Structure Activity Relationship of Human Microsomal Epoxide Hydrolase Inhibition by Amide and Acid Analogues of Valproic Acid

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Purpose. The purpose of this study was to evaluate the *in vitro* inhibitory potency of various amide analogues and derivatives of valproic acid toward human microsomal epoxide hydrolase (mEH).

Methods. mEH inhibition was evaluated in human liver microsomes with 25 μM (S)-(+)-styrene oxide as the substrate. Inhibitory potency expressed as the median inhibitory concentration (IC_{50}) was calculated from the formation rate of the enzymatic product, (S)-(+)-1-phenyl-1,2-ethanediol.

Results. Inhibitory potency was directly correlated with lipophilicity and became significant for amides with a minimum of eight carbon atoms. Branched eight-carbon amides were more potent inhibitors than their straight chain isomer, octanamide. N-substituted valproylamide analogues had reduced or abolished inhibition potency with the exception of valproyl hydroxamic acid being a potent inhibitor. Inhibition potency was not stereoselective in two cases of chiral valpromide isomers. Valproyl glycinamide, a new antiepileptic drug currently undergoing phase II clinical trials and its major metabolite valproyl glycine were weak mEH inhibitors. Acid isomers of valproic acid were not potent mEH inhibitors.

Conclusions. The structural requirements for valproylamide analogues for potent *in vitro* mEH inhibition are: an unsubstituted amide moiety; two saturated alkyl side chains; a minimum of eight carbons in the molecule.

KEY WORDS: microsomal epoxide hydrolase inhibition, valnoctamide, valpromide analogues, valproic acid.

INTRODUCTION

Human microsmonal epoxide hydrolase (mEH) is an important enzyme responsible for detoxifying reactive epoxide intermediates formed by oxidative metabolism of xenobiotics (1–3). Detoxification involves the metabolic hydrolysis of epoxides to their corresponding and generally less reactive trans-dihydrodiols, which can be readily excreted or conjugated. *In vivo* inhibition of mEH has important toxicological implications as several studies have shown that *in vivo* mEH inhibition or low enzyme activity is associated with an increased risk of toxicity (1). Martz et al. showed that co-administration of the antiepileptic drug phenytoin and the mEH inhibitor 1,1,1-trichloropropene-2,3-oxide (TCPO) to pregnant mice, significantly increased the incidence of phenytoin-induced teratogenic effects (4). An increase in phenytoin-induced teratogenicity was also demonstrated in pregnant epileptic women that exhibited low *in vivo* mEH activity (5). The importance of mEH was also demonstrated when the mutagenicity of phenanthrene epoxide metabolites was increased in the presence of TCPO (6). Friedberg et al demonstrated the importance of mEH for arene oxide detoxification in the BHK21 cell line (7). BHK21 cells have extremely low mEH activity as opposed to BHK21-mEH/Mz1 cells (a BHK21 cell clone) which have a 60-fold higher mEH activity. In spite of the fact that both cell lines have similarly high glutathione S-transferase activity, BHK21-mEH/Mz1 cells were significantly better protected against the toxic and mutagenic effects of bezo[a]pyrene-4,5-oxide than the parental BHK21 cells due to the expression of mEH.

Pisani et al. demonstrated that the antiepileptic drug valproic acid (VPA) and its corresponding amide derivative valpromide (VPD), elevated plasma levels of carbamazepine-10,11-epoxide (CBZ-E) in carbamazepine-treated epileptic patients (8). The authors proposed that this *in vivo* interaction might occur due to inhibition of mEH, the key enzyme responsible for hydrolyzing CBZ-E to carbamazepine-10, 11-transdihydrodiol. Subsequently, Kerr et al. demonstrated that both VPA and VPD were *in vivo* and *in vitro* mEH inhibitors (9). The *in vitro* inhibitory constant (Ki) values obtained with CBZ-E and styrene oxide (STO) as substrates were 550 and 261 μM, respectively for VPA and 5.35 and 0.57 μM for VPD. In addition, valnoctamide (Nirvanil®, VCD), an anxyolitic drug available in Europe and a CNS-active chiral isomer of VPD, was also found to inhibit mEH both *in vivo* and *in vitro* with an *in vitro* median inhibitory concentration (IC_{50}) of 15.3 μM (10). In spite of VPD and VCD being more potent mEH inhibitors than VPA, mEH inhibition by VPD, VCD and VPA were achieved at clinically relevant plasma concentrations (9–11). Inhibition of mEH by valproylamide analogues was a surprising finding since most other known mEH inhibitors contain an epoxide moiety and often exert their inhibitory effect by serving as alternative substrates (1–3,12). VPA, VPD and VCD were the first compounds known to be competitive inhibitors of mEH, which were not substrates of the enzyme and in this regard they represented a new class of mEH inhibitors (9–11).

In the last decade, valproylamide analogues have been the subject of many studies conducted in order to develop new anticonvulsants, which are safer and more efficacious than the parent antiepileptic drug, VPA (13–19). Valproylamide analogues (e.g. VPD and VCD) are generally more potent anticonvulsants in animal models than VPA (15). Unlike VPA, several valproylamide analogues were found to be non-teratogenic in murine models (19–21) and in addition, they have favorable pharmacokinetic and metabolic profiles compared to their corresponding acids (15).

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Consequently, in order to explore the structural requirements for mEH inhibition by valproyl analogues, we synthesized and investigated the inhibition potency of: five straight chain aliphatic amides; ten isomers of valpromide; fifteen analogues and derivatives of valpromide; five isomers of valproic acid.

MATERIALS AND METHODS

Chemicals

(S)-(+)-styrene oxide and butyramide were purchased from Fluka, Buchs, Switzerland. (S)-(+)-1-phenyl-1,2-ethanediol, diphenylacetic acid, VPA and decanamide were purchased from Sigma, St. Louis, MO, USA. Pentanamide was purchased from Eastman Kodak Company, Rochester, NY, USA. Hexanamide, cyclohexane carboxamide and benzamide were purchased from Aldrich Chemical Company, Milwakee, WI, USA. Carbamazepine-10,11-cis-dihydrodiol was supplied by Ciba-Geigy Ltd., Switzerland. Felbamate was supplied by Carter-Wallace Inc., Cranbury, NJ, USA. Valpromide and rac-VCD were supplied by Sanofi Pharma International, Paris, France. *tert*-Butyl methyl ether, ethyl acetate, acetonitrile, methanol and n-hexane were purchased from Fisher Scientific, Fairlawn, NJ, USA.

Synthesis of 2-ethyl-2,2-dipropyl acetamide (EDD): acid catalyzed esterification of VPA yielded 2-ethyl valproate which was treated with lithium diisopropylamide and alkylated with ethyl bromide. Hydrolysis of the ester product with potassium hydroxide provided the corresponding acid. The acid product was treated with thionyl chloride, followed by ammonium hydroxide to give the final product, EDD.

Synthesis of 2,2-diphenyl acetamide (DPD) and 2-phenyl-2-propyl acetamide (PPD): the corresponding acids of DPD and PPD were prepared by the malonic acid synthesis from diethyl malonate (22). Subsequently, the acids were treated with thionyl chloride followed by ammonium hydroxide to give their corresponding amide products.

Additional synthetic procedures appear in the following references: TMCD and M-TMCD (17); VGD and VGA (16); Octanamide, DID, DAD, VCA and DBD (14); rac-PID, EBD, APD and MPD (13); VHA, HEV and M-VPD (18); (R)-PID, (S)-PID, (R)-PIA and (S)-PIA (21); (2S,3S)-VCA, (2R,3S)-VCD and (2S,3S)-VCD (23).

Preparation of Microsomes

Whole human livers were obtained from organ transplant donors at the University of Washington Hospital and Harborview Medical Center, Seattle, Washington, USA. Microsomes were prepared from livers by the previously published procedure (24). The protein concentration in the final microsomal preparation was 10–20 mg/ml.

Inhibition Assay

Inhibition studies of mEH were performed in microsomal suspensions prepared by diluting microsomes in 0.1 M sodium phosphate buffer (pH 7.4). Prior to substrate addition, microsomes were incubated in the presence or absence of the inhibitor in deactivated (Prosil-28, PCR Inc., Gainsville, FL, USA) screw top tubes for 1.5 min in a 37°C shaking water bath. The substrate, STO, was added as a 15 μl solution in acetonitrile such that its final concentration was 25 μM, which equals the Km (25). The reaction time was 10 min, and was terminated by addition of 3 ml n-hexane, and placing the tubes on ice.

The final incubation volume was 3 ml and the protein concentration was 5.34 μg/ml. The final concentration of organic solvents (methanol and/or acetonitrile) in the incubations did not exceed 1%. The background (non-enzymatic) hydrolysis of STO was measured by replacing viable microsomal protein with boiled protein. VPD at 5 μM (equal to its IC_{50}) was used as a positive control. TMCD and M-TMCD were evaluated in a similar experimental model except for the use of CBZ-E (250 μM) as substrate, protein concentration was 1 mg/ml and the total incubation volume was 1.5 ml.

Sample Preparation for Chromatography

Following termination of the enzymatic reaction, 100 μl of the internal standard solution (felbamate 16 μg/ml or carbamazepine-10,11-cis-dihydrodiol 5 μg/ml in methanol) was added. The tubes were vortexed (30 sec) and centrifuged at 3000 g (10 min). The aqueous layer was transferred to a new set of deactivated screw-top tubes and 7 ml of *tert*-butyl methyl ether or ethyl acetate were added. The tubes were shaken for 20 min and centrifuged at 3000 g (10 min). The organic phase was transferred to a new set of tubes and dried under nitrogen. The dry residue was reconstituted in 100 μl of the mobile phase, of which 40–80 μl were injected into the HPLC apparatus. The HPLC apparatus and chromatographic conditions are described elsewhere (9,11,25).

Data Analysis

All PED and CBZ-diol formation rates were corrected for non-enzymatic hydrolysis and the maximal enzymatic velocity (absence of inhibitor). Inhibition was evaluated at 3–5 different inhibitor concentrations in triplicates. The remaining activity of the enzyme was plotted vs. the inhibitor concentration and fitted to an exponential inhibitory model (BMDP Statistical Software Inc. Los Angeles, CA, USA). From this fit the IC_{50} and the standard deviation (SD) of the IC_{50} were obtained.

Octanol-Water Partitioning

The partitioning between octanol and water (log P) of the five aliphatic amides is a measure of their lipophilicity. Log P of pentanamide (0.29) and hexanamide (0.79) was taken from Hansch et al (26). Log P for all other straight chain aliphatic amides in Table I was calculated by increasing log P by 0.5 for each added methylene group (27).

RESULTS

In vitro inhibition of mEH by various valproyl derivatives and analogues was examined in microsomal incubations with STO as the substrate. At 25 μM STO concentration and a protein concentration of 5.34 μg/ml, the enzymatic hydrolysis of STO was linear for up to 30 min, therefore a 10-min reaction

was used in all experiments. Under these experimental conditions, substrate depletion did not exceed 7%, whereas the non-enzymatic background hydrolysis was less than 8%. Quality control samples for PED concentrations in the HPLC assay provided accuracy (relative error) of 0.6–4.0% and reproducibility (coefficient of variation) of 1.5–6.8%.

IC_{50} values of all the valproyl derivatives and analogues that were evaluated in this study are presented in Table I, whereas the basic chemical structure is presented in Fig. 1. Butyramide, pentanamide and hexanamide were weak mEH inhibitors with IC_{50} values exceeding 300 μM. Inhibition became significant with octanamide and decanamide where IC_{50} values were below 30μM. VPD and its isomers (MPD, EBD, DID, PID and VCD) had IC_{50} values between 5.1 and 16.2 μM. Inhibition properties of the valproyl hydroxamate derivatives VHA, M-VPD and HEV were distinctly different one from the other. The inhibition of VHA ranged from 91.9% at 102 μM to 99.0% at 1020 μM. M-VPD had an IC_{50} of 1237 μM, whereas HEV was not inhibitory up to 1040 μM. With STO as substrate, M-TMCD caused up to 148% increase in

Table I. Microsomal Epoxide Hydrolase Inhibitory Potencies of Valproyl Derivatives and Analogues.

Compound	Substituents[a]				IC_{50} (μM)[b]
	R_1	R_2	R_3	R_4	
Butyramide	C_2H_5	H	H	NH_2	>1000[c]
Pentanamide	C_3H_7	H	H	NH_2	985 ± 68[d]
Hexanamide	C_4H_9	H	H	NH_2	320 ± 19[e]
Octanamide	C_6H_{13}	H	H	NH_2	25.3 ± 1.3[f]
Decanamide	C_8H_{17}	H	H	NH_2	7.0 ± 0.6[g]
2,2-dipropyl acetamide (valpromide, VPD)	C_3H_7	C_3H_7	H	NH_2	5.1 ± 0.5
2-Methyl-2-pentyl acetamide (MPD)	CH_3	C_5H_{11}	H	NH_2	9.2 ± 0.5
2-Ethyl-2-butyl acetamide (EBD)	C_2H_5	C_4H_9	H	NH_2	13.0 ± 0.7
2,2-Diisopropyl acetamide (DID)	i-C_3H_7	i-C_3H_7	H	NH_2	16.2 ± 0.6
2-Propyl-2-isopropyl acetamide (rac-PID)	C_3H_7	i-C_3H_7	H	NH_2	9.3 ± 0.6[h]
(R)-2-Propyl-2-isopropyl acetamide ((R)-PID)	C_3H_7	i-C_3H_7	H	NH_2	11.8 ± 0.8[h]
(S)-2-Propyl-2-isopropyl acetamide ((S)-PID)	C_3H_7	i-C_3H_7	H	NH_2	8.5 ± 0.6[h]
2-Ethyl-3-methyl valeramide (valnoctamide, rac-VCD)	C_2H_5	sec-C_4H_9	H	NH_2	11.6 ± 1.6
(2R,3S)-2-Ethyl-3-methyl valeramide ((2R,3S)-VCD)	C_2H_5	sec-C_4H_9	H	NH_2	9.6 ± 1.1
(2S,3S)-2-Ethyl-3-methyl valeramide ((2S,3S)-VCD)	C_2H_5	sec-C_4H_9	H	NH_2	13.5 ± 1.3
Valproyl hydroxamic acid (VHA)	C_3H_7	C_3H_7	H	NHOH	—[i]
N-Methoxy valpromide (M-VPD)	C_3H_7	C_3H_7	H	$NHOCH_3$	1237 ± 114
Hydroxyethyl valpromide (HEV)	C_3H_7	C_3H_7	H	NHC_2H_4OH	None[j]
2-Allyl-2-propyl acetamide (APD)	C_3H_5	C_3H_7	H	NH_2	14.3 ± 0.7
2,2-Diallyl acetamide (DAD)	C_3H_5	C_3H_5	H	NH_2	43.9 ± 2.2
2,2-Dimethyl-2-butyl acetamide (DBD)	CH_3	CH_3	C_4H_9	NH_2	29.8 ± 1.1
2-Ethyl-2,2-dipropyl acetamide (EDD)	C_2H_5	C_3H_7	C_3H_7	NH_2	3.9 ± 0.4
2,2-Diphenyl acetamide (DPD)	C_6H_5	C_6H_5	H	NH_2	19.0 ± 0.9
2-Phenyl-2-propyl acetamide (PPD)	C_6H_5	C_3H_7	H	NH_2	22.4 ± 1.5
Cyclohexane carboxamide (CCD)	cyclohexane		H	NH_2	362 ± 17
Benzamide	C_6H_5			NH_2	173 ± 12
2,2,3,3-Tetramethyl cyclopropyl carboxamide (TMCD)	tetramethyl cyclopropyl		H	NH_2	159 ± 14[k]
N-Methyl-2,2,3,3-tetramethyl cyclopropyl carboxamide (M-TMCD)	tetramethyl cyclopropyl		H	$NHCH_3$	—[l]
Valproyl glycinamide (VGD)	C_3H_7	C_3H_7	H	$NHCH_2CONH_2$	2770 ± 690
Valproyl glycine (VGA)	C_3H_7	C_3H_7	H	$NHCH_2COOH$	—[m]
2,2-Dipropyl acetic acid (valproic acid, VPA)	C_3H_7	C_3H_7	H	OH	788 ± 22[n]
(R)-2-Propyl-2-isopropyl acetic acid ((R)-PIA)	C_3H_7	i-C_3H_7	H	OH	784 ± 108
(S)-2-Propyl-2-isopropyl acetic acid ((S)-PIA)	C_3H_7	i-C_3H_7	H	OH	681 ± 191
2-Ethyl-3-methyl valeric acid (valnoctic acid, rac-VCA)	C_2H_5	sec-C_4H_9	H	OH	2112 ± 404
(2S,3S)-2-Ethyl-3-methyl valeric acid((2S,3S)-VCA)	C_2H_5	sec-C_4H_9	H	OH	1643 ± 254

[a] Molecular structure presented in Figure 1.
[b] Mean ± SD.
[c] Butyramide log P: −0.21.
[d] Pentanamide log P: 0.29.
[e] Hexanamide log P: 0.79.
[f] Octanamide log P: 1.79.
[g] Decanamide log P: 2.79.
[h] Rac-PID, (R)-PID and (S)-PID: Data from reference 19.
[i] VHA: 91.9, 98.3 and 99.0% inhibition at 102, 510 and 1020 μM.
[j] HEV: no inhibition observed up to 1040 μM.
[k] TMCD: data obtained from experiments with CBZ-E (250 μM) as substrate.
[l] M-TMCD: Activation and inhibition with STO and CBZ-E as substrates, respectively. See Results section for details.
[m] VGA: 10% inhibition was observed at 1500 μM.
[n] VPA: data from reference 11.

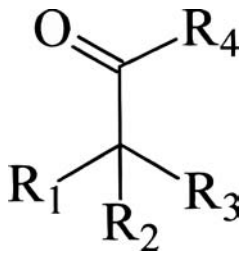

Fig. 1. The general chemical structure of the various amide and acid analogues of valproic acid presented in Table 1.

mEH activity at concentrations up to 650 μM. When M-TMCD was evaluated with CBZ-E as the substrate, it caused 13.5 and 18.9% inhibition at 325 and 650 μM, respectively, which indicated an approximate IC_{50} of 2682 μM. VGD (TV 1901), a new antiepileptic drug currently in phase II clinical trials caused 30% inhibition at 1000 μM, which gives an estimated IC_{50} of 2770 μM. VGA (TV 1900), the major inactive metabolite of VGD, caused 10% inhibition at 1500 μM. (R)-PIA had an IC_{50} of 784 μM and (S)-PIA an IC_{50} of 681 μM. Racemic VCA had an IC_{50} of 2112 μM and (2S,3S)-VCA, one of its four stereoisomers, had an IC_{50} of 1643 μM.

DISCUSSION

Structural requirements for mEH inhibition were explored in a series of 35 compounds most of which are structurally related to VPA and VPD. As an initial screen, this study did not focus on inhibition mechanism(s). In order to make comparisons of IC_{50} values more meaningful, the concentration of the substrate, STO, was kept constant at 25 μM, which equals its Km (25).

Examining inhibitory potency of straight chain aliphatic amides, a significant direct correlation ($r^2 = 0.98$) was observed between log P and IC_{50}, i.e. the more lipophilic compounds were also the more potent mEH inhibitors. Lipophilicity appears to be the single most important determinant of a saturated aliphatic amide's potency as a mEH inhibitor. Dependency of the inhibitory potency on lipophilicity is consistent with the fact that mEH substrates and inhibitors must access the enzyme through lipophilic membranes (which constitute the endoplasmatic reticulum), and the existence of a hydrophobic pocket in the enzyme's catalytic site, as proposed by Robbins et al. (28). These results are also in agreement with observations made by Dent et al. (12) and Mahdalou and Hammock (29) that emphasized the importance of a minimum degree of lipophilicity for meaningful mEH inhibition by epoxide derivatives.

All saturated and branched eight-carbon acetamides, which are structural isomers of VPD were potent mEH inhibitors with IC_{50} values between 5.1–16.2 μM. Thus, there is an apparent increase in inhibition potency compared to octanamide, their straight chain isomer. In addition the inhibitory potency of the aromatic amides DPD and PPD was also comparable to that of the aforementioned VPD isomers. These observations suggest the existence of hydrophobic binding sites on either side of the amide-binding site, which is in agreement with the findings of Mullin and Hammock regarding inhibition of cytosolic epoxide hydrolase by chalcone oxides (30).

PID and VCD are CNS-active chiral isomers of VPD, containing one and two stereogenic centers, respectively. The inhibitory potencies of (2S,3S)-VCD and (2R,3S-VCD), were not appreciably different one from the other, similar to the findings with (R)-PID and (S)-PID (19). As expected, IC_{50} values of rac-PID and rac-VCD were consistent with the values of the single stereoisomers, i.e. IC_{50} of the racemate was in between the values of the individual stereoisomers. Unlike PID enantiomers which were potent mEH inhibitors, both (R)-PIA and (S)-PIA were weak inhibitors, however no stereoselectivity was observed as well. These findings suggest that the absolute configuration of the presently studied valproylamide analogues does not have a significant influence on mEH inhibition thereby reflecting a relative insensitivity of the enzyme's active site/inhibition mechanism to the absolute configuration of the inhibitor.

VHA, the hydroxamic acid derivative of VPA was found to be a potent mEH inhibitor, however, experiments with VHA were carried out at concentrations that were several fold higher than the expected IC_{50} value. As a consequence we could not assume that VHA acts as a competitive inhibitor, and therefore, we could not provide an IC_{50} estimate that will be both accurate and valid. It is therefore clear that a comprehensive characterization of VHA's inhibitory potency and mechanism of inhibition will require additional complementary studies.

The effect of conformational flexibility on mEH inhibition was assessed with unsaturated and cyclic valproylamides. The mono-unsaturated VPD derivative, APD exhibited a three-fold decrease in inhibition potency compared to VPD, whereas DAD the di-unsaturated derivative exhibited a three-fold decrease in inhibitory potency compared to APD. CCD is a cyclic heptanamide and a weak mEH inhibitor, whereas the predicted IC_{50} of heptanamide is about three-fold lower (111 μM). TMCD, a CNS-active analogue of VPD with limited conformational orientations, is a relatively weak mEH inhibitor as well. These findings suggest that reduced conformational flexibility leads to decreased ability of the amide inhibitor to bind to the appropriate inhibitory region of the enzyme.

Experiments with M-TMCD and STO as substrate indicated an apparent activation of mEH-mediated STO hydrolysis, however when CBZ-E was used as the substrate, M-TMCD was found to be a weak inhibitor. *In vitro* activation of mEH is known to occur with several compounds (1,2), among them is the ethyl ester of VPA (28). The mechanism of activation is yet not understood, however it is assumed that binding to an allosteric domain of the enzyme enhances its catalytic activity. In addition, the phenomenon of a compound being an activator and inhibitor of mEH depending on the substrate used in the experiment was previously reported by Seidegard et al. (31). In that study, clotrimazole enhanced mEH-mediated STO hydrolysis up to eight-fold, thus being a potent *in vitro* activator, whereas in the presence of androstene oxide as the substrate it inhibited the activity of mEH. Substituting the amide moiety of VPD reduced and abolished inhibitory potency as was shown with M-VPD and HEV, respectively. Similarly, Kerr and Levy showed that substituting one amide hydrogen of 3-bromocinnamamide with an ethyl or propyl group, reduced inhibition potency 4–13 fold (11). These findings suggest that N-substituted amides will generally have reduced inhibition potency compared to their corresponding unsubstituted amides.

VGD, the conjugate product of glycinamide and VPA, contains an unsubstituted amide moiety and was found to be

a weak mEH inhibitor. VGA, the major metabolite of VGD in humans and animals (16, 32) has no amide moiety and is a very weak mEH inhibitor as well. These findings suggest that compounds which posses two carbonyl groups, as with VGD and VGA, are not expected to be potent mEH inhibitors. Acid isomers of VPA were also weak mEH inhibitors. However, unlike in the case of VPA where *in vivo* inhibition is clinically relevant due to VPA's *in vivo* concentrations (8, 9), inhibition by PIA and VCA should not have clinical importance since VCA is only a minor metabolite of VCD in humans and animals (15,33) and PIA is not a metabolite of PID in several animal species (13,15,19).

The current *in vitro* study suggests that a wide variety of unsubstituted amides are potential inhibitors of mEH. Potent mEH inhibition is associated with unsubstituted amides containing at least eight carbon atoms and two saturated aliphatic alkyl chains attached at the α position to the carbonyl. The absolute configuration of two chiral valproylamides had little effect on inhibitory potency. The limited structural requirements for potent mEH inhibition by valproylamides, suggest that human exposure to potent mEH inhibitors may be more prevalent than previously suspected. In addition the present study suggests that drug candidates with amide moieties should be evaluated for potential inhibition of mEH.

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