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COMPARATIVE STUDIES OF TWO NEW VALPROIC ACID DERIVATIVES ON RAT HEPATOCYTES

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Summary. The cytotoxicity of two new valproic acid derivatives: N-(4-acetylphenyl-2-propyl-pentanamide) (E-31) and 4-((2-propylpentanoyl)aminobenzoic acid) (E-21), with proven anti-epileptic activity, are compared to valproic acid (VPA), using freshly isolated rat hepatocytes, a well-controlled biological model system in vitro with high metabolizing capacity. In rats, as in humans, VPA is metabolized in the liver by cytochrome P-450. As derivatives of VPA, we hypothesize that changes in their structure will result in differences in their metabolism and toxicity. Hepatocytes are treated for two hours with each compound at three concentrations: 0.1 mM; 0.3 mM and 1.0 mM. Effects of E-31 and E-21 on the cells are assessed by measuring cell viability, lactate dehydrogenase (LDH) activity and reduced glutathione level (GSH). E-31 and E-21 are less cytotoxic than VPA in isolated rat hepatocytes most pronounced at the highest concentration (1 mM). This is probably related to the different metabolism of the newly synthesized compounds, compared to VPA.

Key words: valproic acid derivatives, isolated hepatocytes, cell viability, lactate dehydrogenase activity, reduced glutathione

СРАВНИТЕЛНИ ПРОУЧВАНИЯ НА ДВЕ НОВИ ПРОИЗВОДНИ НА ВАЛПРОЕНАТА КИСЕЛИНА ВЪРХУ ХЕПАТОЦИТИ НА ПЛЪХОВЕ

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Резюме. Изследвана е цитотоксичността на две нови производни на валпроената киселина (VPA): N-(4-acetylphenyl-2-propylpentanamide) (E-31) и 4-((2-propylpentanoyl)aminobenzoic acid) (E-21) с доказана антиепилептична активност, върху изолирани хепатоцити от плъх. Валпроената киселина претърпява чернодробен метаболизъм с участието на цитохром P450 както при плъхове, така и при хора. Допуска се, че новосинтезираните производни на валпроената киселина ще претърпят различен метаболизъм и токсичност, свързани с тяхната структура. Изолираните хепатоцити са добре контролиран биологичен модел с висока метаболитна способност. Хепатоцитите са третирани с изследваните вещества в три концентрации: 0.1 mM; 0.3 mM и 1.0 mM, и се инкубират в продължение на 2 часа. Ефектите на E-31 и E-21 се оценяват чрез определяне на клетъчната жизненост, активността на ензима лактатдехидрогеназа (LDH) и нивото на редуциран глутатион (GSH). E-31 и E-21 проявяват по-слаба токсичност от валпроената киселина в най-високата концентрация (1 mM). Това най-вероятно е свързано с различния метаболизъм на новосинтезираните съединения, в сравнение с валпроената киселина.

Ключови думи: деривати на валпроената киселина, изолирани хепатоцити, лактатна дехидрогеназна активност, редукция на глутатиони

In memoriam mag. pharm. Elly Bechar

Introduction

In vitro studies are suitable for assessing new perspective compounds. The perspective new compounds, with proved pharmacological activity and predictable hepatic metabolism, must be examined for cyto- and hepatotoxicity. Isolated hepatocytes are a well-controlled, biological model system with high drug-metabolizing capacities. This *in vitro* system is included in the battery of recommended tests from the European Centre for the Validation of Alternative Methods (ECVAM). The main goal of ECVAM is to promote the acceptance of alternative methods, which are important for reducing, refining and replacing the use of laboratory animals [4].

Valproic acid (VPA) is a commonly used anticonvulsant, prescribed for the treatment of epilepsy and other neurological and psychiatric disorders, such as bipolar affective disorders and migraine headache [5, 14]. However, VPA is hepatotoxic. By making substitutions to the VPA molecule, new compounds with lower toxicity but similar pharmacological potency to VPA are developed [1]. Many metabolic pathways are involved in VPA and VPA analogues biotransformation.

Their metabolism is extremely complex and a variety of overlapping phase I and II metabolic pathways catalyze their biotransformation to over fifty known metabolites [10]. It is well known that metabolism of VPA in the liver includes microsomal cytochrome P-450-mediated hydroxylation/dehydrogenation and mitochondrial β-oxidation. Tang W. et al. [15] find that one particular metabolite, 2-propyl-4-pentenoic acid (4-ene-VPA), received from cytochrome P-450 catalyzed oxidation, structurally resembles the known hepatotoxicants 4-pentenoic acid and methylenecyclopropylacetic acid (a metabolite of hypoglycin) and thereby has been implicated in the VPA associated liver toxicity. In microsomes, 4-ene-VPA is converted by epoxidation to 4, 5-epoxy-VPA, forming a lactone with GSH, which is subsequently depleted. In agreement with this data, in recent years, a number of researchers find that CYP2C9, CYP2A6, CYP2C19, CYP 3A1 and CYP2E1 play a role in the terminal desaturation of valproic acid into toxic metabolite 4-ene-valproic acid [9, 12, 13].

Regarding the information about the VPA toxic effects, Haj-Yehia et al. [10], demonstrate that, by chemical modification of the valproic acid structure, it is possible to obtain new compounds with biological activity comparable to that of VPA, but with lower toxicity and fewer side effects.

The aim of our investigation is to examine the possible cytotoxicity of two derivatives of VPA: N-(4-acetylphenyl-2-propylpentanamide (E-31) and 4-((2-propylpentanoyl) amino) benzoic acid (E-21), with similar anticonvulsant activity to VPA [1] and lower general toxicity, in freshly isolated rat hepatocytes. Since the metabolism of the VPA and their derivatives is complex, quantum mechanical calculations for explaining the influence of the substituents on the localisation of the putative electron acceptor and donor centres are carried out.

Materials and methods

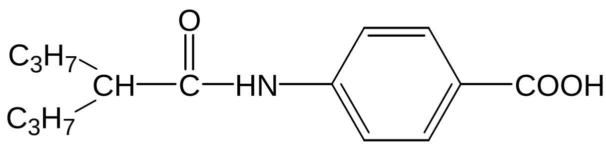
Animals

The International Guiding Principles for Animal Research are strictly adhered to throughout the experiment. Male Wistar rats (b.w. 200 ± 20 g) are housed under standard laboratory conditions (20°C, 12 hours alternating light/dark cycle) with free access to food and water. The animals are purchased from the National Breeding Center – Slivnitza, Bulgaria.

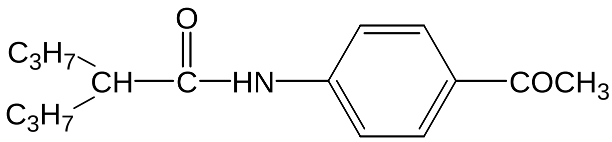
Drugs and chemicals

Synthesis

The compounds used in the study are newly synthesized derivatives of VPA: E-31 and E-21 (Figure 1).



4-[(2-propylpentanoyl)amino]benzoic acid **(E21)**



N-(4-acetylphenyl)-2-propylpentanamide **(E31)**

Fig. 1. *Structures of E-21 and E-31*

E-31 and E-21 are obtained by interaction of valproyl chloride and p-aminobenzoic acid (for E-21) and 4-acetylphenylamine (for E-31) in anhydrous dioxine in the presence of triethylamine (used as an acceptor for the HCl) at O°C and with continuous stirring. Compounds are re-crystallized from solution ethanol/water.

The structures are proved by IR and ^{1}HNMR spectral analysis, as well as by elemental analysis. VPA is used as a reference substance.

In order to investigate the chemical structure of the compounds, basic macroscopic descriptors like partition (Log P) and distribution (Log D) coefficient are calculated, by use of unique additive-constitutive methods, implemented in ACD/Labs^{TM} software. The ACD/Log P algorithm is based on well-characterized Log P contributions of separate atoms, structural fragments and intramolecular interactions between different fragments [6]. The ACD/pKa module uses the Hammett-type equations parameters on the basis of experimental pKa values of 8900 compounds. We calculate the Log D values of the compounds using the expression:

$$\text{Log D} = \text{Log P} - \log(1 + 10^{pH - pKa})$$

Molecular geometry of VPA, E-31 and E-21 is optimized to the energetically most stable conformers. Conformational analysis is performed as normal rule-based search, which excludes conformations with energies higher than 10 kcal above the global energy minimum. The structures are optimized by use of molecular mechanics (MM2 force field) with a 0.0001 energy gradient convergence criterion. The conformers of the global minimum underwent AM1 optimization with key words MMOK and PRECISE. AM1 is a part of MOPAC (Version 6.49), implemented in a Chem-X® computer program. The energies of the highest occupied (HOMO) and the lowest unoccupied molecular orbitals (LUMO), and partial charges (Q) are computed by AM1.

Isolation and incubation of hepatocytes

Rats are anesthetized with sodium pentobarbital (100 mg/kg i.p.), and *in situ* the two step perfusion procedure for isolation of the hepatocytes is performed, using the technique of Berry and Friend [3] with modifications [11]. First, Ca^{2+} is washed out of the liver in order to break Ca^{2+}-dependent cell interactions. Then collagenase is used in Ca^{2+}-containig medium to dissolve intercellular collagen. Cells are diluted with Krebs-Ringer-bicarbonate buffer, enriched with albumine (pH = 7.32) to a final concentration 3 x 10^6 hepatocytes/mL. They are incubated in a shaking water bath, under the atmosphere of carbogen, by gassing every 5-10 min, at 37°C [7]. Cells are treated and incubated with the tested compounds at concentrations: 0.1 mM; 0.3 mM and 1.0 mM) in 25-mL Erlenmeyer flasks. Each flask containes 3 mL of the cell suspension (i.e. 9 x 10^6 hepatocytes). Non-treated hepatocytes are used as a control. Samples were taken at 2 hour of incubation.

For evaluation of the effect of the examined compounds, the following parameters are assessed: cell viability, as assessed by trypan blue (TB) exclusion, lactate dehydrogenase (LDH) leakage into the medium and reduced glutathione level (GSH).

TB exclusion, performed by using the dye trypan blue (0.05 %), is expressed as the percentage of cells viable at zero time – the initial viability and at the 2nd hour after the incubation with the tested compounds [8].

LDH leakage into the medium, which is an indicator for hepatotoxicity, is expressed as nmol/min/10^6 hepatocytes as described by Bergmeyer [2].

At the end of the incubation, cells are pelleted by cenrtrifugation at 400 rpm for 5 min and used to measure the intracellular GSH. GSH is assessed by measuring non-protein sulfhydryl groups, after precipitation of proteins with 5% TCA, followed by measurement of the thiols in the supernatant by the DTNB reagent [8].

Statistical analysis

All quantitative data are presented as mean ± SD (n = 10). One-way Student's t-test is used for statistical comparisons at a significance level of $p < 0.05$.

Results and discussion

The effect of the studied compounds – VPA, E-31 and E-21 at 0.1 mM, 0.3 mM and 1.0 mM on the assessed parameters: TB exclusion, LDH leakage and GSH content, in isolated rat hepatocytes, are shown in Table 1.

The highest TB exclusion is observed at the highest concentration and it's: 50.5% ($p < 0.01$), 58.5% ($p < 0.01$) and 62.5% ($p < 0.01$), for VPA, E-31 and E-21, respectively. The LDH leakage into the medium is mostly increased by 1mM VPA – 230% ($p < 0.01$), followed by E-31 – 197% ($p < 0.01$) and E-21 – 188% ($p < 0.01$). GSH content is depleted by 79% ($p < 0.01$), by 70% ($p < 0.01$) and by 35% ($p < 0.05$), by 1 mM VPA, E-31 and E-21, respectively.

The approximate EC_{50} values of VPA, E-31 and E-21 estimate for the three end points are shown in Table 2.

The results from the theoretical investigation of the chemical structure of the studied compounds are shown in Table 3.

The data show that VPA and E-31 are very weak acids, which are non-ionized at physiological pH. E-21 is a weak acid with two ionization centers, which at pH=7.4, is 99.9% ionized. The results from the quantum mechanical calculations demonstrate that the molecules of E-31 and E-21 have higher HOMO values but lower LUMO values than VPA. The distribution of electron density over carbon atoms in positions 2, 3, 4 and 5 of the alkyl chain is around three times higher than in VPA.

Discussion

In mitochondria, the toxic metabolite – 4-ene-VPA, undergoes β-oxidation to (E)-2-propyl-2, 4-pentadienoic acid ((E)-2,4-diene-VPA) that reacts with GSH, leading to a localized depletion of GSH [13]. It's also suggested the VPA derivatives undergo the similar biotransformation.

Table 1. *Effect of VPA, E-31 and E-21 at three concentrations on TB exclusion, LDH leakage and GSH content, in isolated rat hepatocytes*

Compounds/Concentrations	TB exclusion (% cells)	LDH leakage (nmol/min/10^6cells)	GSH content (nmol/10^6cells)
Control	78.5 ± 2.5	0.119 ± 0.01	32.6 ± 7.5
VPA			
0.1 mM	67.5 ± 6.9*	0.196 ± 0.02**	14.8 ± 3.8*
0.3 mM	61.0 ± 1.4**	0.209 ± 0.04**	11.0 ± 6.5**
1.0 mM	50.5 ± 4.1**	0.274 ± 0.06**	7.00 ± 3.6**
E-31			
0.1 mM	69.5 ± 5.3*	0.204 ± 0.05**	15.6 ± 4.9**
0.3 mM	64.5 ± 4.5*	0.213 ± 0.04**	13.6 ± 4.5**
1.0 mM	58.5 ± 8.2**	0.235 ± 0.03**	10.1 ± 6.4**
E-21			
0.1 mM	70.5 ± 4.9*	0.180 ± 0.04**	19.8 ± 8.1
0.3 mM	65.0 ± 4.5*	0.185 ± 0.07**	16.8 ± 4.4**
1.0 mM	62.5 ± 6.6**	0.224 ± 0.05**	15.4 ± 3.8**

Statistical significance at *p < 0.01; **p<0.001 vs control

Table 2. *Approximate EC50 values of VPA, E-31 and E-21 estimated for the three end points*

Compound	TB exclusion	LDH leakage	GSH content
VPA	1 mM	1 mM	< 0.1 mM
E-31	~ 2 mM	~ 2.3 mM	< 0.1 mM
E-21	~ 4.5 mM	~ 2 mM	~ 0.3 mM

Table 3. *Theoretically calculated properties of the chemical structures of the studied compounds*

Compound	PKa	Log P	Log D	HOMO	LUMO	Q
VPA	16.76	1.77	1.77	-10.46	1.63	C2 0.0375
						C3 -0.0420
						C4 -0.0552
						C5 -0.0653
E-31	14.25	3.97	3.97	-9.01	0.37	C2 0.0380
						C3 -0.0420
						C4 -0.0552
						C5 -0.0653
E-21	$pKa_1 = 4.31$	4.31	1.22	-9.14	0.27	C2 -0.1288
	$pKa_2 = 15.14$					C3 -0.1551
						C4 -0.1656
						C5 -0.2107

In relation to this data, as well as considering the structure of the newly synthesized derivatives of VPA, we determine the hepatotoxicity by measuring the liver cell viability, estimated by TB exclusion, LDH leakage into the medium and GSH content.

For comparison of the relative cytotoxicity of each compound, approximate EC_{50} values are estimated (Table 2). Using the data provided, this actually shows that there is no significant difference in the three compounds.

The effects of the compounds on TB exclusion as well as on the LDH leakage are comparable. GSH conjugation is one of the metabolic pathways for VPA [12]. Both studied compounds deplete GSH at the highest used concentration (1 mM).

Approximate EC_{50} values for VPA and E-31 are < 0.1 mM, and for E-21 - ~0.3 mM respectively. E-31 and E-21 cause GSH depletion similar to that of VPA. This suggests that both VPA derivatives undergo the same GSH conjugation.

Preliminary experiments in vivo [1] show that E-31 has close anticonvulsant activity to that of VPA, while E-21 has low anticonvulsant activity. The observed deviations could be attributed to the structure differences. Lower anticonvulsant activity and lower cytotoxicity of E-21 in the hepatocytes is supported by the higher ionization of this compound, since this property decreases the transport of the molecule through the hepatocyte membrane. Lower uptake into cells results in a more stable compound and a lower clearance by metabolism. As shown in Table 3, the atoms C2, C3, C4 and C5 of the alkyl chain in the molecule of E-21 have higher electron densities (Q) than in E-31 and VPA. It is likely that the introduction of a carboxyl group to the molecule of E-21 leads to an increase in electron density, which may be connected to lower toxicity, because of the lower reactive ability.

Conclusion

The compound E-31 shows close anticonvulsant activity to that of VPA, found in our preliminary experiments. This effect may be connected to the equal level of ionization (the ration between Log P and Log D). In the hepatocytes, E-31 is less cytotoxic than VPA. This may be due to the introduction of acetylphenyl-group in the molecule of E-31, which alters the localization of the putative electron-acceptor center to the benzene ring and decreases the electron-acceptor activity of the compound (Table 3).

E-31 and E-21 have lower toxic effects on the examined parameters than VPA. The structural features of the compounds are probably responsible for the established differences in freshly isolated rat hepatocytes.

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