Aus der Klinik für Neurologie

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des Fachbereichs Medizin der Philipps-Universität Marburg

in Zusammenarbeit mit dem Universitätsklinikum Gießen und Marburg GmbH, Standort Marburg

The influence of levetiracetam and valproate on apoptosis and cytotoxic function of CD8$^+$ T lymphocytes *in vitro*

Inaugural-Dissertation zur Erlangung des Doktorgrades
der gesamten Humanmedizin

dem Fachbereich Medizin der Philipps-Universität Marburg
vorgelegt von

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Marburg, 2011

Angenommen vom Fachbereich Medizin der Philipps-Universität Marburg
am: 27.05.2011

Gedruckt mit Genehmigung des Fachbereichs.

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Table of Contents

1 **Abstract / Zusammenfassung** 5

2 **Abbreviations** 9

3 **Introduction and discussion** 11

3.1 epilepsy and its treatment 11

3.2 Aim of the study 13

3.3 Levetiracetam 16

3.3.1 Pharmacokinetic and pharmacodynamic 16

3.3.2 Mechanism of action 17

3.3.3 Indication and usage 18

3.3.4 Adverse events 19

3.4 Valproate 19

3.5 CD8+ T lymphocytes and its antivirus function 21

3.6 Summary 24

4 **Materials and Methods** 25

4.1 Study population 25

4.2 Antibodies, reagents and peptides 25

4.3 Antiepileptic drugs 26

4.3.1 VPA dilution 26

4.3.2 LEV dilution 27

4.4 Apoptosis evaluation 27

4.4.1 Isolation of peripheral blood mononuclear cells 27

4.4.2 LEV or VPA treatment 28

4.4.3 Apoptosis evaluation with flow cytometer 29

4.5 Functional assay of virus-peptidess-specific CD8+ T lymphocytes 30

4.5.1 Isolation of PBMCs 30

4.5.2 CEF peptides..30

4.5.3 Perforin release..31

4.5.4 CD107 degranulation assay..32

4.5.5 Analysis of $CD8^+$ T lymphocytes Proliferation by CFSE Labeling....................33

4.6 Statistical analysis..36

5 Results 37

5.1 Apoptosis 37

5.2 CEF-peptides-specific activation..40

5.3 Perforin release.. 41

5.4 CD107a/b expression..44

5.5 Proliferation..46

6 Discussion 47

6.1 Methodology..47

6.2 Degranulation and anti-virus function..49

6.3 Perforin release and antiepileptic mechanism..51

6.4 Apoptosis and proliferation..51

6.5 The preliminary observation for SV2A expression in human $CD8^+$ T lymphocytes..52

6.6 Limitations..54

6.7 Conclusion..54

7 Reference..56

8 Lebenslauf 75

9 Acknowledgments..80

1 Abstract：

Purpose: Previous studies showed that epilepsy patients treated with levetiracetam (LEV) had a higher incidence of upper respiratory tract infections and experimental and clinical data suggest also an immunomodulatory actions of valproate (VPA). The aim of this study was, therefore, to investigate the influence of LEV and VPA on apoptosis and cytotoxic function of $CD8^+$ T lymphocytes *in vitro*.

Methods: After isolation of peripheral blood mononuclear cells (PBMCs) in 15 healthy subjects (9 female (60%), age: 35.7±12.1 years), apoptosis and cytotoxic function of $CD8^+$ T lymphocytes were measured *in vitro* using immunofluorescence labeling and flow cytometry. Drug concentrations applied were 5mg/L and 50mg/L for LEV and 10mg/L and100mg/L for VPA, respectively. Apoptosis rates of $CD8^+$ T lymphocytes were determined after incubation of PBMCs with LEV or VPA for 1h or 24h. Apoptotic $CD8^+$ T lymphocytes were deifiined as CD3+/CD8+/ Annexin V+/PI- after applyingwiththe Annexin V Apoptosis Detection Kit® and flow cytometry. Perforin release, CD107a/b expression and proliferation of $CD8^+$ T lymphocytes were measured within in the different groups following activation of $CD8^+$ T lymphocytes with virus peptides, (which were made from cytomegalovirus, Epstein-Barr virus, and influenza virus; （CEF)）. Degranulation of $CD8^+$ T lymphocyte was indicated by perforin release and the increase of CD107a/b expression on the cell surface. Group comparisons were performed with the paired t-test. T and the significance level was set to $p<0.05$.

Results: Both high (50mg/L) and low (5mg/L) concentrations of LEV decreased perforin release (LEV 50 mg/L vs controlCEF : 25.8±12.9% vs 18.2±9.7%, $p<0.01$; LEV 5mg/L vs control CEF: 24.1±13.7% vs 18.2±9.7%, $p<0.01$; n=15) and CD107a/b expression (LEV 50mg/L vs control CEF: 5.3±2.5% vs 11.5±4.7%, $p<0.01$; LEV 5mg/L vs control CEF: 6.7±2.2% vs 11.5±4.7%, $p<0.01$; n=15) of $CD8^+$ T lymphocytes after 2h of virus-peptide induced stimulation. LEV

had no influence on apoptosis and proliferation of CD8+ T lymphocytes ($p > 0.05$). High concentration of VPA (100mg/L) prevented spontaneous apoptosis of CD8+ T lymphocytes after incubation for 24 h (VPA 100mg/L vs control: 7.8±3.4% vs 11.5±4.2%, $p<0.01$, n=15), but had no effects on perforin release or CD107a/b expression ($p > 0.05$).

Conclusions: LEV showed a moderate attenuating effect on degranulation of CD8+ T lymphocytes which may contribute to the increased incidence of upper respiratory tract infections in LEV treated patients. Moreover, it is hypothesized that LEV's attenuating effect on perforin release adds to its anticonvulsant potency via reduction of inflammation in the epileptogenic zone and blood-brain-barrier disruption. Valproate revealed no effects onf the function of CD8+ T lymphocytes function but slowed apoptosis.

KEY WORDS: Levetiracetam, valproate, CD8+ T lymphocytes, apoptosis, perforin, CD107, degranulation, proliferation

Abstrakt

Hintergrund

Epilepsie-Patienten, die Levetiracetam einnahmen, zeigten eine höhere Rate an Infektionen vor allem der oberen Luftwege. Darüber hinaus wiesen experimentelle und klinische Daten auch auf eine immunmodulatorische Wirkung von Valproat hin. Der genaue Mechanismus der Beeinflussung des Immunsystems durch beide Antikonvulsiva ist allerdings nicht vollständig geklärt. Daher war es das Ziel der Arbeit, den Einfluss von Levetiracetam und Valproat auf die Apoptose und zytotoxische Funktion von $CD8^+$ T- Lymphozyten in vitro näher zu charakterisieren.

Methoden

Nach Isolation von peripheren mononukleären Zellen im Blut bei 15 gesunden Probanden (9 weiblich (60%); 35, 7 ± 12,1 Jahre) wurde die Apoptoserate und zytotoxische Funktion von $CD8^+$ T- Lymphozyten mittles durchflusszytometrische Methoden erfasst. Dabei wurde die Apoptoserate über die Bindung von Annexin V festgestellt. Die Funktionstestung beruhte auf dem Nachweis von Perforin und der Expression von CD107 nach Stimulation der Zellen mit viralen Proteinen (Cytomegalovirus, Epstein-Barr Virus, Influenza virus; CEF). Die Messungen wurden ohne und nach Inkubation mit jeweils 5 mg/l Levetiracetam, 50 mg/l Levetiracetam, 10 mg/l Valproat und 100 mg/l Valproat durchgeführt. Die Inkubationsdauer mit den jeweiligen Medikamenten wurden auf 1 h und 24 h festgelegt. Gruppenvergleiche wurden mit t-Tests für abhängige Stichproben durchgeführt bei einem Signifikanzniveau von p=0,05.

Ergebnisse

Die niedrige (5 mg/l) und die hohe (50 mg/l) Konzentration von Levetiracetam verminderten signifikant die Ausschüttung von Perforin (LEV 50 mg/L vs CEF Kontrolle : 25.,8±12,.9% vs 18,.2±9,.7%, p<0,.01; LEV 5mg/L vs KontrolleCEF:

24,.1±13,.7% vs 18,.2±9,.7%, p<0,.01; n=15) und CD107 Expression auf CD8$^+$ T- Lymphozyten (LEV 50mg/L vs KontrolleCEF: 5,.3±2,.5% vs 11,.5±4,.7%, p<0,.01; LEV 5mg/L vs KontrolleCEF: 6,.7±2,.2% vs 11,.5±4,.7%, p<0,.01; n=15) nach Stimulation mit Viruspeptiden (p < 0,01). Levetiracetam zeigte keinen signifikanten Einfluss auf die Apoptoserate von CD8$^+$ T- Lymphozyten. Die hohe Konzentration von Valproat (100 mg/l) zeigte eine signifikante Verminderung der Apoptoserate von CD8$^+$ T- Lymphozyten nach einer Inkubationsdauer von 24 h (VPA 100mg/L vs Kontrollecontrol: 7,.8±3,.4% vs 11,.5±4,.2%, p<0,.01, n=15), hatte aber keinen Effekt auf die Ausschüttung von Perforin oder CD107 Expression (p>0,05).

Schlussfolgerung

Levetiracetam bewirkte eine moderate Verminderung der Degranulation von CD8$^+$ T- Lymphozyten, was ein möglicher Pathomechanismus für die klinisch bemerkte, erhöhte Infektionsrate bei mit Levetiracetam behandelnden Epilepsiepatienten darstellen könnte. Darüber hinaus führen die Ergebnisse zu der Hypothese, dass auch ein Teil der antikonvulsiven Wirkung von Levetiracetam durch die aufgezeigten immunmodulatorischen Effekte bedingt sein könnte, im Sinne einer Reduktion von Entzündung in der epileptogenen Zone und einer Stabilisierung der Bluthirnschranke. Valproat hatte keinen Einfluss auf die Funktion von CD8$^+$ T- Lymphozyten, aber verlangsamte die Apoptoserate.

2 Abbreviations

AED	Antiepileptic drug
APC	Allophycocyanin
AcOH	Acetic acid
BBB	Blood-brain-barrier
CFSE	Carboxyl fluorescein succinimidyl ester
CNS	Central nervous system
CTM	Cell culture medium
EEG	Electroencephalography
EMEA	European Agency for Evaluation of Medicinal Products
FITC	Fluorescein isothiocyanate
GABA	gamma-aminobutyric acid
GTCS	Generalized tonic–clonic seizures
HDAC	Histone deacetylase
IFN	Interferon
IGE	Idiopathic generalized epilepsies
IL-2	Interleukin-2
ILAE	International League Against Epilepsy
JME	Juvenile myoclonic epilepsy
LEV	Levetiracetam
MEG	Magnetoencephalography
MHC	Major histocompatibility complex
MRI	Magnetic resonance imaging
NK	Natural killer
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline

PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PI	Propidium iodide
PS	Phosphatidylserine
SV2A	Synaptic vesicle protein 2A
TNF	Tumour necrosis factor
USFDA	United States Food and Drug Administration
VPA	Valproate
WHO	World Health Organization

3 Introduction and discussion

3.1 Epilepsy and its treatment

Epilepsy is estimated by the World Health Organization to affect 0.8% of the world's population (WHO, 2005). Although epilepsy is a relatively common neurological disorder, there is a debate within the epilepsy community regarding the precise definition of epilepsy. In 2006, the International League Against Epilepsy (ILAE) provided a definition of 'seizure' and 'epilepsy'. A seizure was defined as 'a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain' (Fisher et al., 2005). Epilepsy was defined as 'a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures, and by the neurobiologic, cognitive, psychological, and social consequences of this condition. The definition of epilepsy requires the occurrence of at least one epileptic seizure' (Fisher et al., 2005). The main counterarguments about the proposed definition for 'epilepsy' are that: (1) this definition requires the occurrence of at least one epileptic seizure, however, many persons with a central nervous system (CNS) lesion may never have another seizure, and diagnosing and treating everyone using the proposed definition may lead to overtreatment and unnecessarily labeling many people as having epilepsy (Beghi et al., 2005; Leppik and Birnbaum, 2010); (2) the failure to clarify the concept of 'enduring' is another problem with the proposed definition, and it is very difficult to define or make operational this term (Beghi et al., 2005). In spite of that the the precise definition for 'epilepsy' has often been debated, the consensus on the matter also exists. For example, it is generally accepted that epilepsy is a 'symptom' or 'condition' rather a 'disease', which has a tendency to have recurrent seizures. Moreover, the most widely used classification of 'seizure' and 'epilepsy' is still that proposed by ILAE in 1981 and 1989 (Commission 1981, 1989). Seizures and epilepsies are

differentiated in focal and generalized seizures/epilepsies. Partial seizures are ones in which the first clinical and electrographic changes suggest initial activation limited to part of one cerebral hemisphere. Generalized seizures are those in which the first clinical changes indicate initial involvement of both hemispheres. In addition, the diagnosis of an epileptic syndrome in addition to a classification of seizures is also important for the optimal individual selection of anticonvulsant drugs. An epileptic syndrome is defined as a complex of signs with characteristic seizure types, onset of first manifestation, familial disposition and clinical findings from neurological examination, magnetic resonance imaging (MRI) and electroencephalography (EEG) or magnetoencephalography (MEG) (Stefan and Feuerstein, 2007).

Pharmacologic therapy represents the first line of treatment of epilepsy and is effective in most patients except those develop intractable seizures. The history of effective drug treatment began with the introduction of bromides in 1857 based on later-discredited theories of the cause of epilepsy (Shorvon and Sander, 1996). Until now, more than 20 AEDs have been approved in Europe, United States and elsewhere. The first generation of AEDs were often detected by chance, for example, valproate (described below). Since 1990s, the second generation of AEDs was introduced for clinical use (felbamate, vigabatrin [VBG], LEV and so on). Their antiepileptic effects were often verified by a series of strict, multicenter, randomized, controlled clinical studies, which are often absent for the first generation of AEDs. It still remains unclear for the antiepileptic mechanism of some AEDs. At the molecular level, the majority of AEDs are thought to modify excitatory and inhibitory neurotransmission through effects on voltage-gated ion channels (e.g., sodium and calcium), gamma-aminobutyric acid (GABA)(A) receptors and glutamate-mediated excitatory neurotransmission, respectively (White et al., 2007). But, many

second generation of AEDs often have special target points, for example, the synaptic vesicle protein 2A (SV2A) is probably the antiepileptic target point for LEV. The choice of AED needs to be individualized, mainly on the basis of the patient profile, including seizure or epilepsy type (partial or generalized), tolerability, safety, ease of use and pharmacokinetics (Schmidt, 2009). From a clinical perspective, the ideal AED does not require monitoring of plasma concentrations, is metabolically inert and is not involved in adverse drug interactions, and can be conveniently given once or twice a day (Patsalos and Perucca, 2003). Based on this viewpoint, the second generation of AEDs are often more ideal than the first generation of AEDs because most of new AEDs are less enzyme inducing and are not metabolized by the oxidative cytochrome p450 system. But, adverse side effects can not be absolutely avoided for new AEDs. Insight in these mechanisms of adverse side effects is essential for right usage of new AEDs and even facilitates elucidation of their antiepileptic mechanism.

3.2 Aim of the study

An increasing body of literature suggests that inflammation and immune processes play an important role in focal epilepsy (Vezzani et al., 2002; Heils et al., 2000; Balosso et al., 2008). Inflammatory mediators expressed from glia cells and neurons in the epileptic zone contribute to the notion that these processes may be essential to epilepto- and ictogenesis rather than a mere epiphenomenon (Rizzi et al., 2003; Ravizza et al., 2008).

Levetiracetam (LEV) is a new generation antiepileptic drug (AED) whose efficacy and tolerability in epilepsy treatment is well recognized (Vigevano, 2005; De Smedt et al., 2007). Several clinical trials reported a clear anticonvulsant effect of LEV but also an increased incidence of common cold, pharyngitis and rhinitis in LEV-treated patients (Table 1, Ben-Menachen et al. 2000; Cereghino et al. 2000; Betts et al. 2000;

Shorvon et al. 2000; Peltola et al. 2009). The reason for this finding is unknown. It was reported that white blood cell and neutrophil counts were in the normal range in LEV and placebo treated patients who developed infections (French et al., 2001). Recently, we found in a pilot study that $CD8^+$ T lymphocytes counts tended to decrease in the blood of LEV-treated patients (Nowak et al, 2010). The primary role of $CD8^+$ T lymphocytes is to protect against viral infections by lysing infected cells via degranulation-dependent perforin release, which can be measured by increased expression of surface CD107a and CD107b (Betts et al., 2004), and secretion of soluble factors, such as interferon-γ and tumor necrosis factor-α) (Jin et al., 1999; Schmitz et al., 1999; Haridas et al., 2003; van Lier et al., 2003 ; Mosley et al., 2005; Kaech et al., 2007).

Treatment with other AED including valproate (VPA) was not found to be associated with a higher infection rate (Ben-Menachem et al., 1996; Tassinari et al., 1996; Marson et al., 2007a; Marson et al., 2007b). However, VPA is a potent histone deacetylase (HDAC) inhibitor (Kawagoe et al., 2002; Kuendgen et al., 2006; Bokelmann and Mahlknecht, 2008), and histone modifications play a role in the regulation of the effector functions of memory $CD8^+$ T cells (Araki et al., 2008). Moreover, VPA can induce apoptosis in various leukemia cells *in vivo* and *in vitro* (Kawagoe et al., 2002; Kuendgen et al., 2006; Bokelmann and Mahlknecht, 2008). Valproate influenced postictal blood levels of TNFα and interleukin (IL)-1β as well as decreased $CD4^+$ T cell counts (Bauer et al., 2008; Bauer et al., 2009).

Therefore, we investigated the influence of LEV or VPA on proliferation, apoptosis, CD107 expression or perforin release of CD8+ T lymphocytes in vitro in order to better understand their pharmacological effects and adverse events.

Table 1: Incidence of infection in several large, well-controlled clinical studies of LEV

No.	Study	Therapy type	Treatment	Cases	Incidence of infection# %
1	Ben-Menachen et al. 2000	Add-on, then Monotherapy	Placebo	105	3.8
			LEV 3000mg/day	181	7.2
2	Cereghino et al. 2000	Add-on therapy	Placebo	95	12.6
			LEV 1000mg/day	98	27.6
			LEV 3000mg/day	101	26.7
3	Betts et al. 2000	Add-on therapy	Placebo	39	7.7
			LEV 2000mg/day	42	2.4
			LEV 4000mg/day	38	15.8
4	Shorvon et al. 2000	Add-on therapy	Placebo	112	6.3
			LEV 1000mg/day	106	9.4
			LEV 2000mg/day	106	6.6
5	Peltola et al. 2009	Add-on therapy	Placebo	79	3.8
			LEV 1000mg/day	77	7.8
6	Pooled analyses (including study No. 1-3 and a crossover trial N051 containing study No. 4) by Harden 2001 and French et al. 2001	Monotherapy and add-on therapy	Placebo	439	7.5
			1000-4000mg/day	769	13.4
No.1-6		Monotherapy and add-on therapy	Placebo	518	6.9
			1000-2000mg/day	526	11.4*
			3000-4000mg/day	320	14.4**

#: primarily common cold;

*: $p<0.05$, comparing with placebo group, χ^2 test;

**: $p<0.01$, comparing with placebo group, χ^2 test

3.3 Levetiracetam

Levetiracetam (LEV) (Keppra™, ucb L059, [S]-alpha-ethyl-2-oxo-1-pyrrolidine acetamide, UCB S.A. Pharma Sector, Braine-l'Alleud, Belgium) is a new generation AED. As an S-enantiomer pyrrolidone derivative, its structure is unrelated to other AEDs, with an empirical formula of $C_8H_{14}N_2O_2$ and a molecular weight of 170.21 (Fig. 1) (Cereghino et al., 2000;. De Smedt et al., 2007). Moreover, it is also unique from its predecessors both in mechanism and lack of interaction with other drugs (Patsalos, 2000; Bromfield, 2004; French et al., 2004a; French et al., 2004b).

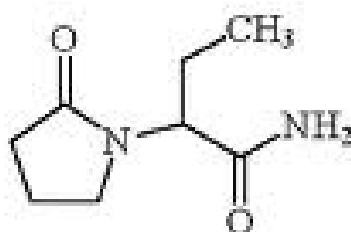


Figure 1. Chemical structures of levetiracetam (LEV)

3.3.1 Pharmacokinetic and pharmacodynamic

LEV has a favorable pharmacokinetic profile with excellent oral absorption, minimal protein binding, lack of hepatic metabolism, and renal excretion. LEV is rapidly and almost completely (>95%) absorbed after oral administration. (Edwards et al. 2004; Patsalos 2004). When taking the drug on an empty stomach, its peak plasma concentration is reached within 1 hour after dosing irrespective of the dose (Radtke, 2001). LEV has linear pharmacokinetics in the therapeutic range of 500–5000 mg (Patsalos 2004). The plasma half-life of oral levetiracetam in adults is 7 ±1 hour. (Radtke, 2001). LEV is insignificantly protein-bound (<10%), and is mainly excreted unchanged in the urine; about 24% is excreted in the urine as its carboxylic

derivative, UCB L057, via cytochrome P450-independent enzymatic hydrolysis (Coupez et al. 2003). In addition, LEV does not increase the activity of the hepatic cytochrome P450 enzyme system. (Coupez et al. 2003; Patsalos 2004).

LEV is unlikely to interact with other drugs, because it has a low protein binding (<10%) and is not cytochrome P450-dependent (Patsalos, 2000; Pellock et al., 2001). Meta-analysis revealed that LEV does not affect the concentrations of carbamazepine, clobazam, clonazepam, diazepam, gabapentin, lamotrigine, phenytoin, phenobarbital primidone, VPA, vigabatrin, and ethosuximide (De Smedt et al., 2007). Conversely, phenytoin, mesuximide, carbamazepine and oxcarbazepine have been shown to lower LEV concentrations (De Smedt et al., 2007).

3.3.2 Mechanism of action

LEV is different in its mechanism of action from that of classic AEDs and unrelated to known mechanisms of neurotransmission. In studies on experimental animals, LEV has no anticonvulsant effect on traditional seizure models—maximal electroshock- and pentylenetetrazol-induced seizures in mice and rats (Gower et al., 1992; Löscher et al., 1993; Klitgaard et al., 1998). Conversely, potent antiepileptic activity is identified in the audiogenic seizureprone mouse model (Gower et al., 1992). Moreover, LEV is also particularly effective in the treatment of absences and tonic seizures in ‘spontaneously epileptic rats’ (Cai et al., 2002). Significant inhibition of pentylenetetrazol kindling in mice and amygdala kindling in rats, which are chronic kindling model, has also been found (Gower et al., 1992; Löscher et al., 1998).

The exact anticonvulsant mechanism of LEV still remains unknown. It does not bind to receptors associated with excitatory or inhibitory neurotransmitters, nor does it act on sodium or calcium channel functions

or interfere with γ-aminobutyric acid (GABA) transaminase or glutamic acid decarboxylase (Vigevano, 2005). SV2A was identified as the binding site of LEV in the brain (Lynch et al., 2004). There is a strong correlation between the affinity of LEV-related compound for SV2A and its ability to protect against seizures in an audiogenic mouse animal model of epilepsy (Lynch et al., 2004). Proepileptic phenotype of SV2A-deficient mice is associated with reduced anticonvulsant efficacy of LEV and even partial SV2A deficiency may lead to increased seizure vulnerability and accelerated epileptogenesis (Kaminski et al., 2009). SV2A is associated with synaptic vesicle fusion, exocytosis, and neurotransmitter release (Crowder et al., 1999). So, the specific effect of LEV binding to SV2A appears to be a reduction in the rate of vesicle release (Yang et al 2007).

3.3.3 Indication and usage

LEV received United States Food and Drug Administration (USFDA) approval in late 1999 as an adjunctive treatment for partial seizures in adults (Harden, 2001). In 2000, LEV monotherapy (1500mg twice daily) was reported to be effective and well tolerated in patients with refractory partial seizures (Ben-Menachem and Falter, 2000). In recent years, double blind studies demonstrated the efficacy of LEV, in comparison to placebo, as an adjunctive treatment in patients with idiopathic generalized epilepsies (IGE) and generalized tonic-clonic seizures (GTCS) and in patients with refractory myoclonic seizures (Berkovic et al. 2007; Noachtar et al., 2008). As of January 2007, European Agency for Evaluation of Medicinal Products (EMEA) guidelines state that LEV is indicated: 1) As monotherapy in the treatment of partial-onset seizures with or without secondary generalization in patients from 16 years of age with newly diagnosed epilepsy, 2) As adjunctive therapy in the treatment of partial-onset seizures with or without secondary generalization in adults and children from four years of age with epilepsy, 3) As adjunctive therapy

in the treatment ofmyoclonic seizures in adults and adolescents from 12 years of age with juvenile myoclonic epilepsy (JME), and 4) As adjunctive therapy in the treatment of primary generalized tonic–clonic seizures in adults and adolescents from 12 years of age with IGE (De Smedt et al., 2007).

3.3.4 Adverse events

Generally speaking, LEV is considered a well-tolerated AED. For LEV-treatment, the most frequent adverse events are somnolence, asthenia, infection and dizziness (Harden, 2001). Several clinical trials reported an increased incidence of infection (primarily common cold), pharyngitis and rhinitis in LEV-treated patients (Ben-Menachen et al. 2000; Cereghino et al. 2000; Betts et al. 2000; Shorvon et al. 2000; Harden 2001; French et al. 2001). The pooled analyses including these studies verified that LEV-treated patients were more prone to infection than the placebo-treated groups (Harden 2001; French et al. 2001, Table 1). Previous researchers have paid little attention to the reasons for raised infection incidence by LEV-treatment. It was reported that white blood cell and neutrophil counts were in the normal range for LEV and placebo patients who developed an infection (French et al., 2001), so, the liability to infection was once thought as the result of an enhanced socialization due to seizure improvement or confusing terminology of ‘infection’ in different investigations (Cramer et al., 2000; French et al., 2001). But, this explanation is contradicted by the fact that many old or new generation AEDs, for example VPA and topiramate, do not increase the infection incidence following with the seizure improvement (Ben-Menachem et al., 1996; Tassinari et al., 1996; Marson et al., 2007a; Marson et al., 2007b). T Therefore, we proposed the alternative hypothesis that LEV maybe has some influence on immune system.

3.4 Valproate

VPA was first synthesized by an American chemist (Burton) in 1882 (Fig. 2) (Burton, 1882). But until 1962, its anti-convulsant properties were serendipitously discovered by French researchers (Eymard et al), when it was being used as a solvent for other compounds (khelline derivatives) whose protective effects against pentylenetetrazol-induced seizures were being investigated (Chapman et al., 1982; Johannessen and Johannessen, 2003). Then, VPA was authorized for use as an AED in 1967 in France (marketed as ‘Depakine’), in 1973 in Great Britain and in 1978 in the USA (Chapman et al., 1982; Lo¨scher, 2002). Today , VPA is a first-line and the most commonly used AED, with a very broad spectrum of activity against both generalized and partial seizures in adults and children (Davis et al, 1994; Chadwick , 1994; Lo¨scher, 2002; Perucca, 2002).

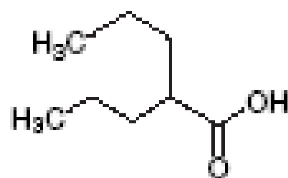


Figure 2. Chemical structures of Valproate (VPA)

Although VPA is a classical old generation AED, its anticonvulsant mechanism still remains unclear and might involve several mechanisms, including enhancement of GABA inhibitory effects in CNS, modulating bioactivity of enzymes related to the metabolic pathways of GABA and cerebral metabolism, attenuation of N-methyl-D-aspartate receptor-mediated excitation, and its interactions with sodium, calcium and potassium channels (Johannessen, 2000; Isoherranen et al., 2003). VPA is rapidly and nearly totally eliminated by hepatic metabolism involving in microsomal oxidation and glucuronidation (CYP2C9, CYP2C19 and CYP2A6) (Peterson and Naunton, 2005). Its half-life is 16–17 h (Chapman et al, 1982). Besides, its pharmacokinetics are also characterized by dose-limited absorption and non-linear plasma protein binding (DeVane, 2003). The main side effects of VPA are idiosyncratic hepatic toxicity,

thrombocytopenia and teratogenicity (Peterson and Naunton, 2005). Side effects are more frequent at higher doses, particularly serious side effects such as hepatotoxicity and thrombocytopenia (Ferrendelli, 2001).The risk for fatal hepatotoxicity is greater in very young children (Ferrendelli, 2001).

VPA was involved in the current study due to three reasons:

1) Treatment with VPA was not found to be associated with a higher infection rate (Ben-Menachem et al., 1996; Tassinari et al., 1996; Marson et al., 2007a; Marson et al., 2007b);
2) VPA has also been described as a potent HDAC inhibitor (Kawagoe et al., 2002; Kuendgen et al., 2006; Bokelmann and Mahlknecht, 2008). HDAC inhibitors promote histone acetylation (Melnick and Licht, 2002; Johnstone and Licht, 2003). Acetylation of histone correlates with a global architectural alteration from hetero- to transcriptionally active euchromatin, which menas chromatin relaxation, uncoiling and allowing for gene transcription (Melnick and Licht, 2002; Johnstone and Licht, 2003; Lin et al, 2006). Interestingly, HDAC inhibitors also display pleiotropic antitumor effects including cell cycle arrest, inhibition of angiogenesis, induction of apoptosis and of differentiation (Bouzar et al., 2009). It has been reported that VPA can induce apoptosis of various leukemia cells *in vivo* and *in vitro* (Kawagoe et al., 2002; Kuendgen et al., 2006; Bokelmann and Mahlknecht, 2008). Moreover, histone acetylation plays a role in the regulation of the effector function of memory $CD8^+$ T lymphocytes through facilitating rapid and robust transcriptional response (Araki et al., 2008).
3) VPA influenced postictal blood levels of TNFα and interleukin (IL)-1β as well as decreased $CD4^+$ T cell counts (Bauer et al., 2008; Bauer et al., 2009).

3.5 $CD8^+$ T lymphocytes and its antivirus function

Both innate immunity and adaptive immunity contributes to resistance to

virus infection. Innate immunity generally plays a role immediately after infection to limit the spread of the pathogen and initiate efficient development of an adaptive immune response (Bertoletti and Gehring, 2007). Innate immune responses during the early phases of viral infections are mainly characterized by production of type 1 interferons (Alexopoulou et al., 2001; Heil et al., 2004), activation of natural killer (NK) (Lanier, 2008), induction of dendritic cell maturation (Dalod et al. 2003), modulation of the quantity of major histocompatibility complex (MHC)-class I molecules on the surface of infected cells (Moretta et al., 2005) and priming of $CD8^+$ T lymphocytes responses (Tough et al., 1996).

Despite innate immunity being a first line of defence against virus infection, adaptive immunity, mainly for cell-mediated immune response, is still crucial for controlling virus infection (Mosley et al., 2005; Thimme et al., 2003; Lukacher and Wilson, 1998). The phenotype of $CD8^+$ T lymphocytes was established in 1975 (Masopust et al. 2007). It has been demonstrated that $CD8^+$ T lymphocytes are pivotal in the resolution of many acute viral infections (Jin et al., 1999; Schmitz et al., 1999; Haridas et al., 2003; Mosley et al., 2005). For example, $CD4^+$ or $CD8^+$ T lymphocytes deletion experiments performed in hepatitis B virus infected chimpanzees showed that $CD8^+$ T lymphocytes were the main cellular subset responsible for viral clearance (Thimme et al., 2003). It was also reported that $CD8^+$ T lymphocytes are critical for the clearance of acute polyomavirus infection in the mouse model (Lukacher and Wilson, 1998). Interestingly, independent of T cell receptor ligation, $CD8^+$ T lymphocytes can mount a response against pathogens by secreting cytokines, indicating that $CD8^+$ T lymphocytes can also play a role in innate immunity (Berg and Forman, 2006). Morover, there is a growing belief that establishing effector memory $CD8^+$ T lymphocytes at common portals of pathogen entry, such as mucosal tissues, may be critical for limiting the initial infectious burst of

certain agents (Masopust et al. 2007). Recently, we found in a pilot study that the counts of $CD8^+$ T lymphocytes decreased in the blood of LEV-treated patients with epilepsy (Nowak et al, 2010). Due to the key role of $CD8^+$ T lymphocytes in antiviral immunity, we decided to further investigate whether LEV or VPA can change the survival and cytotoxic function of $CD8^+$ T lymphocytes.

$CD8^+$ T lymphocytes protect against viral infections by lysing infected cells via degranulation-dependent perforin release and secretion of soluble factors, such as interferon-γ and tumor necrosis factor-α (Walker et al., 1986; Walker et al., 1987; Trapani and Smyth, 2002; van Lier et al., 2003; Kaech et al., 2007). Clonal proliferation of virus-specific $CD8^+$ T lymphocytes can further amplify their cytotoxic effects (Koup et al., 1994; Kostense et al., 2001; Kostense et al., 2002).

Degranulation-dependent lysis is the critical cytotoxic mechanism and correlates directly with cytotoxicity of $CD8^+$ T lymphocytes (Betts et al, 2003). After a killer cell recognizes its target, the cytotoxic granules move to the immunological synapse, where their membrane fuses with the killer cell plasma membrane, then they release their contents into target cells (Hayes et al. 1989; Stinchcombe et al, 2001). The cytotoxic granules of $CD8^+$ T lymphocytes are membrane-bound secretory lysosomes that contain a dense core composed of various proteins, including perforin and granzymes (Peters et al., 1991). The core is surrounded by a lipid bilayer containing lysosomal associated membrane glycoproteins (LAMPs), including CD107a (LAMP-1), CD107b (LAMP-2), and others (Peters et al., 1991). Degranulation can cause positive expression of CD107a/b on the cell surface for a brief period of time before those proteins are internalized (Fukuda, 1991). Perforin is a 70 kDa protein and the key player in the cytotoxic granule of $CD8^+$ T lymphocytes (Russell and Ley, 2002;

Lieberman, 2003). The major function of perforin is to form pores about 16 nm in diameter in the plasma membrane of target cells, facilitating the entry of granzyme which leads to DNA fragmentation (Young et al., 1986; Liu et al., 1995; Kagi et al., 1995a,b). Mice lacking perforin expression were unable to clear infection with the non-cytopathic lymphocytic choriomeningitis virus (Kagi et al., 1994; Kagi et al, 1995b). The lysis of HIV-infected cells by $CD8^+$ T lymphocytes occurs primarily through granule exocytosis, which requires perforin to facilitate the entry of apoptosis-inducing serine protease granzymes into the cytosol of infected target cells (Shankar et al., 1999). Both perforin release and CD107a/b expression on the cell surface may be used for assessing degranulation of $CD8^+$ T lymphocytes (Weren et al., 2004; Betts et al., 2004).

3.6 Summary

In the current study, we investigated the influence of LEV or VPA on proliferation, apoptosis, CD107 expression or perforin release of $CD8^+$ T lymphocytes *in vitro* in order to better understand their pharmacological effects and adverse events. $CD8^+$ T lymphocytes were activated with virus peptides under the presence or absence of LEV or VPA. Apoptosis, perforin release, the increase of CD107a/b expression on cell surface and proliferation of $CD8^+$ T lymphocytes were observed with immunofluorescence labeling and flow cytometry. Perforin release and the increase of CD107a/b expression on cell surface were taken as the main markers of cytotoxicity of virus-peptides-specific $CD8^+$ T lymphocytes.

4 Materials and Methods

4.1 Study population

Fifteen healthy adult volunteers recruited from the staff of Department of Neurology, University of Marburg (9 female, age: range 18-60 years, 35.7± 12.1 years) were included in the study. They fulfilled following inclusion criteria:

1) no infectious diseases within the last 3 months;
2) no neurological diseases;
3) no autoimmune diseases;
4) no malignant tumors;
5) no hematological diseases;
6) no trauma within the last month;
7) no operations within the last month;
8) no immunomodulatory treatment during the last six months;
9) no hepatic or renal insufficiency;
10) no severe psychiatric diseases;
11) no pregnancy;
12) informed consent must be obtained from the volunteer.

The study was approved by the local ethics committee.

4.2 Antibodies, reagents and peptides

The following antibodies, reagents and peptides were purchased from BD Biosciences (San Jose, California, USA): Allophycocyanin (APC) -conjugated to mouse-anti-human CD3 (CD3-APC), peridinin chlorophyll protein (PerCP)-conjugated to mouse-anti-human CD8 (CD8- PerCP), phycoerythrin (PE)-conjugated to mouse-anti-human perforin (cloneδ G9) (perforin-PE) and IgG isotype control antibody (IgG-PE isotype control), fluorescein isothiocyanate (FITC)-conjugated to mouse-anti-human

CD107a/b (CD107a/b-FITC), Perm/Wash Buffer, Cytofix/Cytoperm™ solution and BD GolgiStop (containing monensin). Ficoll-Biocoll separating solution and phosphate buffered saline (PBS) were purchased from Biochrom AG (Berlin, Germany). Annexin V-FITC apoptosis detection kit (containing Annexin V-FITC and propidium iodide (PI)) was purchased from BioVision (Mountain View, California, USA). Carboxyl fluorescein succinimidyl ester (CFSE) was purchased from Invitrogen (Eugene, Oregon, USA). Recombinant human interleukin-2 (rhIL-2) was purchased from ProSpec (Rehovot, Israel). Pooled human AB serum was purchased from 3H Biomedical AB (Uppsala, Sweden). Trypan blue solution (0.4%) was purchased from GIBCO (Paisley, Scotland, UK). RPMI 1640 (containing 20mM HEPES and glutamine) and 1% penicillin-streptomycin were purchased from Sigma (Steinheim, Germany). CTL-CEF-Class I peptide pool “Plus” (CEF peptide) was purchased from Cell Technolgy Ltd (Cleveland, Ohio, USA).

4.3 Antiepileptic drugs

Levetiracetam (Keppra®, UCB Pharma S.A., Brussels, Belgium) and VPA (sodium valproate, Ergenyl® vial, Sanofi Aventis, Frankfurt, Germany) were dissolved in sterile physiological saline to produce fresh solutions as required. When treating cells, the drugs were further diluted in the culture medium to the final concentrations of 5mg/ or 50mg/L for LEV (serum reference range in adults for drug fasting levels: 12－46 mg/L) and 10mg/ or 100mg/L for VPA (serum reference range in adults for drug fasting levels: 50mg/－100mg/L) (Patsalos et al., 2008).

4.3.1 VPA dilution

(1) was added 4 ml H_2O → 100 mg/ml (dilution 1)

(2) took 500 μl of dilution 1 and was added 9.5 ml H_2O → 5 mg/ml (dilution 2)

(3) took 1 ml of dilution 2 and was added 9 ml H2O → 500 μg/ml

(dilution 3)

4.3.2 LEV dilution

(1) LEV stock solution: Keppra® contains 5 ml solution of 100 mg/ml LEV per bottle (dilution 1).

(2) taken 250 µl of dilution 1 and was added 9.75 ml H2O → 2.5 mg/ml (dilution 2)

(3) taken 1 ml of dilution 2 and was added 9 ml H2O → 250 µg/ml (dilution 3)

When treating the cells, the drugs were further diluted in the culture medium to the final concentrations of 5 or 50 mg/L for LEV (reference range in adults, drug fasting levels: 12-46 mg/L) and 10 or 100 mg/L for VPA (reference range in adults, drug fasting levels: 50-100 mg/L) (Patsalos et al., 2008).

4.4 Apoptosis evaluation

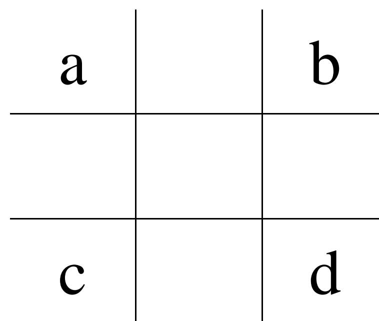
4.4.1 Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated with Ficoll-Biocoll Separating Solution by density gradient centrifugation (1500rpm, 20 ^{o}C, 30 min, without brake) of heparinized blood (10 ml) obtained by venepuncture of the heathy volunteers. The viability of PBMCs obtained was always >95%, as determined by 0.4% trypan blue staining. After double washing in cold PBS, the PBMCs were cultured with RPMI 1640 supplemented with 10% heat-inactivated pooled human AB serum and 1% penicillin-streptomycin.

PBMCs were isolated by Ficoll-Biocoll Separating Solution as the followings:

- Cell culture medium (CTM): RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin
- mixed 10 ml heparin blood 1:1 with 10 ml PBS in 50 ml tube
- stacked above 5 ml of Ficoll-Biocoll separating solution

- centrifuge (1500 rpm, 30 min, 20°C , without brake)
- picked up supernatant
- isolated lymphocytes and the lymphocytes were resuspend in 20 ml sterile PBS
- centrifuge (1500 rpm, 12 min, with brake)
- discarded supernatant
- repeated 'resuspending in 20 ml sterile PBS, centrifuge and discarding supernatant' for two times
- the lymphocytes were resuspended in 2ml CTM
- under microscope, cell count was performed and the viability of cell was determined by trypan blue staining (which was always >95% in the formal experiments). The method for counting cell under microscope was as following:



Cell number/ml = (a+b+c+d/4)×2×10^4

2: cell suspension was diluted by trypan blue (1:1)

10^4: 1.0mm (length)×1.0mm(width)×0.1mm(depth)=0.1mm^3; 1ml=10^3 mm^3

- diluted the cell suspension with CTM. The final density is 2-3x 10^6 cells/ml.

4.4.2 LEV or VPA treatment

- 100µl of cell suspension was seeded at 2-3x 10^5 cells /well in 96-well plates
- 96µl of CTM was added into each well
- was added 4 µl of drugs or PBS
  - o 4 µl of VPA dilution 2 (for end concentration of 100 mg/l)

- o 4 μl of VPA dilution 3 (for end concentration of 10 mg/l)

  - **OR** -

- o 4 μl of LEV dilution 2 (for end concentration of 50 mg/l)
- o 4 μl of LEV dilution 3 (for end concentration of 5 mg/l)

  AND

- o 4 μl of PBS to control samples
- incubation for 1h or 24h at 37°C in a humidified, 5% CO_2-air incubator

4.4.3 Apoptosis evaluation with flow cytometer

At the end of the culture under each condition, plates were centrifuged (1200rpm, 20 ^{o}C, 4 min, with brake) and supernatants were aspirated. Then, the cells were stained with CD3-APC and CD8- PerCP for 30 min in the dark on ice. Annexin V Apoptosis Detection Kit was used to detect apoptosis. Briefly, after washing with culture medium and centrifugation (1200rpm, 20 ^{o}C, 4 min, with brake), the cells were resuspended in 200μl of Annexin V binding buffer and incubated with 2μl of Annexin V-FITC and 2μl of PI for 10 min in the dark at room temperature. Then, apoptosis was measured on a four-colour flow cytometer (FACSCalibur®, CellQuest® software, Becton Dickinson). Data from at least 50 000 events per well were acquired. After gating lymphocyte characteristics using both forward and sideward scatter, apoptotic CD8^{+} T lymphocytes were defined as CD3+/CD8+/Annexin+/PI-. Spontaneous apoptosis was observed in cells untreated with LEV or VPA after incubation for 1 h and 24 h.

- Annexin V and PI: In the current study, Annexin V and PI were used to distinguish between live, apoptotic and necrotic cells based on differences in membrane permeability.The hydrophilic protein Annexin V selectively binds to phosphatidylserine (PS), which was translocated from the inner face of the plasma membrane to the cell surface soon after initiating apoptosis (Koopman et al., 1994; van Engeland et al., 1998). So, Annexin V staining can be used for distinguishing apoptotic

cells from their intact counterparts, but it can not distinguish apoptotic cells from necrotic cells because PS also exist on the surface of the latter. PI is impermeable to cells with an intact plasma membrane, and thus live and early apoptotic cells are not stained with PI (Darzynkiewicz et al., 1992; Vermes et al., 1995; Vermes et al., 2000). In late apoptotic and necrotic cells, nuclear membrane integrity was disrupted (Faleiro and Lazebnik, 2000; Kroemer et al., 1998). PI gains a access to the nucleus and intercalates into DNA, rendering the nucleus highly fluorescent (Darzynkiewicz et al., 1992; Vermes et al., 1995; Vermes et al., 2000). So, PI can be used for distinguishing early apoptotic cells from late apoptotic and necrotic cells. In the current study, apoptotic cells were defined as CD3+/CD8+/Annexin+/PI-. In fact, this definition was suitable for ealy apoptotic cells.

4.5 Functional assay of virus-peptidess-specific CD8$^+$ T lymphocytes

4.5.1 Isolation of PBMCs

PBMCs were isolated with Ficoll-Biocoll Separating Solution by density gradient centrifugation (1500rpm, 20 ^{o}C, 30 min, without brake) of heparinized blood (20 ml) obtained by venepuncture of the heathy volunteers. PBMCs were isolated by Ficoll-Biocoll Separating Solution as described in **4.4.1** (final cell density : 2-3x 10^6 cells/ml).

4.5.2 CEF peptides

In the functional assays, CD8$^+$ T lymphocytes were activated with CTL-CEF-Class I peptide pool “Plus”, which contained 32 peptides, each corresponding to a defined HLA class I restricted T-cell epitope from cytomegalovirus, Epstein-Barr virus and influenza virus. Most humans have been previously exposed to these pathogens. The fresh PBMCs were incubated with 64 µg/mL of CEF peptide at 37°C in a humidified, 5% CO_2 incubator for different time intervals. A negative control (without activation

by CEF peptide) was included in every experiment.

- Working solution of CEF peptides (64 µg/mL)

o Flicked tube to ensure that all powder was at the bottom of the tube.

o Added 10µl tissue culture grade DMSO followed by 40µl of sterile double distilled water. Vortex briefly and watch sterile handling.

o Stock solution: was added 450µl of tissue culture grade PBS and vortexed briefly. Flicked tube to ensure that all liquid was at the bottom of the tube.

o Before use, added 500µl of stock solution into 4500µl CTM as the working solution (1:10)

o The unused stock solution was stored at -20°C for less than 2 weeks.

4.5.3 Perforin release

Perforin release was measured as previously described with some modifications (Weren et al., 2004).

- 96µl of PBMCs suspension was seeded at 2-3x 10^5 cells /well in 96-well plates
- was added 4 µl of drugs or PBS

o 4 µl of VPA dilution 2 (for end concentration of 100 mg/l)

o 4 µl of VPA dilution 3 (for end concentration of 10 mg/l)

\- **OR** -

o 4 µl of LEV dilution 2 (for end concentration of 50 mg/l)

o 4 µl of LEV dilution 3 (for end concentration of 5 mg/l)

AND

o 4 µl of PBS to CEF-treated-control group

o 4 µl of PBS to negative control group

- incubation for 10 min (37°C； 5%CO_2)
- added 100 µl of working solution CEF peptides into AED-treated groups and CEF-treated-control group; added 100 µl of CTM in negative control group
- incubation for 2h 37°C in a humidified, 5% CO2-air incubator

After activation of $CD8^+$ T lymphocytes for 2 h in the presence or absence of antiepileptic drugs, the cells were first stained with CD3-APC and CD8-PerCP for 30 min and were then resuspended in BD Cytofix/Cytoperm solution for 20 min at 4°C. After washing and centrifugation (1200rpm, 20 ^{o}C, 4 min, with brake), cells were labeled with perforin-PE or IgG-PE isotype control in Perm/Wash buffer solution. Then, perforin expression was measured via flow cytometry. Data from at least 50,000 events per well were acquired. The frequency of perforin expression was analyzed in the CD3+/CD8+ gate. The baseline of perforin expression was measured in unstimulated cells immediately after isolation.

4.5.4 CD107 degranulation assay

$CD8^+$ T lymphocyte degranulation can be measured by increased expression of surface CD107a and CD107b (Betts et al., 2003; Betts et al., 2004). CD107 expression was measured as previously described with some modifications (Betts et al., 2003).

- added 216µl of CD107a/b-FITC into 480µl of PBMCs suspension (2-3x 10^6 cells/ml)
- added 116µl of PBMCs suspension mixed with CD107 into each cell
- was added 4 µl of drugs or PBS

o 4 µl of VPA dilution 2 (for end concentration of 100 mg/l)

o 4 µl of VPA dilution 3 (for end concentration of 10 mg/l)

- **OR** -

o 4 µl of LEV dilution 2 (for end concentration of 50 mg/l)

o 4 µl of LEV dilution 3 (for end concentration of 5 mg/l)

AND

o 4 µl of PBS to CEF-treated-control group

o 4 µl of PBS to negative control group

- incubation for 10 min (37°C; 5%CO_2)
- added 80 µl of working solution CEF peptides into AED-treated groups and CEF-treated-control group; added 80 µl of CTM into negative control group

- incubation for 50min （37°C； 5%CO_2）
- was added 0.5 μl monesin (BD GolgiStop)
- incubation for 1h

After activation of $CD8^+$ T lymphocytes for 2h in the presence or absence of antiepileptic drugs, the cells were stained with CD3-APC and CD8-PerCP for 30 min at 4° C in the dark. After washing, centrifuge and resuspending in 130μl of CTM, cells were analyzed on the four-colour flow cytometer. At least 50,000 events were collected per cell. The frequency of CD107a/b expression was analyzed in the CD3+/CD8+ gate. The baseline of CD107a/b expression was measured in unstimulated cells immediately after isolation. The cells were stained with CD107a/b-FITC, CD3-APC and CD8- PerCP at the same time.

Monensen was used in this experiment because the expression of CD107a/b on the cell surface caused by degranulation is transient before those proteins are internalized (Fukuda, 1991). Monensin is a carboxylic ionophore specific for monovalent cations and can intercalate into membranes and exchange protons for potassium ions (Tartakoff, 1983; Mellman et al., 1986). Thereby, monensin can neutralize the pH within endosomes and lysosomes, which prevents degradation of fluorochrome conjugates of endocytosed anti-CD107 antibodies (Betts and Koup, 2004).

4.5.5 Analysis of $CD8^+$ T lymphocytes Proliferation by CFSE Labeling

In 1994, Lyons and Parish set up the method that investigated the kinetics of lymphocyte proliferation with the fluorescein related dye CFSE and flow cytometry (Lyons and Parish, 1994). This approach has proved suitable for *in vitro* and *in vivo* study of T lymphocytes (Lyons and Parish, 1994; Kurts et al., 1997; Gett and Hodgkin, 1998). Its working principle was introduced by Lyons (Lyons, 2000.): CFSE consists of a fluorescein molecule containing two acetate moieties and a succinimidyl ester functional group. In this form, it is membrane permeant and non-fluorescent. After diffusion

into the intracellular environment, endogenous esterases remove the acetate groups, rendering the molecule highly fluorescent and non-permeant to the cell membrane. In addition, the succinimidyl ester reacts with free amine groups of intracellular proteins, forming dye–protein adducts. Proteins which have a low turnover rate, including some cytoskeletal components, are thought to be responsible for the very long lived staining afforded by CFSE. Importantly, cellular differentiation turned out to be unaffected by the dye CFSE (Hodgkin et al., 1996; Gett and Hodgkin, 1998).

The protocol was as following:

- preparation for working solution of IL2
  - o 20mM AcOH：added 10μl of 100% AcOH into 8715μl sterile double distilled water
  - o stock solution of IL2: added 10μg of IL2 into 100μl of 20mM AcOH. The concentration was 100μg/ml (1.3 x 10^6IU/ml)
  - o aliquot stock solution of IL2 was stored at -20°C for less than 3 weeks
  - o when using, added 1μl of stock solution into 129μl of PBS，the latter concentration of IL2 was 10 000IU/ml. Then, added 0.8ul of 10000IU/ml IL2 into 200μl CTM, the final working concentration of IL2 was about 40IU/ml
- 6ml PBMC-CTM : added 3ml CTM into 3ml of cell suspension (2-3x 10^6 cells/ml)
- CFSE preparation (CellTrace™ CFSE Cell Proliferation Kit): prepared a 1.25 mM CellTrace™ CFSE stock solution immediately prior to use by dissolving the contents of one vial (Component A) in 72 μL of the DMSO provided (Component B).
- added 1.9μl of CFSE stock solution into 6ml PBMC-CTM（the working

concentration of CFSE is 0.4μM）

- incubation at 37°C for 10 min in the dark
- the free CFSE was quenched with 30ml of ice-cold CTM for 5 min on ice
- centrifuge for 4min,1500rpm
- discarded supernatant
- added 30ml of fresh CTM medium into the cells
- centrifuge for 4min,1200rpm
- discarded supernatant
- repeated wash again
- cells stained with CFSE were resuspended in 1ml of CTM
- 96μl of cell suspension (stained with CFSE) was seeded into 96-well plates
- was added 4 μl of drugs or PBS

o 4 μl of VPA dilution 2 (for end concentration of 100 mg/l)
o 4 μl of VPA dilution 3 (for end concentration of 10 mg/l)

- **OR** -

o 4 μl of LEV dilution 2 (for end concentration of 50 mg/l)
o 4 μl of LEV dilution 3 (for end concentration of 5 mg/l)

AND

o 4 μl of PBS to CEF-treated-control group
o 4 μl of PBS to negative control group

- incubation for 10 min (37°C； 5%CO_2)
- added 100 μl of working solution CEF peptides into AED-treated groups and CEF-treated-control group; added 100 μl of CTM into negative control group
- added 0.8ul of 10000IU/ml IL2 into AED-treated groups and CEF-treated-control group (the working concentration of IL2 was about 40IU/ml)
- incubation for 5d at 37°C in a humidified, 5% CO_2-air incubator

After the incubation, cells were washed with culture medium, next, cell

surface was stained with CD3-APC and CD8- PerCP as described above. Then, cells were analyzed on the four-colour flow cytometer. At least 50,000 events were collected per cell. Cells proliferation was measured by the percantage of low CFSE cells in CD3+/CD8+ gate (in the upper left quadrant of each FACS plot). The definition for low CFSE cells was defined according to the distribution of CFSE dye in baseline, which was measured in unstimulated cells. CFSE decrease was a result of dye dilution in each cell division. Less than 1% CFSE dim (proliferating) cells were observed in the negative control groups.

4.7 Statistical analysis

All results are expressed as mean±standard deviations. Depending group comparisons were performed with the paired t-test. In this explorative study, the significance level was set to $p<0.05$.

5 Results

5.1 Apoptosis

In the high-concentration VPA group (100 mg/L), the percentage of apoptotic $CD8^+$ T lymphocytes was lower than in the control group after 24h of incubation (VPA 100mg/L vs control: 7.8±3.4% vs 11.5±4.2%, $p<0.01$, n=15, Table 2, Fig.3). Levetiracetam had no significant influence on apoptosis of $CD8^+$ T lymphocytes after 1h or 24h.

A representative example is shown in Fig. 4.

Table 2: Comparative analysis for the percentage of spontaneous and drug-treated apoptosis in $CD8^+$ T lymphocytes

Incubation	LEV (5 mg/l)	LEV (50 mg/l)	VPA (10 mg/l)	VPA (100mg/l)	Spontaneous
1h	8.32±3.56	8.50±3.66	8.09±3.10	8.43±3.59	7.52±2.78
24h	11.76±4.37	12.60±4.39	12.05±4.61	7.82±3.41**	11.52±4.16

** $p<0.01$ (compared with spontaneous group, paired t-test)

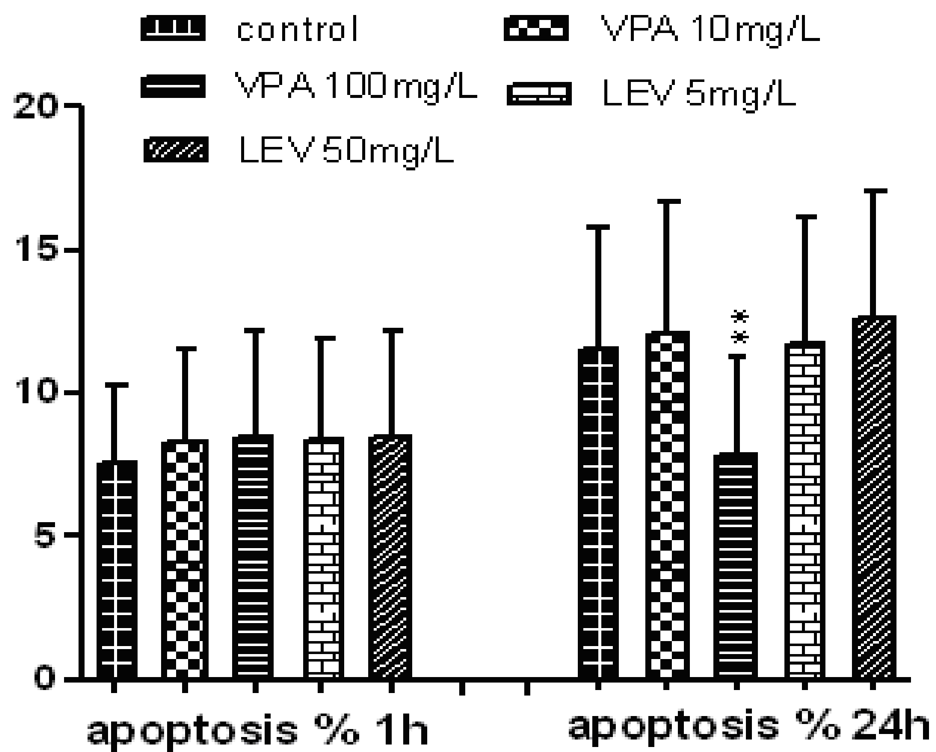


Figure 3.

Percentage of spontaneous (control) and drug-mediated (VPA 10mg/L, VPA 100mg/L, LEV 5mg/L and LEV 50mg/L) apoptosis in $CD8^+$ T lymphocytes (data given as mean ± SD (standard deviation)). Two asterisks indicate $p<0.01$ comparing the control condition and the high dose VPA condition (n=15).

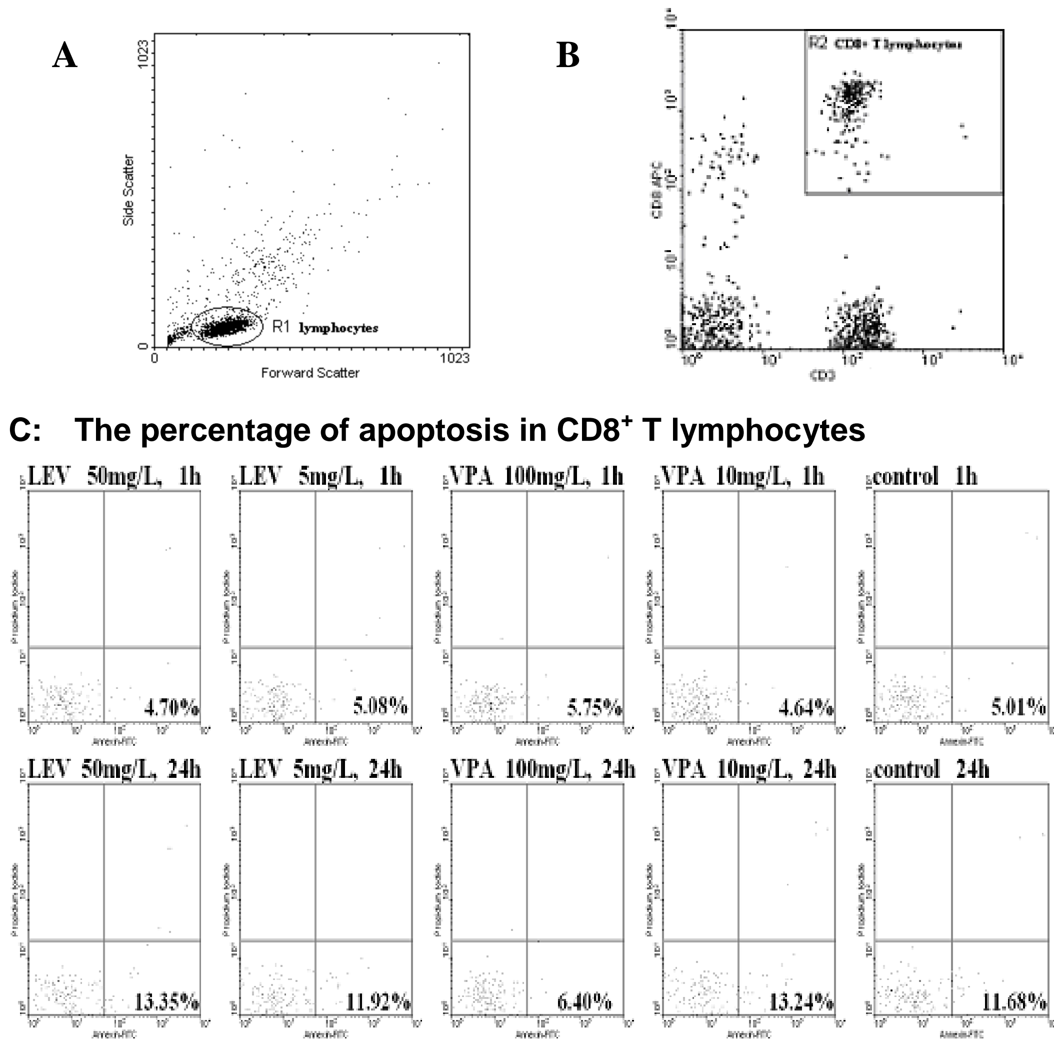


Figure 4.

Flow cytometry analysis of spontaneous, VPA-treated and LEV-treated apoptosis of $CD8^+$ T lymphocytes. These representative dot-plots are from a healthy volunteer's result. PBMCs were tested after 1h and 24h incubation. **A:** The lymphocytes region (R1) is shown in forward-scatter (FSC) versus side-scatter (SSC) dot plot. **B:** CD3 versus CD8 fluorescence dot plot shows $CD8^+$ T lymphocytes region (R2) in lymphocytes gate (the R1 logical gate). **C:** Annexin V/FITC versus propidium iodide (PI) fluorescence dot plot shows the percentage of apoptosis in $CD8^+$ T lymphocytes gate (the R1 AND R2 logical gate). Note decreased percentages of apoptotic cells (Annexin+/PI-) in high concentration (100mg/L) of VPA-treated group relative to other groups after 24h incubation.

5.2 CEF-peptides-specific activation

CEF-peptides-specific activation for $CD8^+$ T lymphocytes was effectively induced in all volunteers. Perforin release, CD107a/b expression and proliferation were significantly increased in the CEF-peptides stimulated group as compared to control measurements after incubation for 2h and 5days, respectively (control vs CEF : $perforin^+$ cells 40.9±10.9% vs 18.2±9.7%, $p<0.01$; $CD107^+$ cells 0.7±0.4% vs 11.5±4.7%, $p<0.01$; low CFSE cells 1.0±0.4% vs 6.9±2.9%, $p<0.01$; n=15, Fig. 5). In addition, after 2h measurements, there was a small, but significant drop in $perforin^+$ cells and increase of $CD107^+$ cells in the control group as compared to baseline indicating little degranulation of $CD8^+$ T lymphocytes *in vitro* culture (control vs baseline : $perforin^+$ cells 40.9±10.9% vs 47.0±12.3%, $p<0.05$; $CD107^+$ cells 0.7±0.4% vs 0.5±0.2%, $p<0.05$; n=15, Fig. 5).

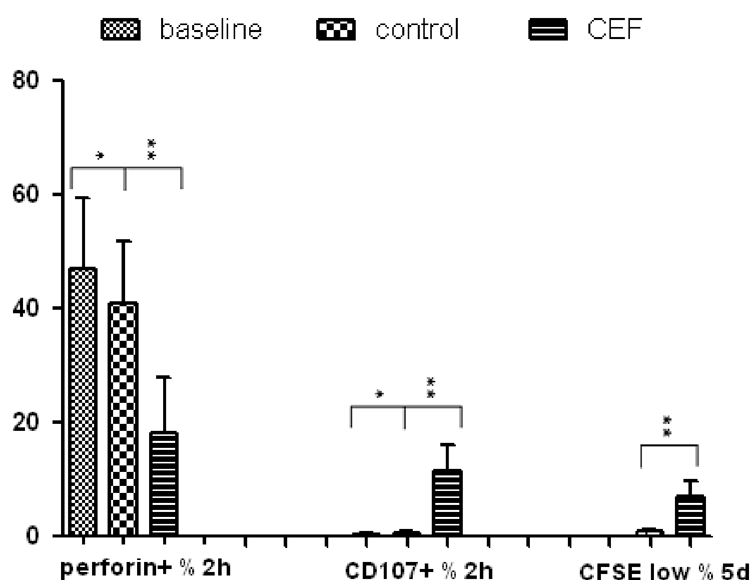


Figure 5.
Percentage of $perforin^+$, $CD107^+$ and low CFSE $CD8^+$ T lymphocytes at baseline, under control condition and after CEF-stimulatation. No LEV or VPA was added. (data given as mean ± SD (standard deviation); one asterisk: $p<0.05$; two asterisks: $p<0.01$).

5.3 Perforin release

Both high and low concentration of LEV decreased perforin release in $CD8^+$ T lymphocytes after CEF-peptide stimulation of 2 h (LEV 50 mg/L vs CEF : 25.8±12.9% vs 18.2±9.7%, p<0.01; LEV 5mg/L vs CEF: 24.1±13.7% vs 18.2±9.7%, p<0.01; n=15, Table3, Fig. 6). Adding valproate in a low or high concentration did not change perforin release (p>0.05; Table3, Fig. 6) A representative example is shown in Fig. 7.

Table 3: Comparative analysis of perforin$^+$ cells, CD107$^+$ cells and CFSE low cells in the CEF-stimulated group versus CEF-drug-treated groups

	CEF-LEV (50mg/l)	CEF-LEV (5 mg/l)	CEF-VPA (100 mg/l)	CEF-VPA (10mg/l)	CEF
2h perforin$^+$	25.8±12.9**	24.1±13.7**	19.0±11.9	20.3±13.0	18.2±9.7
2h CD107$^+$	5.3±2.5**	6.7±2.2**	11.0±5.5	10.9±5.0	11.5±4.7
5d CFSE low	6.9±3.4	6.6±3.1	7.0±3.5	6.8±3.0	6.9±2.9

* * p<0.01 (compared with CEF group, paired t-test).

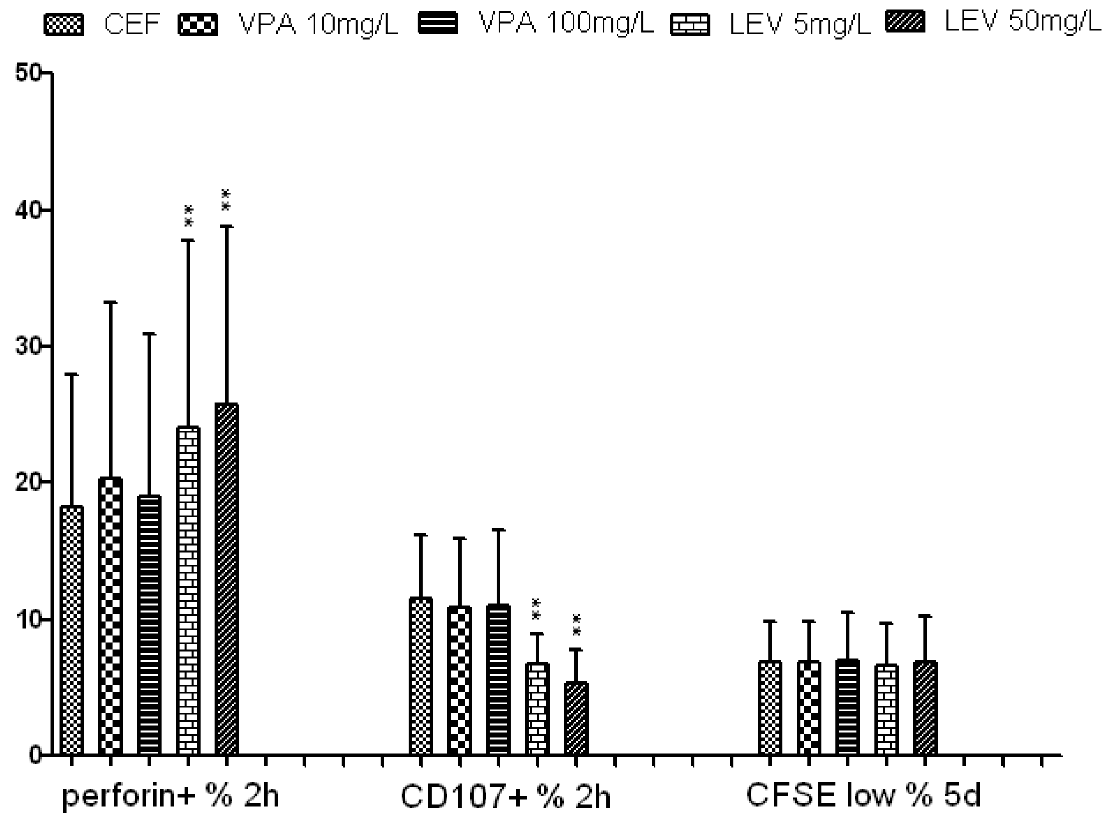


Figure 6.

Perforin$^+$, CD107$^+$ and low CFSE CD8$^+$ T lymphocytes in the CEF-stimulated group and in CEF-stimulated groups in the presence of LEV or VPA at different concentrations, including VPA 10mg/L, VPA 100mg/L, LEV 5mg/L and LEV 50mg/L. (Data given as mean ± SD (standard deviation); two asterisks: $p<0.01$).

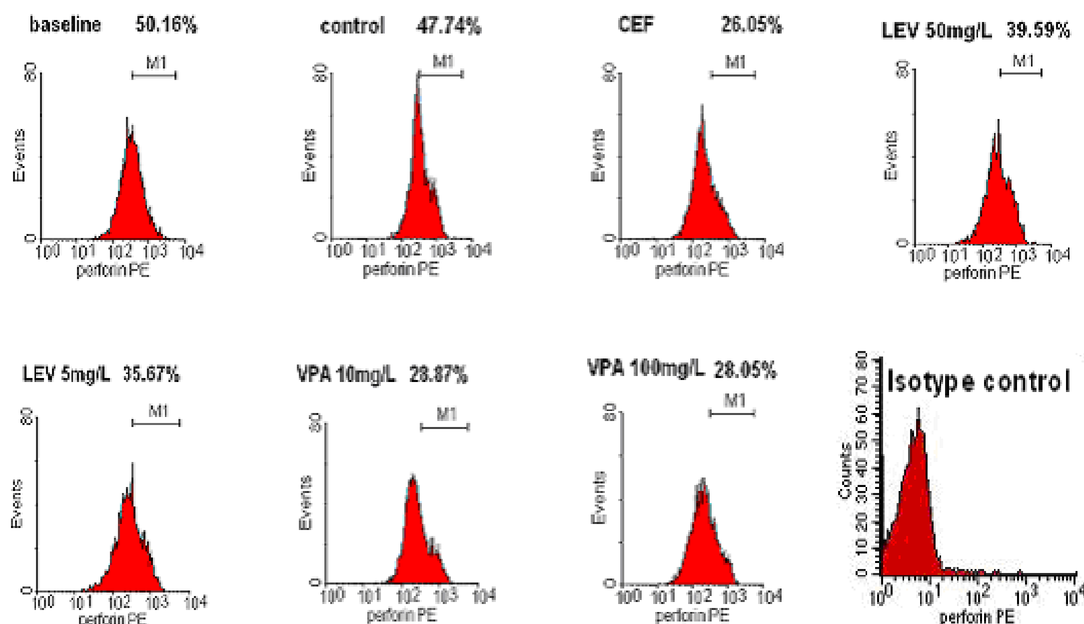


Figure 7.

Representative examples of histograms analysis of perforin release in CD8$^+$ T lymphocytes gate. Lymphocytes gate and CD8$^+$ T lymphocytes gate were defined as described above. Due to the unimodal distribution of perfoin, the mark for perforin$^+$ cells was set up on the middle point of the baseline distribution. PBMCs were stimulated by CEF peptide for 2h in the presence or absence of antiepileptic drugs. Perforin release was observed by comparing the the percentage of perforin$^+$ cells in baseline group with that in the control group or CEF-stimulated group (including CEF, LEV 5mg/L, LEV 50mg/L, VPA 10mg/L and VPA 100mg/L). Note higher percentage of perforin$^+$ cells in LEV-treated group (5mg/L and 50mg/L) relative to other CEF-stimulated groups (CEF, VPA 10mg/L and VPA 100mg/L).

5.4 CD107a/b expression

After 2h of stimuation, both high (50mg/L) and low (5mg/L) concentrations of LEV decreased CD107a/b expression on $CD8^+$ T lymphocytes compared with the CEF-peptides stimulated group (LEV 50mg/L vs controls: 5.3±2.5% vs 11.5±4.7%, p<0.01; LEV 5mg/L vs controls: 6.7±2.2% vs 11.5±4.7%, p<0.01; n=15, Table3, Fig. 6). CD107 expression on cell surface and perforin release are two important methods for degranulation assay (Betts et al., 2003; Betts et al., 2004; Weren et al., 2004). Moreover, CD107a/b expression was concomitant with a loss of perforin (Betts et al., 2003). In our study, both data from CD107a/b expression experiments and data from perforin release experiments drawed a same conclusion that LEV decreases degranulation of CD8+ T lymphocytes induced by CEF-peptide stimulation *in vitro*.

Valproate did not reveal a significant effect on CD107a/b expression.

A representative example of LEV decreasing the CD107a/b expression is shown in Fig. 8.

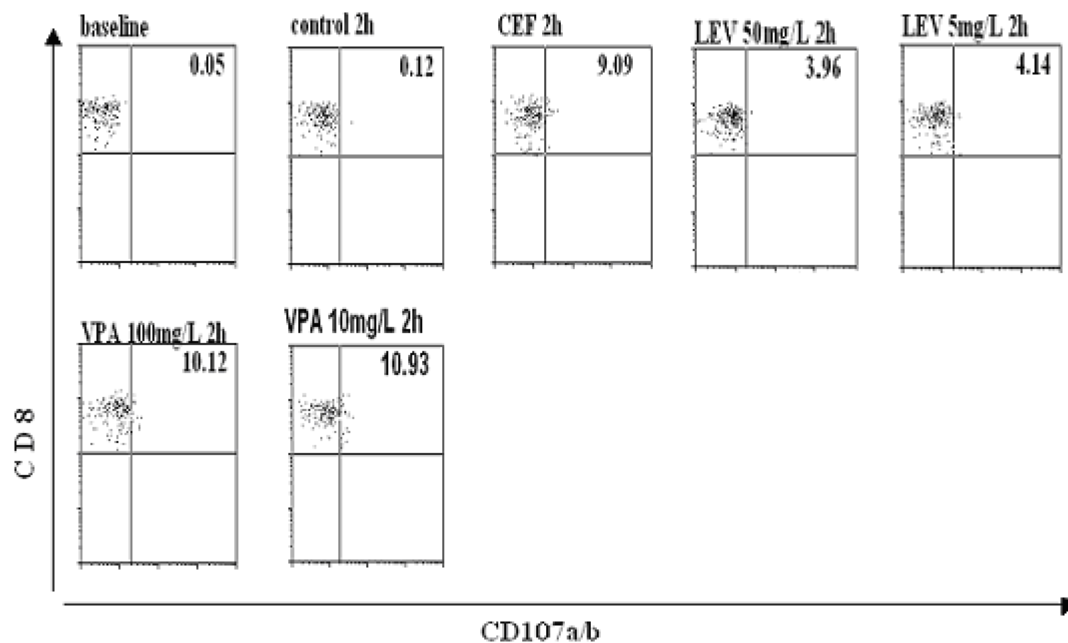


Figure 8.

Degranulation analysis of $CD8^+$ T lymphocytes were performed with cell surface CD107a/b expression. In this representative example, lymphocytes gate and $CD8^+$ T lymphocytes gate were defined as described above. PBMCs were stimulated by CEF peptide for 2h in the presence or absence of antiepileptic drugs. Degranulation was observed by comparing the the percentage of CD107a/b^+ cells in baseline group with that in the control group or CEF-stimulated group (including CEF, LEV 5mg/L, LEV 50mg/L, VPA 10mg/L and VPA 100mg/L). After 2h incubtion, LEV-treated group (5mg/L and 50mg/L) showed lower percentages of CD107a/b^+ cells than other CEF-stimulated groups (CEF, VPA 10mg/L and VPA 100mg/L).

5.5 Proliferation

Levetiracetam and VPA did not influence proliferation of CD8+ T lymphocytes induced by CEF-peptide stimulation ($p>0.05$, n=15, Table3, Fig. 6).

A representative example of proliferation of CD8+ T lymphocytes induced by CEF-peptides-stimulation is shown in Fig. 9.

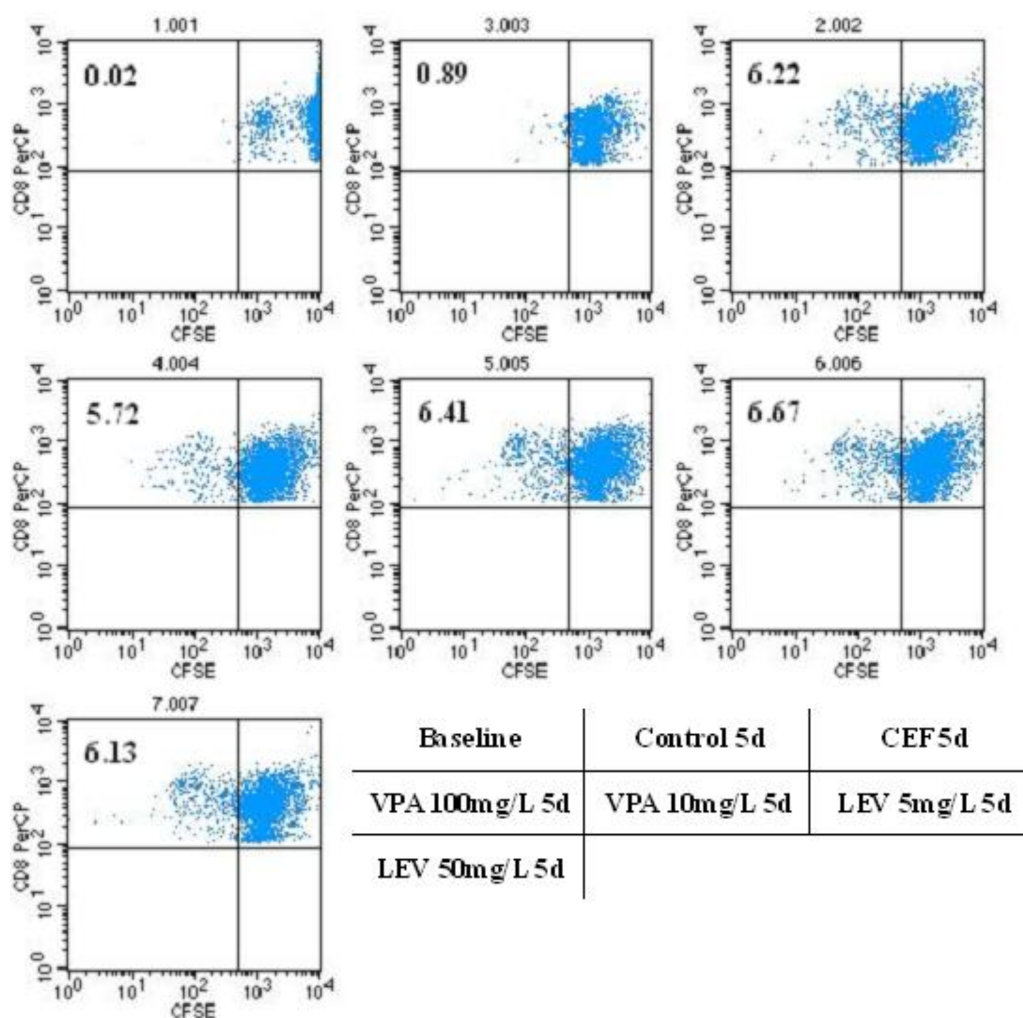


Figure 9.

In this representative example, lymphocytes gate and CD8+ T lymphocytes gate were defined as described above. Proliferation of CD8+ T lymphocytes was measured in the upper left quadrant of each FACS plot, which reflected CFSE dilution. No obvious difference was found between CEF-peptides-stimulated group and CEF-drug-treated groups.

6 Discussion

The main finding of this study was that LEV had a attenuating effect on degranulation of $CD8^+$ T lymphocytes as indicated by reduced perforin release and decreased CD107a/b expression after CEF-peptide stimulation *in vitro*. Levetiracetam did not appear to influence apoptosis or proliferation of $CD8^+$ T lymphocytes. In contrast, high concentrations of VPA prevented spontaneous apoptosis of $CD8^+$ T lymphocytes *in vitro* but did not have any effects on proliferation or perforin release.

6.1 Methodology

Because $CD8^+$ T lymphocytes play a key role in immune surveillance for eliminating of virus infected cells, certain tumor cells, and MHC class I incompatible cells (Hussell et al., 1997; Trapani and Smyth, 2002; Lieberman, 2003), many different types of cytotoxicity assay have been developed. These methods can be divided into groups: the first group lays particular emphasis on measuring target cell death and the second focus on observing the cytotoxic pathways. The former includes the ^{51}chromium release assay (Pearson et al., 1969), the detection of DNA-degradation (Brawn et al., 1975) and the release of fluorescent dyes such as bis-carboxyethyl-carboxyfluorescein (Kolber et al., 1988). These techniques are cumbersome, semi-quantitative, and potentially insensitive (Betts et al., 2003). More recently, many new methods based on flow cytometry have been established to observe intracellular cytokine production, CFSE-based proliferation and degranulation (Brenchley et al., 2002; Betts et al., 2003; Weren et al., 2004). This second group of tests directly examines the functional state of $CD8^+$ T lymphocytes rather than the death of target cells. These assays are often simple, rapid and sensitive. Therefore, we chosed the second this method for the current study.

As described in the introduction, degranulation-dependent mechanism is

principal compared with granule-independent pathway. So, in the current study, degranulation was taken as the main marker of cytotoxicity of virus-peptides-specific $CD8^+$ T lymphocytes. Degranulation assay can be performed with at least two classical kinds of methods: perforin release and the increase of CD107 expression on cell surface (Betts et al., 2003; Betts et al., 2004; Weren et al., 2004).

Perforin is a key component of the lytic granules machinery in cytotoxic $CD8^+$ T lymphocytes and perforin release plays a critical role in cell-mediated cytotoxicity against viral infection (Barry and Bleackley, 2002; Russell and Ley, 2002; Lieberman, 2003). In the previous studies, granule-mediated killing occured within minutes to hours of target cell recognition. The following reconstitution or upregulation of intracellular perforin could not be detected until cellular proliferation (Sandberg et al., 2001; Migueles et al., 2002; Meng et al., 2006). However, Hersperger et al. recently found that antigen-specific CD8 T lymphocytes rapidly upregulate perforin after activation for 6h if B-D48 clone anti-perforin antibody, but not δ G9 clone antibody, was employed (Hersperger et al., 2008). In the current study, we used δ G9 clone anti-perforin antibody and perforin was detected after CEF-peptides-stimulation of 2h. So, the intracellular perforin reduction only reflected perforin release. In baseline, perforin had the unimodal distribution. So, the mark for $perforin^+$ cells was set up on the middle point of the baseline distribution in most cases, which is consistent with some previous studies (Appay et al., 2000; Zhang et al. 2003; Harari et al., 2009).

There is a difference in staining methods between intracellular perforin staining and cell surface staining of CD107. Intracellular perforin staining needs cell fixation and permeabilization, which possibly incerease perforin loss and degranulation. The fixation and permeabilization are unnecessary

for cell surface staining of CD107. So, perforin staining possibly showed more obvious degranulation than cell surface staining of CD107, which was once reported in the previous study (Weren et al., 2004). In our study, the potential discrepancy caused by methodology did not result in the inconsistent conclusion. Both data from CD107a/b expression experiments and data from perforin release experiments led to the same conclusion that LEV decreases degranulation of CD8+ T lymphocytes induced by CEF-peptide stimulation *in vitro*.

6.2 Degranulation and anti-virus function

In clinical studies, LEV treatment increased the incidence of common cold, pharyngitis and rhinitis which was associated with a slight but significant drop in white blood cell counts (Harden, 2001). Similarly, a study on interictal alterations of leukocytes and cytokines in the blood of patients with active epilepsy found a trend towards decreased counts of $CD8^+$ T lymphocytes (Nowak et al, 2010).

It is well known that $CD8^+$ T lymphocytes play a central role in the defense against viral infections, especially for the clearance of virus (Lukacher and Wilson, 1998; Thimme et al., 2003; Mosley et al., 2005). However, there was once a debate regarding the immediate cytotoxic function of $CD8^+$ T lymphocytes (McMichael et al., 1986; Lau et al., 1994; Bachmann et al.,1999). Recently, the immediate induction for cytotoxic function, involving cell cloning and lysis of target cells, was reported in human influenza-specific $CD8^+$ T lymphocytes (Touvrey et al., 2009), which was greater than previously described in mice (Selin and Welsh, 1997; Kedl and Mescher, 1998). Moreover, $CD8^+$ T lymphocytes was also found to play a role in innate immunity (Berg and Forman, 2006). Our results support the existence of immediate cytotoxic function of $CD8^+$ T lymphocytes, which was shown by degranulation and perforin release. So, $CD8^+$ T lymphocytes

is not only crucial for the clearance of virus, but also plays an important role for preventing virus infection.

Although this study did not confirm a significant effect of LEV on apoptosis of $CD8^+$ T lymphocytes *in vitro*, the results provided clear evidence that levetiracetam compromised the function of $CD8^+$ T lymphocytes. Cytotoxicity of $CD8^+$ T lymphocytes is mainly mediated by release of pre-formed cytolytic granules which contain perforin, other less important pathways involve the secretion of soluble cytokines and Fas/FasL interaction (Walker et al., 1986; Walker et al., 1987; Barry and Bleackley, 2002; van Lier et al., 2003; Kaech et al., 2007). The major function of perforin is to insert into the target membrane and to form pores of about 16 nm in diameter by polymerization in order tofacilitate the entry of granzyme which leads to DNA fragmentation (Young et al., 1986; Liu et al., 1995). Mice deficient in perforin were severely immunocompromised, and so were humans with familial hemophagocytic lymphohistiocytosis (HLH) due to mutations of the perforin gene (Kagi et al., 1994). Degranulation leading to perforin release is the key process of cytotoxic function of $CD8^+$ T lymphocytes (Barry and Bleackley, 2002; Russell and Ley, 2002; Lieberman, 2003). The cytotoxic granule of $CD8^+$ T lymphocytes are membrane-bound secretory lysosomes that contain a dense core composed of various proteins, including perforin and granzymes (Peters et al., 1991). The core is surrounded by a lipid bilayer containing lysosomal associated membrane glycoproteins (LAMPs), including CD107a (LAMP-1), CD107b (LAMP-2), and others (Peters et al., 1991). Degranulation can cause positive expression of CD107a/b on the cell surface for a brief period of time before these proteins are internalized (Fukuda, 1991). So, the expressions of CD107 on the $CD8^+$ T lymphocytes can become a marker for degranulation. The attenuating effect of LEV on perforin release and degranulation may contribute to the higher infection rates in LEV-treated patients. The exact

time course of the interaction of LEV and cytotoxicity of $CD8^+$ T lymphocytes *in vivo* remains to be established.

6.3 Perforin release and antiepileptic mechanism

A series of studies have supported the view that transient openings of the blood-brain-barrier (BBB) facilitate both behavioral and electrographic seizures and may establish a link between the systemic immune system and the epileptogenic zone (Van Vliet et al., 2007; Marchi et al., 2007; Kleen and Holmes, 2008). Disruption of BBB permeability is a possible pathway for cytokines influencing seizures and epilepsy. Several studies revealed BBB 'failure' after administration of IL-1, IL-6, TNFα, and interferon-γ (De Vries et al., 1996; Wong et al., 2004; Candelario-Jalil et al., 2007). In a mouse model of epilepsy induced by pilocarpine, Fabene et al., found that leukocyte-endothelial interactions were altered by seizures and played an important role in BBB damage and seizure generation (Fabene et al, 2008).

Recently, it was reported that $CD8^+$ T lymphocytes can lead to BBB dysfunction through a perforin-dependent process (Suidan et al., 2010). Similarly, Kim et al. observed a reduction of CNS vascular permeability in perforin-deficient-mice infected with lymphocytic choriomeningitis virus (Kim et al., 2009). Therefore, perforin also plays a role in BBB permeability. It may be hypothesized that LEV's attenuating effect on perforin release may add to its anticonvulsant potency via reduction of BBB disruption.

6.4 Apoptosis and proliferation

This study did not reveal any effects of LEV on apoptosis and proliferation of $CD8^+$ T lymphocytes, which refused our initial hypothesis that LEV could decrease the amount of $CD8^+$ T lymphocytes.

We also found that VPA had neither influence on proliferation of $CD8^+$ T

lymphocytes nor influence on degranulation of $CD8^+$ T lymphocytes. However, 100mg/L of VPA reduced spontaneous apoptosis of $CD8^+$ T lymphocytes in healthy volunteers. Similarly, it was previously reported that healthy donor $CD3^+$ T cells were significantly less sensitive to apoptosis induction by VPA than primary chronic lymphocytic leukemia cells (Bokelmann and Mahlknecht, 2008). This insensitivity was more prominent for high concentrations (3 mM) of VPA than low concentration (0.1 and 1 mM), although the antiapoptotic effect of VPA was not overemphasized by the authors. Moreover, a few studies indicated the antiapoptotic effect of VPA and other HDAC inhibitors on ischemic neurons and non-small cell lung cancer cells (Mayo et al., 2003; Chuang, 2005). In contrast to the antiapoptotic effects, more evidence from *in vitro* culture, animal model studies and clinical observations has shown that VPA can render various cancer cells more susceptible to apoptosis by HDAC inhibition, reactive oxygen species production, DNA damage, caspase-dependent pathway and other mechanisms (Kawagoe et al., 2002; Chen et al., 2006; Bokelmann and Mahlknecht, 2008; Lin et al, 2008; Ozaki et al, 2008). Therefore, these results support the view that VPA has anti- as well as proapoptotic effects depending on the target cell type . It is less clear for the current study whether these effects of VPA contribute to its anticonvulsant or adverse effects.

6.5 The preliminary observation for SV2A expression in human $CD8^+$ T lymphocytes

The main finding of this study was that LEV had a attenuating effect on degranulation of $CD8^+$ T lymphocytes. For disclosing the mechanism of this depressive effect, it was hypothesized for from our experiments that SV2A, the binding site of LEV, exist in human $CD8^+$ T lymphocytes.

Synaptic vesicle protein 2 (SV2) is a highly glycosylated protein involved in

exocytosis (Buckley and Kelly, 1985; Portela-Gomes et al., 2000). Vertebrates have three distinct genes encoding highly homologous proteins referred to as SV2A, -B and -C isoforms (Bajjalieh et al., 1992; Bajjalieh et al., 1993; Feany et al., 1992). SV2A is the most widely distributed isoform, being nearly in all types of neurons, as well as being present in endocrine cells (Buckley and Kelly,1985; Bajjalieh et al., 1993; Bajjalieh et al., 1994; Xu and Bajjalieh, 2001). SV2A knockout mice fail to grow, develop severe seizures and die within the first weeks of life, – a phenotype that suggests both neurological and endocrine deficits (Crowder et al., 1999; Janz et al., 1999). In neurons, SV2A is associated with synaptic vesicle fusion, exocytosis, and neurotransmitter release (Crowder et al., 1999). SV2A was identified as the binding site of LEV in the brain (Lynch et al., 2004). The specific effect of LEV binding to SV2A appears to be a reduction in the rate of vesicle release (Yang et al 2007). In adrenal chromaffin cells, SV2A is associated with calcium-dependent exocytotic burst (Xu and Bajjalieh, 2001).

To test the above mentioned hypothesis that SV2A is also involved in the degranulation, we preliminarily observed measured SV2A expression in human $CD8^+$ T lymphocytes in one healthy person:. The freshly isolated PBMCs were first stained with CD3-APC, CD8- PerCP for 30 min. Then, the stained PBMCs were resuspended in BD Cytofix/Cytoperm® solution for 20 min at 4°C. After washing and centrifugation (1200rpm, 20 oC, 4 min, with brake), PBMCs were labeled with SV2A-FITC (GmbH, Aachen, Germany) in Perm/Wash buffer solution. IgG isotype control (FITC) was used for negative control. Then, SV2A expression was measured via flow cytometry. Our The preliminary results showed that iSV2A was probably likely to be expressed in human $CD8^+$ T lymphocytes. A representative example is shown in Fig. 10. Therefore, SV2A inhibition possibly explains the depressive effects of LEV on degranulation of $CD8^+$ T lymphocytes.

More work is being currently performed to verify this conclusion.

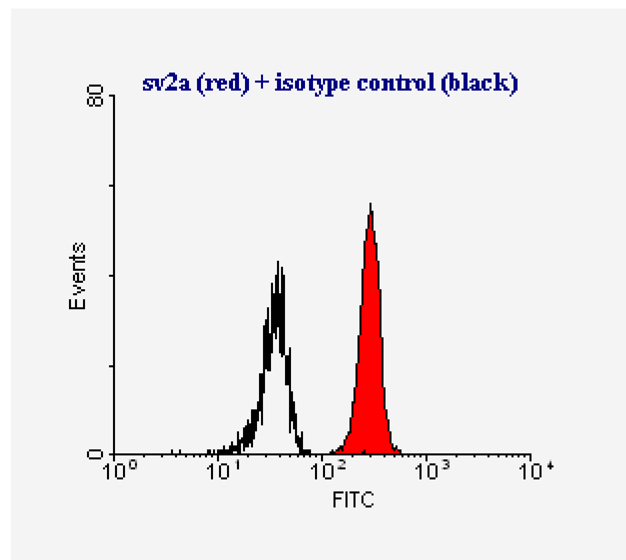


Figure 10.

In this representative example, lymphocytes gate and CD8+ T lymphocytes gate were defined as described above. The histogram of SV2A-FITC and IgG isotype control (FITC) were overlapped.

6.6 Limitations

A limitation of this study was that CD8+ T lymphocytes were taken from healthy volunteers and not from epilepsy patients. Alterations in immune cells and cytokine expression have been observed both in human epileptic patients and in animal models of epilepsy (Plata-Salamán et al., 2000; Ravizza and Vezzani 2006; Lehtimäki et al., 2007; Bauer et al., 2008, 2009). A study to confirm the present results in epilepsy patients is, therefore, underway.

6.7 Conclusion

Taken together, our studies found that LEV had moderate depressive effect on degranulation of CD8+ T lymphocytes, indicating that LEV can disturb the anti-virus function of immune system. This effect is likely to explain the

increased incidence of infection in LEV-treated patients with epilepsy, and also shed light on a new antiepileptic mechanism of LEV in the sense of reduction of BBB disruption caused by perforin release. There is a great need for further studies to verify these conclusions in future studing epileptic patients *in vivo* or animal models.

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9 Acknowledgments

A list of people contributed in some way to this thesis, for whom I would like to express my grateful acknowledgment. I would like kindly acknowledge:

My wife has always encouraged and inspired me, gave me unconditional love and support throughout the years.

Professor Hamer, my supervisor, not only a Professor, a boss, a friend, somebody to be admired, made this work possible. He afforded the scholarship for me and invited my family to his home every year, which made my stay in Marburg a wonderful experience. Thank you.

Dr. Sebastian Bauer collected blood samples and ordered the staff for this study. More importantly, he took his time to review this work and gave me many good suggestions.

Dr. Mareike Nowak helped in collections of blood samples and she is a good technical director.

Kerstin Schlegel, Michael Happel and Paul Philippi gave me many technical help and advice which were very advantageous to me .

All volunteers selflessly afforded their blood which was essential for finishing this study.

Ehrenwörtliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Humanmedizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel

"The influence of levetiracetam and valproate on apoptosis and cytotoxic function of $CD8^+$ T lymphocytes *in vitro*"

in der Klinik für Neurologie unter der Leitung von Herrn Prof. Dr. H. M. Hamer ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe.

Ich habe bisher an keinem in- und ausländischen Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt.

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(Ort, Datum) (Unterschrift)