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# INHIBITION OF MEDIUM-CHAIN FATTY ACID β-OXIDATION IN VITRO BY VALPROIC ACID AND ITS UNSATURATED METABOLITE, 2-n-PROPYL-4-PENTENOIC ACID

Susan M. Bjorge and Thomas A. Baillie[1]

Department of Medicinal Chemistry
School of Pharmacy, BG-20
Seattle, Washington 98195

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Valproic acid and its unsaturated metabolite, 2-n-propyl-4-pentenoic acid, were found to inhibit strongly the metabolism of decanoic acid in homogenates of rat liver. Reductions in decanoate consumption in response to inhibitors were paralleled by decreases in the formation of octanoic and hexanoic acids, two products of decanoate β-oxidation. In contrast, 4-pentenoic acid, an established inhibitor of long-chain fatty acid β-oxidation, had little effect on the metabolism of decanoate. It is concluded that the title compounds are potent, broad-spectrum inhibitors of fatty acid β-oxidation, a property which may be of key toxicological importance in the pathology of valproate-induced liver injury. 

Valproic acid (VPA; Fig. 1)[2] is a relatively new anticonvulsant drug which is used widely in the management of several forms of epilepsy, particularly in childhood (1). Although especially valuable in the treatment of absence seizures, VPA is known to cause serious liver damage in a small proportion of recipients (2-5) and more than 80 VPA-related fatalities have now been recorded (6). Histological examination of liver tissue obtained from 23 of these fatal cases revealed extensive hepatic steatosis, in some cases accompanied by hepatic cirrhosis and necrosis (3). While the mechanism of VPA-induced liver injury remains obscure, the similarity between the steatosis caused by VPA and the liver damage associated with Jamaican Vomiting Sickness and Reye's Syndrome has led to speculation that these different hepatotoxicities may, in fact, share a similar biochemical etiology with inhibition of

[1]Author to whom correspondence should be addressed.

[2]Abbreviations used: VPA, 2-n-propylpentanoic acid, valproic acid; $\Delta^4$-VPA, 2-n-propyl-4-pentenoic acid; CoASH, coenzyme A; BSTFA, bis(trimethylsilyl)-trifluoroacetamide; TMS, trimethylsilyl; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; FID, flame ionization detector.

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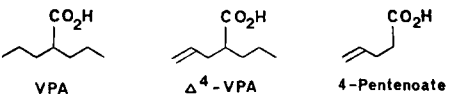


Figure 1. Structures of VPA, $\Delta^4$-VPA and 4-pentenoic acid.

fatty acid β-oxidation as a common link (3). Indeed, a number of recent studies have shown that VPA inhibits the β-oxidation of long-chain fatty acids both *in vitro* and *in vivo* (7-13) and that high doses of the drug may cause hepatic steatosis in rats (14,15).

One theory to account for these findings proposes that a known metabolite of valproate, $\Delta^4$-VPA, may be the actual hepatotoxic species (2,3). This hypothesis stems from the close structural resemblance of $\Delta^4$-VPA to 4-pentenoic acid (Fig. 1), a powerful inhibitor of long-chain fatty acid β-oxidation (16) which produces a Reye-like syndrome in rats (17). In support of this theory, Kesterson *et al.* (10) demonstrated recently that $\Delta^4$-VPA is a potent steatogenic agent in the rat, and that it inhibits the β-oxidation of long-chain fatty acids *in vivo*. In order to examine in more detail this parallel between $\Delta^4$-VPA and 4-pentenoic acid, and to establish whether VPA and $\Delta^4$-VPA can block the β-oxidation of medium-chain, as well as long-chain fatty acids, we have developed new analytical methodology to study the influence of these compounds on the catabolism of decanoic acid in homogenates of rat liver. The preliminary findings reported in this communication demonstrate that VPA and (to an even greater degree) $\Delta^4$-VPA are potent inhibitors of medium-chain fatty acid β-oxidation *in vitro*, although they suggest that the two unsaturated compounds, $\Delta^4$-VPA and 4-pentenoate, act by different mechanisms.

## MATERIALS AND METHODS

VPA was purchased from the Aldrich Chemical Co. (Milwaukee, WI), (±)$\Delta^4$-VPA was prepared by synthesis (18) and all straight-chain fatty acids were obtained from the Sigma Chemical Co. (St. Louis, MO). Co-factors for metabolic studies were also purchased from Sigma. D-Carnitine was supplied by Chemical Dynamics Corp. (South Plainfield, NJ), BSTFA was obtained from Supelco, Inc. (Bellefonte, PA) and bovine serum albumin was from Calbiochem-Behring Corp. (La Jolla, CA).

Gas chromatography (GC) was carried out using a Hewlett-Packard Model 5890 instrument, equipped with a fused silica capillary column (60 m x 0.32 mm i.d.) coated with the bonded stationary phase DB-5 (J and W Scientific, Rancho Cordova, CA). Samples were injected using the splitless mode of operation (injection port temperature 250°C) and "cold trapped" on the column, which was held at 60°C for the first 4 min of the analysis. The temperature of the oven was then raised linearly at 6°C $min^{-1}$ to 186°C. Components eluting from the column were detected by an FID, maintained at 280°C, and recorded by means of a Hewlett-Packard Model 3392A integrator. Gas chromatography-mass spectrometry (GC-MS) was performed on a VG 70-70H instrument as described in detail elsewhere (19).

Biological experiments employed male Sprague-Dawley rats (150-200 g) which were fasted for 24 hr before use to stimulate β-oxidation activity and suppress hepatic fatty acid biosynthesis (20). Water was supplied ad libitum. Animals were sacrificed by decapitation and homogenates of liver were prepared according to a published procedure (21). Incubation mixtures contained the following: ATP (5.0 mM), CoASH (0.1 mM), L-carnitine (2.0 mM), $NAD^+$ (1.0 mM), cytochrome c (12.5 μg), FAD (0.1 mM), malate (0.5 mM), dithiothreitol (12 mM), bovine serum albumin (9.7 mg) and homogenate protein (10 mg), all in 20 mM Hepes buffer (1.35 ml) (21). These mixtures were held at 37°C for 15 min and then cooled on ice before adding substrate (decanoic acid) in aqueous solution (100 μl) to give a final concentration of 0.4 mM. Incubations were then carried out at 37°C for 10 min. Studies on the effects of inhibitors on the β-oxidation of decanoate were performed in a similar fashion, although the inhibitor (0.2 mM) was added at the start of the 15 min preincubation period. Cofactor requirements for metabolism of decanoate were assessed by the addition of buffer in place of ATP or CoASH, and by substitution of D-carnitine for L-carnitine. Homogenates were denatured by heating in a boiling water bath for 30 min. Protein concentrations were measured by the method of Lowry et al. (22).

Incubations were terminated by cooling on ice and were quenched with 0.10 M $Ba(OH)_2$ solution (1.0 ml). Internal standard (nonanoic acid) was then added in buffer solution (50 μl) to a final concentration of 0.96 mM and the samples were allowed to stand overnight at room temperature to hydrolyze coenzyme A and carnitine esters. Samples were acidified to pH 1 with 20% $H_2SO_4$ and extracted with ethyl acetate (7 ml, followed by 2 x 3.5 ml). The combined organic extracts were dried ($MgSO_4$), concentrated under a stream of dry $N_2$ (to approx. 25 μl) and treated with BSTFA (100 μl) at 100°C for 2 hr. Aliquots (1 μl) of these final derivatized samples were analyzed directly by GC. Quantitative measurements of decanoate, octanoate and hexanoate in post-incubation extracts were based on the ratios of GC peak areas (metabolite: internal standard) which were related to calibration curves prepared using the respective synthetic compounds. The calibration curve for each fatty acid was linear over the concentration ranges examined (100-400 μM for decanoate, 25-100 μM for octanoate and hexanoate). The identity and homogeneity of the GC peaks of interest were verified by GC-MS analysis.

## RESULTS

Incubation of decanoic acid with rat liver homogenates which had been fortified with the cofactors necessary for β-oxidation led to a time-dependent loss of total decanoate (free plus esterified forms) from incubation media. The rate of substrate consumption was linear with time over the 10 min incubation period, was dependent upon added ATP and CoASH (necessary for the bio-

synthesis of decanoyl-CoA) and was abolished when boiled homogenates were used, demonstrating the need for functional liver enzymes in the process. Metabolism was also inhibited when L-carnitine was replaced by D-carnitine, which blocks the entry of fatty acids into mitochondria (data not shown). The disappearance of decanoate in these liver preparations was accompanied by the formation of octanoic and hexanoic acids, two products of decanoate β-oxidation. Both residual substrate and its metabolites were quantified by capillary GC analysis (Fig. 2), using a modification of a published GC-MS assay for

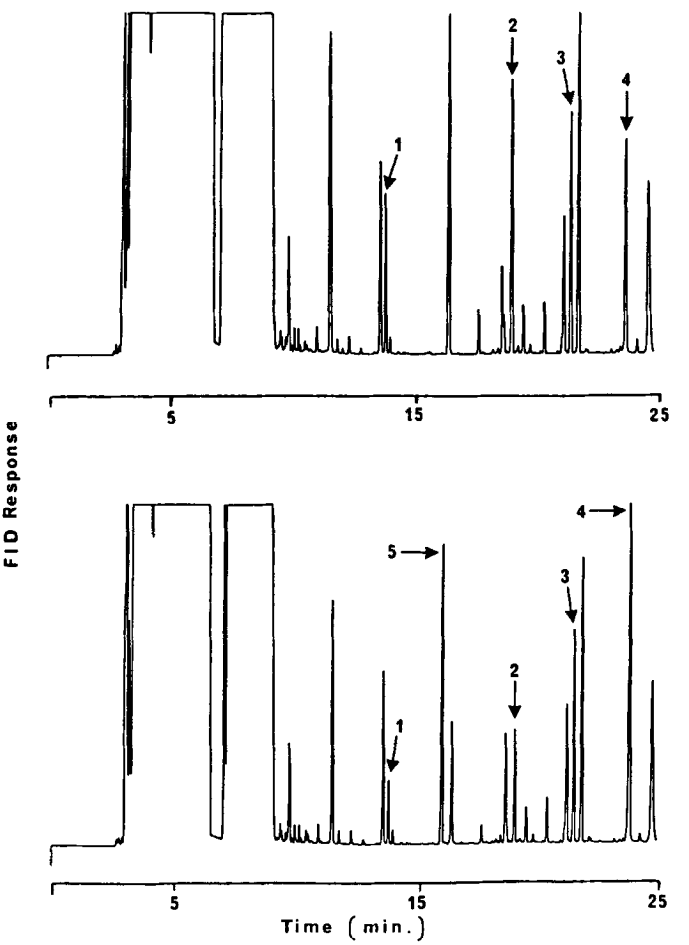


Figure 2. Gas chromatographic separation of decanoic acid and two of its products of β-oxidation, octanoic and hexanoic acids, in an isolate of rat liver homogenate. Acids were extracted, converted to TMS derivatives and analyzed by capillary GC as described under Materials and Methods. The traces depict decanoate and its metabolites formed in the absence (upper chromatogram) and presence (lower chromatogram) of $\Delta^4$-VPA (0.2 mM). Numbered peaks correspond to the following compounds: 1, hexanoic acid; 2, octanoic acid; 3, internal standard (nonanoic acid); 4, decanoic acid; 5, $\Delta^4$-VPA.

these compounds (21). Decanoic, octanoic and hexanoic acids were present in only trace amounts in liver homogenate which had not been fortified with exogeneous decanoate.

As indicated in Table 1, approx. 70% of the decanoate consumed in control incubations could be accounted for by the formation of octanoic and hexanoic acids; no attempt was made to detect products of further β-oxidation of hexanoate. (Hydroxy acids derived from cytochrome P-450-mediated reactions were found by GC-MS analysis, but accounted for < 3% of the substrate consumed). When homogenates were exposed to VPA (0.2 mM) prior to incubation with decanoate, marked inhibition of β-oxidation activity was observed, as reflected by a 28% decrease in the consumption of substrate and slightly greater decreases in the formation of products (Table 1). This inhibition was even more pronounced when homogenates were preincubated with an equivalent concentration of $\Delta^4$-VPA, which blocked the consumption of decanoate by 44% relative to control values (Table 1). 4-Pentenoic acid, on the other hand, proved to be a weak inhibitor of medium-chain fatty acid β-oxidation when preincubated at 0.2 mM;

TABLE 1

Effects of Inhibitors on the β-Oxidation of Decanoic Acid

| Inhibitor[a] | Consumption of Decanoate[b] | Formation of Products[b] | |
|---|---|---|---|
| | | Octanoate | Hexanoate |
| None (control) | 38.3 ± 1.2 (100)[c] | 15.9 ± 0.2 (100) | 11.2 ± 0.5 (100) |
| VPA | 27.5 ± 1.7 (71.8) | 9.8 ± 0.5 (61.6) | 6.4 ± 0.7 (57.1) |
| $\Delta^4$-VPA | 21.5 ± 0.9 (56.1) | 7.5 ± 0.6 (47.2) | 4.9 ± 0.5 (43.8) |
| 4-Pentenoate[d] | 33.1 ± 0.3 (86.4) | 14.3 ± 0.3 (89.9) | 9.2 ± 0.5 (82.1) |
| 4-Pentenoate (1.0 mM) | 26.5 ± 1.1 (69.2) | 11.2 ± 0.2 (70.4) | 7.9 ± 0.5 (70.5) |

[a] Inhibitor concentration was 0.2 mM unless otherwise noted.

[b] Rate of substrate consumption and product formation are expressed in nmol $mg^{-1}$ protein per 10 min incubation and represent mean values ± S.D. (N = 6).

[c] Numbers in parentheses denote the fraction of substrate consumed or product formed as a percentage of the control values.

[d] Number of replicate incubations in this experiment, N = 4.

concentrations of 4-pentenoate in excess of 1.0 mM would have been required to match the degree of inhibition achieved with 0.2 mM VPA (Table 1). The corresponding saturated straight-chain compound, valeric acid (0.2 mM), had no effect on the β-oxidation of decanoate in this system (data not shown) and served as a negative control.

## DISCUSSION

The results of this study demonstrate that VPA, even at a concentration of 0.2 mM (which is approx. one-third of the therapeutic plasma level in man), strongly inhibits the β-oxidation of the medium-chain fatty acid, decanoic acid. Taken together with literature data on the effect of VPA on the metabolism of long-chain fatty acids (7-13), it may be concluded that this drug is an unusually potent and broad-spectrum inhibitor of fatty acid β-oxidation in mammalian liver. This conclusion is supported by the fact that otherwise healthy epileptic patients given VPA excrete abnormally large amounts of $C_6$-$C_{10}$ dicarboxylic acids in urine, indicative of an impairment of hepatic fatty acid β-oxidation activity (8). Under physiological conditions (e.g. starvation) or disease states (e.g. diabetes) where energy metabolism is dependent upon catabolism of fatty acids, the inhibitory effects of VPA on β-oxidation would be expected to be marked and the hepatotoxic potential of the drug maximized. Indeed, some fatal episodes of VPA toxicity have been preceded by anorexia (2,23).

The unsaturated metabolite of valproate, $\Delta^4$-VPA, proved to be the most effective inhibitor of decanoate β-oxidation tested in this study. Under normal circumstances, $\Delta^4$-VPA appears to be a very minor metabolite of VPA in man with circulating concentrations in the nanomolar range (24); however, greatly elevated plasma levels of $\Delta^4$-VPA have been noted in human subjects receiving high doses of the drug (24) and in a patient in the terminal phase of VPA-induced hepatic failure (25). Whether these high concentrations of $\Delta^4$-VPA contribute directly to the development of hepatic steatosis, or merely result from the altered metabolic state of an already-damaged liver, is not

known. It seems likely, however, that enhanced production of $\Delta^4$-VPA from VPA could lead to severe impairment of fatty acid β-oxidation activity in vivo.

In contrast to the title branched-chain compounds, 4-pentenoic acid proved to be a weak inhibitor of decanoate β-oxidation. This finding is consistent with a published report that 4-pentenoic acid strongly inhibits mitochondrial metabolism of the long-chain substrate, palmitic acid, but not of the medium-chain compound, octanoic acid (16). 4-Pentenoic acid is believed to undergo metabolic activation in mitochondria to a chemically-reactive intermediate which alkylates, and thereby destroys, the terminal enzyme of the β-oxidation complex, 3-ketoacyl-CoA thiolase (EC 2.3.1.16) (26,27). Based on structural considerations, it has been speculated that $\Delta^4$-VPA might act in a similar fashion (3) and, indeed, the results of a recent study on the metabolic fate of $\Delta^4$-VPA in rat liver lend support to this hypothesis (18). However, 4-pentenoate and $\Delta^4$-VPA differ in their respective abilities to block the β-oxidation of endogeneous fatty acids of different chain lengths; 4-pentenoate is a potent inhibitor with long-chain substrates (16,28-30) but is only weakly active with medium-chain compounds (16 and this work), whereas $\Delta^4$-VPA strongly inhibits the metabolism of both classes of fatty acids (10 and this work). It would appear, therefore, that either the mechanism by which $\Delta^4$-VPA inhibits fatty acid metabolism is fundamentally different from that of 4-pentenoate, or that multiple mechanisms operate for $\Delta^4$-VPA.

Finally, it should be noted that, under the experimental conditions employed in this study, no metabolism of VPA or $\Delta^4$-VPA could be detected in liver homogenates (although CoA or carnitine derivatives, if formed, would have been hydrolyzed in the work-up and not observed). Modification of the VPA skeleton by metabolism, therefore, is not required for the generation of inhibitors of medium-chain fatty acid β-oxidation.

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