOVA with Dunnett's multiple comparison test. Significance levels: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. Abbreviation: WT, wide type, VHC, vehicle, FFA, fenfluramine, norFFA, norfenfluramine.

norFFA in humans typically follows a step-wise increase to steady state concentrations after long-term repetitive drug administration.[33]

Other studies reported the uptake of compounds in heads and brain of larval zebrafish after 1 h incubations.[25,34] Of interest, when measuring head uptake of haloperidol (clog P value: 4.3) or diphenhydramine (clog P value: 3.3) after incubating 5 dpf zebrafish larvae for 1 h with 15 µg/ml of the compounds, the recovered concentrations were 21.6 µg/g and 115.5 µg/g, respectively.[34] As the lipophilicity of compounds is a crucial determinant for the uptake in body and brain tissue,[25,35] and the clog P values of FFA and norFFA are 3.47 and 2.68, respectively, the aforementioned data are in line with the results obtained after 1-4 h incubations in this study. Significantly, when comparing (+)-FFA with the less lipophilic (+)-norFFA, the data clearly show that the nor-metabolite of FFA is taken up less than the parent compound.

4.3 Antiseizure activity of enantiomers of FFA and norFFA in the zebrafish *scn1Lab*$^{-/-}$ mutant model

As uptake of compounds in larval heads was maximal after 22 h, we proceeded to use this prolonged incubation condition to explore the pharmacological activity of the FFA and norFFA enantiomers, as performed previously.[23,24] To investigate the potency of the compounds to prevent the epileptiform activity exhibited by the *scn1Lab*$^{-/-}$ mutants, they were first examined by a behavioral assay, at a wide range of concentrations. As illustrated in Figure IV-5, all enantiomers of FFA and norFFA effectively counteracted abnormal locomotor activity of mutant larvae at their MTC, 1/4 MTC and 1/40 MTC (1/20 MTC for (-)-FFA), whereas lower concentrations were not active. In addition, all drugs displayed their maximum effects at their respective 1/4 MTCs (Figure IV-5, $P \leq 0.0001$).

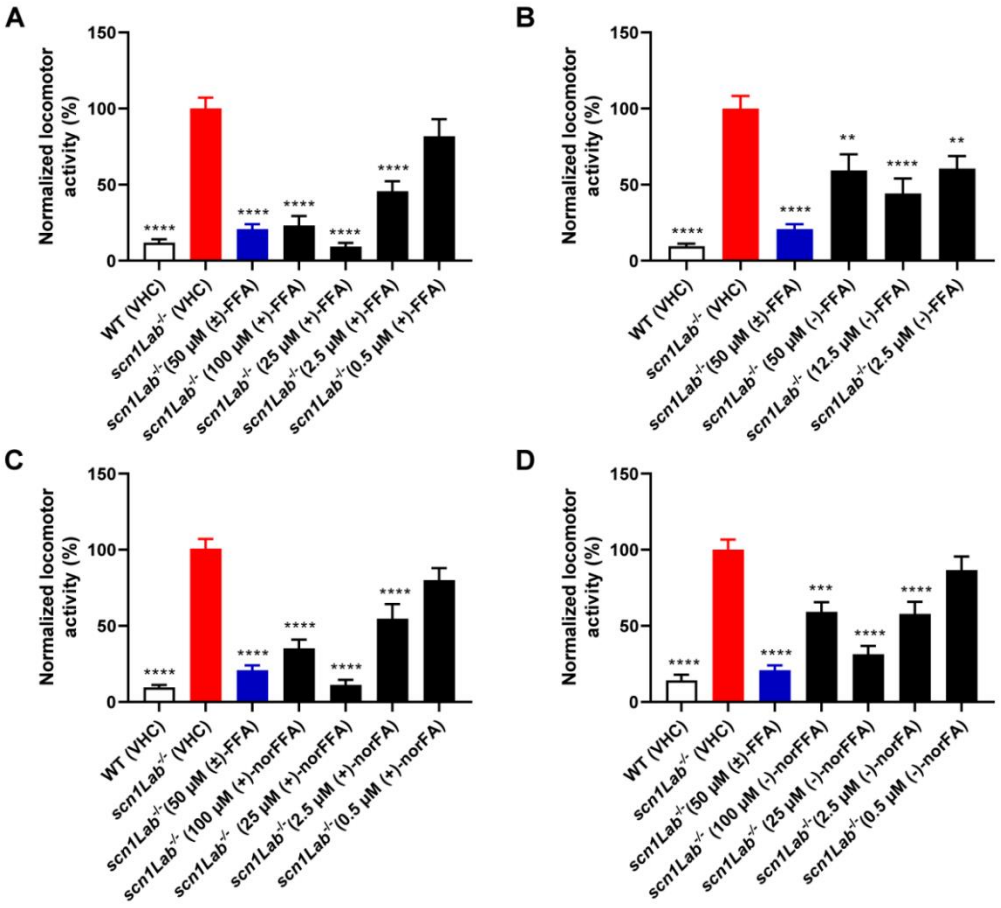

Figure IV-5. Behavioral antiseizure activity of (+)-FFA (A), (-)-FFA (B), (+)-norFFA (C) and (-)-norFFA (D) in the zebrafish *scn1Lab*$^{-/-}$ mutant model, and (±)-FFA (colored in blue, A-D), used as a positive control. (A) Locomotor activity of larvae pre-exposed to different concentration of enantiomers of FFA and norFFA for 22 hours. Locomotor activity was normalized against VHC-treated *scn1Lab*$^{-/-}$ mutant larvae (colored in red), and displayed as a percentage (± SEM). Results were pooled from 3-4 independent experiments, with 67-75 larvae for each VHC-treated group, 21-28 larvae for each compound-treated group. Statistical analysis: one-way ANOVA with Dunnett's multiple comparison test. Significance levels: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. Abbreviation: WT, wide type, VHC, vehicle, FFA, fenfluramine, norFFA, norfenfluramine.

Subsequently, we examined the effect of the compounds on the epileptiform discharges of the mutant larvae by recording local field potentials (LFP) of the brain. Representative traces of brain activity during the recordings are shown in Figure IV-6. As depicted in Figure IV-7, all compounds except for (-)-norFFA significantly reduced the frequency and cumulative duration of the epileptiform events. The results therefore confirm most of the results obtained using the locomotor assay, although some concentration-related discrepancies exist, especially in the case of (-)-norFFA. A different outcome between the two assays has also been reported by others,[22,24,36] possibly the result of some extra peripheral off-target effects of compounds affecting the locomotor read-out.

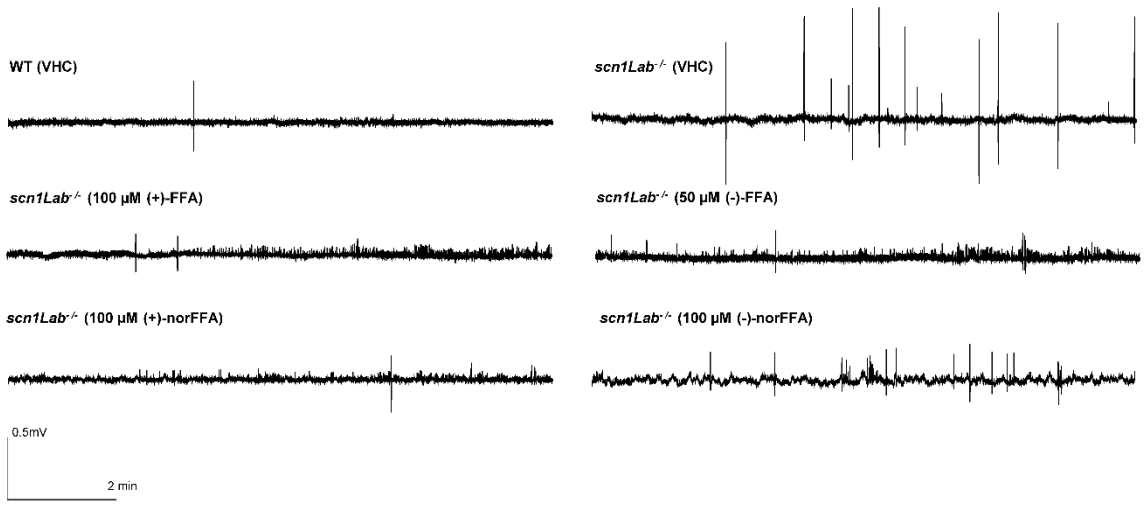

Figure IV-6. Representative local field potential recordings. Ten-min noninvasive local field potential (LFP) recordings from the optic tectum of larvae pre-exposed (+)-FFA, (-)-FFA, (+)-norFFA and (-)-norFFA for 22 hours. Abbreviation: WT, wide type, VHC, vehicle, FFA, fenfluramine, norFFA, norfenfluramine.

Overall, the outcome seems to indicate that (+)-FFA and especially (+)-norFFA exhibited inhibitory profiles that were more consistent and concentration-dependent than the (-)-enantiomers, for both locomotor and LFP read-outs.

Obviously, the activity of the compounds is determined by their relative uptake in brain tissue in combination with their underlying molecular mechanisms. Of importance, an increasing number of reports has indicated that low 5-HT (serotonin) brain levels are involved in epileptogenesis and/or seizure propagation,[37,38] and a 5-HT deficit was also reported in the heads of homozygous *scn1Lab*$^{-/-}$ mutants.[24] Significantly, as shown in Table IV-2, both enantiomers of FFA and norFFA are potent substrates for 5-HT transporter proteins, with EC_{50}

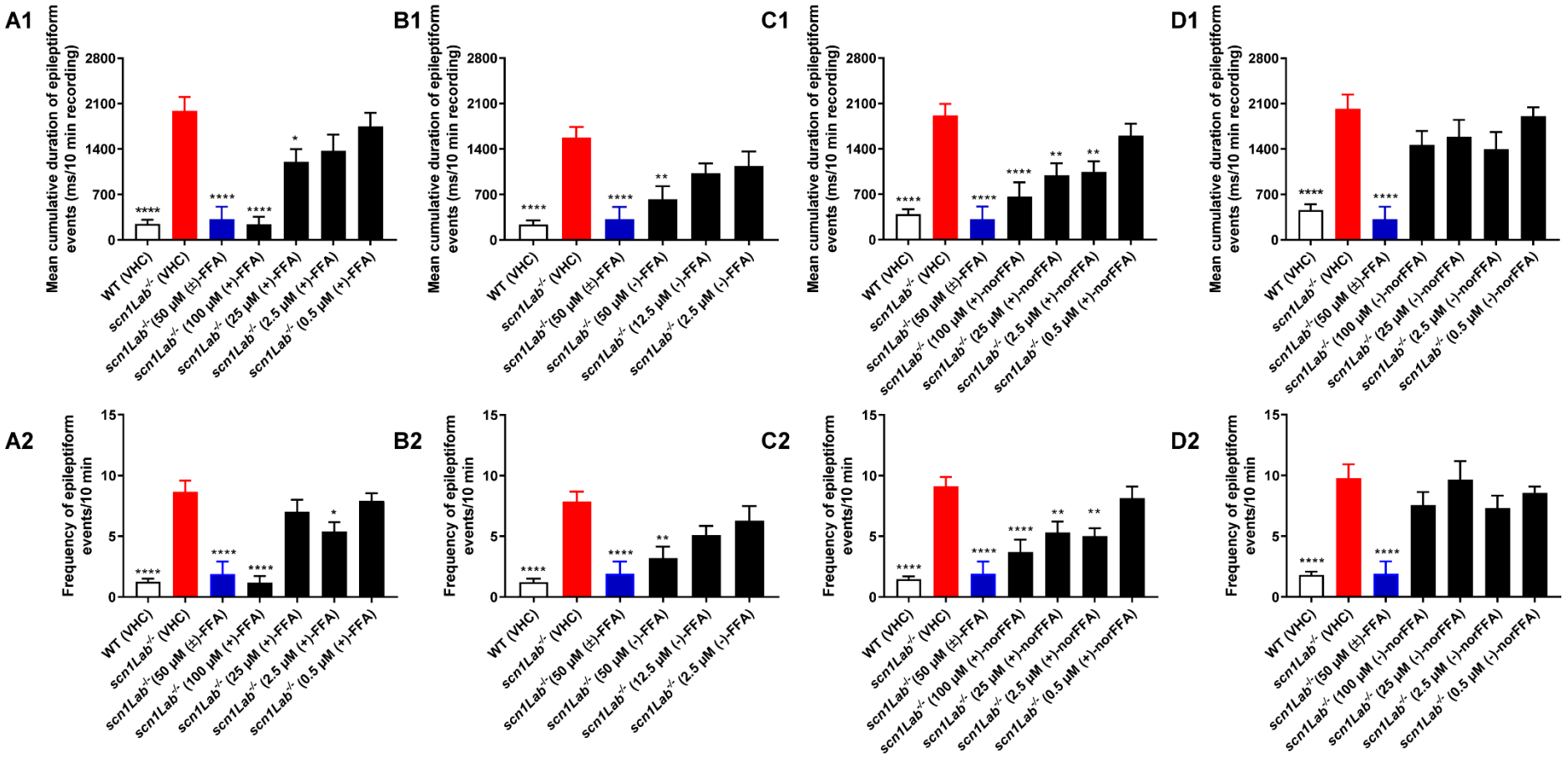

Figure IV-7. Electrophysiological antiseizure activity of (+)-FFA (A1-2), (-)-FFA (B1-2), (+)-norFFA (C1-2) and (-)-norFFA (D1-2) in the *scn1Lab*$^{-/-}$ mutant model, and (±)-FFA (colored in blue, A-D) used as a positive control. (A1-2, B1-2, C1-2, D1-2) Noninvasive local field potential (LFP) recordings from the optic tectum of larvae pre-exposed to (+)-FFA, (-)-FFA, (+)-norFFA and (-)-norFFA for 22 hours. Epileptiform discharges are quantified by the cumulative duration (mean ± SEM) (A1, B1, C1, D1) and frequency (mean ± SEM) (A2, B2, C2, D2) of events per 10-min recording. With 27-45 larvae for the VHC-treated group, 10-16 larvae for each compound-treated group. Statistical analysis: one-way ANOVA with Dunnett's multiple comparison test. Significance levels: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. Abbreviation: WT, wide type, VHC, vehicle, FFA, fenfluramine, norFFA, norfenfluramine.

values ranging from 52 to 287 nM. Other effects of the enantiomers relate to potent serotonin uptake inhibition and agonistic effects on 5-HT_2 subtype receptors (Table IV-2).[13,39] Effects on dopamine and norepinephrine uptake and release have also been documented, although a wide range of potencies were found for the different FFA and norFFA enantiomers (Table IV-2). In addition, the (+)-enantiomers of FFA and norFFA can diminish glutamatergic N-methyl-D-aspartate (NMDA) neurotransmission through disrupting its association with type 1 sigma (σ1) receptors, thereby acting as highly potent σ1R antagonists.[15] Unfortunately, the activity of the individual enantiomers of FFA and its metabolites were not examined in the latter study. Significantly, using similar conditions as in this study (i.e. 25 µM, 22 h incubation), the racemic mixture (±)-FFA was found to exert its anti-seizure activity in DS zebrafish mainly through its modulation of 5-HT_{2C}-R, 5-HT_{1D}-R, sigma-1-R and possibly 5-HT_{2A}-R,[23] thereby confirming the aforementioned data obtained with mammalian cell-based assays.

Table IV-2. Potency of enantiomers of FFA and norFFA

Compound	EC_{50} values (nM) for release			K_i values (nM) for uptake inhibition			K_{act} values (nM) for 5-HT_2 receptor subtypes		
	DA	NE	5-HT	DA	NE	5-HT	5-HT_{2A}	5-HT_{2B}	5-HT_{2C}
(+)-FFA	>10,000	302 ± 20	51.7 ± 6.1	>20,000	1,286 ± 52	150 ± 5	>10,000	379 ± 70	362 ± 64
(-)-FFA	>10,000	>10,000	147 ± 19	>20,000	7,187 ± 559	714 ± 31	5,279 ± 587	1,248 ± 252	360 ± 91
(+)-norFFA	924 ± 112	72.7 ± 5.4	59.3 ± 2.4	2,312 ± 87	205 ± 19	214 ± 9	630 ± 141	18.4 ± 5.3	13 ± 2.4
(-)-norFFA	>10,000	474 ± 40	287 ± 14	19,194 ± 1,048	2,052 ± 297	1,175 ± 89	1,565 ± 190	357 ± 105	18 ± 3.5

(adapted from Rothman *et al*., 2002, 2003)[13,39]

Taken together, the data available so far show that the enantiomers of FFA and norFFA possess somewhat different pharmacological potencies on a subset of receptors that have been implicated in their anti-epileptic activity. However, in view of the larval head concentrations of the individual compounds found in this study, one would not anticipate any major difference in outcome for both locomotor and LFP read-outs. Moreover, as the pharmacological fingerprint of (-)-norFFA is not substantially different from the one of (-)-FFA, it is rather surprising that the former compound exerted a less conclusive inhibitory activity in the DS zebrafish model. Evidently, the slightly conflicting results might be explained by enantiomers' relative affinities for zebrafish proteins differing to those for their mammalian counterparts. However, the mechanism-of-action of the antiseizure effect of FFA is multi-dimensional, also involving σ1-receptors and possibly other targets, further complicating the interpretation of the

finding. Clearly more investigations are needed to better understand the relationship between the pharmacology of the compounds and their respective antiseizure activities against DS.

5. Conclusions

Taken together, our study is the first to validate the *scn1Lab*$^{-/-}$ mutant model by using a combined treatment of ASDs, further supporting the application of the zebrafish-based model as a rapid screening platform to find precision medicine for DS, and possibly for other difficult-to-treat epilepsies. In addition, our results show that (+)-FFA, (-)-FFA and (+)-norFFA displayed significant antiseizure effects in the preclinical model, and thus can be considered as compounds actively contributing to the clinical efficacy of FFA. In case of (-)-norFFA, the results were less conclusive. Whether this inconsistency is related to a different pharmacological fingerprint is presently unexplored and warrants further investigation.

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CHAPTER V

General Discussion

Drug-resistant epilepsy is the major challenge for the pharmacotherapy of epilepsy and affects one third of epilepsy patients. Currently, as well as modern antiseizure drugs (ASDs), surgical procedures, neurostimulation devices and ketogenic diets have also been incorporated into epilepsy management. Even so, the occurrence of drug-resistant epilepsy has not been substantially reduced.[7,125] Therefore, programs to develop innovative ASDs for therapy-resistant epilepsies remain of the utmost importance, requiring also the introduction of novel models and drug discovery strategies.[76]

Since 2005, when the first PTZ zebrafish seizure model was introduced,[158] the utilization of zebrafish for *in vivo* drug screening has grown substantially within the epilepsy field. This is likely due to the fact that seizure-like behavioral and neurophysiological responses in zebrafish can be easily induced with pharmacological or genetic modulation,[134,172] and their responses can be analyzed in a time-efficient and data-dense manner.[172] Besides, zebrafish can capture the complex pathological background of refractory epilepsy and have exhibited drug-resistant properties.[140,225,226] Moreover, their small size and fast reproduction rate are advantageous to support high-throughput studies.

In this chapter, we will further discuss the results obtained from the doctoral research projects described in chapters III and IV, and highlight zebrafish-based drug discovery in the era of precision medicine. In parallel, the emergent readout assays for the zebrafish screening pipeline will be reviewed.

1. The application of zebrafish models to identify antiseizure compounds

1.1 Using the EKP-induced seizure model

In the first project (Chapter III), the ethylketopentenoate (EKP)-induced zebrafish seizure model was used. EKP is an inhibitor of rate-limiting enzyme glutamate decarboxylase (GAD), and a lower activity of GAD was documented to be associated with several drug-resistant epilepsies.[156,161,163,164] Seizures elicited in EKP-treated zebrafish demonstrate a high level of resistance against commercially available ASDs.[161] Finally, the utilization of this model in our antiseizure compound discovery strategy has led to the successful identification of hits from medicinal plants, namely, magnolol and honokiol, as well as their structurally related allyl biphenolic, methylhonokiol. Besides, magnolol was active in the 6 Hz psychomotor mouse test, thereby confirming its efficacy in a mammalian model of therapy-resistant partial seizure.

Hence, the identified hits can be expected to display a novel or specific mechanism-of-action accounting for their antiseizure activity. Of interest, a literature search revealed that the compounds exhibit a multi-target profile encompassing $GABA_A$, cannabinoid and AMPA receptors. Additionally, they are known to exert other effects, such as anti-oxidative and anti-inflammatory activities.[227–229] In particular, both magnolol and honokiol can scavenge oxygen-derived free radicals directly,[230] and using microglial cells it was shown that they exhibit significant inhibitory effects on the production of cytokine-induced reactive oxygen species (ROS).[227] Furthermore, it was found that the compounds can reverse the overexpression of a proinflammatory cytokine, thereby suppressing neuroinflammation.[231,232]

Of interest, a relationship between oxidative stress and epileptic seizures has been evidenced in numerous animal models.[233,234] The main underlying mechanism relates to the overproduction of ROS that induces mitochondrial disfunction and disruption of intracellular Ca^{2+} homeostasis. Afterwards this phase is followed by neuronal excitability and synaptic transmission, eventually resulting in the initiation and progression of epilepsy.[233,234] Regarding neuroinflammation, it commonly occurs in epileptogenic brain regions and is typically activated by acute brain injuries.[235] Moreover, inflammatory mediators such as proinflammatory cytokines were reported to significantly contribute to neurotoxicity, seizure generation and epileptogenesis.[236] Finally, evidence gathered from animal experiments and clinical cases has suggested that add-on anti-inflammatory and anti-oxidative treatments might be beneficial to drug-resistant seizures and epilepsies.[234,235]

Therefore, although other yet unknown targets might play a substantial role, we hypothesize that the antiseizure activity of the allyl biphenolic compounds as found in the zebrafish and mouse tests, relates to their unique pharmacological action on multiple targets, involving a distinct set of neurotransmitter receptors in combination with an anti-inflammatory component.

Taken together, our findings further show that screening ethnomedically used plants using a relevant zebrafish-based discovery platform is an interesting paradigm to identify antiseizure compounds that are promising in the fight against pharmacoresistant epilepsies. For obvious reasons, our study only represents the initial stage of the development of new antiseizure compounds that can be deployed clinically. Since the overall antiseizure activity of magnolol and honokiol has been described previously, the compounds cannot be patented. However, efforts are underway to generate a new generation of allyl biphenolics and structurally related compounds with improved water-solubility and efficacy.

1.2 Using genetic models

The application of zebrafish genetic epilepsy models mirroring specific human disease offers a great opportunity for the discovery of precision therapeutics tailored to subgroups of epilepsy patients. In the second study (chapter IV), the *scn1Lab* double indemnity (*didy*[552]) mutant model was employed that reflects well the genetic basis and characteristics of Dravet syndrome (DS).[167] First of all, the pharmacological activity of commonly used ASDs was explored in the *scn1Lab*$^{-/-}$ mutants. The results show that the compounds exert a similar activity profile as observed in the clinic, thereby pharmacologically validating the model.

In 2020, FDA has approved fenfluramine (FFA) for treatment of seizures associated with DS. FFA used in the clinic is a racemic mixture that is substantially metabolized in humans to norfenfluramine (norFFA). Using a *scn1Lab*$^{-/-}$ mutant platform, in this study we examined the relative antiseizure contribution of each of the four enantiomers, and showed that (+)-FFA, (-)-FFA and (+)-norFFA were active, whereas in case of (-)-norFFA the results were less conclusive. Hence, as far as therapeutic activity is concerned, a major conclusion drawn from this study is that there is no need to separate the racemic mixture into its enantiomers, and the choice to go for racemic FFA as a potential therapeutic against DS was an appropriate one.

As far as the potential adverse effects are concerned, the use of high doses of FFA as an anorectic drug has been associated with unusual cardiac valvular morphology.[54] This chemically-induced pathology is related to the agonistic activity of FFA at the 5-HT_{2B} receptor.[237] Of interest (+)-norFFA, the main metabolite of (+)-FFA, is the most potent 5-HT_{2B}-R agonist among the FFA and norFFA enantiomers (Table IV-2). Conversely, it has been shown that the use of (-)-FFA causes lethargy and sleepiness, and as a consequence purified (+)-FFA (Redux) was therapeutically used once as an anorectic to substitute for racemic FFA.[238] In the most recent clinical trial proving the efficacy of racemic FFA in the treatment of DS patients, lethargy was also observed,[58] possibly due to the presence of 50 % of (-)-FFA in the racemic mixture. However, the valve function was within the normal physiological range in all patients during the trial and no signs of pulmonary arterial hypertension were observed.[58] So although one could have opted to use (-)-FFA in the clinic, thereby reducing the risk to induce valvulopathy, probably by using small doses the adverse effect could be avoided.

1.3 The role of power spectral density (PSD) analysis

Local field potential (LFP) recordings that monitor epileptiform electrical discharges in the zebrafish brain have been widely used to validate novel zebrafish seizure and epilepsy models, and to evaluate the efficacy of new drug candidates. However, visual analysis of LFP recordings is time-consuming and prone to subjectivity. Alternatively, automated methods such as PSD analysis, can save time and provide an objective assessment. Within the study on constituents in Magnolia extracts, PSD analysis of the recordings was developed and performed using MatLab R2018 (MATrix LABoratory, USA) software. In brief, by using fast Fourier analysis, LPF recording signals could be decomposed in a spectrum of frequencies based on their amplitude, and by using the Welch's method, the power spectral density could be estimated as a function of frequency.

In the zebrafish epilepsy and seizure models, especially in the chemically-induced seizure model, e.g. PTZ/EKP model, typically around 20-30 epileptiform electrical discharges per recording appear, and an average of 10 recordings per condition is needed. With visual analysis, those recordings take hours to be analyzed. By contrast, with the computational PSD analysis, they can be analyzed in just a few minutes. Significantly, PSD results (Figure III-5) are in agreement with the visual analysis (Figure III-4) of LFP recordings in the PTZ/EKP model. Hence, the availability of software to conduct PSD analysis as performed for the first time on LFP recordings of the zebrafish brain is a great asset fur future studies, reducing dramatically the time spent in post-recording analysis, and improving the overall objective quantification of the electrophysiological signals of the brain acquired during the recordings. A drawback of the PSD analysis is that the validity of the outcome depends on the seizure or epilepsy zebrafish model used. For instance, in case of the zebrafish *scn1Lab*$^{-/-}$ mutant model of DS we were not able to optimize the system yet, and as a consequence the LFP recordings are still examined visually.

2. The zebrafish models-based precision medicine discovery in epilepsy

2.1 Precision medicine in epilepsy

According to the Precision Medicine Initiative (NIH, USA), precision medicine is defined as "an emerging approach for disease treatment and prevention that takes into account individual variability in genes, environment, and lifestyle for each person." In contrast to the "one-size-fits-all" approach, precision medicine aims to develop tailor-made treatments for individuals

or subgroups who fail to respond to currently available therapeutics.[239] With the technological advances in genomic discovery, especially the emergence of next-generation sequencing, the expense of the genome sequencing have been dramatically decreased, and throughput has been significantly enhanced. According to a published report, the cost of sequencing a whole genome from research laboratories is now around US$1,000.[240,241] These advances have accelerated progression towards the discovery of causative genes of disease, and thus, are allowing precision medicine treatments to be developed that can reverse or circumvent the specific gene mutations and related pathophysiological consequences.[242] A notable example for such strategies is the specialty of cancer, where many precision medicine treatments have been already realized from bench to bedside.

Besides cancer, epilepsy research is also well suited to the development of precision medicine due to its strong genetic basis. It is estimated that 70-80% of all epilepsies are caused by genetic factors.[240,243] Additionally, since the identification of the first epilepsy-related gene in 1995, a growing number of disease-associated genes have been discovered in epilepsy patient cohorts,[17] and over 140 epilepsy-associated genes or loci have been published in the Online Mendelian Inheritance in Man® database.[242–244] Of particular interest, some "disease-specific" precision treatments targeting associated biological mechanisms of causative gene mutations, already displayed efficacy in various genetically defined epilepsies. For instance, with next-generation sequencing, gene mutations in *SLC2A1* encoding the glucose transporter type 1 (GLUT1) protein of the blood-brain barrier, were found to be associated with glucose transporter deficiency syndrome, which could be treated by ketogenic diet (KD).[240,245,246] Another example of effective precision therapy in genetic epilepsies is the management of pyridoxine-dependent epilepsy, generally caused by mutations in the *ALDH7A1* gene, by pyridoxine (vitamin B6).[19,20] Overall, precision medicine of epilepsy is rapidly developing, and we anticipate more effective treatments based on new genetic discoveries can be translated into clinical practice in the near future.

2.2 The generation of novel zebrafish models for drug discovery in the era of precision medicine

Translation of genetic causes into precision medicine relies on effective experimental model systems. Recently, many excellent *in-vitro* and *in-vivo* model systems, which are able to capture the pathogenesis of a specific genetic mutation, have been generated and developed.

Among whole-animal models, zebrafish models have gained a lot of attention for reasons mentioned before.

Table V-1. Overview of morpholino or CRISPR/Cas9 knockdown of zebrafish epilepsy models

Model	Genetic tool	Human gene	Functional target or mechanism	Seizure or EEG or molecular biomarkers of seizure
lgila morphant	morpholino	*LIGI1*	Synapse transmission	Yes
lgilb morphant		*LIGI1*		Not observed
kcnq3 morphant		*KCNQ3*	Kv7.3 channel	Yes
pk1a morphant		*PK1*	Neuronal migration, Axonal outgrowth	Not investigated
kcnj10a morphant		*KCNJ10*	Kir4.1 channel	Yes
chd 2 morphant		*CHD2*	Gene transcription modification	Yes
stx1b morphant		*STX1B*	GABA and glutamate release	Yes
cnnm2a and *cnnm2b* morphant		*CNNM2*	Cyclin M2	Not investigated
scn1Lab morphant		*SCN1A*	$Na_V1.1$ channel	Yes
cacna1aa morphant		*Cacna1a*	$Ca_V2.1$ channel	Yes
pgap3 morphant		*PGAP3*	Glycosylphosphatidylinositol (GPI)-specific phospholipase	Yes
stxbp1b mutant	CRISPR/Cas9	*STXBP1*	Neurotransmitter release	Yes
*aldh7a1*mutant		*ALDH7A1*	Lysine degradation	Yes
*gabra1*mutant		*GABRA1*	Inhibitory synaptic network	Yes
plpbp mutant		*PLPHP*	Neurotransmitter biosynthesis	Yes
pnpo mutant		*PNPO*	Vitamin B6 metabolism	Yes
got2a mutant		*GOT2*	Aspartate aminotransferase	Yes

(adapted and updated from Copmans *et al*., 2017, Karnebeek *et al*., 2019, Ciapaite *et al*., 2019, and Gawel *et al*., 2019, Da'as *et al*., 2019, and Johnstone *et al*., 2019)[134,247–251]

With the introduction of morpholino tools and CRISPR/Cas9 methodologies to the zebrafish community, the generation of specific zebrafish mutations or knockdowns can now be realized in a short time, in large numbers.[252–254] For example, morpholinos can be injected to freshly fertilized eggs, in order to splice target genes up to 5dpf. Such knockdowns can be obtained in hundreds of embryos in one single experiment.[252] Importantly, most organs of zebrafish are fully functional in 5 dpf, and therefore mutant zebrafish larvae can be used for phenotypic large-scale drug or genetics screening projects. However, several drawbacks of morpholino-mediated knockdown were also be reported, including the transience of morpholinos and potential off-target effects.[255] More recently, the implementation of CRISPR/Cas9 technology in the zebrafish field allows for more straightforward and precise genome editing by targeting specific DNA sequences.[256] If the microinjection of guide RNA and Cas9 protein are performed in one-cell stage embryos, the highest mutagenesis efficiency can be obtained.[256] As CRISPR/Cas9-based genome editing can be undertaken in specific tissue or protein domains, it has the advantage of providing more detailed information regarding drug-target interactions

for phenotypic drug screening studies.[256] To date, a growing number of epilepsy-associated genes knockdown zebrafish lines have been successfully established in both our lab and other research groups (Table V-1). Overall, these new gene-editing techniques speed up the generation of mutant zebrafish lines for all known and yet-to-be-discovered human epilepsy genes, emphasizing the utility of those models for precision medicine in genetic epilepsies.

2.3 The application of zebrafish models for precision medicine

As shown in Figure V-1, the incorporation of the disease-specific zebrafish models into effective discovery of precision medicine drugs for difficult-to-treat epilepsies can be mainly categorized into two different approaches.

1) By directly using those disease-specific zebrafish models, and their biological features (e.g. behavioral profiling), as the basis for conducting phenotypic drug screenings which aim to identify new ASDs.[141,256] For example, phenotype-based random screens of chemical libraries and re-purposing screens of existing drugs have been successfully conducted in *scn1Lab*$^{-/-}$ mutant models, and potential antiseizure hits have been identified.[257,258] Of particular interest, one identified hit, lorcaserin, was reported to lead to a substantial reduction of seizure frequency in three of five patients with DS.[259]

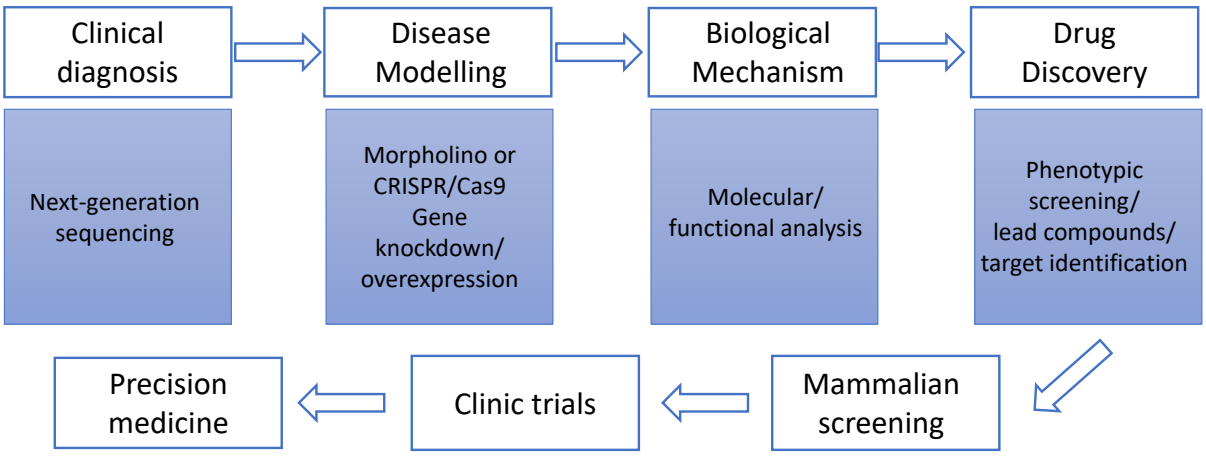

Figure V-1. A work plan for using zebrafish models in precision medicine strategies. (adapted from Griffin *et al*., 2016, Gut *et al*., 2017, Baxendale *et al*., 2017)[146,260,261]

2) Candidate druggable targets can also be found from genomic screenings on human patient populations,[262] and/or from gene functional analysis (e.g. transcriptomics) of disease-specific experimental models.[263] Then, high-throughput genetic screens (e.g. morpholino screens) on zebrafish can further validate genes that are important to reverse the disease progression, and

sequentially define potential targets for precision medicine. Once such information is confirmed, then targeted therapeutics-based screens of compound libraries can be conducted in the zebrafish models.

2.4 The application of rodent models for precision medicine

Similar as in case of zebrafish models, with the advent of morpholino tools and CRISPR/Cas9 methodologies, a large number of genetic rodent models that mirror specific epileptic pathophysiology have been generated.[127,264127,265] For instance, the transgenic DS mice model, which carries the *Scn1a* variant and exhibits spontaneous seizures and early mortality, has been used in the discovery of precision therapeutics.[91] One identified hit, the venom peptide Hm1a that selectively potentiates Nav1.1 channels, turned out to be of particular interest in the potential treatment of DS patients.[265] Another example is the *Scn8a*$^{D/+}$ mutant mice model which is designed to recapitulate the clinical features of *SCN8A* epileptic encephalopathy. Using this model, a Nav1.6-selective sodium channel modulator, Prax330, has been successfully identified, and suggested as a precision therapeutic for *SCN8A* epileptic encephalopathy.[265,266]

However, the application of genetic rodent models in precision medicine is still facing some practical problems like scalability and costs, especially when it comes to the screening of large libraries.[241] Therefore, rodent models could pair with *in vitro* pluripotent stem cell methodology or zebrafish models that inherently have high-throughput capacity to address those limitations.[241] Consequently, the so-obtained pre-rodent results could be further validated in rodent models before moving to clinical trials in human beings.[91,240,247,266]

3. The emerging readout assays in the zebrafish models

Besides the developments in zebrafish model generation, the advances in zebrafish movement tracking, electrophysiological recording and brain activity mapping also further promote zebrafish models as ideal high-throughput screening platforms for innovative antiseizure therapeutics discovery. To date, the most commonly used video tracking devices are limited in recognizing and extracting rich and complicated seizure behavioral information (e.g. twitching or abnormal body position), which limits the application of certain zebrafish models with subtle seizure-related behavior in high-throughput drug screening projects. Recently, a computer-based three-dimensional behavioral analysis of zebrafish has been developed. This allows recording of zebrafish locomotion in XYZ coordinates by imaging both top and side

views.[267,268] However, it has only been applied to adult zebrafish, and the throughput capacity is so far quite limited.

Furthermore, the conventional LFP recordings have only a moderate throughput of six detectable larvae per hour. To improve this limitation, some multichannel electrophysiology systems have been developed to measure brain activity of several zebrafish at once, such as the high-throughput LFP recording platform developed by Eimon *et al.*,[269] and the integrated zebrafish analysis platform (iZAP) created by Hong *et al.*[270] The LFP platform was shown to predict the efficacies and side effects of the potential drugs on the larvae, through analyzing the LFP pattern complexity with an independent component analysis (ICA).[269] Meanwhile, the iZAP platform utilizes additional electrodes for recording each zebrafish, in order to capture various epileptic episodes by electroencephalography, electromyography, or electrooculography and audiology.[270] To date, these two platforms have been used to successfully monitor electrographic seizures events in both *scn1Lab*$^{-/-}$ mutant model and the PTZ model.

Functional neuroimaging techniques depending on activity-dependent bioluminescent/fluorescent reporters have also been introduced to zebrafish models, providing an opportunity to visualize their neuronal microcircuits and brain-wide network.[271–273] The most common approach is genetically-encoded calcium indicators (GECIs)-based brain imaging (e.g. GCaMP). When used with specific neuronal *elavl3* or *NeuroD* promoters, this enables imaging of neural networks of transgenic zebrafish lines. Also, GECIs offer a rapid readout of calcium dynamics of single cells in the whole brain network by using fluorescence microscopy.[274,275] Based on the correlation between dynamic intracellular calcium levels and frequency of the seizures in the CNS, the simultaneous fluorescence change of the zebrafish whole brain can directly reflect neural activity during seizures.[276] Such imaging technologies can compensate for the drawbacks of behavioral and electrophysiological measurements in evaluating effect of potential ASDs.[272,276]

4. General conclusion

In conclusion, the evidence from our research group and others has demonstrated the value of zebrafish seizure and epilepsy models in identifying effective therapies for difficult-to-treat epilepsies. Furthermore, the physiological features of zebrafish are highly conserved across vertebrates. Therefore, zebrafish models can recapitulate mammalian drug metabolism features, and predict safety liabilities of drugs.[141,256] It is anticipated that with more personalized genetic

zebrafish models and new emerging screening approaches being generated and incorporated in the screening workflow, the discovery of finding precision medicine drugs will be highly accelerated, paving the way for the most effective antiseizure drug to be identified for each therapy-resistant epilepsy patient.

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Summary

With 70 million people affected worldwide is epilepsy one of the most common neurological diseases characterized by unpredictable, unprovoked, recurrent seizures. Moreover, the disease is often accompanied by psychiatric and cognitive comorbidities, affecting dramatically the quality of life of patients.

So far, pharmacological intervention is the first-line treatment for epilepsy. Unfortunately, about 30% of patients experience seizures that cannot be well controlled with the current marketed antiseizure drugs (ASDs).

To find novel compounds a wide variety of preclinical animal epilepsy and epileptic seizure models has been generated and employed in phenotypic drug discovery projects. For instance, in 2015, the updated Epilepsy Therapy Screening Program (ETSP) (NIH, USA) has incorporated several rodent seizure models with drug-resistant signature in its working flow. Although those models have the potential to identify innovative ASDs with efficacy in as yet ASDs-resistant patients, the newly screening pipeline is labor-intensive, and has limited throughput. More recently, zebrafish models, especially those with a drug-resistant profile and high-throughput capacity, have gained an increasing popularity in drug discovery. Zebrafish display an excellent compromise between system complexity of the vertebrate organism and the practical simplicity of the *in vitro* models. Moreover, they allow to find candidate ASDs at a lower cost and time, and thus can be used in the early-stage drug screening to speed up innovative ASDs discovery.

In this doctoral research two distinct zebrafish models, a chemically-induced and genetic zebrafish seizure and epilepsy model that proved to exhibit high pharmacoresistant profiles were used for the discovery of new hits and the identification of compounds of interest.

Compared to random screening of compounds, a medicinal plant-based approach has been suggested to result in a faster and cheaper identification of active ingredients, as these plants have been pre-selected through centuries of use by ethnomedical practitioners. Traditional Chinese Medicine (TCM) is one of the most widely practiced forms of botanical therapy in the world, including also multiple recipes of medicinal plants against epilepsy and seizures.

Therefore, in the first project 42 extracts of medicinal extracts from fourteen neuroprotective and antiseizure TCM plants were prepared, and a phenotype-based screening was performed using a combination of acute zebrafish seizure models (PTZ and EKP) and a rodent seizure model (mouse 6-Hz psychomotor seizure model).

Both the zebrafish EKP seizure and the mouse 6 Hz (44 mA) psychomotor seizure models represent a high potential to identify ASDs with a novel mechanism-of-action. Our strategy led to the identification of magnolol and honokiol, main constituents *Magnolia officinalis*, as well as the structurally related allyl biphenolic methylhonokiol as potent antiseizure agents. In addition, magnolol was able to protect mice from seizures induced by 6-Hz electrical stimulation in a dose-dependent manner, thereby confirming its antiseizure activity in a mammalian model.

Dravet syndrome (DS) is a catastrophic genetic epilepsy of childhood characterized by a variety of drug-resistant seizures initially often induced by fever. Of interest, distinct *de novo* mutations in the *SCN1A* gene are found in about 85% patients with DS. A genetic zebrafish Dravet model based on *scn1Lab*$^{-/-}$ mutants, has been developed to accurately reflect the genetic basis and characteristics of DS. This model was used in our second study with the aim to explore the antiseizure activity of enantiomers of fenfluramine (FFA) and its presumedly active metabolite norfenfluramine (norFFA), expanding on the clinical treatment of DS patients with racemic FFA. Firstly, we validated the model pharmacologically by using both single and combined ASDs. The results were in accordance with the updated clinical treatment algorithm for DS. Thereafter, the antiseizure activities of the compounds were confirmed showing a concentration-response relationship, thereby demonstrating that both the enantiomers of FFA but also their norFFA metabolites contribute to the antiseizure activity of racemic FFA in DS patients.

Taken together, our work discovered three potential hits against therapy-resistant epilepsies and revealed the value of medicinal plants as an interesting resource for ASDs discovery. Furthermore, evidence is provided that the use of zebrafish as high-throughput model of treatment-resistant seizures can be deployed in innovative ASDs discovery.

Samenvatting

Met wereldwijd 70 miljoen patiënten is epilepsie een van de meest voorkomende neurologische aandoeningen. De ziekte wordt gekenmerkt door onvoorspelbare, niet-uitgelokte, terugkerende aanvallen. Bovendien gaat de ziekte vaak gepaard met psychiatrische en cognitieve comorbiditeiten, die de kwaliteit van leven van patiënten dramatisch beïnvloeden.

Farmacologische interventie is tot dusver de eerstelijnsbehandeling voor epilepsie. Helaas vertoont ongeveer 30% van de patiënten epileptische aanvallen die slecht behandelbaar zijn met de huidige op de markt verkrijgbare anti-epileptica.

Om nieuwe verbindingen te vinden, is een breed scala aan preklinische diermodellen voor epileptische aanvallen en epilepsie ontwikkeld en gebruikt in projecten voor het fenotypisch ontdekken van nieuwe geneesmiddelen. In 2015 bijvoorbeeld werden verschillende farmaco-resistente knaagdiermodellen in de werkstroom van het vernieuwde Epilepsie Therapie Screening Programma (ETSP) (NIH, VS) opgenomen.

Hoewel deze modellen de mogelijkheid bieden om innovatieve anti-epileptica te identificeren die werkzaamheid vertonen bij farmacoresistente patiënten, is de nieuwe screeningpijplijn arbeidsintensief en is ze gelimiteerd door de beperkte doorvoer. Meer recentelijk hebben zebravismodellen, vooral die met een farmacoresistent profiel en een hoge doorvoercapaciteit, aan populariteit gewonnen bij de ontdekkingstocht naar nieuwe geneesmiddelen. Zebravissen vertonen een uitstekend compromis tussen de systeemcomplexiteit van gewervelde organismen, maar tevens ook de praktische eenvoud van de in vitro modellen. Bovendien maken ze het mogelijk om kandidaat-antiepileptica te vinden tegen lagere kosten en tijd, en kunnen ze dus worden gebruikt in de vroege fase van geneesmiddelenonderzoek om de ontdekking van innovatieve antiepileptica te versnellen.

In dit doctoraatsonderzoek werden twee verschillende farmacoresistente zebravismodellen, een chemisch geïnduceerd en een genetische epilepsie-model, gebruikt voor de ontdekking van nieuwe hits en voor het nagaan van de activiteit van geselecteerde verbindingen.

In vergelijking met een willekeurige screening van moleculen, wordt een benadering gebaseerd op medicinale planten verondersteld efficiënter te verlopen ter identificatie van actieve verbindingen. Dit is het gevolg van het feit dat deze planten een selectie vertegenwoordigen na eeuwenlang gebruik van hun (veronderstelde) therapeutische waarde. Traditionele Chinese geneeskunde (TCM) is een van de meest toegepaste vorm van fytotherapie in de wereld, inclusief meerdere recepten gebaseerd op planten die gebruikt worden bij epilepsie. In het eerste project werden 42 extracten van medicinale extracten van veertien neuroprotectieve en antiseizure TCM-planten bereid, en werd een fenotypische screening uitgevoerd met zebravismodellen van epileptische aanvallen (PTZ en EKP) en een knaagdiermodel (muis 6-Hz psychomotorisch epileptische aanval-model).Zowel het zebravis EKP- als het 6 Hz psychomotorische model vertonen een hoog potentieel om anti-epileptica te identificeren met een nieuw werkingsmechanisme.

Onze strategie leidde tot de identificatie van magnolol en honokiol, hoofdbestanddelen van *Magnolia officinalis*, evenals van het structureel verwante allylbifenolische methylhonokiol als potente verbindingen tegen epileptische aanvallen. Bovendien was magnolol in staat om de muis op een dosisafhankelijke manier te beschermen tegen aanvallen geïnduceerd door 6-Hz elektrische stimulatie, waardoor de antiepileptische activiteit van de verbinding in een zoogdiermodel werd bevestigd.

Het Dravet Sydroom (DS) is een catastrofale genetische epilepsie die zich manifesteert vanaf de kindertijd en gekenmerkt wordt door een verscheidenheid aan farmacoresistente aanvallen die aanvankelijk vaak worden veroorzaakt door koorts. Interessant is dat verschillende *de novo* mutaties in het *SCN1A*-gen worden gevonden bij ongeveer 85% van de patiënten met DS. Een genetisch zebravis Dravet-model gebaseerd op *scn1Lab*$^{-/-}$ mutante werd eerder ontwikkeld om de genetische basis en kenmerken van DS nauwkeurig te reproduceren. Dit model werd gebruikt in onze tweede studie met als doel de antiepileptische activiteit van de enantiomeren van fenfluramine (FFA) en zijn verondersteld actieve metaboliet norfenfluramine (norFFA) te onderzoeken. Bedoeling was na te gaan welke van de twee enantiomeren aanwezig in het racemische FFA mengsel, klinisch gebruikt bij de behandeling van DS-patiënten, actief zijn. Ten eerste hebben we het model farmacologisch gevalideerd door zowel enkelvoudige als als gecombineerde anti-epileptica therapie te gebruiken. De resultaten waren in overeenstemming met het klinische behandelingsalgoritme voor DS. Daarna werd bevestigd dat de anti-epileptische activiteit van de verbindingen een concentratie-responsrelatie vertoonden,

waarmee werd aangetoond dat zowel de enantiomeren van FFA als hun norFFA-metabolieten bijdragen aan de therapeutische activiteit van racemisch FFA bij DS patiënten.

Samenvattend kan gesteld worden dat het uitgevoerde onderzoekswerk drie mogelijke hits tegen farmacoresistente epilepsie identificeerde, en dat het de waarde van medicinale planten als een interessante bron voor de ontdekking van nieuwe anti-epileptica aantoonde. Verder wordt bewezen dat het gebruik van zebravismodellen als hoog doorvoermodel van therapieresistente aanvallen kunnen worden ingezet bij het ontdekken van innovatieve anti-epileptica.

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Leuven

2020.11

Curriculum vitae

Jing Li

Date of birth: 27 Sep. 1990

Nationality: Chinese

Tel.: +32 (0)483391149

Email: jing.li0927@gmail.com

Education

11/2016 – 12/2020 **PhD**, Pharmaceutical Sciences
Katholieke Universiteit Leuven, Leuven, Belgium

09/2013 – 07/2016 **Master**, Pharmaceutical Sciences
Tianjin University, Tianjin, China

09/2009 – 07/2013 **Bachelor**, Pharmaceutical Sciences
South-Central University for Nationalities, Wuhan, China

Research Experience

2016-2020, Katholieke Universiteit Leuven, Leuven, Belgium

The application of in vivo seizure and genetic epilepsy models as platform for antiseizure drug discovery

- Evaluation of acute zebrafish and rodent seizure models; and, using screening approaches (i.e. quantifiable behavioral and electrographic assays) to identify novel antiseizure compounds; Plus, to study the structure-activity relationship of the hits.
- Conduct "precision medicine"-based screening, utilizing zebrafish genetic epilepsy model with the pathophysiology of a specific epileptic syndrome; and investigate the uptake kinetics of the hits in the zebrafish.

The identification of potential agonist and antagonist acting on Transient receptor potential channels (TRP channels)

- Identify new ligands (potential agonist and antagonist for TRP channels), depends on calcium fluorescence assay of the cell lines expressing recombinant human TRP channels (i.e. TRPM3 and TRPM5).

2013-2016, Tianjin University, Tianjin, (China)

The establishment and optimization of the medicinal plant bio-culture system

- Establish and optimization of medicinal plant bio-culture system; to quantify the production of the active composition of the plants by using the HPLC-MS^n; to evaluate the activity of the plant metabolites by using the animal model.

Skills

Experimental	Animal experimentation (zebrafish, mouse); Electrophysiological assay and analysis; Fluorescence microscope; Behavioral study; Molecular biology; Cell culture, Calcium fluorescence assays; Drug screening and discovery; Plant extraction and purification; HPLC-MS; Statistical analysis of scientific data; etc.
Software	Adobe-Illustrator; GraphPad Prism, MarvinSketch, Clampex, Clampitfit, Microsoft-Office; etc
Language	Chinese (native), English (proficient)
Other	Scientific writing and presentation

Training and Teaching

Trainings	Laboratory Animal Science module (FELASA B); Scientific writing for biomedical science; VIB training: Drug discovery; Training course: Genetic epileptic encephalopathies in infancy and childhood; etc.
Teaching	Supervisor in "Laboratory session of Biopharmaceutical research (KU Leuven)" at 2017-2018 semester 1, 2018-2019 semester 1 and 2019-2020 semester 1.

Publications

Journal articles

- **Jing Li,** Daniëlle Copmans, Michèle Partoens, Borbála Hunyadi, Walter Luyten, and Peter de Witte**.** Zebrafish-based screening of antiseizure plants used in Traditional Chinese Medicine: *Magnolia officinalis* extract and its constituents magnolol and honokiol exhibit potent anticonvulsant activity in a therapy-resistant epilepsy model. *ACS Chem Neurosci*. 2020,11(5):730-742
- Juan Wang [1] & **Jing Li [1]** (co-first author), Xiaolei Wu, Shujie Liu, Hongfa Li, Wenyuan Gao. Assessment of genetic fidelity and composition, mixed elicitors enhance triterpenoids and flavonoids biosynthesis of *Glycyrrhiza uralensis* Fisch. tissue cultures. Biotechnol Appl Biochem. 2017, 64(2):211-217.
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In preparation

- **Jing Li**, *et al*., Antiseizure activity of enantiomers of fenfluramine and norfenfluramine in a zebrafish model of Dravet syndrome.

Poster Presentation

- 15th EILAT Conference on New Antiepileptic Drugs and Devices - VIRTUAL Conference, July 2020, Madrid, Spain.

Personal Contributions

Chapter III: Jing Li performed the zebrafish and mice experiments, data analysis and wrote the manuscript. Daniëlle Copmans was involved in data analysis and experimental design. Michèle Partoens coordinated the mice experiments. Borbála Hunyadi designed the PSD analysis software. Walter Luyten was involved in the experimental design, and the preparation of the manuscript and Figures. Peter A. M. de Witte was responsible for the experimental design, data analysis, and preparation of the manuscript and Figures. All authors edited and approved the final version of the manuscript.

Chapter IV: Jing Li performed the experiments, data analysis and wrote the manuscript. Jo Sourbron was assisted experimental design. Maxim Nelis coordinated the LC-MS experiments. Lieven Lagae and Deirdre Cabooter were involved in the experimental design, and the preparation of the manuscript. Peter A. M. de Witte was responsible for the experimental design, data analysis, and preparation of the manuscript and Figures.

Conflict of Interest Statement

No potential conflict of interest was identified.