

*Chapter IV*

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## **Homocysteine and Apoptotic Factors in Epileptic Patients Treated with Anti-Epileptic Drugs**

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*Urszula Lagan-Jedrzejczyk<sup>1</sup>, Margarita Lianeri<sup>2</sup>,  
Wojciech Kozubski<sup>1</sup> and Jolanta Dorszewska<sup>2\*</sup>*

<sup>1</sup>Chair, Department of Neurology,

<sup>2</sup>Laboratory of Neurobiology, Department of Neurology,  
Poznan University of Medical Sciences, Poznan, Poland

### **Abstract**

An elevated plasma homocysteine (Hcy) level is an established risk factor for many diseases such as vascular disease, neurodegenerative diseases, and fetal developmental defects. The literature data indicate that therapy with antiepileptic drugs (AEDs) may induce numerous plasma molecular changes, including an increase in the concentration of Hcy and apoptotic factors. Hyperhomocysteinemia (HHcy) occurs in 10-40% of the epileptic population, and AEDs pharmacotherapy has a fundamental effect on the plasma Hcy level. Moreover, several studies have shown that Hcy may stimulate the apoptotic process of cells.

One of the AEDs, valproic acid (VPA), may induce apoptosis in numerous neoplastic cell lines derived from leukemia, hepatic and gastric cancers, suggesting a potential anticancer drug effect. On the other hand, some studies have shown that VPA can also stimulate the anti-apoptotic Bcl-2 protein in neuronal cell lines, suggesting a potential neuroprotective role in the treatment of neurodegenerative diseases. This pro- or anti-apoptotic activity of VPA is dependent on the dose of the drug and the type of cell line, but further investigation is needed.

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\* Corresponding author: Jolanta Dorszewska, MDs, PhD, Laboratory of Neurobiology, Department of Neurology, Poznan University of Medical Sciences, 49 Przybyszewskiego Street, 60-355 Poznan, Poland. Phone: + 48 61 86 91 439; Fax: + 48 61 86 91 697; Email: dorszewskaj@yahoo.com

Our study of peripheral lymphocytes of 23 epileptic patients aged 18-69 and treated with AEDs demonstrated increased plasma Hcy levels, analyzed by high performance liquid chromatography (HPLC), no difference in expression of the apoptotic proteins p53, Bax, and Bcl-2, analyzed by the Western Blot method, and increased numbers of apoptotic cells, analyzed by flow cytometry. Analyses were completed before and during AEDs treatment in monotherapy with VPA, carbamazepine (CBZ) or lamotrigine (LTG), and in polytherapy, compared to 22 controls aged 22-61.

Our results demonstrate an increased plasma Hcy level, greater than 16  $\mu\text{M}$ , in epileptic patients treated with AEDs in monotherapy with VPA and CBZ, and in polytherapy. Moreover, in the epileptic patients with HHcy, we observed a lower expression of p53 protein, a higher level of Bax/Bcl-2 ratio, and an increased number of apoptotic cells compared to patients with a plasma Hcy level of less than 16  $\mu\text{M}$ .

Our conclusion is that some AEDs may generate HHcy, involving the apoptotic process in epileptic patients. The process by which apoptosis is induced by AEDs needs further study.

**Keywords:** Homocysteine, apoptotic proteins, apoptosis, anti-epileptic drugs, epilepsy

## Introduction

### Apoptosis in the Cell

Apoptosis is suicidal, programmed cell death (PCD), and is an active physiological process, requiring an output of energy and the activation of many genes. As the opposing process to mitosis, apoptosis is responsible for regulating the number of cells in an organism and for maintaining homeostasis within the organism. Apoptosis is also of great significance in embryogenesis, organogenesis, and the involution of organs, as well as in the elimination of degenerative, mutated or pre-neoplastic cells. Inappropriate cell death, either excessive or insufficient, may form the basis of neurodegenerative, auto-immunological or neoplastic diseases. The term *apoptosis* comes from a Greek word which means “dropping off leaves or flowers”, and the term was first used in the literature in 1972 [61].

Apoptosis is a highly sophisticated process that occurs in an organized way and is subject to regulation by multiple factors. The cellular morphological changes, which are visible in electron or light microscopy, include shrinkage of the cell nucleus (pyknosis), chromatin condensation, nuclear fragmentation (karyorrhexis), ultrastructural modifications of organelles, as well as formation of apoptotic bodies and their phagocytosis. Moreover, cell membrane integrity is maintained until the last stages of apoptosis [61,71]. Since the apoptotic death of cells does not result in a release of proteolytic enzymes, the process is not accompanied by an inflammatory reaction. Thus, elimination of individual cells by apoptosis does not lead to tissue destruction.

There are two main apoptotic pathways: the intrinsic mitochondrial pathway, and the extrinsic receptor pathway. An additional pathway is the pseudo-receptor, sphingomyelin-ceramide and stress-induced pathway [108].

## The Extrinsic Apoptotic Pathway

The extrinsic signalling pathway is activated by ligand stimulation of death receptors. These receptors are members of the tumour necrosis factor (TNF- $\alpha$ ) receptor gene super-family. The death receptors also play a role in trans-membrane proteins containing an intracellular domain called the *death domain* (DD). To date, the best characterized ligands and corresponding receptors include FasL/FasR (CD95/Apo1), TNF- $\alpha$ /TNFR1, Apo3L/DR3, Apo2L/DR4, and Apo2L/DR5 [34,108]. When the ligand binds with the death receptor, the death signal is transmitted to the adapter protein, either the Fas-associated death domain protein (FADD) or the TNF receptor-associated death domain (TRADD), which can associate with procaspase-8 or procaspase-10, form the death-inducing signalling complex (DISC). This complex may cause proteolysis of procaspase-8 to caspase-8, which is a direct activator of caspase-3 [69,108]. Depending on the amount of caspase-8 production by the cell, we can distinguish two types of cells: type-1 cells, in which a great release of caspase-8 directly induces caspase-3, thus initiating the apoptotic process; and type-2 cells, producing a small amount of caspase-8. The apoptotic signal is increased by Bid protein activation, leading to stimulation of the intrinsic pathway. A truncated Bid (t-Bid) causes aggregation of other pro-apoptotic proteins and release of cytochrome c from mitochondria [95].

## The Intrinsic Apoptotic Pathway

The intrinsic signalling pathway involves direct participation of mitochondria. The activation of this process may be a result of many factors, such as an elevated level of reactive oxygen species (ROS), oxidative stress, oncogenes, an elevated level of calcium ions, disturbance in transport of electrolytes, ischemia, and DNA damage [55,75]. These factors contribute to specific changes in the inner mitochondrial membrane and opening of the mitochondrial permeability transition pores (MPTP), producing a reduction of mitochondrial trans-membrane potential and leading to the release of two main pro-apoptotic groups of proteins from the intermembrane space into the cytosol [99]. The first group contains cytochrome c, Smac/Diablo, and serine protease HtrA2/Omi. Cytochrome c binds and activates the apoptotic protease activating factor-1 (Apaf-1) and procaspase-9, thus forming the apoptosome, which then leads to the activation of caspase-9, the initiating caspase of the mitochondrial pathway. Apoptosis inducing factor (AIF), endonuclease G, and caspase-activated deoxyribonuclease (CAD) constitute the second group of pro-apoptotic factors, which acts in a caspase-independent manner. These substances may translocate to the nucleus, leading to DNA fragmentation and peripheral chromatin condensation. Caspase-independent apoptosis has some characteristic features of normal apoptosis, but it lasts much longer [69].

## Other Apoptotic Pathways

The pseudo-receptor pathway occurs with the involvement of granzyme A and B proteins, and has been observed in cytotoxic T lymphocytes as well as in natural killer (NK)

cells. The intrinsic, extrinsic and granzyme B pathways have in common the fact that initiation of the executive phase is connected with caspase-3 activation, whereas granzyme A activates caspase-independent apoptosis [34,112].

The sphingomyelin-ceramide pathway is connected with an increased level of cell ceramides as the result of the activation of acid or neutral sphingomyelinase. The ceramide in this process plays a role of secondary lipid death messenger [66,108].

Some studies have shown a different stress-induced pathway of apoptosis, which is mainly connected with the endoplasmic reticulum as the regulation center of cell death and with caspase-12 [75,87,108].

### The Role of p53 Protein in Apoptosis

The p53 protein, a product of gene *TP53*, plays the dominant role in the regulation of apoptosis and has been described as “*the guardian of the genome*”. This gene, one of the best known tumour suppressor genes, is located on the short arm of chromosome 17: *17p13.1q* [27,30]. Its vital role is prevention of the transmission of genetic disturbances to the daughter cells by extending the G1 phase of the cell cycle, enabling repair of the damaged DNA fragments by the repair enzymes, poly(ADP-ribose) polymerase (PARP), DNA-glycosylase and endonucleases [30]. Excessive damage to genetic material targets a cell for the apoptotic process.

There are two forms of the p53 protein. The healthy, inactive form, which is described as “wild-type,” and the mutated form, which is deprived of its suppressing function [33]. In physiological conditions, all the cells of the organism contain an inactive form of p53 protein at a low concentration [69]. However, cell stress may result in p53 activation by oligomerization and phosphorylation, leading to an increased p53 level [120]. This activation induces the ability of p53 to promote gene transactivation and transrepression, and, according to the extent of cell damage, it may lead either to cell cycle arrest or to apoptosis. The active p53 protein may also be directly involved in DNA repair [109].

While inducing the apoptotic process, the protein product p53 translocates to the mitochondria, where it influences mitochondrial membrane permeability, leading to the release of cytochrome c by inducing the expression of pro-apoptotic genes, including Bax, Bak, Puma, and Noxa. The p53 protein also influences the extrinsic pathway by its participation in the translocation of death receptors onto the cell surface. Moreover, p53 protein is a well-known inhibitor of the expression of the anti-apoptotic proteins, Bcl-2, Bcl-xl and IAP [28,108].

The protein product of the *MDM2* gene plays a fundamental role in controlling the level of p53 protein; Mdm2 may bind and inhibit the p53 protein, whereas p53 positively regulates the expression of Mdm2. Thus both of the proteins form a feedback loop, in which p53 protein may self-regulate its amount in the cell [109].

### The Role of the Bcl-2 Protein Family in Apoptosis

The Bcl-2 protein family is the best characterized regulator of apoptosis. This group of substances contains the inhibitors, Bcl-2, Bcl-xL, Bcl-w, Mcl-1, and the inducers of

apoptosis, Bid, Bad, Bak, Bax, Noxa, and Puma [108]. The name of the Bcl-2 family comes from the cell line from which these proteins were first isolated, B-cell leukemia/ lymphoma-2. The proteins from the Bcl-2 family contain at least one of the 4 regions, referred to as the homology BH domains, BH1, BH2, BH3, and BH4. All of these domains are present only in anti-apoptotic proteins. The pro-apoptotic Bcl-2 proteins, Bax and Bak, show homologous domains with BH1, BH2, and BH3. Bik, Bad, Bid, Bim, Noxa, and Puma are the so-called BH3-only proteins. The BH3 domain is essential during induction of apoptosis, because it takes part in binding the pro- and anti-apoptotic proteins together [98,128].

The Bcl-2 family of proteins is localized in the mitochondrial membrane, and their function is to regulate membrane permeability by opening of the voltage-dependent anion channels (VADC), the so-called mitochondrial pores. Bcl-2 proteins interact together, forming dimers, both homo- and heterodimers. Cell viability depends on the majority of either inhibitory proteins or pro-apoptotic proteins [67].

## The Role of Caspases in Apoptosis

Caspases are enzymes belonging to the cysteine protease group, the members of which participate both in the induction and execution of apoptosis. Among them there are caspases that induce the apoptotic process (-2, -8, -9, -10), and caspases that execute the apoptotic process (-3, -6, -7). Some caspases are also involved in inflammatory processes (-1, -4, -5, -11, -12, -13, -14). Caspases are present in the cells in the form of inactive zymogens (pro-caspases), containing 3 subunits that are combined with a link, which is a point of proteolysis, oligomerization, and activation [68]. Induction of the caspase cascade may be a result of stimulation of both the extrinsic and intrinsic apoptotic pathways.

All caspases, because of their structure, take part in the cleavage of cytoplasmic and nuclear proteins, leading to the activation or inhibition of their functions. Among proteins altered by caspases during apoptosis are the nuclear proteins, PARP, retinoblastoma protein (RB protein), A and B laminin, topoisomerases I and II (chromatin structure stabilizing proteins), protein kinases, PAK and FAK, as well as the cytoplasmic proteins, fodrin and actin. Moreover, the potential substrates for caspases are also the proteins which are responsible for the cell cycle and signal transmission [69, 86].

## Apoptosis and Antiepileptic Drugs

Epilepsy is not a disease in itself but rather a collection of somatic, vegetative or mental symptoms, which may be the result of a morphological or metabolic change in the brain. The etiology of the disease is varied, and among the most common causes are head trauma, tumours, vascular, degenerative, demyelinating, inflammatory and toxic brain diseases. However, in 65-75% of epileptic patients, the reasons for the disease are unknown [54].

The antiepileptic drugs (AEDs) are among the most common medications used in neurology. AEDs are prescribed not only for the treatment of epilepsy, but also for headache therapy (migraine, cluster headache), neuropathic pain, and bipolar affective disorder. The older generation of AEDs consists of carbamazepine (CBZ), valproic acid (VPA), phenytoin (PHT), phenobarbital (PB), ethosuximide (ESM), and benzodiazepines. The new generation

(NG) consists of lamotrigine (LTG), topiramate (TPM), gabapentin (GBP), oxcarbazepine (OXCZB), levetiracetam (LEV), tiagabine (TGB) and vigabatrin (VGB). AEDs may also be classified according to whether or not they induce liver cytochrome P450, an essential factor in drug interactions [37,106].

The current concept is that epileptic seizures are the result of a disturbed balance between stimulating and suppressing neurotransmitters, glutamic acid neurotransmitters and gamma-aminobutyric acid (GABA), respectively, in the epileptogenic focus.

## Older Generation AEDs and Apoptosis

### Valproic Acid

VPA is a well known teratogen, and taking the drug during the first trimester of pregnancy increases the risk of foetal developmental abnormalities, including neural tube, heart, and skeletal defects, as well as intrauterine growth arrest and development of the foetal valproate syndrome [92,113]. The teratogenic process of VPA remains unclear, but suppression of histone deacetylase, altered gene expression, and increased oxidative stress may be involved [92]. VPA appears to have a pro-apoptotic influence on foetal cells [113], since *in vitro* and *in vivo* research on mouse cells revealed a significant VPA influence on the production of ROS and an increased expression of the apoptotic markers, active caspase-3 and PARP, contributing to the production of neural tube defects. In addition, the enzyme catalase attenuates the effects of VPA on ROS production. Other studies have shown that in growth factor deficiency, VPA may protect neuronal progenitor cells from apoptosis both by activation of the NF- $\kappa$ B pathway and by increasing the level of the anti-apoptotic Bcl-xL protein, thereby disturbing the natural process of cell death during neurogenesis [45].

Epigenetic alterations, such as histone acetylation and DNA methylation, play a vital role in the regulation of genetic expression, which is connected with the cell cycle and apoptosis. Changes in chromatin structure are physiologically modulated by two enzymes, acetyltransferase and histone deacetylase. Some oncogenes and viral oncogenic molecules may affect these enzymes, leading to carcinogenesis [39]. Since VPA is a histone deacetylase inhibitor, it may have anti-neoplastic effects by inducing cell cycle arrest, suppressing growth, and triggering apoptosis in neoplastic cells. Notably, VPA affects leukemic cells by triggering an apoptotic process, changing expression of anti-apoptotic genes, and inducing pro-apoptotic genes. For example, VPA activates the extrinsic apoptotic pathway in chronic lymphoid leukemia cells (CLL), triggering the caspase-8-dependent cascade and TRAIL factor [76]. In other studies using a thyroid medullary carcinoma cell line, VPA increased the expression of the *Notch1* gene, leading to growth arrest and induction of apoptosis [46]. Moreover, VPA at a dose of 300  $\mu$ M causes apoptosis of rat hepatocellular carcinoma cells with the characteristic changes of chromatin condensation, DNA fragmentation, increase of caspase-11 expression, and FAS receptor activation [96]. Furthermore, studies on gastric cancer cells demonstrated that VPA stimulated acetylation of histone H3,  $\alpha$ -tubulin and p21WAF1, and these modulations were accompanied by an increase in active caspases-3 and -9 and a decreased level of Bcl-2, cyclin D1 and survivin proteins. In an animal model these changes were accompanied by a reduction of tumour volume of 36% and this anticancer

effect was independent of p53 protein levels [122]. Other studies showed that induction of apoptosis by VPA in gastric cancer is probably combined with induction of both the intrinsic and extrinsic pathways [127].

VPA also has the ability to induce apoptosis in both androgen-dependent and androgen-independent prostate cancer cells [39]. In small doses, VPA may enhance the radio-sensitivity of prostate cancer cells by p53-dependent modulation of mitochondrial membrane potential [19]. Moreover, retinoblastoma cells are sensitized to radiotherapy by the synergistic use of VPA [60]. VPA caused arrest of cell proliferation and increased activity of caspase-3 in human choriocarcinoma cells [74]. Exposure of human glioma cells to combined treatment with VPA and other anticancer drugs, such as taxol or nanotaxol, leads to activation of caspase-8 and tBid protein, an increased Bax/Bcl2 ratio, and release of cytochrome c and AIF factor from mitochondria [97]. VPA may have anticancer effects by acting as an apoptosis inducing factor for many other tumours such as lung cancer [56], gallbladder cancer [24], pancreatic cancer, and cholangiocarcinoma [53]. Moreover, VPA may interact synergistically with other cytotoxic substances, such as non-steroidal anti-inflammatory drugs (NSAIDs), celecoxib in neuroblastoma cells [20], etoposide in human glioblastoma cells [24] as well as in neuroblastoma cells, and paclitaxel (PTX) in gastric cancer cells [118]. In pleural mesothelioma cells, VPA had the opposite effect, since no increase in the number of apoptotic cells was demonstrated, and the decrease of neoplastic cell invasion was most probably an effect of autophagy [123].

The influence of VPA on neoplastic cells is dependent upon the type of cell line and on the dose of the applied drug that was used in the study. VPA affected activated lymphocytes with a bidirectional influence on cell viability, and a low dose of the drug was protective, whereas a high dose (5 mM) stimulated the apoptotic pathway [18].

Apart from its anticancer effects, VPA may also play a neuroprotective role. In rodents, both in *in vivo* and in *in vitro* studies, the neuroprotective and neurotrophic effects of VPA are partially connected with changes in glial cell function and with selective stimulation of the caspase-3 dependent apoptotic pathway. However, that finding was not confirmed in human cells [43]. In a study of rat neurons *in vitro*, the neuroprotective properties of VPA are a consequence of N-methyl-D-aspartate (NMDA) receptor inactivation, protection from an excito-toxicity process, and activation of the factors promoting cell viability, including PI3, heat-shock protein (HSP) and Bcl-2 protein.

In an animal model of ischemic stroke, the application of VPA caused a reduction of both the ischemic area and the neurologic deficit, combined with a decrease of caspase-3 activation and an increased level of Hsp70 [21]. VPA influences cultured human neuroblastoma cells in a time- and dose-dependent manner by an increasing levels of the anti-apoptotic Bcl-2 protein, suggesting the that VPA may have the properties of a neurotrophic factor and may lead to an increased level of Bcl-2 by activation of both the ERK1/2 and PI3K pathways [23]. Moreover, VPA may directly or indirectly suppress GSK-3 kinase [23]. VPA may also play a protective role in the ischemic damage of retina by suppression of the intrinsic apoptotic pathway [126]. On the basis of these studies, VPA may be found to be useful in future treatment of neurodegenerative diseases, because of its neuroprotective properties.

### Carbamazepine

CBZ is an anticonvulsant that inactivates voltage-gated sodium channels. Its pro-apoptotic effect has been described in *in vitro* research with cerebellar cells [42], but an apoptotic effect was not observed in another study of rat limbic cells [38].

### Phenytoin

PHT blocks conduction in the voltage-gated sodium channels. Its pro-apoptotic influence was demonstrated in studies on rodents. PHT at the concentration of an average effective antiepileptic dose in human subjects caused apoptosis of neurons in the developing rat brain [8]. Furthermore, this drug stimulated apoptosis of neurons of the striatum, thalamus and cerebral cortex [58], of Purkinje cells, as well as granular layer cells in the developing mouse brain [90], and also had a pro-apoptotic effect on rat nucleus accumbens cells in an *in vivo* study [38]. Moreover, PHT-induced gingival enlargement may be associated with insufficient apoptosis of fibroblasts [57].

### Phenobarbital

The antiepileptic properties of PB involve an increase of GABA activity, glutaminergic pathway suppression, and a small influence on the conduction in sodium, potassium and calcium channels [107]. PB stimulated apoptosis of cells in the developing rat brain [8,38], and, in combination with LTG, stimulated apoptosis mainly in the thalamus and striatum neurons [58]. In contrast, an anti-apoptotic influence of PB was observed in mouse liver cells where it stimulated their proliferation after 3 and 10 days of treatment [111].

## **Newer Generation Antiepileptic Drugs and Apoptosis**

### Levetiracetam

The action of LEV consists of binding to a synaptic vesicle protein SV2A [107]. In a study on choriocarcinoma cells, no influence on cell proliferation was observed; however, after a longer exposure of 96 hours, the researchers found that caspase-3 activity was decreased [74]. Even at high doses, LEV did not induce apoptosis in rat neonatal neurons [63,64]. However, the opposite results were demonstrated in human ovarian cells: after exposure to LEV, an increased level of active caspase-3 was demonstrated, and the effect was greater than the effect seen with exposure to VPA [110].

## Oxcarbazepine

OXCZ is a voltage-gated sodium channel blocker. In *in vivo* studies on rat ovarian and uterine cells, OXCZ was pro-apoptotic and stimulated degenerative processes [16]. OXCZ also induced apoptosis in the process of folliculogenesis in female rats and decreased the expression of p53 protein as well as the number of cells in spermatogenesis compared to controls [15]. However, these results were not statistically significant [17]. In a culture of rat hippocampal neurons, OXCZ caused increased caspase-3 expression and condensation of chromatin [2]. In contrast, potential pro-apoptotic properties of the drug were not confirmed [24].

## Lamotrigine

LTG is also a blocker of the voltage-gated sodium channels [107]. In monotherapy, a maximal dose of 50 mg/kg of this anticonvulsant did not induce apoptosis, whereas a significant increase in the number of apoptotic cells was observed at 100 mg/kg. Moreover, polytherapy with LTG and PHT or PB stimulated an apoptotic process compared to monotherapy [58]. LTG weakens the pro-apoptotic effect of kainic acid on rat hippocampal neurons [24] and suppresses the apoptotic death of neurons caused by methamphetamine in prefrontal cortex neurons [88]. In an animal model of Parkinson's disease (PD), the neuroprotective properties of LTG caused a decreased mortality of dopaminergic cells [77]. Therefore, based on recent studies, LTG seems to be the safest drug for women of child-bearing age and during pregnancy.

## Topiramate

TPM is an antiepileptic drug that, apart from blocking the voltage-gated sodium channels, suppresses the activity of calcium channels, enhances the GABA blocking effect, and decreases the activity of the glutamatergic system [107]. TPM leads to the apoptosis of rat neurons at a dose of more than 50 mg/kg, which is much higher than the therapeutic dose [44]. In an animal model of ischemic stroke, TPM caused a reduction of neurologic deficit and neuronal loss, and a decrease in both the caspase-3 level and the Bax/Bcl2 ratio [82].

TPM protected dopaminergic neurons and enhanced their viability in an animal model of PD [50]. TPM suppressed the apoptotic process in an oxidative damage model of rat hippocampal neurons [73]. Moreover, the neuroprotective properties of TPM were observed in an animal model of subarachnoid hemorrhage (SAH) [101].

## Gabapentin

GBP binds to the  $\alpha_2\gamma$  subunit and changes the activity of the affected calcium channels [107]. In an experimental model of stroke, GBP reduced the volume of the ischemic area and concomitant oedema, an effect that is most probably connected with an increased expression

of Hsp70 [65]. However, in that study GBP did not reduce the number of cells with active caspase-3, compared to controls, and thus did not prevent the apoptotic process. Moreover, in an animal model of peripheral neuropathy, GBP did not suppress the apoptosis of sciatic nerve cells [26].

### Pregabalin

PGB influences a change in calcium channel activity. In rats after spinal cord injury, PGB was neuroprotective, causing a decreased caspase-3 level and decreased expression of p38 and MAP kinases [47].

### Vigabatrine

VGB, a selective, irreversible inhibitor of GABA transaminase, stimulates the apoptotic process in the developing rat brain [8]. Visual field deficits, a side effect of VGB, may be a result of induction of apoptosis in retinal photoreceptors [32].

### Zonisamide

The action of ZNM is complex. ZNM inhibits the activity of the voltage-gated sodium and calcium channels, the glutaminergic pathway, and carbonic anhydrase, and stimulates a change of GABA-A receptor activity [107]. The anti-apoptotic influence of ZNM was demonstrated both in studies on human neuroblastoma cells [59] and in rat hippocampal neurons that had previously been treated with kainic acid [24].

## Homocysteine and Apoptosis

One of the factors leading to the induction of apoptosis is an increase in plasma levels of Hcy [78]. HHcy causes changes in the central nervous system (CNS) and is a risk factor for cardiovascular diseases, secondary stroke, and neurodegenerative diseases [89]. The latter observation documents a direct neurotoxic effect of Hcy. The mechanism of this effect is complex and not clearly understood. Apoptosis, neuronal death, oxidative stress, glutamate receptor hyperactivity, mitochondrial dysfunction, and activation of caspases are all involved in the pathological mechanism of neurodegenerative diseases [13,89]. HHcy, secondary to abnormal folate metabolism, is implicated in excito-toxicity [72,85]. Excito-toxicity is a phenomenon in which neuronal damage and degradation by glutamate and related compounds occurs during the excessive activation of NMDA and  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors by glutamate. It is believed that too high a concentration of glutamate can cause excito-toxicity by increasing the number of calcium ions entering the cell [12]. The influx of calcium ions into cells activates a number of enzymes, including phospholipases, endonucleases and proteases such as calpain, which damage the

components of the cytoskeleton, cell membrane, and DNA [79]. Hcy is an agonist of NMDA receptors and a weak NMDA channel activator [81]. Homocysteic acid, an oxidized metabolite of Hcy, is also responsible for the activation of NMDA receptors. Hcy, acting as an agonist of the NMDA receptor by excessive stimulation, can lead to oxidative stress and apoptosis [52, 85].

## Methods

### Homocysteine Levels and Antiepileptic Drugs in Epileptic Patients

The aim of this study was to analyze the plasma levels of the sulfur-containing amino acid Hcy in epileptic patients with newly diagnosed epilepsy before AEDs treatment; in patients receiving VPA, CBZ, and newer generation AEDs, either in mono- and polytherapy; and in controls.

#### Patients

The study group consisted of 23 Caucasian patients with a diagnosis of cryptogenic epilepsy, 7 women and 16 men aged 18-69 years (mean age,  $37.1 \pm 13.7$ ). Among this group, 20 of the patients were treated with AEDs, including 6 women and 14 men aged 21-69 years (mean age,  $35.9 \pm 12.0$ ). Three patients were observed before starting anticonvulsive therapy, 1 woman and 2 men aged 18-65 years (mean age,  $45.0 \pm 24.3$ ). The control group consisted of 22 healthy individuals aged 22-61 years (mean age,  $35.7 \pm 13.3$ ).

Among the patients treated with AEDs, 3 epileptic patients were taking VPA (15%), 4 CBZ (20%), and 5 were receiving newer generation AEDs, mainly LTG (25%); the remaining 8 patients were on polytherapy, either VPA or CBZ with newer generation AEDs (40%). Ten of the epileptic patients had been receiving AEDs for more than 5 years.

In the group of patients treated with AEDs, the mean levels of VPA and CBZ were within the reference values.

The diagnosis of epilepsy was made according to the criteria and terminology recommended by the Commission on Classification and Terminology of The International League against Epilepsy (1989).

None of the epileptic patients and control subjects was diagnosed with impaired liver or kidney function. None of the patients or controls had been receiving B group vitamin or folic acid supplementation, and testing was conducted a few hours before the patients took the patients' AEDs dose.

None of the control subjects had verifiable symptoms of dementia or neurological disorders. None consumed alcohol or smoked.

A Local Ethical Committee approved the study, and written consent of all patients or their caregivers was obtained.

## Analysis of Plasma Hcy Concentration

The analyzed plasma thiol compound (Hcy, Fluka Germany) was diluted with water at a 2:1 ratio and reduced using 1% TCEP [Tris-(2-carboxyethyl)-phosphin-hydrochloride; Applichem, Germany] at a 1:9 ratio. Subsequently, the sample was deproteinized using 1M HClO<sub>4</sub> (at a 2:1 ratio) and applied to the HPLC/EC system.

## Determination of Hcy Concentration

The samples were fed to the HPLC system (P580A; Dionex, Germany) coupled to an electrochemical detector (CoulArray 5600; ESA, USA). The analysis was performed in Termo Hypersil BDS C18 column (250mm x 4.6mm x 5µm) [Germany] in isocratic conditions, using the mobile phase of 0.15 M phosphate buffer, pH 2.9, supplemented with 12.5-17% acetonitrile for estimation of Hcy [1].

The system was controlled and the data were collected and processed using Chromeleon software (Dionex, Germany).

## Results

Our study demonstrated that the level of plasma Hcy was significantly increased in epileptic patients treated with AEDs compared to the control group (one-way ANOVA test,  $p < 0.001$ ) [Table 1].

**Table 1. Concentrations of Hcy in epileptic patients before AEDs treatment AEDs(-), after AEDs treatment AEDs(+), and in controls**

Analyzed compound	Control group	Epileptic patients		P
		AEDs(-)	AEDs(+)	
Hcy [µM]	12.3 ±4.2	14.5 ±1.6	18.2 ±5.5*	0.0011

Results are presented as mean ± SD.

One-way ANOVA for independent variables was used.

Statistically significant differences at \* $p < 0.001$  between Hcy concentration in AEDs (+) and controls.

The highest concentration of plasma Hcy was observed in individuals taking VPA and CBZ and also when polytherapy was implemented [Table 2]. The plasma level of Hcy was within the normal limits only in patients taking newer AEDs (one-way ANOVA test,  $p < 0.05$  between patients treated with VPA and newer generation AEDs).

The concentration of plasma Hcy was higher in patients during the first 5 years of therapy, compared to patients with more than 5 years of therapy (Mann-Whitney test,  $p < 0.05$  compared to patients treated with AEDs for less than 5 years [Table 3].

**Table 2. Concentrations of Hcy in epileptic patients treated with VPA, CBZ, NG AEDs and polytherapy, before starting AEDs(-) treatment, and in controls**

Analyzed compound	Control group	AEDs(-)	Epileptic patients				P
			VPA	CBZ	NG AEDs	Poly-therapy	
Hcy [ $\mu$ M]	12.3 $\pm$ 4.2	14.5 $\pm$ 1.6	23.2 $\pm$ 7.8	20.7 $\pm$ 4.2	13.4 $\pm$ 3.5*	19.2 $\pm$ 3.4	0.0351

Results are presented as mean  $\pm$  SD.

One-way ANOVA for independent variables was used.

Statistically significant differences at \* $p < 0.05$  between patients treated with VPA and newer generation (NG) AEDs.

**Table 3. Concentrations of Hcy in epileptic patients treated with various AEDs, depending on length of treatment**

Analyzed compound	Epileptic patients treated with AEDs		P
	Less than 5 years	More than 5 years	
Hcy [ $\mu$ M]	19.5 $\pm$ 6.9	17.0 $\pm$ 3.5*	0.1088

Results are presented as mean  $\pm$  SD.

Non-parametric Mann-Whitney test for independent variables was used.

Statistically significant difference at \* $p < 0.05$  compared to patients treated with AEDs for less than 5 years.

HHcy is a well-known risk factor for a wide range of pathological conditions, such as cardiovascular and neurodegenerative diseases, whereas during pregnancy HHcy may lead to repeated miscarriages, neural tube defects, intrauterine growth retardation and preeclampsia [10]. High doses of exogenous Hcy produce seizures in experimental animals [48,85]. Thus, HHcy might also be connected with poor control of epileptic attacks and development of refractory epilepsy [11]. The plasma concentration of Hcy depends on dietary and genetic factors [102] and Hcy is metabolized in two pathways, remethylation and transsulfuration, requiring folate, vitamin B6 and vitamin B12 as cofactors [29,31,106].

HHcy occurs in 10-40% of epileptic patients, and the risk of HHcy is attributed to AEDs pharmacotherapy rather than to the epilepsy itself [103]. This observation was also demonstrated by studies in the Polish population, patients aged 18-70 years [106], in Greek epileptic children, patients aged 4.5 to 14 years, treated with AEDs for 20 weeks [4], in epileptic adult patients treated with AEDs in mono- and polytherapy for over 30 days [100], and in patients awaiting treatment with AEDs [114]. Elevated levels of Hcy in epileptics may be secondary to polymorphisms in the *MTHFR* gene, resulting in reduced activity of the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR), as well as secondary to dietary deficiency of the main cofactors of its metabolism, including folate, vitamin B12 and vitamin B6. Moreover, AEDs may disturb absorption of dietary folate by changing the gastrointestinal pH [3,11,106].

Several published studies and our current study indicate that the older generation of AEDs, especially VPA and CBZ, may lead to HHcy and coexisting health problems as a side effect [5,93,115]. In our study, the concentration of plasma Hcy in patients receiving AEDs was also increased in comparison to patients before initiation of treatment. While

supplementation of B vitamins, especially folic acid, may decrease the Hcy level, there is still no consensus regarding the exact dosage of supplements that may be effective in the treatment of HHcy. Moreover, as HHcy is an important risk factor for cardiovascular diseases, there are concerns about the benefits for patients taking vitamin B supplementation. Some clinical trials suggest that vitamin B supplementation does not alter the recurrence of stroke and death from cardiovascular events [14,82]. However, meta-analyses confirm the effectiveness of folic acid supplementation in stroke prevention [51,116]. It is therefore vital to identify the target population that may benefit the most from vitamin B therapy and to establish a consensus for the effective doses of these supplements [105].

The literature reports contradictory results concerning NG AEDs and their influence on Hcy production. Anticonvulsants such as LTG and LEV do not induce HHcy [7], as confirmed in our current study. However, OXCBZ and TPM may lead to an increased plasma Hcy level, most likely because of the ability of these drugs to induce liver enzymes [7,35]. Further studies are warranted to investigate the influence of AEDs on plasma Hcy concentration, in order to optimize treatment of HHcy in patients with epilepsy.

## **Apoptotic Proteins, Apoptotic Cells and Antiepileptic Drugs in Epileptic Patients**

The aim of this study was to analyze the levels of the apoptotic proteins, p53, Bcl-2 and Bax, and the number of apoptotic cells in peripheral lymphocytes of epileptic patients before and after treatment with the AEDs VPA, CBZ, and newer AEDs, in mono- and polytherapy, compared to controls.

### **Patients**

The patient and control populations are described in the previous section of this review.

Apoptotic proteins and apoptotic cells were analyzed in peripheral lymphocytes of epileptic patients and controls.

### **Isolation of Apoptotic Proteins**

Blood was layered onto Gradisol L at a 1:1 ratio and centrifuged. The interphase was collected, rinsed in PBS buffer (0.9% NaCl in phosphate buffer), and centrifuged. The lymphocyte precipitate was rinsed with radioimmunoprotein assay (RIPA) buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% IGEPAL CA-630, 0.05% SDS, and 1% sodium deoxycholate) supplemented with protease inhibitor cocktail (Sigma), homogenized in a mixture of RIPA with protease inhibitor cocktail (16:1) and 0.5 µl phenylmethanesulfonyl fluoride (PSMF) [Sigma] in isopropanol (10 mg/100 µl), and centrifuged for analysis of the supernatant [89].

## Western Blot Analysis

The Bax and Bcl-2 proteins were analyzed in 12% and p53 in 7.5% polyacrylamide gel. Equivalent amounts of protein (30 µg protein/lane) were loaded to the wells. The gel-separated proteins were electrotransferred to nitrocellulose filter in a semidry Western Blot analysis apparatus (Apelex, France). The p53, Bax, and Bcl-2 proteins were identified using anti-p53 (IgG-2a, 200 µg/1.0 ml; Santa Cruz, USA), anti-Bax (IgG-2b, 200 µg/1.0 ml; Santa Cruz, USA) and anti-Bcl-2 (IgG-1, 200 µg/1.0 ml; Santa Cruz, USA) mouse monoclonal antibody, respectively, diluted to 1:500. Subsequently, individual sheets of nitrocellulose filter were incubated with a second antibody, goat anti-mouse IgG-HRP (200 µg/0.5 ml; Santa Cruz, USA) at a dilution of 1:2000. To stain immunoreactive bands, peroxidase BMB was added (BM blue POD substrate precipitation; Roche, Germany). The surface area of the immunoreactive bands was analyzed using a densitometer (GS-710; Bio-Rad, Hercules, CA) in the Quantity One System.

## Analysis of Apoptotic Cells

The peripheral lymphocytes in apoptosis were determined by the detection of active caspase-3 with the use of a commercial set of reagents (ApoFluor® Green Caspase Activity Assay, MP Biomedicals, LLC, USA) containing substrates that are cleaved by caspase-3. Simultaneously, the blood cells were stained with propidium iodide (PI, Sigma, P4170) to assess cell membrane integrity. Four types of samples were prepared: (1) unstained cells, (2) cells stained only with ApoFluor® Green, (3) cells stained only with PI, and (4) cells stained with both ApoFluor® and PI. Cells were stained with ApoFluor® in the optimal cell concentration ( $1 \times 10^6$  cells/ml) and then gently mixed and incubated in a dark space for 1 hour at 37°C and 5 % CO<sub>2</sub> saturation. 2 ml buffer was added and samples were mixed and centrifuged for 5 min at room temperature. After removing the upper layer, the cell precipitates were rinsed again with 400 µl buffer, then 2 µl PI was added to all of the samples. After 30 min of incubation samples were analyzed by flow cytometry.

## Flow Cytometry Analysis

The analysis utilized flow cytometry FACScan (BD Biosciences, USA) with argon laser emitting light wave 488 nm, and the results were presented as histograms and cytograms.

## Results

There was no significant difference in p53, Bax or Bcl-2 protein levels or the Bax/Bcl-2 ratio in epileptic patients regardless of AEDs therapy, compared to the control group. However, in epileptic patients the number of apoptotic cells significantly increased after AEDs therapy (one-way ANOVA test,  $p < 0.05$ ) as compared to controls [Table 4].

**Table 4. Levels of apoptotic proteins (% area immunoreactive bands) and apoptotic cells (%) in epileptic patients treated with AEDs(+), before starting AEDs(-) treatment, and in controls**

Analyzed compound	Control group	Epileptic patients		P
		AEDs(-)	AEDs(+)	
P53	49.5 ± 9.2	44.0 ± 2.6	44.0 ± 8.2	0.1416
Bax	64.7 ± 20.0	63.7 ± 14.5	55.0 ± 17.6	0.2451
Bcl-2	44.6 ± 16.6	46.7 ± 9.1	43.1 ± 14.5	0.9076
Bax/Bcl-2	1.7 ± 1.2	1.4 ± 0.2	1.4 ± 0.7	0.5977
Apoptotic cells	0.98 ± 0.38	1.07 ± 0.80	1.44 ± 0.44*	0.0357

Results are presented as mean ± SD.

One-way ANOVA for independent variables was used.

Statistically significant difference at \*p<0.05 between the number of apoptotic cells in controls and AEDs(+).

The number of apoptotic cells was increased in all epileptic patients regardless of the type of AED used as compared to controls. CBZ therapy and polytherapy stimulated the greatest number of apoptotic cells, while VPA had no pro-apoptotic potential [Table 5].

**Table 5. The levels of apoptotic proteins (% area immunoreactive bands) and apoptotic cells (%) in epileptic patients treated with VPA, CBZ, NG AEDs, and polytherapy**

Analyzed compound	Control group	Epileptic patients				P
		VPA	CBZ	NG AEDs	Polytherapy	
p53	49.5 ± 9.2	48.8 ± 8.8	42.4 ± 4.8	46.1 ± 10.4	41.9 ± 7.5	0.6345
Bax	64.7 ± 20.0	59.0 ± 4.4	44.0 ± 30.0	61.8 ± 14.9	52.4 ± 13.8	0.5020
Bcl-2	44.6 ± 16.6	34.8 ± 12.0	35.2 ± 11.3	48.4 ± 10.0	46.1 ± 18.9	0.4188
Bax/Bcl-2	1.7 ± 1.2	1.8 ± 0.7	1.5 ± 1.3	1.3 ± 0.4	1.3 ± 0.6	0.7241
Apoptotic cells	0.98 ± 0.38	1.18 ± 0.15	1.75 ± 1.06	1.33 ± 0.35	1.47 ± 0.74	0.7204

Results are presented as mean ± SD.

One-way ANOVA for independent variables was used. No statistically significant differences are demonstrated.

VPA has been reported to induce apoptosis in numerous neoplastic cell lines derived from leukemia, hepatic or gastric cancer [96,122] suggesting potential anticancer effects of the drug. However, there are conflicting reports concerning the process by which PCD is stimulated. Some reports have shown that apoptosis induction by VPA may occur via the extrinsic pathway [76,96], some by the intrinsic pathway, and some studies have revealed that both pathways may be involved [127]. In the current study, the pro-apoptotic properties of VPA were not observed compared to CBZ and polytherapy treatment. This discrepancy may

be due to the small group of patients studied, the type of the cells being investigated, the dose of the drug, and the duration of treatment [20].

Other reports have shown that VPA stimulates the anti-apoptotic protein, Bcl-2, in neuronal cell lines [21,43], suggesting a possible neuroprotective role in the treatment of neurodegenerative diseases. This pro- or anti-apoptotic activity of VPA seems to be dependent on the dose of the drug, the time of treatment, and the type of cell line.

Our study shows that CBZ stimulates apoptosis of lymphocytes, confirming the previous study, where pro-apoptotic properties of CBZ were described in *in vitro* research on cerebellum cells [42]. However, in another study on rat limbic cells, such an influence was not observed [38].

## **Homocysteine, Apoptotic Proteins, Apoptotic Cells and AEDs in Epileptic Patients**

The aim of the study was to analyze the level of Hcy and apoptotic proteins in peripheral lymphocytes (p53, Bcl-2 and Bax) of the epileptic patients awaiting treatment with AEDs, treated with AEDs (VPA, CBZ, NG AEDs), and those in mono- and polytherapy, and in the control group, as well as the level of lymphocyte apoptotic cells in all analyzed groups.

### **Patients**

Hcy levels and the effect of AEDs were studied in epileptic patients, as described in the previous section of this review.

### **Analysis of Hcy, Apoptotic Proteins and Apoptotic Cells**

Hcy levels, apoptotic proteins and apoptotic cells were studied in epileptic patients, as described in the previous section of this review.

### **Results**

The levels of p53, Bax, Bcl-2 proteins, the Bax/Bcl-2 ratio, and the number of apoptotic cells were compared in patients with HHcy and in patients with Hcy less than 16  $\mu\text{M}$  [Figure 1].

In patients with HHcy, the number of apoptotic cells was increased the most in patients treated with CBZ and in patients on polytherapy. The greatest increase in Hcy and the lowest number of apoptotic cells was observed in the group treated with VPA [Figure 2].

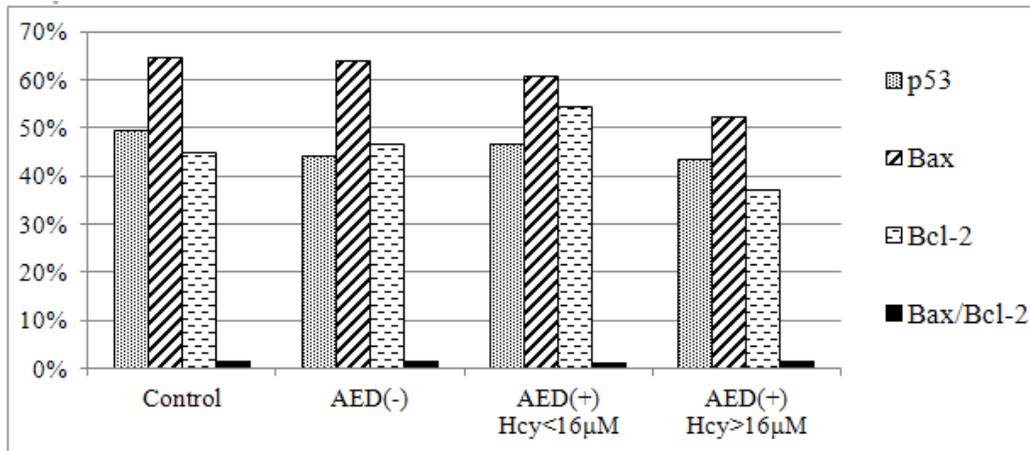


Figure 1. The levels of apoptotic proteins (% of immunoreactive bands) in epileptic patients before treatment AEDs(-), and beginning AEDs(+) treatment with homocysteine more than 16  $\mu\text{M}$  and less than 16  $\mu\text{M}$  and controls, Hcy- homocysteine, AED- antiepileptic drugs, p53, Bax, Bcl-2- apoptotic proteins.

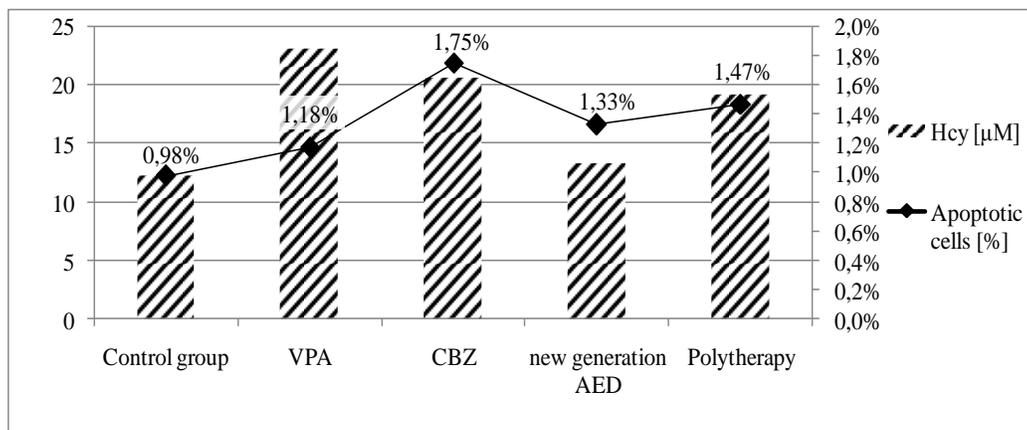


Figure 2. The concentrations of Hcy and the levels of apoptotic cells in epileptic patients treated with different AEDs, and controls. Hcy - homocysteine, VPA - valproic acid, CBZ - carbamazepine, AED - antiepileptic drug.

An increased number of apoptotic cells in patients treated with AEDs with HHcy, compared to patients on AEDs therapy with normal Hcy, suggests a correlation between Hcy and induction of apoptosis. It has previously been demonstrated that an elevated level of Hcy may stimulate apoptosis in many cell types, and several processes involving DNA strand breakage, have been suggested as mediators of this effect [49,84].

Some studies have shown that generation of ROS is necessary for induction of apoptosis by Hcy. For example, in an investigation of neuroblastoma cells, exogenous Hcy stimulated NADPH oxidase leading to superoxide anion production and apoptosis [41]. Moreover, the pro-apoptotic effects of Hcy are believed to be based on its ability to promote excito-toxicity by a stimulation of NMDA receptors, increasing ionized calcium levels and ROS generation, thereby triggering apoptosis [12, 85].

Another potential process for Hcy-mediated cell death is associated with the mitogen-activated protein kinases (MAPK) signaling pathway. In several studies, p38 MAPK was found to play a vital role in transmitting Hcy effects. In *in vitro* studies of cardiomyocytes it was observed that induction of cell death is associated with activation of p38 MAPK and ROS generation, as well as a down-regulation of antioxidant proteins such as thioredoxin (TRX) [119]. These effects were prevented by adding p38 MAPK inhibitor. Interestingly, in this process Hcy stimulated mitochondrial membrane potential changes, suggesting the involvement of the intrinsic signaling pathway [119]. Similar effects have been observed in the study of glomerular mesangial cells, where activation of p38 MAPK by Hcy was combined with increased p38 MAPK nuclear translocation via an oxidative stress-dependent mechanism, causing DNA damage and apoptosis [104]. The involvement of other MAPK kinases, such as ERK and JNK, has also been confirmed in rat cardiomyocytes [80]. Another risk factor for cardiovascular diseases, asymmetric dimethylarginine (ADMA), has also been suggested as playing a role in Hcy-induced apoptosis in vascular smooth muscle cells by leading to intracellular ROS generation and activation of the JNK/p38 MAPK signaling pathways [124].

NF- $\kappa$ B, a nuclear transcription factor, is known to be involved in inflammatory and immune processes. The NF- $\kappa$ B pathway may be involved in Hcy-induced cell death, but its role still remains controversial. In a study on neuroblastoma cell cultures, the activation of NF- $\kappa$ B was associated with an increase of early apoptotic markers such as Bax, caspase-3 and p53 proteins [36]. Similar effects were demonstrated in a study of a human bone marrow stromal cell line, where apoptosis induced by Hcy was associated with NF- $\kappa$ B activation and the ROS-mediated apoptotic pathway [62]. However, other studies have shown that this protein may have neuroprotective properties, suggesting that its inhibition might lead to neurodegeneration [40]. The exact role of NF- $\kappa$ B in Hcy-induced apoptosis remains controversial and is open to debate.

Another compound, 14-3-3 $\epsilon$  protein, has been implicated in transmitting some effects of Hcy [118]. The investigators of that study, on rat hippocampal neurons, found that Hcy may induce apoptosis by down-regulating the concentration of 14-3-3 $\epsilon$  protein, thereby stimulating calcineurin formation.

There are reports that Hcy may induce apoptosis via an apoptotic pathway connected with the endoplasmic reticulum, the stress-induced apoptotic pathway. Hcy induces caspase activation in platelets of patients with diabetes independently of extracellular calcium anion level [125]. The same effect was observed in osteoblastic cells, which are considered to be more susceptible to Hcy-mediated apoptosis than any other cell type [94].

Some studies suggest that some of the statin drugs may inhibit Hcy-induced apoptosis. For example, pretreatment of endothelial progenitor cells with atorvastatin attenuated the influence of Hcy on apoptosis, possibly by suppressing oxidative stress and down-regulating the p38MAPK/caspase-3 pathway [6]. In another study simvastatin up-regulated c-IAP1 and c-IAP2 in human umbilical vein endothelial cells (HUVECs) pretreated with Hcy, suppressing Hcy induced apoptosis [121].

The possible pathways leading to cell death in HHcy are illustrated in Figure 3.

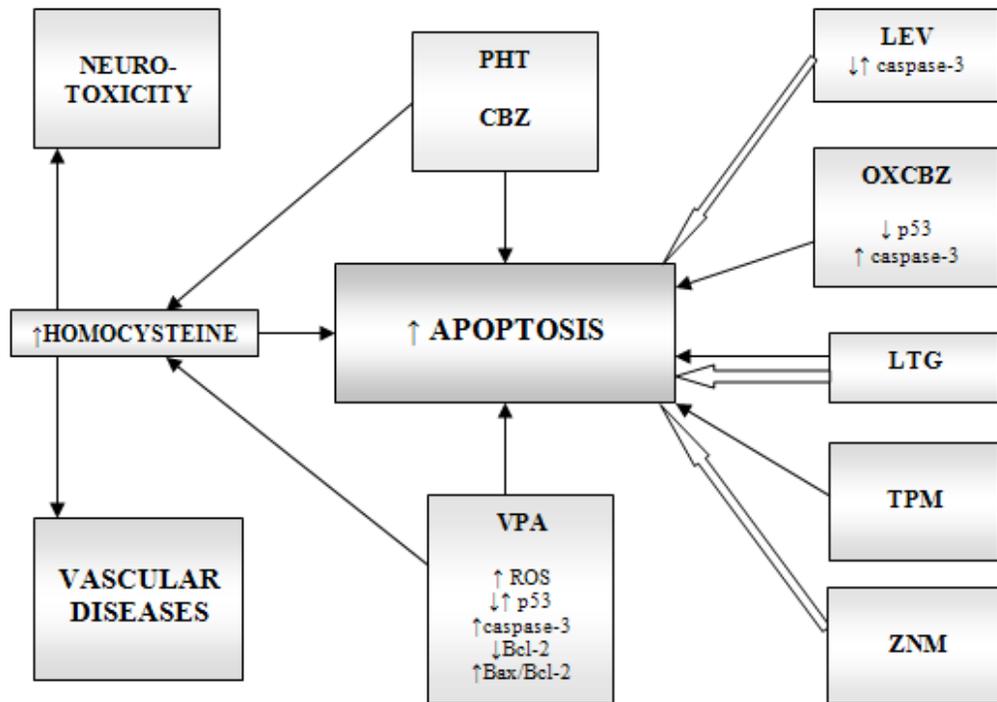


Figure 3. Apoptosis, Hcy, and AEDs. Antiepileptic drugs (AEDs): carbamazepine (CBZ), valproic acid (VPA), phenytoin (PTH), lamotrygine (LTG), topiramate (TPM), oxcarbazepine (OXCZB), levetiracetam (LEV), zonisamide (ZNM). (↑) An increase and (↓) a decrease in the activity of biochemical parameters and molecular processes. (→) Black color: activated/generation. White color: inactivated process.

## Conclusion

The present study demonstrates that pharmacotherapy with AEDs leads to increased plasma Hcy levels in epileptic patients. Data from the literature indicate that the older generation AEDs (VPA, PTH, CBZ) may lead both to generation of increased Hcy levels and induction of apoptosis.

The newer AEDs (TPM, LTG, OXCZB) most likely do not affect the level of Hcy but may only induce apoptosis. AEDs may induce apoptosis involving p53, Bcl-2, Bax proteins, caspase-3, and Hcy. The present study suggests that Hcy may be involved in the induction of apoptosis caused by CBZ. Induction of apoptosis by Hcy is one of the important effects of this thiol, and further studies are needed to determine the exact pathways leading to this process. Thus, the toxic effect of AEDs not only involves production of neurotoxic Hcy, but also involves induction of apoptosis with the participation of the apoptotic factors p53, Bax, Bcl-2 proteins, and caspase-3.

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