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Levetiracetam but not valproate inhibits function of CD8$^+$ T lymphocytes

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ABSTRACT

Purpose: To further elucidate possible immune-modulatory effects of valproate (VPA) or levetiracetam (LEV), we investigated their influence on apoptosis and cytotoxic function of CD8$^+$ T lymphocytes in humans.

Methods: In 15 healthy subjects (9 female (60%), 35.7 ± 12.1 years), apoptosis and cytotoxic function of CD8$^+$ T lymphocytes were measured using flow cytometry following *in vitro* exposure to LEV (5 mg/L and 50 mg/L) and VPA (10 mg/L and 100 mg/L). Apoptosis rates were determined after incubation with LEV or VPA for 1 h or 24 h. Cytotoxic function was assessed following 2 h stimulation with mixed virus peptides, using perforin release, CD107a/b expression and proliferation. The presence of synaptic vesicle protein 2A (SV2A) was investigated in human CD8$^+$ T lymphocytes by flow cytometry analysis, Western blot and real time polymerase chain reaction (rtPCR).

Results: High concentration of LEV decreased perforin release of CD8$^+$ T lymphocytes (LEV 50 mg/L vs. CEF only: 21.4% (interquartile range (IQR) 16.5–35.9%) vs. 16.6% (IQR 12–24.9%), $p = 0.002$). LEV had no influence on apoptosis and proliferation ($p > 0.05$). VPA (100 mg/L) slowed apoptosis of CD8$^+$ T lymphocytes after 24 h (VPA 100 mg/L vs. control: 7.3% (IQR 5.4–9.5%) vs. 11.3% (IQR 8.2–15.1%), $p < 0.001$), but had no effects on perforin release ($p > 0.05$). SV2A protein was detected in CD8$^+$ T lymphocytes.

Conclusion: LEV decreased degranulation of CD8$^+$ T lymphocytes which may contribute to the increased incidence of upper respiratory tract infections in LEV treated patients. Inhibition of SV2A may be responsible for this effect.

1. Introduction

Levetiracetam (LEV) is a new generation antiepileptic drug whose efficacy and tolerability in epilepsy treatment is well recognized.[1,2] Synaptic vesicle protein 2A (SV2A), an intracellular protein, is the binding site of levetiracetam in the brain.[3] Several clinical trials reported a clear anticonvulsant effect of LEV but also an increased incidence of pharyngitis and rhinitis in LEV-treated patients.[4–9] The reason for this latter finding remains unknown. It was reported that white blood cell and neutrophil counts were in the normal range in levetiracetam-treated patients who developed infections[8] but were significantly decreased during treatment compared to the pretreatement status.[9] Similarly, we found interictally no clear effect of LEV on B lymphocytes as well as

Abbreviations: CEF, CTL-CEF-Class I peptide pool "Plus"; CFSE, carboxyl fluorescein succinimidyl ester; IFNγ, interferon gamma; IL, interleukin; IQR, interquartile range; LEV, levetiracetam; PBMC, peripheral blood mononuclear cell; rtPCR, real time polymerase chain reaction; SV2A, synaptic vesicle protein 2A; TNFα, tumor necrosis factor alpha; VPA, valproate.

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on CD4+ and CD8+ T lymphocyte counts in the blood of LEV-treated patients.[10]

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 CD107a and CD107b on the cell surface[11] and secretion of soluble factors, such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNFα).[12–15] Treatment with valproate (VPA) was not found to be associated with a higher infection rate.[16] However, VPA is a potent histone deacetylase (HDAC) inhibitor[17–19] and histone modification plays a role in the regulation of the effector functions of memory CD8+ T cells.[20] Moreover, VPA can induce apoptosis in various leukemia cells *in vivo* and *in vitro*.[17–19] VPA also influenced postictal blood levels of TNFα and interleukin (IL)-1β as well as CD4+ T cell counts.[21,22]

In this study, we investigated the influence of LEV and VPA on proliferation, apoptosis, CD107 mobilization and perforin release of CD8+ T lymphocytes *in vitro* in order to better understand their pharmacological effects and adverse events.

2. Materials and methods

2.1. Study population

Fifteen healthy adult volunteers recruited from the staff of the Department of Neurology, University of Marburg (9 female (60%), age: 35.7 ± 12.1 years; range: 18–60 years) were included in the study. None of the volunteers took any medication. Immediately after venous blood drawing (10 mL), the experiments were started. The study was approved by the local ethics committee.

2.2. Antibodies, reagents and peptides

We used the following antibodies, reagents and peptides: CD3-APC, CD8-PerCP, perforin-PE, IgG2b-PE isotype control, CD107a/b-FITC, Perm/Wash Buffer™, Cytofix/Cytoperm™ solution, BD GolgiStop™ (all BD Bioscience), recombinant human interleukin-2 (rhIL-2) (ProSpec), SV2A-FITC (ByOrbit), IgG-FITC isotype control (antibodies-online GmbH), Polyclonal antibody to SV2A-Aff-Purified (Acris Antibodies GmbH), IgG-HRP (Santa Cruz Biotechnology) Annexin V-FITC apoptosis detection kit (Mountain View), carboxyl fluorescein succinimidyl ester (CFSE) (Invitrogen), pooled human AB serum (3H Biomedical AB), trypan blue solution (0.4%) (GIBCO), RPMI 1640 and 1% penicillin–streptomycin (Sigma) and CTL-CEF-Class I peptide pool "Plus" (Cell Technolgy Ltd.).

2.3. Anticonvulsant drugs

Levetiracetam (Keppra®, UCB Pharma S.A., Brussels, Belgium) and 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 5 mg/L or 50 mg/L for LEV (serum reference range in adults for drug fasting levels: 12–46 mg/L) and 10 mg/L or 100 mg/L for VPA (serum reference range in adults for drug fasting levels: 50–100 mg/L), respectively.[23]

2.4. Isolation of peripheral blood mononuclear cells

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

2.5. Activation of CD8+ T lymphocytes

In the functional assays, CD8+ T lymphocytes were activated with CTL-CEF-Class I peptide pool "Plus", which contains 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 one or more of these pathogens, respectively. One hundred microliter PBMCs ($2–3 \times 10^6$ cells/mL) were incubated with 64 μg/mL of CEF peptide (100 μL) at 37 °C in a humidified, 5% CO_2 incubator for 2 h. Costimulatory antibodies (BD FastImmune™ CD28/CD49d costimulatory reagent) were added according to the manufacturer's protocol. A negative control (only anti-CD28/CD49d) was included in every experiment.

2.6. Perforin release

Perforin release was measured as previously described.[24] After activation of CD8+ T lymphocytes for 2 h either in the presence or absence of anticonvulsant drugs, 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 (1200 rpm, 20 °C, 4 min), cells were labeled with perforin-PE in Perm/Wash buffer solution. IgG2b-PE isotype control was used for negative control. Perforin expression was measured by flow cytometry. The baseline of perforin expression was measured in unstimulated cells immediately after isolation. A reduction in degranulation was indicated by a higher percentage of perforin+ cells remaining after stimulation.

2.7. CD107 mobilization

CD8+ T lymphocyte degranulation can also be measured by increased expression of surface CD107a and CD107b.[11,25] CD107 mobilization was measured as previously described.[30] Briefly, CD107a/b-FITC and monensin (BD GolgiStop, used according to manufacturer's instructions) were added before activation of CD8+ T lymphocytes in the presence or absence of anticonvulsant drugs. After incubation for 2 h, cells were washed and stained with CD3-APC- and CD8-PerCP-antibodies as described above followed by characterization of cells in the flow cytometer. The baseline of CD107a/b expression was measured in unstimulated cells immediately after isolation.

2.8. Analysis of CD8+ T lymphocyte proliferation by CFSE labeling

PBMCs were labeled with 0.4 μM CFSE at 37 °C for 10 min in the dark. Free CFSE was quenched with ice-cold culture medium for 5 min. Following two washes, the labeled cells were resuspended in the culture medium. Costimulatory antibodies (CD28/CD49d) and 100 μL of CEF peptides were added into 100 μL of CFSE-labeled PBMCs ($2–3 \times 10^6$ cells/mL) in the presence or absence of anticonvulsant drugs. Recombinant human IL-2 (40 IU/mL) was added at the time of peptide stimulation. The negative control consisted of cultures of CFSE-labeled, unstimulated cells. After incubation in a 5% CO_2 incubator at 37 °C for 5 days, cells were washed with culture medium and cell surfaces were stained with CD3-APC and CD8-PerCP. Cells were analyzed by flow cytometry. The definition for low CFSE cells was defined according to the distribution of CSFE in unstimulated cells.

2.9. Expression of SV2A in human CD8+ T lymphocytes measured by flow cytometry, Western blot and PCR

Fresh isolated PBMCs were first stained with CD3-APC and CD8-PerCP and incubated for 30 min. Then, stained PBMCs were resuspended in BD Cytofix/Cytoperm solution for 20 min at 4 °C. After washing and centrifugation (1200 rpm, 20 °C, 4 min), cells were labeled with SV2A-FITC in Perm/Wash buffer solution. IgG-FITC isotype control was used as negative control. Expression of SV2A was measured by flow cytometry in 3 samples because the study focus lay on possible alterations of function induced by levetiracetam and not on modes of action.

To confirm flow cytometry results, Western blot analysis and rtPCR were additionally performed. First, we used FICOLL as described above for isolation of PBMCs and further $CD8^+$ T lymphocytes for separation into $CD8^+$ cell populations with MicroBeads (human CD8 MikroBeads, Miltenyi Biotec, Bergisch Gladbach, Germany) using magnetic activated cell sorting (MACS, Miltenyi Biotec) according to the manufacturer's instructions. This led to a count of 3 million $CD8^+$ cells. Protein was extracted with mammalian protein extraction reagent (10 μL, MPER, Pierce) according to manufacturer's instructions. Additionally protease inhibitor complex (Protease Inhibitor Cocktail Tablets, Boehringer Ingelheim, Germany) was used for prevention of protein degradation.

For Western blot analysis, protein was separated on 10% SDS-PAGE gels (Rotiphorese 10× SDS PAGE, Rotiphorese Gel 30, TRIS HCl, Roth, Karlsruhe, Germany) by electrophoresis. Gels were transferred to nitrocellulose membranes (Invitrogen, Germany) and processed as described for protein assays. Briefly, Roti-Block blocking buffer (Roth, Karlsruhe, Germany) was used for blocking the blots, incubation with the SV2A antibody (1:1000) overnight at 4 °C followed. After washing with PBS/0.05% Tween 20, the blots were incubated for 2 h with HRP-conjugated secondary antibody (1:1000, rabbit anti goat IgG-HRP) at room temperature.

Actin-HRP antibody (Goat anti actin HRP) served as loading control.

Total RNA was isolated from PBMCs with RNeasy Mini Kit (Quiagen Inc.) according to manufacturer's instructions. Total RNA (2 μg) was reverse transcribed with 200 U/μL of Superscipt II Rnase H-Reverese Transcription (Invitrogen), using $Oligo(dT)_{12-18}$ primers (Pd(T)12–18: #277858-25UT) according to the manufacturer's instructions.

SV2a mRNA levels were determined by using the Applied Biosystems 7900 HT Thermal Cycler. The amplification protocol consisted of first step at 50 °C for 2 min, a denaturation step at 95 °C for 10 min, followed by 50 cycles with a 95 °C denaturation step for 15 s and 60 °C annealing for 60 s. The following primers were used for specific amplification.

SV2a: Hs00372069_m1 (Applied Biosystems) and β-Actin: TaqMan b-actin Detection Reagent (Applied Biosystems).

PCR products were analyzed by melting curve analysis. A negative control (no template) was measured in each of the PCR runs. The cDNA copy number of SV2a was calculated in relation to the amplification product amount of internal standard.

2.10. Apoptosis evaluation

Fresh PBMCs were seeded at 2–3 × 10^5 cells/well in 96-well plates and LEV (5 mg/L or 50 mg/L) or VPA (10 mg/L or 100 mg/L) was added, respectively. PBMCs without addition of anticonvulsants served as controls. After incubation for 1 h or 24 h at 37 °C in a humidified, 5% CO_2–air incubator, plates were centrifuged (1200 rpm, 20 °C, 4 min) and supernatants were aspirated. 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 (1200 rpm, 20 °C, 4 min), the cells were resuspended in 200 μL of Annexin V binding buffer and incubated with 2 μL of Annexin V-FITC and 2 μL of PI for 10 min in the dark at room temperature. Then, Annexin-binding was measured on a four-color flow cytometer (FACSCalibur®, CellQuest® software, Becton Dickinson).

2.11. Statistical analysis

Intra-individual group comparisons were performed with nonparametric Wilcoxon matched-pairs test because Gaussian distribution could not assumed. The data are presented as median values and interquartile ranges (IQR). Because of multiple testing and the explorative nature of the study, the significance level was set to $p < 0.01$.

3. Results

3.1. Perforin release

Perforin release, CD107a/b expression and proliferation were significantly increased in the CEF-peptide stimulated cells as compared to controls after incubation for 2 h, respectively (control vs. CEF: remaining perforin: 40.2% (IQR 31.9–45.6%) vs. 16.6% (IQR 12–24.9%), $p < 0.001$; CD107a/b: 0.7% (IQR 0.3–1%) vs. 10.5% (IQR 8.4–13.9%), $p < 0.001$; low CFSE: 1.8% (IQR 1.4–2.7%) vs. 7% (IQR 4.3–9.7%), $p < 0.001$).

Perforin release was decreased in high concentration of LEV (LEV 50 mg/L vs. CEF only: 21.4% (IQR 16.5–35.9%) vs. 16.6% (IQR 12–24.9%), $p = 0.002$) and showed a trend toward decreased release in the low-concentration group (LEV 5 mg/L vs. CEF only: 23% (IQR 11.5–36.3%) vs. 16.6% (IQR 12–24.9%), $p = 0.048$) without changes after addition of VPA (VPA 10 mg/L vs. CEF only: 18.9% (IQR 8.7–33%) vs. 16.6% (IQR 12–24.9%), $p = 0.33$; VPA 100 mg/L vs. CEF only: 17.3% (IQR 8.2–27.6%) vs. 16.6% (IQR 12–24.9%), $p = 0.93$) (Fig. 1).

3.2. CD107a/b expression

After 2 h of stimulation, both high and low concentrations of LEV decreased CD107a/b expression on $CD8^+$ T lymphocytes compared with the CEF-peptide stimulated group without LEV (LEV 50 mg/L vs. CEF only: 5.5% (IQR 2.7–7.2%) vs. 10.5% (IQR 8.4–13.9%), $p < 0.001$; LEV 5 mg/L vs. CEF only: 6.6% (IQR 4.8–8.3%) vs. 10.5% (IQR 8.4–13.9%), $p < 0.001$). VPA did not reveal any effect on CD107a/b expression (VPA 100 mg/L vs. CEF only: 9.9% (IQR 6.7–14%) vs. 10.5% (IQR 8.4–13.9%), $p = 0.64$; VPA 10 mg/L vs. CEF only: 10.1% (IQR 6.8–14.2%) vs. 10.5% (IQR 8.4–13.9%), $p = 0.15$) (Fig. 2).

3.3. Proliferation of CD8+ T lymphocytes

LEV and VPA did not influence proliferation of $CD8^+$ T lymphocytes induced by CEF-peptide stimulation (LEV 50 mg/L vs. CEF only: 6.2% (IQR 4.4–8.7%) vs. 7% (IQR 4.3–9.7%), $p = 0.85$; LEV 5 mg/L vs. CEF only: 5.9% (IQR 4.2–8.7%) vs. 7% (IQR 4.3–9.7%), $p = 0.43$; VPA 100 mg/L vs. CEF only: 5.9% (IQR 3.8–9.4%) vs. 7% (IQR 4.3–9.7%), $p = 0.78$; VPA 10 mg/L vs. CEF only: 5.7% (IQR 4.3–9%) vs. 7% (IQR 4.3–9.7%), $p = 0.81$).

3.4. Expression of SV2A in human CD8+ T lymphocytes

SV2A could be detected in human $CD8^+$ T lymphocytes by both flow cytometry and Western blot analysis. The rtPCR could verify that mRNA for SV2A exists in PBMCs.

3.5. Apoptosis

Spontaneous apoptosis was observed in cells untreated with LEV or VPA after incubation for 1 h and 24 h. In the high-concentration VPA group, the percentage of apoptotic $CD8^+$ T lymphocytes was significantly lower than that in the control group after 24 h of incubation (VPA 100 mg/L vs. control: 7.3% (IQR 5.4–9.5%) vs. 11.3% (IQR 8.2–15.1%), $p < 0.001$). None of the other treatment groups showed significant changes (VPA 10 mg/L vs. control: 13.3% (IQR 7.6–14.9%) vs. 11.3% (IQR 8.2–15.1%), $p = 0.42$; LEV 5 mg/L vs. control: 10.8% (IQR 9.3–13.1%) vs. 11.3% (IQR 8.2–15.1%), $p = 0.85$; LEV 50 mg/L vs. control: 12.5% (IQR 9.3–14.4%) vs. 11.3% (IQR 8.2–15.1%), $p = 0.17$) (Fig. 3). After 1 h, there was no significant decrease in apoptosis of $CD8^+$ T lymphocytes in any of the groups (VPA 10 mg/L vs. control: 8.1% (IQR 5.4–11%) vs. 7.4% (IQR 5.6–10.8%), $p = 0.09$; VPA 100 mg/L vs. control: 8.5% (IQR 5.8–10.4%) vs. 7.4% (IQR 5.6–10.8%), $p = 0.04$; LEV 5 mg/L vs. control: 7.6% (IQR 6.6–10.3%) vs. 7.4% (IQR 5.6–10.8%), $p = 0.17$; LEV 50 mg/L vs. control: 7.9% (IQR 6.2–10.9%) vs. 7.4% (IQR 5.6–10.8%), $p = 0.11$).

4. Discussion

The main finding of this study was that LEV had an attenuating effect on degranulation of $CD8^+$ T lymphocytes as indicated by reduced perforin release and decreased CD107a/b mobilization after CEF-peptide stimulation *in vitro*. SV2A was found to exist in human $CD8^+$ T lymphocytes. Therefore, it can be hypothesized that the inhibiting effect of LEV on the function of $CD8^+$ T cells is mediated by interaction of LEV with SV2A protein. LEV 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.

In clinical studies, LEV treatment increased the incidence of pharyngitis and rhinitis which was associated with a slight but significant drop in white blood cell counts.[9] Similarly, a study on interictal alterations of leukocytes and cytokines in the blood of patients with active epilepsy found a trend toward decreased counts of $CD8^+$ T lymphocytes.[10]

While this study did not confirm a significant effect of LEV on apoptosis of $CD8^+$ T lymphocytes *in vitro*, the results suggest that LEV compromised the function of $CD8^+$ T lymphocytes.

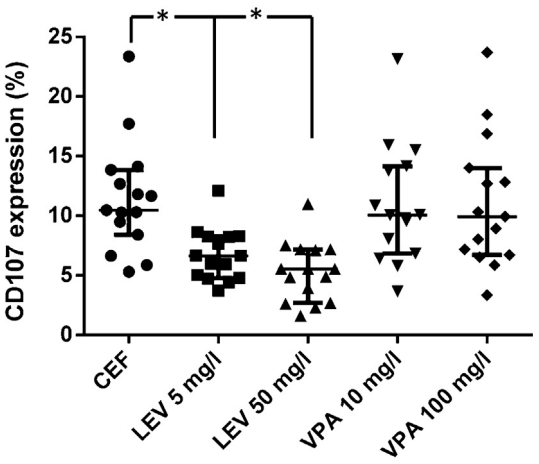

Fig. 2. CD107 expression on $CD8^+$ T lymphocytes after 2 h of CEF-peptide stimulation dependent on low and high concentrations of LEV or VPA. Data are presented as median and interquartile range (IQR). CEF = CTL-CEF-Class I peptide pool, LEV = levetiracetam, VPA = valproate, $^*p < 0.01$.

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.[12–15]

Degranulation can cause positive expression of CD107a/b on the cell surface for a brief period of time before these proteins are internalized again.[26] Therefore, expression of CD107 on $CD8^+$ T cells can be a marker for degranulation. Consequently, expression of CD107 on the cell surface was reduced in this study in the presence of LEV paralleling the reduction in perforin release. This attenuating effect of LEV on perforin release may help explaining the higher rates of pharyngitis/rhinitis in LEV-treated patients.[5]

In addition to its immunological effects, perforin plays also a role in blood–brain-barrier (BBB) permeability. Recently, it was reported that $CD8^+$ T lymphocytes can lead to BBB dysfunction through a perforin-dependent process.[27] In recent experiments, Marchi and co-workers found reduced BBB damage in perforin-deficient pilocarpine-treated epileptic mice.[28] Inducing BBB disruption may establish a link between the systemic immune system and the brain.[29–32] This leads to the hypothesis that levetiracetam's attenuating effect on perforin release adds to its

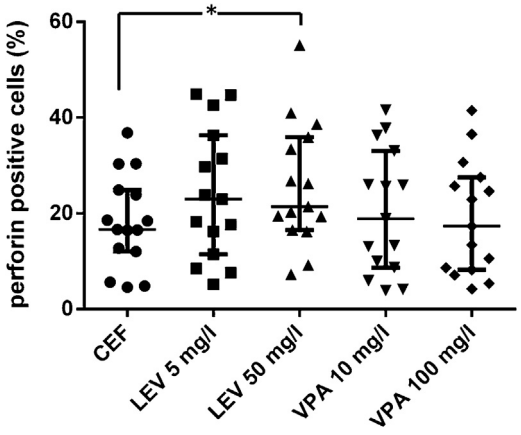

Fig. 1. Perforin release of $CD8^+$ T lymphocytes dependent on low and high concentrations of LEV or VPA after 2 h of CEF-peptide stimulation. A reduction in degranulation was indicated by a higher percentage of perforin+ cells remaining after stimulation. Data are presented as median and interquartile range (IQR). CEF = CTL-CEF-Class I peptide pool, LEV = levetiracetam, VPA = valproate, $^*p < 0.01$.

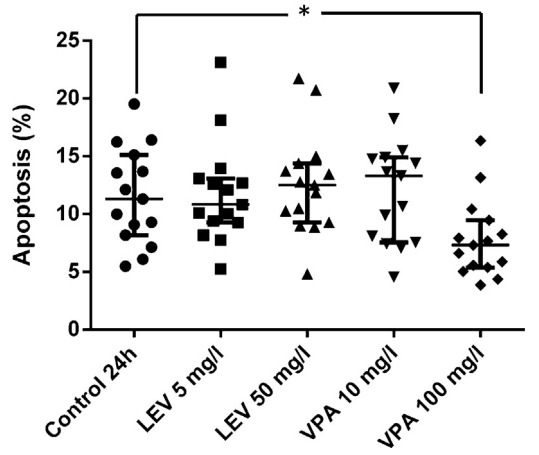

Fig. 3. Apoptosis of $CD8^+$ T lymphocytes after 24 h of incubation dependent on low and high concentrations of LEV and VPA. After 24 h, cells treated with VPA 100 mg/l showed significantly lower apoptosis rate compared to the control group. Data are presented as median and interquartile range (IQR). LEV = levetiracetam, VPA = valproate, $^*p < 0.01$.

anticonvulsant potency via reduction of BBB disruption. Further studies in this field are warranted.

The attenuated degranulation of $CD8^+$ T lymphocytes by LEV is probably caused by binding to SV2A, the presence of which we could prove in human $CD8^+$ T lymphocytes. These results are preliminary and because of the limited number of samples future examination is warranted for this respect. But this detection is in line with a recently published study where detection of SV2A mRNA in T lymphocytes of mice could be documented.[33]

SV2A was identified as the binding site of LEV in the brain.[3] It has been reported that SV2A exists in a variety of neurons and endocrine cells.[34–37] In neurons, SV2A is associated with synaptic vesicle fusion, exocytosis and neurotransmitter release.[38] Further studies are required to verify that SV2A inhibition explains the depressive effects of LEV on degranulation of human $CD8^+$ T lymphocytes.

This study did not reveal any effects of VPA on degranulation of $CD8^+$ T lymphocytes but could show that 100 mg/L of VPA reduced spontaneous apoptosis of $CD8^+$ T lymphocytes in healthy volunteers. Similarly, it was reported that healthy donor T cells were significantly less sensitive to induction of apoptosis in the presence of VPA than primary chronic lymphocytic leukemia cells.[19] This insensitivity was more prominent for high concentrations of VPA than low concentrations. Valproate was found to induce apoptosis of various leukemia cells *in vivo* and *in vitro* contrasting its effects on healthy T cells.[17–19] Therefore, our results support the view that VPA has antiapoptotic effects.

In conclusion, we could show that LEV had a moderate depressive effect on degranulation of $CD8^+$ T lymphocytes, indicating that LEV can disturb the antiviral function of the immune system. This effect is possibly mediated by SV2A inhibition and may help to explain the increased incidence of upper respiratory tract infections in LEV-treated patients.

Conflict of interest

All authors declare that there is no conflict of interest concerning the submitted manuscript.

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