Prediction of Embryotoxic Effects of Valproic Acid-Derivatives with Molecular *in vitro* Methods

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Summary
Therapy with the antiepileptic drug valproic acid (2-propyl-pentanoic acid, VPA) during early pregnancy can cause similar teratogenic effects (neural tube defects) in humans and mice. In this study a new molecular bioassay is presented using following endpoints: differentiation of F9 teratocarcinoma cells, altered cell morphology, induction of possible targed genes, and the induction of viral RSV-promoter. The induction of a transiently transfected viral (RSV) promoter driven luciferase gene by VPA was used to screen a set of VPA-derivatives. Structure-activity investigations showed: the longer the aliphatic side chain the more the induction of the RSV-reporter gene. The specific induction was stereoselective. The teratogenic enantiomer S-4-yn-VPA (2-propyl-4-pentynoic acid) induced the RSV-driven reporter gene while the non teratogenic R-4-yn-VPA does not. Heptyl-4-yn-VPA was the most potent teratogen in vitro and in vivo. Non teratogenic VPA-derivatives like R-4-yn-VPA and 2-en-VPA (2-propyl-2-pentenoic acid) were ineffective in this system. Thus, the teratogenic effect of VPA and VPA-derivatives in the mouse correlated with the specific induction of the viral RSV-promoter controlled reporter in F9-cells. Acid compounds such as fatty acids are known to interact with peroxisome proliferator-activated receptors (PPARs). To test structure-acitivity relationships by VPA or its derivatives we used CHO cells stably expressing hybrid proteins of the ligand-binding domain of either of the PPARs. The teratogen VPA and the teratogenic derivatives of VPA activated the PPAR-δ construct in a very specific structure- and stereoselective way which correlated well with the activities in the reporter gene assay (bioassay) and those in vivo. No such correlation was found with respect to activation of PPAR-α or PPAR-γ. These structure-activity relationships indicate that PPAR-δ may be a potential mediator of VPA-induced differentiation of F9 cells and may possibly be involved in the mechanism of teratogenicity of VPA in vivo. Furthermore two bioassays were designed with clearly defined endpoints, amenable to automation and screening of great number of compounds. The test system allows to replace animal experiments in the preclinical development of new antiepileptic drugs with reduced teratogenic risk.
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Zusammenfassung: Die Vorhersage Embryotoxischer Eigenschaften von Derivaten der Valproinsäure mit Hilfe molekularbiologischer *in vitro* Testmethoden
Die Pharmakotherapie mit dem Antiepileptikum Valproinsäure (VPA) kann in der frühen Phase der Schwangerschaft beim Menschen wie auch bei der Maus zu schweren Entwicklungsanomalien führen. Wir präsentieren ein neues molekulares Testsystem zur Früherkennung von teratogenen Effekten mit folgenden Endpunkten: Differenzierung von F9 Teratokarzinomazellen, die Veränderung der Zellmorphologie, die Induktion möglicher Zielgene und die Induktion des viralen RSV-Promoters. Die Induktion viraler Reporter durch VPA, welche nach transienter Transfektion gemessen werden kann, wurde mit einer Reihe von VPA-Derivaten untersucht. Struktur-Aktivitätsmessungen zeigten: je länger die aliphatische Seitenkette des VPA-Derivates, desto stärker die Induktion des RSV-Promoters. Die Induktion war stereospezifisch. Das teratogen S-4-yn-VPA induzierte den RSV-kontrollierten Reporter, während das nichtteratogen R-4-yn-VPA dies nicht konnte. Heptyl-4-yn-VPA war das stärkste teratogen in vitro und in vivo. Nicht teratogene VPA-Derivate wie z.B. 2-en-VPA aktivierten den RSV-Reporter nicht. Die Teratogenität von VPA bzw. von VPA-Derivaten in der Maus korrelierte mit der Induktion des RSV-Reporters in F9 Zellen. Fettsäuren aktivieren Peroxisomen Proliferator aktivierende Rezeptoren (PPAR-α, PPAR-γ, PPAR-δ). Wir haben CHO Zellen, die mit der jeweiligen ligandenbindenden Domäne von den PPAR-subtypen stabil transfiziert wurden, eingesetzt, um Struktur-Aktivitätsmerkmale in der Aktivierung von PPARs durch VPA-Derivate zu untersuchen. Es zeigte sich, daß nur die teratogene Valproinsäure bzw. teratogene VPA-Derivate in der Lage waren PPAR-δ zu aktivieren. Nicht teratogene VPA-Derivate aktivierten PPAR-δ nicht. Die Aktivierung PPAR-δ – nicht jedoch die von PPAR-α oder PPAR-γ -korrelierte gut mit Aktivierung des RSV kontrollierten Reporters und der Teratogenität in vivo. Diese Daten lassen den Schluß zu, daß PPAR-δ ein möglicher Vermittler der durch Valproinsäure verursachten Differenzierung von F9 Zellen ist, und daß es möglicherweise im Mechanismus der durch Valproinsäure induzierten Teratogenität eine bedeutende Rolle spielt. Damit wurden zwei Testsysteme entwickelt, die in der Lage sind, teratogene Substanzen vom VPA-typ zu detektieren. Diese Tests haben den Vorteil, daß sie automatisierbar sind und damit für das Screening von einer größeren Anzahl an Substanzen eingesetzt werden könnten. Somit können sie als Testverfahren bei der Suche nach neuen Antiepileptika mit geringerem teratogenen Risiko Tierversuche ersetzen.
Dieses Projekt wurde unterstützt durch das BgVV-ZEBETT (Berlin).

Keywords: 3R, F9 cells, CHO cells, valproic acid, differentiation, teratogenicity, PPAR

1 Introduction

Valproic acid (2-n-propylpentanoic acid, VPA, Fig.1) is an antiepileptic drug particularly used for the treatment of several forms of epilepsy, of bipolar disorders and as migraine prophylaxis. Although originally considered to be of low toxicity, VPA unfortunately proved to have considerable teratogenic potential in the human. VPA is known to cause spina bifida aperta in 1 - 2 % of human fetuses exposed to the compound during early pregnancy. VPA-analogues (Fig.1) with varying teratogenic activity have been synthesized and evaluated in the mouse *in vivo* (Nau et al., 1991). Studies with these VPA-analogues indicate that the teratogenic potency depends strictly on the structure of the administered compound. It is remarkable that the teratogenic effect of VPA and VPA-derivatives is stereospecific. The molecular mechanisms behind the teratogenic effects of VPA are unclear. Recently Werling et al. (1999) reported that VPA as well as the teratogenic derivative S-4-yn-VPA activate intracellular receptors, the peroxisome proliferator-activated receptors (PPARs). Furthermore it was shown that VPA induces differentiation of F9 teratocarcinoma cell (Werling et al., 1999). The changes taking place during the differentiation closely mimic events of early mouse embryogenesis. The goals of this study were to develop assay systems suitable for high through-put screening for the VPA-induced changes in F9 cells based on an easily detecable enzymatic reaction. Both activation of PPARs and differentiation of F9 cells were used to test a series of VPA-related compounds with known gradual differences in regard to induction of teratogenic effects and embryonic lethality.

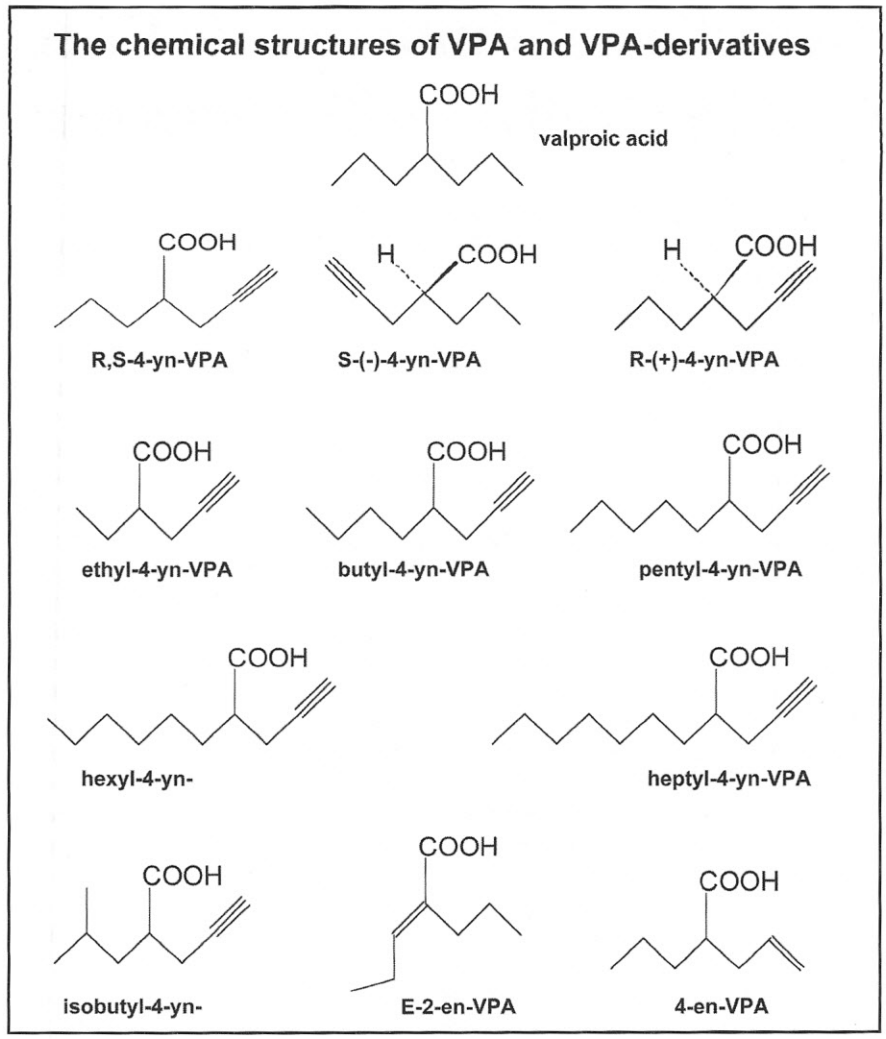


Figure 1: Structure of VPA and VPA-derivatives.

2 Results

2.1 VPA induces viral promoter

Viral promoters are repressed in undifferentiated embryonal carcinoma cells like the F9 cells, and are activated during differentiation (Sleigh et al. 1987). To determine whether VPA and VPA analogues induce cell differentiation and activation of viral promoters we transiently transfected F9 cells with pRSV-Luc, or pUbi-Luc (non-viral) as control. The cells were then exposed for 20 h to various concentrations of VPA and VPA-derivatives. The activity of the transiently transfected RSV-promoter-driven luciferase reporter gene was induced by 1 mM VPA more than 10-fold, whereas expression of a similar reporter gene driven by the housekeeping gene promoter of the ubiquitin C gene (pUbi-Luc) was unaltered or even slightly reduced (Fig. 2).

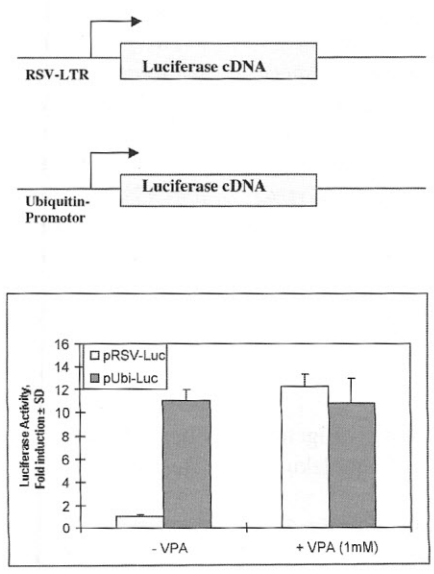


Fig. 2: VPA treatment induces viral (RSV) promoter but not the housekeeping (ubiquitin) promoter in F9 cells. F9 cells were transiently transfected with pRSV-Luc or pUbi-Luc expression plasmids. Cells were treated with or without 1 mM VPA for 20 hours and cell extracts were subsequently assayed for luciferase activities (Bottom panel). Upper panel: schematic sketch of the plasmids used in the bioassay.

2.2 *In vitro* screening of VPA-derivatives

The induction of the RSV-luciferase reporter gene was then developed as an *in vitro* screening model to compare a number of VPA-derivatives with known teratogenic potency in the mouse embryo. From each substance a concentration-activity relationship was obtained by exposure of the F9 cells to different concentrations (0 to 1.5 mM) after transient transfection in the reporter gene assay. VPA-derivatives known to be teratogenic *in vivo* (butyl-4-yn-VPA, pentyl-4-yn-VPA, hexyl-4-yn-VPA, heptyl-4yn VPA, 4-en-VPA, S-4-yn-VPA, R,S-4-yn-VPA) induced the reporter gene under the control of the viral promoter in such a way that the structure-activity relationship seen *in vivo* is well reflected (Fig. 3, data at 300 µM). The induction of RSV-driven reporter gene increased with increasing length of the side chain up to heptyl-4-yn-VPA. The relative potency of the tested compounds was (at 300 µM tested): heptyl-4-yn-VPA > hexyl-4-yn-VPA > pentyl-4-yn-VPA > S-4-yn-VPA > butyl-4-yn-VPA > R,S-4-yn-VPA > 4-en-VPA, VPA. Heptyl-4-yn-VPA was the most potent drug, 300 µM induced the RSV-luciferase reporter gene 37-fold. The lowest concentrations which induced a two-fold induction of the RSV-driven reporter gene were 15 µM for heptyl-4-yn-VPA, 30 µM for S-4-yn-VPA, and 45 µM for R,S-4-yn-VPA. These substances were also the most potent teratogens *in vivo*.

A strong stereoselective effect was found *in vitro* which correlated well with the *in vivo* situation: 300 µM of the teratogenic S-enantiomer of 4-yn-VPA induced the luciferase reporter gene by a factor 7 while the R-enantiomer showed no induction.

And additionally, *in vivo* non teratogenic VPA-analogues (E-2-en-VPA (= E-2-propyl-2-pentenoic acid), isobutyl-4-yn-VPA, and ethyl-4-yn-VPA) did not induce the viral promoter-driven reporter gene. Thus, the teratogenic effect of VPA and the VPA-derivatives in the mouse correlated with the specific induction of the viral RSV-promoter-controlled reporter as seen in F9 cells. The same ranking order (S-4-yn-VPA > R,S-4-yn-VPA > VPA > R-4-yn-VPA) was observed with the induction of the RSV-luciferase reporter gene *in vitro* and the rate of exencephaly induced *in vivo*.

2.3 Specific activation of PPAR-δ

In a second approach the VPA-derivatives were used to define a potential intracellular target for VPA action by structure-activity relationships. Acid compounds such as fatty acids are known to interact with peroxisome proliferator-activated receptors (PPARs), which belong to the nuclear receptor superfamily. To test activation of PPARs by VPA or its derivatives we used CHO cells stable expressing hybrid proteins of the ligand-binding domain (LBD) of either of the PPARs fused to the DNA-binding domain of the glucocorticoid receptor (GR). Activation of these hybrid receptors was detected by expression of secreted alkaline phosphatase (AP). VPA and S-4-yn-VPA were used as a positive control and isobutyl-4-yn-VPA and R-4-yn-VPA as negative controls (Werling et al., 1999). The teratogen VPA and the teratogenic derivatives of VPA (butyl-4-yn-VPA, pentyl-4-yn-VPA, hexyl-4-yn-VPA, heptyl-4-yn- VPA, 4-en-VPA S-4-yn-VPA, R,S-4-yn-VPA) activated the PPAR-δ construct in the same very specific structure-dependent way as seen *in vivo* and in the F9 cell bioassay. The activation of the PPAR-δ was increased with the length of the side chain again up to heptyl-4-yn-VPA (Fig. 4). The ranking of activation potency was heptyl-4-yn-VPA > hexyl-4-yn-VPA > pentyl-4-yn-VPA > S-4-yn-VPA > butyl-4-yn-VPA > R,S-4-yn-VPA > VPA, 4-en-VPA. The results were comparable to the results obtained in F9 differentiation bioassay as well as *in vivo*. Non teratogenic derivatives like isobutyl-4-yn-VPA, E-2-en-VPA, and ethyl-4-yn-VPA were not able to activate PPAR-δ. A stereoselective activation of PPAR-δ was seen when the S-4-yn-VPA and the R-enantiomer were compared. Only the teratogenic S-4-yn-VPA activated the PPAR-δ construct. Specific PPAR-α or PPAR-γ activators like clofibrate, or indomethacin did not induce the PPAR-δ construct whereas 2-bromopalmitate did activate PPAR-δ. To sum it up: The activities of the VPA analogues in the reporter gene assay for PPAR-δ activators correlated well with the teratogenic activities *in vivo* and with the results seen in the F9 bioassay.

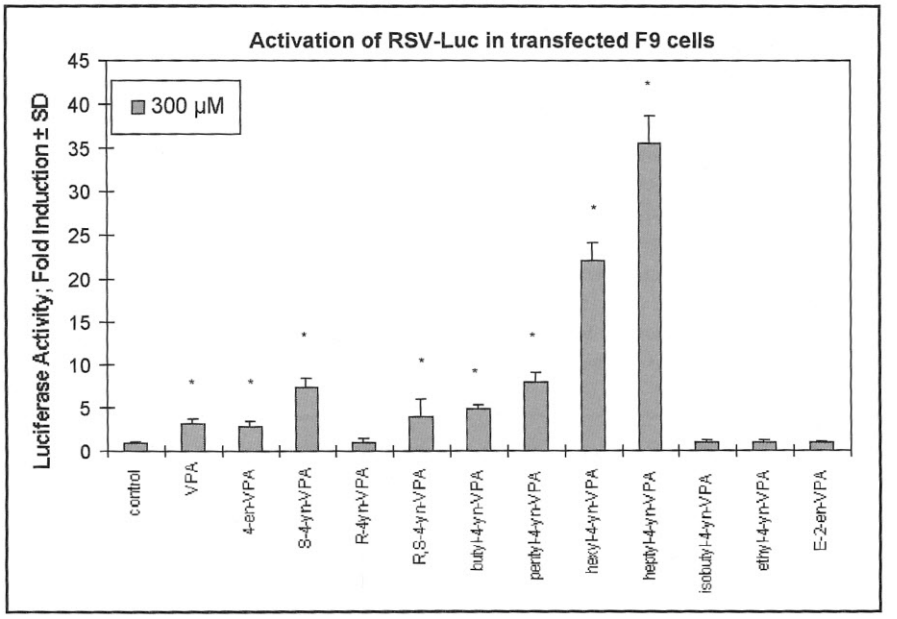


Fig. 3: *In vitro* screening of VPA-derivatives for correlation with their teratogenic potency. F9 cells were transiently transfected with pRSV-Luc or pUbi-Luc-GH (control) plasmids. Cells were treated with or without test compounds for 20 hours and cell extracts were subsequently assayed for luciferase activities and protein concentrations. Values represent means ± SD from triple determinations, with asterisks indicating a significant difference ($p < 0,005$; ANOVA) from untreated cultures. Comparison with 300 µM test compound concentration.

3 Conclusions

We present a structure-activity relationship study with two molecular bioassays for the estimation of the teratogenic potency of

VPA-related compounds. A correlation between the *in vivo* teratogenic potency of VPA-derivatives and differentiation of F9 cells in vitro was made by using a reporter gene assay with viral promoter sequences. VPA and known teratogenic VPA-derivatives induced differentiation of F9 cells characterized by morphological changes to polygonal cells. VPA induced a viral (RSV) but not a housekeeping gene (ubiquitin C) promoter driven reporter gene after transient transfection of F9 cells. The bioassay can distinguish between all known teratogenic and non-teratogenic derivatives of VPA, including enantiomers. There was a highly significant correlation between teratogenic potency of VPA-derivatives *in vivo* and activities *in vitro*. In the second assay, VPA and teratogenic derivatives were shown to activate the PPAR-δ-hybrid-receptor in the same specific structure-dependent way as seen in the F9 bioassay and *in vivo*; no such specific activation was seen with the PPAR-α and PPAR-γ-hybrid receptor. Therefore the specific activation of PPAR-δ can be used as a second in vitro assay for the detection and estimation of the teratogenic potential of VPA-derivatives. The F9 bioassay is a rapid, sensitive, and reproducible method for determining the teratogenic potency of VPA-analogues. The advantage of the system compared to other *in vitro* methods is the use of an embryonic stem cell line and a transient transfection assay with clearly defined endpoints, amenable to automation and screening of a great number of compounds.

Abbreviations

VPA: valproic acid; butyl-4-yn-VPA: 2-(2-propinyl)-hexanoic acid; pentyl-4-yn-VPA: 2-(2-propinyl)-heptanoic acid; hexyl-4-yn-VPA: 2-(2-propinyl)-octanoic acid; heptyl-4-yn-VPA: 2-(2-propinyl)-nonanoic acid; isobutyl-4-yn-VPA: 2-(2-methylpropyl)-4-pentynoic acid; 4-yn-VPA: 2-n-propyl-4-pentynoic acid; E-2-en-VPA: 2-n-propyl-2-pentenoic acid; 4-en-VPA: 2-n-propyl-4-pentenoic acid; ethyl-4-yn-VPA: 2-ethyl-4-pentynoic acid; PPAR: peroxisomal proliferator-activated receptor;

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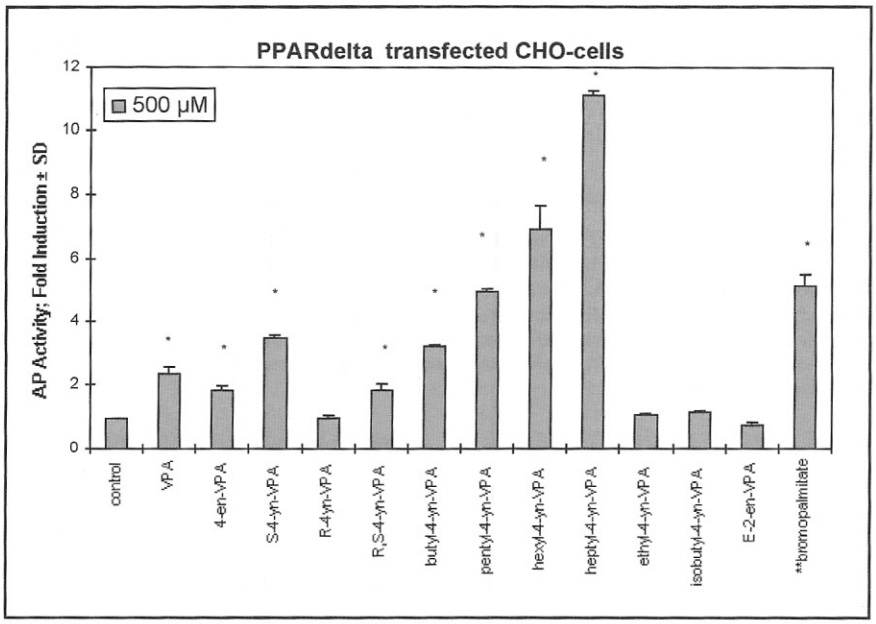


Fig. 4: Activation of the hGR/PPAR-δ hybrid receptor by VPA or VPA-derivatives. Relative alkaline phosphatase activity (AP) in culture supernatants was measured by increase in A_{405} per hr per culture. CHO cells containing stable expressed hGR/PPAR-δ were seeded at a density of 20000 cells per culture well and grown for 24 hr. Cells were grown for an additional 48 hr in the presence of test compounds prior to assaying the culture supernatants for AP activity. Values represent means from triple determinations ± SD. Comparison with 500 µM test compound, asterisks indicating a significant difference ($p < 0,005$; ANOVA) from untreated cultures; Two asterisks indicate 50 µM.

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