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Original paper

Synthesis and anticonvulsant activity of enaminones Part 7. Synthesis and anticonvulsant evaluation of ethyl 4-[(substituted phenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylates and their corresponding 5-methylcyclohex-2-enone derivatives

Natalie D. Eddington [a], Donna S. Cox [a,1], Manoj Khurana [a], Noha N. Salama [a], James P. Stables [b], Sylvia J. Harrison [c], Abraham Negussie [c], Robert S. Taylor [c], Uy Q. Tran [c], Jacqueline A. Moore [c], Judith C. Barrow [d], K.R. Scott [c,*]

[a] *Department of Pharmaceutical Sciences, Pharmacokinetics Biopharmaceutics Laboratory, School of Pharmacy, University of Maryland at Baltimore, Baltimore, MD 21201-4403, USA*
[b] *Epilepsy Branch, Division of Convulsive, Developmental and Neuromuscular Disorders, National Institute of Neurological Disorders and Stroke, Bethesda, MD 20892, USA*
[c] *Department of Pharmaceutical Sciences, School of Pharmacy, Howard University, Washington, DC 20059, USA*
[d] *Department of Chemical Engineering, College of Engineering, Architecture and Computer Science, Howard University, Washington, DC 20059, USA*

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Abstract

Further investigation of the potential anticonvulsant activity of the enaminones was attempted to discern the possible role of metabolites as the active/co-active entities of the esters of the enaminones. A series of 5-methyl-2-cyclohexene enaminones, the hypothesised metabolites corresponding to a sequence of active and inactive esters were synthesised and evaluated for anticonvulsant activity. With two exceptions, ethyl 4-[(4-cyanophenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate (**1k**), and 3-[*N*-(4-cyanophenyl)amino]-5-methyl-2-cyclohexenone (**3g**), and ethyl 4-(phenylamino)-6-methyl-2-cyclohexenone (**1n**), and 3-*N*-(phenylamino)-5-methyl-2-cyclohexenone (**3j**), anticonvulsant screening data were parallel, with the ester and their putative decarboxylated analogue displaying similar activity. The most active analogue evaluated in this series, ethyl 4-[(4-chlorophenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate (**1e**), which displayed an ED_{50} of 16.7 mg kg^{-1} and a TD_{50} of 110.7 mg kg^{-1} (protective index, $PI = TD_{50}/ED_{50} = 6.6$) in the maximal electroshock seizure (MES) test in mice and an ED_{50} of 3.0 mg kg^{-1} and a $TD_{50} > 250$ mg kg^{-1} ($PI > 83.3$) in rats in the same evaluation, making this compound the most potent enaminone emanating from our laboratories. Pharmacokinetic evaluation of compound **1e** in rats using LC/MS analysis unequivocally provides evidence that this compound is converted into the decarboxylated analogue **3a** in the brain and the urine.

Keywords: Enaminones; Maximal electroshock seizure test; Anticonvulsant activity; Structure–activity relationship; LC/MS analysis

Abbreviations: MES, maximal electroshock seizure; TD_{50}, toxic dose for 50% of test animals; ED_{50}, effective dose for 50% of test animals; ip, intraperitoneal; PI, protective index, TD_{50}/ED_{50}; scPTZ, subcutaneous pentylenetetrazol; Tox, neurologic toxicity; ADD, Antiepileptic Drug Development; NINDS, National Institutes of Neurological Disorders and Stroke; TTE, threshold tonic extension; AUC, area under the curve; Clog *P*, calculated log *P*; [^{3}H]BTX-B, [^{3}H]batrachotoxinin A 20 α-benzoate; IC_{50}, 50% in vitro inhibition of binding of [^{3}H]BTX-B to sodium channels in rat brain synaptoneurosomes; m.p., melting point; b.p., boiling point; EtOAc, ethyl acetate; 2-PrOH, 2-propanol.

* Corresponding author. Present address: Department of Pharmaceutical Sciences, College of Pharmacy, Howard University, Washington, DC 20059, USA.
E-mail address: kscott@fac.howard.edu (K.R. Scott).
[1] Present address: DuPont Pharmaceuticals, Drug Metabolism and Pharmacokinetics Section, S112/36, 1090 Elkton Road, P.O. Box 30, Newark, DE 19713, USA.

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1. Introduction

Despite optimal use of available antiepileptic drugs, many patients with epilepsy fail to experience seizure control and other do so only at the expense of significant toxic side effects. Estimates suggest that available medication controls the seizures in only 50% of patients or decreases the incidence in only 75% of patients [1]. In a continuing study of potential enaminone anticonvulsants [2–17], we have postulated a biotransformation pathway in which the carboalkoxy substituent **1** (Fig. 1) was a bioactive intermediate, and that a two step reaction involving deesterification **2** and decarboxylation would occur, producing a second active enaminone metabolite **3** [8]. Specifically, compound **1**, being more lipophilic than enaminone **3**, would more readily penetrate the blood–brain barrier to exert an initial anticonvulsant effect. Subsequently, the ester **1**, would react with non-specific esterases to form the unstable β-keto acid intermediate **2**, which would subsequently decarboxylate to form the corresponding enaminone **3**. Although this hypothesis was previously demonstrated in vitro with methyl 4-[(4-chlorophenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate (**1a**, $R = CH_3$, $R^1 = 4\text{-Cl}$) [8], it was not apparent in vivo [9]. In the initial study, only the corresponding $R^1 = 4\text{-Cl}$ ketone (**3a**, Table 1), was synthesised [8]. However, this current study includes an extensive synthesis and anticonvulsant evaluation of the potential 5-methyl metabolites (**3a**–**3t**, Table 1) emanating for the esters (**1e**–**1x**, Table 1). In addition, we herein report the results of the LC/MS analysis that verified the biotransformation of ethyl 4-[(4-chlorophenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate (**1e**) to the 5-methyl ketone (**3a**), in the brain and urine of rats.

2. Chemistry

2.1. Synthesis

The synthesis of the enaminones that appear in Table 1 is indicated in Fig. 2. The requisite β-hydroxy keto esters (**4a**, $R = C_2H_5$, or **4b**, $R = C(CH_3)_3$), were prepared as previously reported [2,4,5,8–11,16] and were refluxed with appropriate aniline derivatives under standard conditions. Enaminones (**3a**–**3t**, Table 1) were prepared from 5-methylcyclohexane-1,3-dione (**5**), formed by the decarboxylation of 4-carbo-*tert*-butoxy-5-methylcyclohexane-1,3-dione (**4b**, $R = C(CH_3)_3$) [18]. These reactions are shown in Fig. 2. As previously reported by Friary [18] and by us [6], the stereochemistry of the esters (**1e**–**1x**, Table 1) indicated that they exist as *trans* racemates. The NMR analyses of the enaminones at 300 MHz were consistent with their assigned structures.

3. Pharmacology

3.1. Preliminary pharmacological testing

The Antiepileptic Drug Development (ADD) Program, Epilepsy Branch, Neurological Disorders Program, National Institute of Neurological Disorders and Stroke (NINDS) have provided preliminary pharmacological testing of the reported compounds. These testing procedures have been described [19–21]. Phase I studies of the reported enaminones involved three tests: maximal electroshock seizure (MES), subcutaneous pentylenetetrazol (scPTZ), and neurologic toxicity (Tox). The Anticonvulsant Screening Project (ASP) classifications are as follows: class 1 = activity at 100 mg kg^{-1} or less; class 2 = activity >100 mg kg^{-1}, but less than 300 mg kg^{-1}; class 3 = no activity at doses up to and including 300 mg kg^{-1}. As previously reported [2,3,7–9], class 1 or 2 active compounds in the Phase I evaluation were advanced to Phase II mice quantitation for ED_{50} and TD_{50} evaluation. Further, to differentiate the results between distinct rodent species, the active analogues were subsequently evaluated for oral (po) activity (Phase VIA) in the rat. Data for these compounds is shown in Table 2. Quantitation of the Phase I intraperitoneal (ip) and/or po activity (Phase VIB) is provided, where appropriate, as well as the protective index ($PI = TD_{50}/ED_{50}$). In one case, ethyl 4-[(3-iodophenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate (**1q**), an initially inactive compound in Phase I was

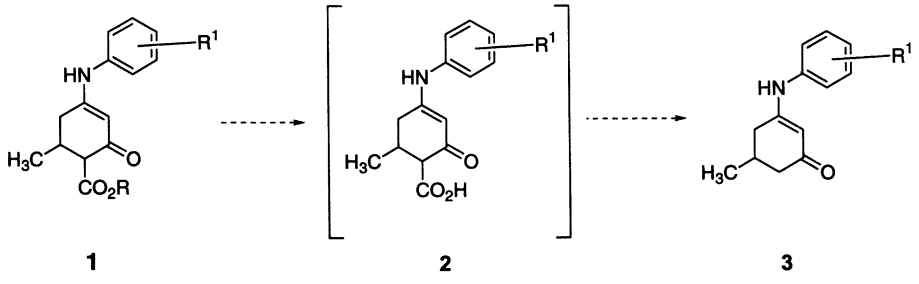
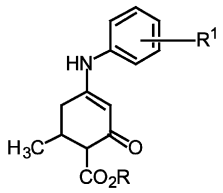


Fig. 1.

Table 1
Enaminone esters, **1**, and enaminones, **3** [a]



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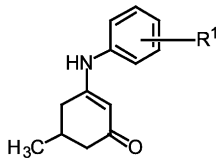

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Compound **1** ($R = C_2H_5$)				Compound **3**			
Compound	R^1	Clog *P* [b]	M.p. (°C)	Compound	R^1	Clog *P* [b]	M.p. (°C)
1e	4-Cl	3.46	161–163 [c]	**3a**	4-Cl	3.07	198–199.5 [c]
1f	4-Br	3.61	151–154 [c]	**3b**	4-Br	3.22	213–215 [d]
1g	4-I	3.87	160.5–162.5 [e]	**3c**	4-I	3.48	225–227 [d]
1h	4-F	2.89	150–151.5 [c]	**3d**	4-F	2.50	185–187 [f]
1i	4-CF_3	3.86	184.5–186 [c]	**3e**	4-CF_3	3.47	205–206 [d]
1j	4-OCF_3	3.66	162–164 [c]	**3f**	4-OCF_3	3.27	174–175 [f]
1k	4-CN	2.58	190–193 [e]	**3g**	4-CN	2.19	234–236 [f]
1l	4-NO_2	−0.84	173–175 [c]	**3h**	4-NO_2	−1.24	208–209 [f]
1m	4-CH_3	2.95	134.5–135.5 [c]	**3i**	4-CH_3	2.55	194–195 [d]
1n	H	2.45	155–158 [c]	**3j**	H	2.06	155 (soften); 158–160 [f]
1o	3-Cl	3.46	137–138 [e]	**3k**	3-Cl	3.07	177–179 [f]
1p	3-Br	3.61	135–137 [e]	**3l**	3-Br	3.22	172–174 [f]
1q	3-I	3.87	120–124 [e]	**3m**	3-I	3.48	185–187 [f]
1r	3-F	2.89	138–140 [e]	**3n**	3-F	2.50	163–165 [f]
1s	3-CF_3	3.86	164–166 [e]	**3o**	3-CF_3	3.47	179–180.5 [f]
1t	3-OCF_3	3.66	151–152 [e]	**3p**	3-OCF_3	3.27	156–160 [f]
1u	3-CN	2.58	165–167 [e]	**3q**	3-CN	2.19	175.5–177 [f]
1v	3-NO_2	−0.84	155–157 [e]	**3r**	3-NO_2	−1.24	187–189 [f]
1w	3-CH_3	2.95	125–127 [e]	**3s**	3-CH_3	2.55	147–148.5 [f]
1x	3-OCH_3	2.55	110–113 [e]	**3t**	3-OCH_3	2.16	140–142 [f]

[a] The infrared and ^{1}H-NMR spectra (300 MHz) were consistent with assigned structures. All compounds gave satisfactory C, H, N, and halogen (when required) analyses (±0.4%). Recrystallisation solvents as indicated.
[b] Calculated from Ref. [24].
[c] Ref. [8].
[d] 2-Propanol.
[e] Ref. [9].
[f] Ethyl acetate.

subsequently shown to be active in the threshold tonic extension (TTE) test [22]. The TTE evaluation was also employed in the evaluation of ethyl 4-[(3-cyanophenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate (**1u**); however, this compound remained inactive.

3.2. Metabolism and pharmacokinetics

Ethyl 4-[(4-chlorophenyl)amino]-6-methyl-2-oxo-3-cyclohexene-1-carboxylate (**1e**) was found to be the most promising enaminone analogue in the aniline series in both the rat and mouse models during preliminary pharmacological evaluation providing an ED_{50} 16.7 mg kg^{-1} ip in mice, ED_{50} 3.0 mg kg^{-1} po in rats, almost twice as effective than previously for the reported prototype analogue, methyl 4-[(4-chlorophenyl)amino]-6-methyl-2-oxo-3-cyclohexene-1-carboxylate (**1a**, R = CH_3, R^1 = 4-Cl; ED_{50} 26.2 mg kg^{-1} ip in mice, ED_{50} 5.9 mg kg^{-1} po in rats) [2,3]. Based on the promising pharmacological results obtained with compound **1e**, the present studies were conducted to understand its distribution in the target organ, the brain, and its metabolism and pharmacokinetics in rats. Based on the pharmacological activity data obtained with ip administration, and to verify the hypothesis delineated in Fig. 1, a disposition study was carried out via the same route of administration in rats. Therefore, this data will provide relevant information on the uptake of the target compound **1e**, by the target organ, its metabolism and pharmacokinetic profile and aid in further development of the target compound as a candidate drug. Preliminary in vitro studies on target compound **1e** were performed. Treatment of compound **1e** with porcine liver esterase (EC 3.1.1.1) at pH 7.4 at

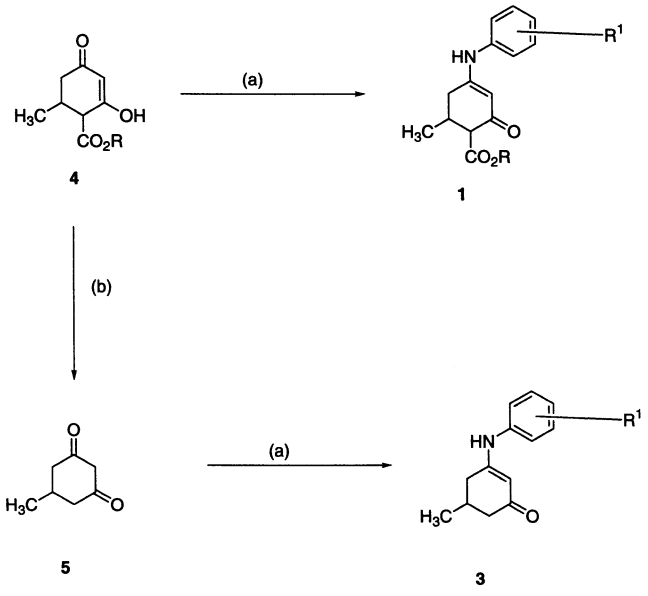

Fig. 2. Conditions: (a) Δ, amine; (b) R = $C(CH_3)_3$, H_2SO_4, Δ.

37 °C and monitoring by ultraviolet analysis resulted in a 30% conversion of compound **1e** to enaminone **3a**, in 8 h. This was consistent with previous studies in our laboratories with methyl 4-[(4-chlorophenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate (**1a**, R = CH_3, R^1 = 4-Cl) which was also converted into the identical enaminone **3a** and was metabolised to the extent of 20% over 24 h [8]. As noted by us earlier, the enaminones are quite stable at pH 7.0 and 7.4 in the absence of esterase [8]. Edafiogho et al., in a stability-indicating assay of methyl 4-[(4-bromophenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate (**1b**, R = CH_3, R^1 = 4-Br) noted that the compound was stable in both 0.1 M NaOH as well as in neutral media, but decomposed rapidly in acidic (0.1 M HCl) media [23].

Data on the pharmacokinetic parameters of ethyl 4-[(4-chlorophenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate (**1e**) following a single ip dose (10 mg kg^{-1}) in rats is shown in Table 3, data on mass spectra and daughter ion spectra of compound **1e** is shown in Table 4, the brain and plasma concentrations of compound **1e** following a single ip dose (10 mg kg^{-1}) is shown in Fig. 3, a representative mass spectra of compound **1e** and its metabolites is shown in Figs. 4 and 5 provides the major metabolic pathways of compound **1e** in rats.

3.3. Sodium channel binding

Ethyl 4-[(4-chlorophenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate (**1e**) was evaluated in a synaptoneurosomal preparation in N1E-115 neuroblastoma cells [25–29]. This assay was similar to literature procedure [29] and measured the ability of the test compound to inhibit the specific binding of [^{3}H]batra-

Table 2
Anticonvulsant evaluation of enaminone esters, **1**, enaminones, **3** in mice and rats

Compound	Quadrant [a]	ASP class [b]	Anticonvulsant results [c]
Esters			
1e	+σ, +π	1	Phase II: ED_{50} 16.7, TD_{50} 110.7, PI 6.6, TPE 0.25 Phase VIB: ED_{50} 3.0, TD_{50} > 250, PI > 83.3, TPE 1.0
1f	+σ, +π	1	Phase II: ED_{50} 7.9, TD_{50} 86.9, PI 10.9, TPE 0.25
1g	+σ, +π	1	Phase VIB: ED_{50} 6.6, TD_{50} > 220, PI > 33.3, TPE 0.5
1j	+σ, +π	1	Phase II: ED_{50} 37.1, TD_{50} > 400, PI > 10.8, TPE 1.0 Phase VIB: ED_{50} 6.6, TD_{50} > 500, PI > 75.8, TPE 2.0
1k	+σ, −π	1	Phase II: ED_{50} 63.1, TD_{50} 159.5, PI 2.1, TPE 0.25
1o	+σ, +π	1	Phase VIB: ED_{50} 11.5, TD_{50} > 424, PI > 36.9, TPE 0.25
1u	+σ, −π	1 [d]	ED_{50} (rats) 7.7, TD_{50} not determined, TPE 0.25
5-Methyl ketones			
3a	+σ, +π	1	Phase II: ED_{50} 40.4, TD_{50} 93.6, PI 2.3, TPE 0.25 Phase VIB: ED_{50} 14.7, TD_{50} > 186, PI > 12.7, TPE 1.0
3b	+σ, +π	1	Phase VIB: ED_{50} 7.6, TD_{50} > 500, PI > 65.8, TPE 2.0
3c	+σ, +π	1	Phase II: ED_{50} 77.0, TD_{50} 343.6, PI 4.5, TPE 0.25 Phase VIB: ED_{50} 17.6, TD_{50} > 500, PI > 28.3, TPE 2.0
3e	+σ, +π	1	Phase VIB: ED_{50} 10.3, TD_{50} > 500, PI > 48.4, TPE 1.0
3f	+σ, +π	1	Phase VIB: ED_{50} 6.5, TD_{50} > 400, PI > 62.0, TPE 0.5
3h	+σ, +π	1	Phase VIB: ED_{50} 18.4, TD_{50} > 500, PI > 27.4, TPE 4.0
3t	+σ, +π	2	Phase VIB: ED_{50} 39.4, TD_{50} > 500, PI > 12.7, TPE 0.5

[a] Refs. [34,35].
[b] Phase I activity in mice-class 1 = activity at 100 mg kg^{-1} or <; class 2 = activity between 100 and 300 mg kg^{-1}; class 3 = no activity up to and including 300 mg kg^{-1}.
[c] All results vs. MES, Phase II in mice, Phase VIB in rats; ED_{50}, median effective dose in mg kg^{-1}; TD_{50}, median toxic dose in mg kg^{-1}; TPE, time of peak effect in hours.
[d] Result of TTE evaluation (Ref. [33]).

Table 3
Pharmacokinetic parameters of ethyl 4-[(4-chlorophenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate (**1e**) in plasma and brain values and C_{max} observed after single ip dose (10 mg kg^{-1}) in male Sprague–Dawley rats

Parameter	Plasma	Brain
C_{max} (μg L^{-1})	11 042 ± 2973	10 394 ± 1387
T_{max} (h)	0.25	0.25
$AUC_{0-\infty}$ (μg h L^{-1})	17 487	15 660
Cl F^{-1} (L h^{-1} kg^{-1})	0.57	0.63
V_d F^{-1} (L kg^{-1})	0.62	0.80
$t_{1/2}$ (h)	0.75	0.84
MRT (h)	1.9	1.9

C_{max}, mean maximum concentration; T_{max}, time to reach maximum concentration; AUC, area under the curve; Cl F^{-1}, plasma clearance; $t_{1/2}$, time required to reach 0.5 maximum concentration; MRT, mean residence time.

Table 4
ESI mass spectra and daughter ion spectra of ethyl 4-[(4-chlorophenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate (**1e**) and its metabolites excreted in rat urine

Retention time (min)	*m/z* of parent ion [M+1]	*m/z* of daughter ions [M+1]
16.5	280	262, 234
19.2	280	262, 234
27.7	236	193, 178
45.8	308	262, 234

chotoxinin A 20 α-benzoate ([^{3}H]BTX-B) to neurotoxin site 2 of the voltage-dependent sodium channel [9]. Local anesthetics, class I antiarrhythmics and class 1 anticonvulsants bind at pharmacologically relevant concentrations to a site (or sites) on the sodium channel that is allosterically linked to neurotoxin site 2, resulting in the inhibition of binding of [^{3}H]BTX-B [25–29]. It was previously shown that methyl 4-[(4-chlorophenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate (**1a**, R = CH_3, R^1 = 4-Cl) and methyl 4-[(4-trifluoromethoxyphenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate (**1c**, R = CH_3, R^1 = 4-OCF_3) were inhibitors of the specific binding of [^{3}H]BTX-B; the IC_{50} for the 4-chloro analogue **1a** at 489 μM; the 4-trifluoromethoxy analogue **1c**, at 170 μM [9]. The results of the sodium channel study on compound **1e** are shown in Table 5.

3.4. Hippocampal slice

Several reported 5-methyl ketones [4-chloro (**3a**), 4-bromo (**3b**), 4-iodo (**3c**), 4-trifluoromethyl (**3e**), 4-trifluoromethoxy (**3f**), and 4-nitro (**3h**)] were evaluated in isolated hippocampal slices [19,30–33]. This method has the advantage of recording important electrical activity under a variety of experimental conditions and tissue penetration by the drug. However, this evaluation has the disadvantages of cell viability and the lack of interactions with other neural circuits found in the intact animal [33]. The hippocampal results for these analogues are shown in Table 6. Seizures were scored according to the following criteria: stage 1 = mouth and facial clonus; stage 2 = stage 1 plus head nodding; stage 3 = stage 2 plus forelimb clonus; stage 4 = stage 3 plus rearing; stage 5 = stage 4 plus repeated rearing and falling [32].

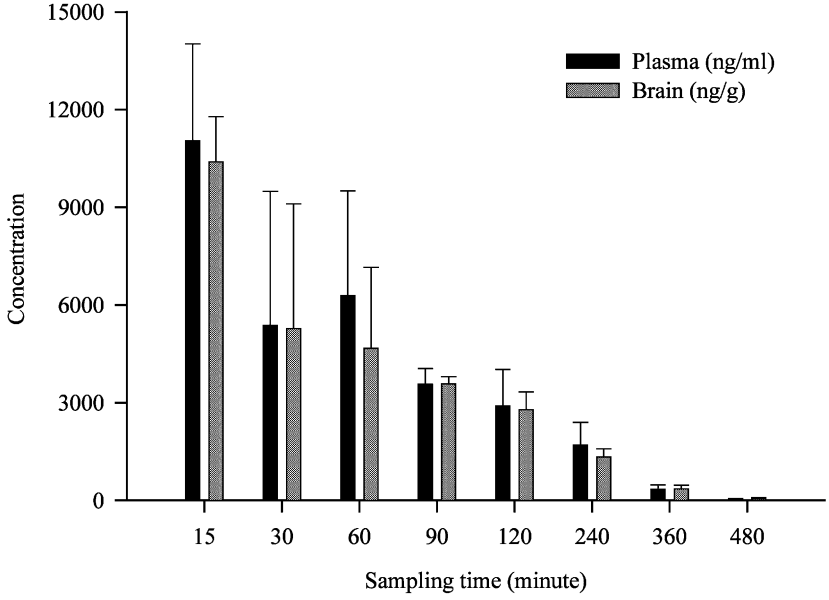

Fig. 3. Brain and plasma concentrations of ethyl 4-[(4-chlorophenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate (**1e**) after administration (10 mg kg^{-1}) to rats (mean ± SD, n = 3).

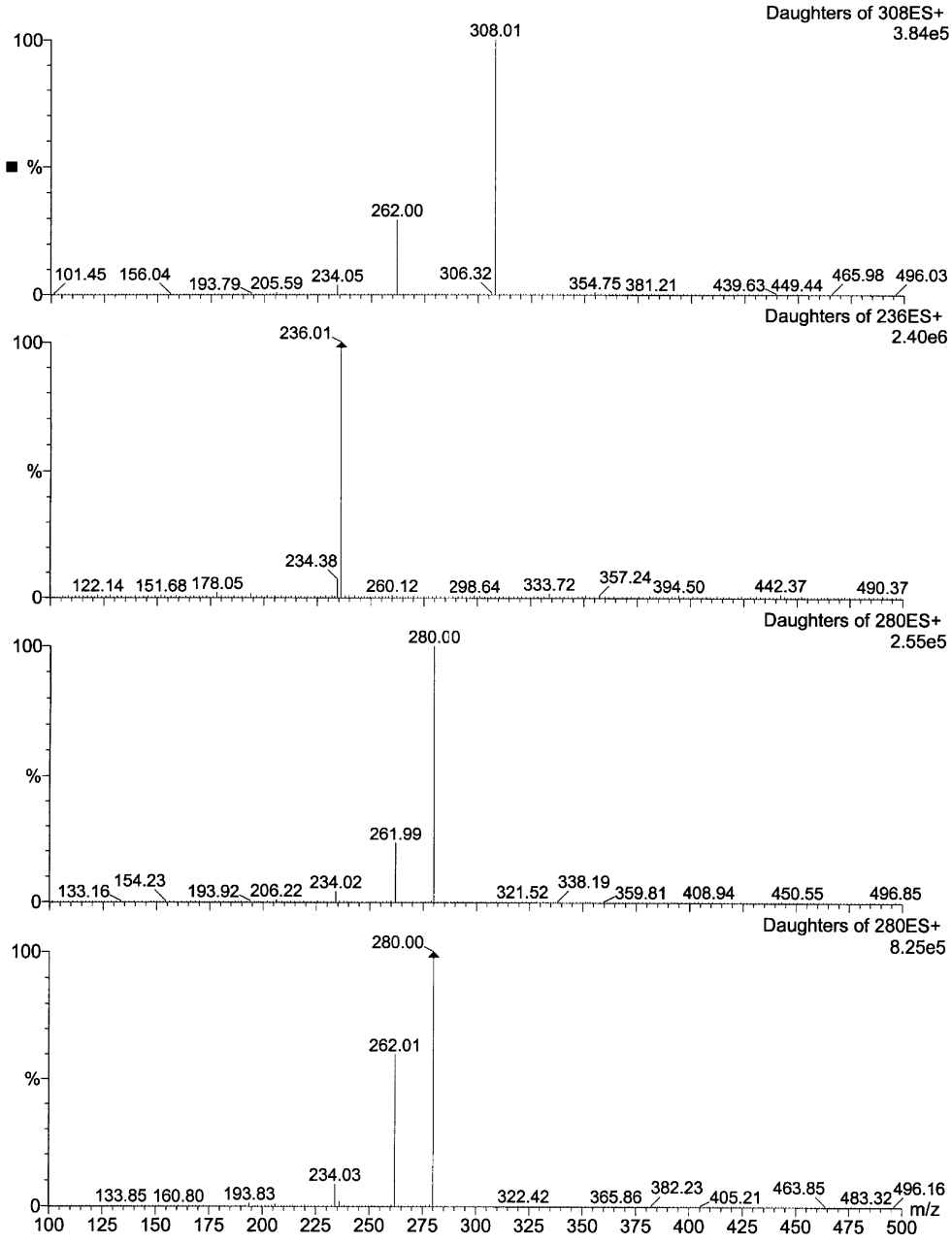

Fig. 4. Representative mass spectra of ethyl 4-[(4-chlorophenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate (**1e**) and its metabolites in rat urine analysed via LC-MS/MS.

4. Results and discussion

4.1. Structure activity relationship

The ester analogues chosen for this study provided a range of anticonvulsant activity from the highly active (class 1) [(**1e**–**1j**), (**1l**), (**1o**–**1q**), and (**1v**)], to moderately active (class 2) [(**1k**), (**1m**), (**1s**) and (**1t**)], to inactive [(**1n**), (**1r**), (**1v**) and (**1w**)]. In addition, these analogues represent the following positions in the Craig plot [38,39]: [(+σ, +π) (**1e**–**1j**), (**1l**), (**1o**–**1t**), (**1v**), and (**1x**)]; [(+σ, −π) (**1k**), and (**1u**)], [(−σ, +π) (**1m**), and (**1w**)]; and [(0σ, 0π) (**1n**)]. The activity or inactivity for compounds **3a**–**3t** should therefore parallel these data. As noted in Table 1, Clog *P* [24] data indicates that analogues **3a**–**3t** are more hydrophilic than the corresponding enaminones **1e**–**1x**. Thus, the activity or inactivity of the former enaminones **3a**–**3t** may also be attributed to this physiochemical phenomena as well as to an efflux mechanism previously reported [12,14,16]. The lipophilic nature of the enaminone ester **1** (Fig. 1), permits passive diffusion across the blood–brain barrier; however, the active efflux mechanism militates against a sustained anticonvulsant activity. Biotransfor-

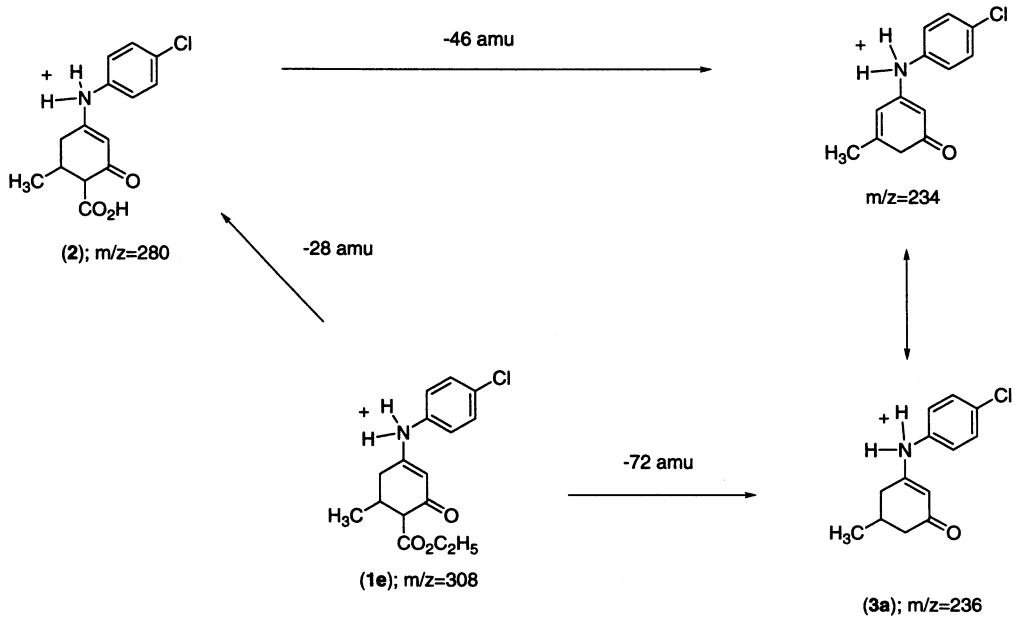

Fig. 5. Major metabolic pathways of ethyl 4-[(4-chlorophenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate (**1e**) in rats.

mation within the brain (Fig. 1), however, could produce the corresponding enaminone **3**, the more hydrophilic moiety that opposes the efflux mechanism and provides a sustained response. Experimental data with the isomeric chloro analogues illustrates this point. As noted in Fig. 6, comparing the time course of anticonvulsant effect in rats for the 4-chloro analogues: ester **1e**; and the putative metabolite **3a**. As was expected, 75% of the rats were protected by the ester **1e** at 15 min and remained the same at 30 min, increasing to a 100% maximum at 1 h, thereafter the activity declined rapidly. The more hydrophilic enaminone **3a**, however, developed its protective effect slowly, but sustained its activity for 2 h, declining slower than the more lipophilic ester **1e**. In Fig. 7, the results were more dramatic, the 3-chloro ester **1o** being immediately active at 15 min, but declined rapidly thereafter, while the corresponding enaminone **3k**, maintained significant anticonvulsant activity up to 2 h.

5-Methyl ketone analysis—an ip study in mice of the 4-chloro analogue **3a**, revealed notable toxicity at 60 mg kg^{-1} with 4/16 animals displaying neurologic deficit, at 90 (3/8), 120 (12/16) and 200 mg kg^{-1} (7/8). This factor may explain the toxic effects previously reported with the carbomethoxy analogue (**1a**, $R = CH_3$, $R^1 = 4\text{-Cl}$) in the ip toxicity evaluation at 100 mg kg^{-1} over a 24 h period [8]. In comparing the other 4-substituted halo ketones, the 4-bromo analogue **3b**, appeared to be more toxic than the 4-iodo compound **3c** in Phase I analysis, however, Phase VIB ED_{50} data (Table 2) disclosed that the 4-bromo compound **3b** was more potent than the iodo compound **3c**. The 4-fluoro compound **3d**, while moderately active, did not possess much separation between the protective and toxic effects in the MES screen to be considered further. The trifluoromethyl analogue **3e** was quite active in both mice and rats and was more active than the isomeric 3-substituted analogue **3o**. However, as noted in Table 2, the rat po ED_{50} for this compound **3e** was less active than the 4-trifluoromethoxy derivative **3f**, one of the most active compounds in the series. This latter compound **3f** possessed potency paralleled the ED_{50} of the putative parent ester **1j** [(ED_{50} 6.5 mg kg^{-1}, PI >62.0 for **3f** vs. ED_{50} 6.6 mg kg^{-1}, PI >75.8 for **1j**] in rats. The 3-methoxy keto analogue **3t** displayed moderate potency (class 2) comparable to the parent ester (**1x**, class 2) and provided a reasonable ED_{50} in rats (Table 2). As noted previously, the 4-trifluoromethoxy compound **3f** was more active than its 3-substituted isomer **3p**. This consistent pattern was also noted within other unrelated

Table 5
Effect of ethyl 4-[(4-chlorophenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate (**1e**) on voltage-dependent sodium channels in N1E-115 neuroblastoma cells

Drug concentration (μM)	Holding potential (mV)	Number of cells	% Control (±S.E.M.)
100	−60	5	85±5 [a]
100	−90	5	98±4

[a] Significantly different from control, $P < 0.05$.

Table 6
Preliminary hippocampal kindling screen in rats (ip)

Compound	Dose (mg kg^{-1})	Time (min)	Seizure score		ADD [a]	
			Pre-drug	Drug	Pre-drug	Drug
3a	100	165	Rat #1: 5 Rat #2: 5	Rat #1: 4 [b] Rat #2: 3 [b]	Rat #1: 57–67 Rat #2: 50–84	Rat #1: 103 Rat #2: 68
3b	100	165	Rat #1: 4–5 Rat #2: 4	Rat #1: 4 Rat #2: 3	Rat #1: 76–87 Rat #2: 52–81	Rat #1: 66 Rat #2: 66
3c	100	15–135	Rat #1: 5 Rat #2: 4–5	Rat #1: 4–5 Rat #2: 4–5	Rat #1: 30–80 Rat #2: 19–68	Rat #1: 50–61 Rat #2: 47–99
3e	30	15–135	Rat #1: 4–5 Rat #2: 4–5	Rat #1: 4 Rat #2: 4–5	Rat #1: 42–62 Rat #2: 60–73	Rat #1: 54–74 Rat #2: 62–87
3f	30	165	Rat #1: 5 Rat #2: 4–5	Rat #1: 1 Rat #2: 4	Rat #1: 45–52 Rat #2: 42–64	Rat #1: 26 Rat #2: 57
3h	100	105	Rat #1: 5 Rat #2: 4–5	Rat #1: 3 Rat #2: 4	Rat #1: 68–76 Rat #2: 74–80	Rat #1: 71 Rat #2: 58
Carbamazepine [c]	30	[e]	5 [d]	0.8	54–59	23–28
Phenytoin [c]	30	[e]	5 [d]	3	59–65	46–54

[a] After-discharge duration (s).
[b] Toxic.
[c] Data from Ref. [32].
[d] $N = 5$ animals.
[e] Not stated.

compounds in the ADD database [36]. By far the most active, in terms of time of peak effect (TPE), was the 4-nitro analogue **3h**, which provided activity up to 4 h and displayed an ED_{50} of 18.4 mg kg^{-1} with no evidence of toxicity up to 500 mg kg^{-1}. As was expected, the isomeric 3-nitro analogue **3r**, was inactive in mice, but

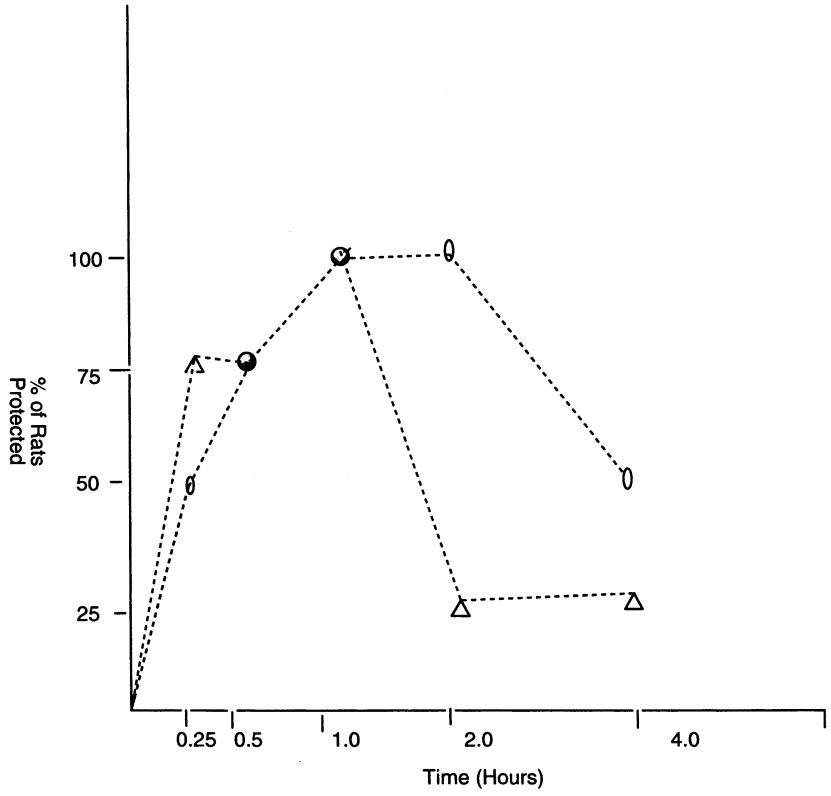

Fig. 6. Time vs. MES protection graph. $N = 4$ rats. Δ, Ethyl 4-[(4-chlorophenyl)amino]-6-methyl-2-oxocyclohex-3-en-1-carboxylate (**1e**, 10 mg/kg, ip); 0, 3-[N-(4-chlorophenyl)amino]-5-methylcyclohex-2-enone (**3a**, 40 mg/kg, ip); ◕, points of coincidence.

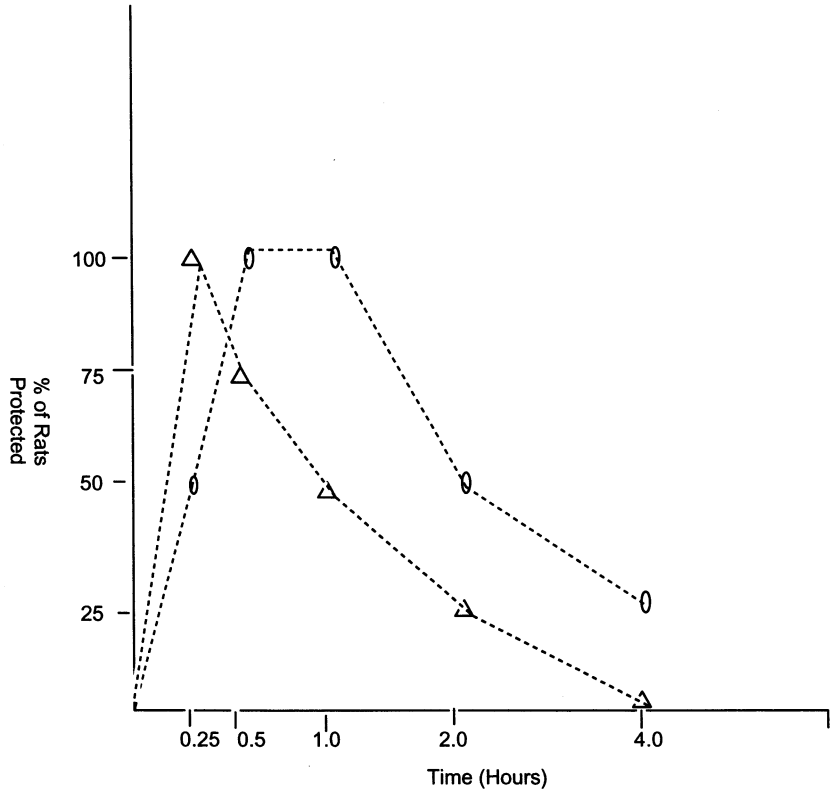

Fig. 7. Time vs. MES protection graph. $N = 4$ rats. Δ, Ethyl 4-[(3-chlorophenyl)amino]-6-methyl-2-oxocyclohex-3-en-1-carboxylate (**1o**, 50 mg/kg, ip); 0, 3-[N-(3-chlorophenyl)amino]-5-methylcyclohex-2-enone (**3k**, 50 mg/kg, ip).

was somewhat active in rats. Nonetheless, an ED_{50} could not be obtained for compound **3r** because of poor suspension properties.

Anomalous results were obtained in two instances: (1) the 4-cyano analogues: ester **1k**, and ketone **3g**; and (2) the unsubstituted analogues: ester **1n**, and ketone **3j**. In the 4-cyano series, the enaminone ester **1k** was active, while the decarboxylated entity **3g** was inactive. It should also be noted that the ester **1k** displayed dual activity, being active in the scPTZ test at 30 (1/5), 100 (3/5) and at 300 mg kg^{-1} (5/5) and in the MES evaluation at 100 (3/3) and 300 mg kg^{-1} (1/1). It is suspected that due to the strong electron withdrawing property of the nitrile group, the decarboxylation reaction was inhibited. This fact is substantiated by the work of Dougherty et al. [37–39] who has shown that in the simple molecule, benzonitrile, the electron withdrawing character of the cyano group was deactivating, more so than fluorine. In the second case, i.e. the unsubstituted pair ester **1n** and ketone **3j**, the reverse trend was noted, with the parent ester **1n** being inactive, while the decarboxylated analogue **3j** being highly active. It should also be noted that the carbomethoxy analogue (**1d**, $R = CH_3$, $R^1 = H$) was initially determined to be inactive (class 3 in Phase I analysis in mice) [2], but was subsequently shown to have a po rat ED_{50} of 7.8 mg kg^{-1} and a TD_{50} > 500 mg kg^{-1} (PI > 64.2) [12]. It was concluded that the oral route apparently activated the compound **1d**. The 5-methyl compound **3j** was the most toxic of all decarboxylated ketone derivatives in the study; one animal died in the scPTZ evaluation at 300 mg kg^{-1} without seizure and one died at 300 mg kg^{-1} in the toxicity evaluation.

4.2. Pharmacokinetics

The results of the pharmacokinetic evaluation of ethyl 4-[(4-chlorophenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate (**1e**) in rats revealed after ip administration a rapid absorption into the systemic circulation with C_{max} appearing at 15 min post dose. The mean plasma and brain concentration versus time profiles for compound **1e** after ip dose is presented in Fig. 3 and the pharmacokinetic parameters are summarised in Table 3. After an ip administration, compound **1e** was rapidly absorbed into the systemic circulation with C_{max} appearing at 15 min post dose. Compound **1e** was eliminated from plasma with a half-life of ca. 0.75 h and a plasma clearance (Cl F^{-1}) was around 0.57 L h^{-1} kg^{-1}. Mean maximum plasma and brain concentrations of 11 042–2973 μg L^{-1} and 10 394–1387 μg kg^{-1}, respectively were observed at 15 min post dose indicating a rapid uptake of the drug in the target organ. The Cl F^{-1} and $t_{1/2}$ were found to be 0.63 L h^{-1} kg^{-1} and 0.84 h, respectively in the brain. A scatter plot (not shown) [17] of brain concentration versus plasma

concentration observed in individual rats at various time points revealed a good correlation ($r^2 = 0.998$) between the two variables. The overall brain to plasma ratio compound **1e** obtained from brain to plasma concentration ratios of individual rats was found to be 1.0–0.3, which was similar to the ratio (0.9) obtained by comparison of $AUC_{0-\infty}$ in brain versus plasma. The metabolite ratio calculated as $AUC_{\text{compound 1e}}/AUC_{\text{Compound 3a}}$ in plasma was found to be 0.008.

The results of mass spectral analysis are shown in Fig. 4 and Table 4. Fig. 2 shows the daughter ion spectra of metabolite(s) M1 (*m/z* 280), M2 (*m/z* 280), the diasteromeric acids, **2**, noted in the HPLC analysis (not shown), M3 (*m/z* 236), the 5-methyl ketone **3a**, the putative metabolite, and target compound **1e** (*m/z* 308). In ESI-MS and MS/MS spectra of target compound **1e** and its metabolites, the presence of Cl resulted in *m/z* values with typical isotopic pattern for the [M+H] ion. Inversely, the presence of this pattern was used to identify the possible metabolites during the LC-MS/MS study. The typical fragmentation of target compound **1e** in positive ion mode resulted in a major fragment with *m/z* 234 (loss of 28 amu; –CO) forming carboxylic acid **2**. The fragmentation of metabolites M1 and M2 [17] also resulted in an ion with *m/z* of 234 (loss of 28; –CO), which was similar to the pattern observed with the parent compound **1e**. This pattern is shown in Fig. 5. Of theoretical concern was the absence of the isomeric ^{37}Cl (m+2) fragment for compound **1e**. As noted, however, carboxy metabolite **2** and keto metabolite **3a** did produce the isomeric fragments. In a separate in vitro study, the LC-MS analysis of compounds **1a**, **1b** and **3a** was performed [40]. In all cases we did find the two tops for the halogens. We can only attribute the absence to the differences in the scanning modes.

4.3. Sodium channel binding

As noted in Table 5, ethyl 4-[(4-chlorophenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate (**1e**) was evaluated for sodium channel binding activity. While target compound **1e** provided a statistically significant block ($P < 0.05$) at −60 mV, the blockade was not strong enough to conclude that this was its principal mechanism of action, however.

4.4. Hippocampal slice

The hippocampal results for the test analogues **3a**–**3c**, **3e**, **3f** and **3h** are shown in Table 6 with carbamazepine and phenytoin included for comparative purpose [32]. In all cases, including that provided for compound **3c**, these data suggest only a weak ability to block the expression of fully kindled seizures for these analogues. Further testing would be required before any definitive conclusion regarding efficacy against focal seizures could be drawn. As indicated by Lothman et al. [32], the time period for selection of the hippocampal test is also critical. Clearly, more extensive testing with this procedure is warranted. We had initially proposed [2] that the enaminones possessed an anticonvulsant profile similar to phenytoin. The data presented in Table 6 further confirms this hypothesis.

5. Conclusions

The pharmacokinetic evaluation of ethyl 4-[(4-chlorophenyl)amino]-5-methyl-2-oxocyclohex-3-ene-1-carboxylate (**1e**) conclusively indicates its conversion into the 5-methyl analogue **3a**. Analysis of the anticonvulsant activity of the various enaminone esters (**1e**–**1x**, Table 1) and their comparable 5-methyl analogue metabolites (**3a**–**3t**, Table 1) is pervasive and differed in only two cases, one of which can be theoretically explained, while the second may have resulted from an error in animal testing. It is clear that although the 4-chloro enaminone ester **1e**, was active in the sodium channel assay, in a similar manner as to ester homologues **1a** ($R = CH_3$, $R^1 = 4\text{-Cl}$) and **1c** ($R = CH_3$, $R^1 = 4\text{-}OCF_3$), this action alone cannot fully explain its principal mechanism of action.

6. Experimental

6.1. Chemistry

Melting points were determined on a Thomas–Hoover capillary melting point apparatus and were uncorrected. Observed b.p.s were also uncorrected. IR spectra were recorded in Nujol, as diluted $CHCl_3$ solutions in matched NaCl cells, or neat on a Perkin–Elmer 1660 series FTIR spectrophotometer. ^{1}H-NMR spectra were recorded on a General Electric QE 300-MHz spectrometer in deuterated solvents using TMS as an internal reference. Coupling patterns are described as follows: s, singlet; bs, broad singlet; d, doublet; dd, doubled doublet; t, triplet; q, quartet; m, multiplet; and 1H, 2H, 3H, etc. as the number of hydrogens integrated within a given coupling pattern. The chemical shifts were measured to two decimal points, while the coupling constants were rounded off to one decimal place. TLC analysis employed EtOAc–cyclohexane (3:1) elution solvent mixture and 5×10 and 5×20 cm^2 fluorescent plates (Whatman silica gel 60A). Elemental analyses (C, H, N, and halogen, where appropriate) were performed by Schwarzkopf Microanalytical Laboratory, Woodside, NY 11377. The analytical results for the elements were within ±0.4% of the theoretical values. Enzymatic hydrolysis studies were conducted on a Milton Roy 1201

UV spectrophotometer. HPLC analysis was performed on a Waters-Millipore (Milford, MA) model 515 HPLC system supplied with a Waters-Millipore model 717 autosampler. Solutes were detected with a Waters-Millipore model 486 UV detector ($\lambda = 307$ nm). Chromatograms were recorded on a Hewlett-Packard (Rockville, MD) model 3390A integrator. All samples were run on a reverse phase ODS 'Luna' C_{18} column (250 × 4.5 mm, 5 μm, 440 m^2 g^{-1}, 19% carbon load, Phenomenex, Torrance, CA) equipped with a guard column (Phenomenex). The LC-MS comprised of a Micromass Quattro triple quadrupole mass spectrometer coupled to a Waters 2695 separation module equipped with a Waters dual wavelength tunable absorbance detector in series. The separations were achieved at ambient temperature on a reverse phase C_{18} column (Waters XTERRA, 150 × 4.6 mm^2, i.d. 5 μ) preceded by a Supelguard™ guard cartridge column 20 mm, 5 μ. 4-Carbo-*tert*-butoxy-5-methylcyclohexane-1,3-dione (**4**, Fig. 2, R = $C(CH_3)_3$) [18], 4-carbethoxy-5-methylcyclohexane-1,3-dione (**4**, R = C_2H_5) [2] and 5-methyl-1,3-cyclohexandione (**5**) [18] were prepared by literature methods. Aniline was distilled under reduced pressure from NaOH pellets before use. Enaminone esters **1e**, **1f**, **1o**–**1q** [8], **1g** [2], and **1h**–**1n**, **1r**–**x** [9], were prepared by literature methods. 3-*N*-[(4-Chloro)phenylamino]-5-methyl-2-oxocyclohexenone (**3a**), was prepared by the literature method [8].

6.2. General procedure for the preparation of 5-methyl-2-cyclohexene enaminones

6.2.1. 3-[*N*-(4-Bromophenyl)amino]-5-methyl-2-cyclohexenone (*3b*)

A mixture of 5-methyl-1,3-cycohexanedione (**5**) [18] (3.41 g, 27 mmol) and 4-bromoaniline (5.70 g, 33 mmol), were added to a mixture of C_6H_6 (50 mL) and EtOAc (15 mL) in a 100 mL single neck flask connected to a Dean-stark trap and the mixture stirred and refluxed until the evolution of water ceased (ca. 3 h). The mixture was concentrated to ca. 1/2 its volume and allowed to stand overnight at room temperature (r.t.). Crystallisation occurred spontaneously and after separation and drying, three recrystallisations from 2-PrOH provided an analytical sample of **3b** as amber crystals, m.p. 213–215 °C (6.4 g, 84%). ^{1}H-NMR (DMSO-d_6): δ 1.06 (3H, d, $J = 6.7$ Hz, CH_3), 1.89–2.55 (5H, m, cyclohexene ring), 5.52 (1H, s =CH), 6.68 (1H, s, NH), 6.35–7.04 (4H, m, C_6H_4). Anal. Calc. for ($C_{13}H_{14}BrNO$) C, H, Br, N.

6.2.2. 3-[*N*-(4-Iodophenyl)amino]-5-methyl-2-cyclohexenone (*3c*)

In a similar procedure, ketone **5** and 4-iodoaniline produced the 4-iodo enaminone **3c**. After three recrystallisations from 2-PrOH, the product occurred as bright yellow plates, m.p. 225–227 °C (64%). ^{1}H-NMR (DMSO-d_6): δ 1.05 (3H, d, $J = 6.15$ Hz, CH_3), 2.10–2.95 (5H, m, cyclohexene ring), 5.53 (1H, s =CH), 6.27–7.40 (4H, m, C_6H_4), 8.87 (1H, s, NH). Anal. Calc. for ($C_{13}H_{14}INO$) C, H, I, N.

6.2.3. 3-[*N*-(4-Fluorophenyl)amino]-5-methyl-2-cyclohexenone (*3d*)

In a similar procedure, ketone **5** and 4-fluoroaniline produced the 4-fluoro enaminone **3d**. After three recrystallisations from EtOAc, the product occurred as yellow crystals, m.p. 185–187 °C (44%). ^{1}H-NMR (DMSO-d_6): δ 1.06 (3H, d, $J = 6.55$ Hz, CH_3), 1.90–2.92 (5H, m, cyclohexene ring), 5.54 (1H, s =CH), 6.47–6.74 (4H, m, C_6H_4), 8.24 (1H, s, NH). Anal. Calc. for ($C_{13}H_{14}FNO$) C, H, F, N.

6.2.4. 3-[*N*-(4-Trifluoromethylphenyl)amino]-5-methyl-2-cyclohexenone (*3e*)

In a similar procedure, ketone **5** and 4-trifluoromethylaniline produced the 4-trifluoromethyl enaminone **3e**. After three recrystallisations from 2-PrOH, the product occurred as a gray amorphous powder, m.p. 205–206 °C (31%). ^{1}H-NMR (DMSO-d_6): δ 1.06 (3H, d, $J = 6.15$ Hz, CH_3), 1.95–2.90 (5H, m, cyclohexene ring), 5.50 (1H, s =CH), 6.37–7.22 (4H, m, C_6H_4), 8.91 (1H, s, NH). Anal. Calc. for ($C_{14}H_{14}F_3NO$) C, H, F, N.

6.2.5. 3-[*N*-(4-Trifluoromethoxyphenyl)amino]-5-methyl-2-cyclohexenone (*3f*)

In a similar procedure, ketone **5** and 4-trifluoromethoxyphenylaniline produced the 4-trifluoromethoxyphenyl enaminone **3f**. After three recrystallisations from EtOAc, the product occurred as a gray amorphous powder, m.p. 174–175 °C (39%). ^{1}H-NMR (DMSO-d_6): δ 1.00 (3H, d, $J = 6.45$ Hz, CH_3), 1.95–2.92 (5H, m, cyclohexene ring), 5.54 (1H, s =CH), 6.35–6.55 (4H, m, C_6H_4), 8.71 (1H, s, NH). Anal. Calc. for ($C_{14}H_{14}F_3NO_2$) C, H, F, N.

6.2.6. 3-[*N*-(4-Cyanophenyl)amino]-5-methyl-2-cyclohexenone (*3g*)

In a similar procedure, ketone **5** and 4-aminobenzonitrile produced the 4-cyanophenyl enaminone **3g**. After three recrystallisations from EtOAc, the product occurred as an orange–brown powder, m.p. 234–236 °C (14%). ^{1}H-NMR (DMSO-d_6): δ 0.98 (3H, d, $J = 6.15$ Hz, CH_3), 1.92–2.90 (5H, m, cyclohexene ring), 5.60 (1H, s =CH), 6.64–7.25 (4H, m, C_6H_4), 8.98 (1H, s, NH). Anal. Calc. for ($C_{14}H_{14}N_2O$) C, H, N.

6.2.7. 3-[*N*-(4-Nitrophenyl)amino]-5-methyl-2-cyclohexenone (*3h*)

In a similar procedure, ketone **5** and 4-nitroaniline produced the 4-nitrophenyl enaminone **3h**. After three recrystallisations from EtOAc, the product occurred as a

bright yellow plates, m.p. 208–209 °C (59%). ^{1}H-NMR (DMSO-d_6): δ 1.00 (3H, d, $J = 6.29$ Hz, CH_3), 1.92–2.94 (5H, m, cyclohexene ring), 5.50 (1H, s =CH), 6.28–6.68 (4H, m, C_6H_4), 8.22 (1H, s, NH). Anal. Calc. for ($C_{13}H_{14}N_2O$) C, H, N.

*6.2.8. 3-N-[(4-Tolyl)amino]-5-methyl-2-cyclohexenone (**3i**)*

In a similar procedure, ketone **5** and 4-toluidine produced the 4-tolyl enaminone **3i**. After three recrystallisations from EtOAc, the product occurred as a brown crystals, m.p. 194–195 °C (79%). ^{1}H-NMR (DMSO-d_6): δ 1.00 (3H, d, $J = 6.35$ Hz, CH_3), 1.89–2.85 (5H, m, cyclohexene ring), 2.35 (3H, s, CH_3 on ring), 5.50 (1H, s =CH), 6.20–6.85 (4H, m, C_6H_4), 8.38 (1H, s, NH). Anal. Calc. for ($C_{14}H_{17}NO$) C, H, N.

*6.2.9. 3-N-(Phenyl)amino]-5-methyl-2-cyclohexenone (**3j**)*

In a similar procedure, ketone **5** and aniline (freshly distilled under reduced pressure) produced the unsubstituted phenyl enaminone **35**. After three recrystallisations from EtOAc, the product occurred as a yellow powder, m.p. 155 °C (soften), 158–160 °C (32%). ^{1}H-NMR (DMSO-d_6): δ 0.98 (3H, d, $J = 6.56$ Hz, CH_3), 1.94–2.55 (5H, m, cyclohexene ring), 5.35 (1H, s =CH), 6.34 (1H, s, NH), 6.44–7.00 (5H, m, C_6H_4). Anal. Calc. for ($C_{13}H_{15}NO$) C, H, N.

*6.2.10. 3-[N-(3-Chlorophenyl)amino]-5-methyl-2-cyclohexenone (**3k**)*

In a similar procedure, ketone **5** and 3-chloroaniline produced the 3-chlorophenyl enaminone **3k**. After three recrystallisations from EtOAc, the product occurred as a yellow crystalline mass, m.p. 177–179 °C (44%). ^{1}H-NMR (DMSO-d_6): δ 1.08 (3H, d, $J = 6.35$ Hz, CH_3), 1.90–2.95 (5H, m, cyclohexene ring), 5.35 (1H, s =CH), 6.34 (1H, s, NH), 6.44–7.00 (4H, m, C_6H_4). Anal. Calc. for ($C_{13}H_{14}ClNO$) C, H, Cl, N.

*6.2.11. 3-[N-(3-Bromophenyl)amino]-5-methyl-2-cyclohexenone (**3l**)*

In a similar procedure, ketone **5** and 3-bromoaniline produced the 3-bromophenyl enaminone **3l**. After three recrystallisations from EtOAc, the product occurred as a yellow crystalline mass, m.p. 172–174 °C (84%). ^{1}H-NMR (DMSO-d_6): δ 1.06 (3H, d, $J = 6.70$ Hz, CH_3), 1.89–2.55 (5H, m, cyclohexene ring), 5.52 (1H, s =CH), 6.68 (1H, s, NH), 6.35–7.04 (4H, m, C_6H_4). Anal. Calc. for ($C_{13}H_{14}BrNO$) C, H, Br, N.

*6.2.12. 3-[N-(3-Iodophenyl)amino]-5-methyl-2-cyclohexenone (**3m**)*

In a similar procedure, ketone **5** and 3-iodoaniline produced the 3-iodophenyl enaminone **3m**. After three recrystallisations from EtOAc, the product occurred as a white powder, m.p. 185–187 °C (44%). ^{1}H-NMR (DMSO-d_6): δ 1.00 (3H, d, $J = 6.3$ Hz, CH_3), 1.90–2.90 (5H, m, cyclohexene ring), 5.50 (1H, s =CH), 6.47–7.00 (4H, m, C_6H_4), 8.79 (1H, s, NH). Anal. Calc. for ($C_{13}H_{14}BrNO$) C, H, I, N.

*6.2.13. 3-[N-(3-Fluorophenyl)amino]-5-methyl-2-cyclohexenone (**3n**)*

In a similar procedure, ketone **5** and 3-fluoroaniline produced the 3-fluorophenyl enaminone **3n**. After three recrystallisations from EtOAc, the product occurred as a white powder, m.p. 163–165 °C (77%). ^{1}H-NMR (DMSO-d_6): δ 0.98 (3H, d, $J = 6.55$ Hz, CH_3), 1.90–2.90 (5H, m, cyclohexene ring), 5.54 (1H, s =CH), 6.55–7.00 (4H, m, C_6H_4), 8.47 (1H, s, NH). Anal. Calc. for ($C_{13}H_{14}FNO$) C, H, F, N.

*6.2.14. 3-[N-(3-Trifluoromethylphenyl)amino]-5-methyl-2-cyclohexenone (**3o**)*

In a similar procedure, ketone **5** and 3-trifluoromethylaniline produced the 3-trifluorophenyl enaminone **3o**. After three recrystallisations from EtOAc, the product occurred as a gray powder, m.p. 179–180.5 °C (69%). ^{1}H-NMR (DMSO-d_6): δ 1.08 (3H, d, $J = 6.67$ Hz, CH_3), 1.92–2.90 (5H, m, cyclohexene ring), 5.54 (1H, s =CH), 6.46–6.94 (4H, m, C_6H_4), 8.79 (1H, s, NH). Anal. Calc. for ($C_{14}H_{14}F_3NO$) C, H, F, N.

*6.2.15. 3-[N-(3-Trifluoromethoxyphenyl)amino]-5-methyl-2-cyclohexenone (**3p**)*

In a similar procedure, ketone **5** and 3-trifluoromethoxyaniline produced the 3-trifluoromethoxyphenyl enaminone **3p**. After three recrystallisations from EtOAc, the product occurred as off-white crystals, m.p. 156–160 °C (38%). ^{1}H-NMR (DMSO-d_6): δ 1.06 (3H, d, $J = 6.16$ Hz, CH_3), 1.92–2.92 (5H, m, cyclohexene ring), 5.55 (1H, s =CH), 6.00–6.90 (4H, m, C_6H_4), 8.28 (1H, s, NH). Anal. Calc. for ($C_{14}H_{14}F_3NO_2$) C, H, F, N.

*6.2.16. 3-[N-(3-Cyanophenyl)amino]-5-methyl-2-cyclohexenone (**3q**)*

In a similar procedure, ketone **5** and 3-aminobenzynitrile produced the 3-cyanophenyl enaminone **3q**. After three recrystallisations from EtOAc, the product occurred as light yellow crystals, m.p. 175.5–177 °C (54%). ^{1}H-NMR (DMSO-d_6): δ 1.00 (3H, d, $J = 6.60$ Hz, CH_3), 1.79–2.88 (5H, m, cyclohexene ring), 5.50 (1H, s =CH), 6.85–7.55 (4H, m, C_6H_4), 8.49 (1H, s, NH). Anal. Calc. for ($C_{14}H_{14}N_2O$) C, H, N.

*6.2.17. 3-[N-(3-Nitrophenyl)amino]-5-methyl-2-cyclohexenone (**3r**)*

In a similar procedure, ketone **5** and 3-nitroaniline produced the 3-nitrophenyl enaminone **3r**. After three recrystallisations from EtOAc, the product occurred as

yellow crystalline plates, m.p. 187–189 °C (68%). ^{1}H-NMR (DMSO-d_6): δ 0.98 (3H, d, $J = 6.55$ Hz, CH_3), 1.90–2.90 (5H, m, cyclohexene ring), 5.50 (1H, s =CH), 6.47–7.00 (4H, m, C_6H_4), 8.79 (1H, s, NH). Anal. Calc. for ($C_{14}H_{13}N_2O_3$) C, H, N.

6.2.18. 3-[N-(3-Tolyl)amino]-5-methyl-2-cyclohexenone (**3s**)

In a similar procedure, ketone **5** and 3-toluidine produced the 3-tolyl enaminone **3s**. After three recrystallisations from EtOAc, the product occurred as brown crystals, m.p. 147–148.5 °C (79%). ^{1}H-NMR (DMSO-d_6): δ 0.98 (3H, d, $J = 6.68$ Hz, CH_3), 1.90–2.90 (5H, m, cyclohexene ring), 2.35 (3H, s, CH_3 on ring), 5.54 (1H, s =CH), 6.27–6.90 (4H, m, C_6H_4), 8.24 (1H, s, NH). Anal. Calc. for ($C_{14}H_{17}NO$) C, H, N.

6.2.19. 3-[N-(3-Methoxyphenyl)amino]-5-methyl-2-cyclohexenone (**3t**)

In a similar procedure, ketone **5** and 3-anisidine produced the 3-methoxyphenyl enaminone **45**. After three recrystallisations from EtOAc, the product occurred as an off-white powder, m.p. 140–142 °C (44%). ^{1}H-NMR (DMSO-d_6): δ 0.98 (3H, d, $J = 6.55$ Hz, CH_3), 1.90–2.90 (5H, m, cyclohexene ring), 3.74 (3H, s, methoxy group on ring), 5.54 (1H, s =CH), 5.98–6.99 (4H, m, C_6H_4), 8.62 (1H, s, NH). Anal. Calc. for ($C_{14}H_{17}NO_2$) C, H, N.

6.3. Pharmacology

Initial evaluations for anticonvulsant activity were performed by the ADD Program, Epilepsy Branch, Neurological Disorders Program, NINDS performed initial evaluations for anticonvulsant activity and included Phases I, II, VIA and VIB test procedures which have been described [19–21]. These tests were performed either in male Carworth Farms no. 1 (CF1) mice (weighing 18–25 g) or male Sprague–Dawley rats (weighing 100–150 g). Phase I, a qualitative anticonvulsant ip evaluation in mice included three tests: MES, scPTZ and the rotorod test for Tox. Compounds were suspended in 0.5% aq. methylcellulose and were administered at three dosage levels (30, 100, and 300 mg kg^{-1}) with anticonvulsant activity and motor impairment noted 30 min and 4 h after administration. Phase VIA was a similar qualitative evaluation to the Phase I evaluation, however the test drug was administered orally (po) in rats utilizing the three tests noted previously. Phase II test quantitated the anticonvulsant activity and motor impairment observed for the most promising compounds in Phase I while Phase VIB quantitated the activity obtained in Phase VIA, respectively. An ip test in rats was performed at 30 mg kg^{-1} for activity and toxicity. The TPE ip in mice and po rats was also noted for the determination of ED_{50} and TD_{50} values. All test data are listed in Table 2.

6.4. Sodium channel binding

The procedure for the sodium channel study is as follows. The N1E-115 neuroblastoma cell line was maintained at 35 °C in Dulbecco's modified Eagles Medium supplemented with 5% fetal calf serum, 20 mM HEPES, 80 μg mL^{-1} gentamicin, and 4 mM glutamine. Prior to electrophysiological studies, cells were plated and incubated for 3–5 days in a differentiation medium similar to the above solution, however, with reduced (2.5%) fetal calf serum and 2% DMSO. Recordings were carried out at r.t. in a bath solution containing 130 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 5 mM HEPES. The solution also contained 0.1 mM $CdCl_2$ and 25 mM tetraethylammonium chloride to block voltage-gated Ca^{2+} and K^+ channels, respectively. Whole-cell recordings were obtained using patch electrodes (1–2 MΩ) filled with the intracellular solution described above. The currents were filtered at 5 kHz and analysed on a computer using PClamp 6 (Axon Instruments, Union City, CA, USA). Series resistance and capacitance currents were compensated using the internal clamp circuitry. The series resistance was 3–5 mΩ and 80–90% of the series resistance was compensated. Cells were voltage clamped [41] at −70 mV and ethyl 4-[(4-chlorophenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate (**1e**) was applied using the perfusion system described above. To activate voltage-gated Na channels, cells were hyperpolarised to −90 mV for 90 ms and then depolarised to potentials from −80 to +60 mV in 10 mV increments in control and at 1 and 2 min following incubation with target compound **1e**. The process was repeated, hyperpolarizing at −60 mV. The results from the individual cells were averaged, the S.E.M. was calculated, and statistical significance was determined using the Student's t-test. Significance was taken to be in the range of P values < 0.01. Data for target compound **1e** is shown in Table 5.

6.5. Hippocampal slice

The procedure for hippocampal slices is as follows. A bipolar stimulating electrode was stereotactically implanted in the ventral hippocampus (AP −3.6, ML 4.9, DV −5.0 from dura, incisor bar +5) of adult male Sprague–Dawley rats (250–300 g) under ketamine–xylazine anesthesia. Three anchor screws were attached to the skull and the electrode assembly anchored to the skull with dental acrylic cement. After the incision was closed with sutures, the animal received a single dose of bicillin (60 000 U, im) and returned to the home cage to recover. Animals were kindled according to the proce-

dure of Lothman and Williamson [42]. Briefly, after 1 week, animals were stimulated with suprathreshold trains of 200 μA for 10 s, 50 Hz, every 30 min for 6 h on alternate days until the animals were fully kindled. One week later the effect of a single dose of test substance (50 mg kg^{-1}, ip) on the behavioral seizure score (BSS) and after-discharge was assessed in a single group of kindled rats ($n = 6–8$) at 15, 45, 75, 105, 135, 165, and 195 min after drug administration. Results obtained at the various time points were compared with the last control stimulus delivered 15 min prior to drug administration. Thus, each animal served as its own control. Seizures were scored according to the following criteria: Stage 1—mouth and facial clonus; Stage 2—stage 1 plus head nodding; Stage 3—stage 2 plus forelimb clonus; Stage 4—stage 3 plus rearing; Stage 5—stage 4 plus repeated rearing and falling [32]. When a drug treatment was observed to significantly lower seizure score and decrease after-discharge, a dose–response study was initiated. The following 5-methyl keto enaminones were evaluated: 4-Chloro (**3a**), 4-bromo (**3b**), 4-iodo (**3c**), 4-trifluoromethyl (**3e**), 4-trifluoromethoxy (**3f**) and 4-nitro (**3h**), and the data is shown in Table 6.

6.6. Metabolism

A solution of target compound, ethyl 4-[(4-chlorophenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate (**1e**), was dissolved in 95% EtOH and diluted to 50 mL in the same solvent. Tris buffer (0.01 M, pH 8.0), and esterase (EC 3.1.1.1, 200 units mg^{-1}, 19 mg, dissolved in Tris buffer and diluted to 10 mL), was employed. All experiments were performed in triplicate. A volume of the stock EtOH solution of compound **1e** (0.2 mL), 1 mL buffer, and 0.1 mL of enzyme solution were placed in a Teflon-capped reaction vial and incubated at 37 °C for the following time periods: 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 16.0 and 24.0 h. The blank (time = 0 h) contained 0.2 mL of stock solution, 1 mL of buffer but no enzyme. After each time period, the vials were removed from the incubator and quenched at −80 °C until ready for analysis. As previously reported [8], differential ultraviolet analysis of target compound **1e** and the 5-methyl ketone metabolite **3a** provided two distinct λ_{max} values that were used to calculate the % conversion.

6.7. Pharmacokinetics

6.7.1. Animals

Male Sprague–Dawley rats (300–350 g body weight) was purchased from Harlan (Indianapolis, IN, USA) and was provided free access to rodent chow and water ad libitum. Care and use of the animals followed the *Guide for the Care and Use of Laboratory Animals* [43]. Dosing solution for ip administration in animals was prepared by dissolving an appropriate amount of ethyl 4-[(4-chlorophenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate (**1e**) in a solution containing 19% DMSO, 49% propylene glycol and 32% PBS.

6.7.2. Pharmacokinetics and brain distribution in rats

Pharmacokinetics and brain distribution of target compound **1e** was evaluated in male Sprague–Dawley rats. Overnight fasted rats were administered with a single ip dose of target compound **1e** (10 mg kg^{-1}). Cohorts of three animals were sacrificed by CO_2 asphyxiation at 15, 30, 60, 90, 120, 240, 480 and 1440 min post dose. Blood was collected using heparinised syringes via cardiac puncture into heparinised polypropylene centrifuge tubes (15 mL), which were later centrifuged for 10 min to separate plasma. Brain tissue samples were collected, blotted dry on a tissue paper and weighed. The plasma and brain tissue samples were flash frozen, stored at −70 °C and analysed using a validated HPLC assay method described in Ref. [17].

6.7.3. Urinary excretion study in rats

In a separate study, a single ip dose of 10 mg kg^{-1} of target compound **1e** was administered to five male Sprague–Dawley rats and the animals were housed in individual metabolism cages designed to separately collect feces and urine. Urine was collected for a period of 8 h post dose and stored at −70 °C until analysis.

6.7.4. Analytical procedures

Rat plasma and brain samples were assayed using a validated HPLC–UV method for simultaneous analysis of target compound **1e** and the putative metabolite **3a** [17]. Carbamazepine was used as the internal standard. In brief, 0.5 mL of plasma sample was extracted with two mL of methyl *tert*-butyl ether. The organic phase was separated by freezing the aqueous layer and evaporated under a gentle stream of nitrogen. The dried residue was reconstituted in 0.25 mL of mobile phase, vortexed and centrifuged The 50 μL volume of supernatant was injected into the HPLC. Brain tissue 0.1 g (blank, spiked or test) was homogenised in 0.5 mL of mobile phase using a tissue tearor (PowerGen 125 Homogeniser) for 30 s. The homogenate was centrifuged at 14 000 rpm for 1 min using a tabletop microcentrifuge (Eppendorf, Model 5415). The 50 μL volume of supernatant was injected into the HPLC. The HPLC system was a Model 515 Pump (Waters-Millipore, Milford, MA, USA) coupled with a 717 Waters autosampler. Analytes were separated at ambient temperature on a reverse phase C_{18} column (Waters XTERRA, 150 × 4.6 mm^2, i.d. 5 μ) preceded by a Supelguard™ guard cartridge column 20 mm, 5 μ. Analytes were detected using a Waters Model 486 Tunable absorbance detector in the ultraviolet mode ($\lambda_{max} = 307$ nm). Chromato-

graphic data acquisition and analysis was performed on a Millenium Chromatography Manager (Waters Version 3.2). The mobile phase consisted of MeCN–0.05 M NaH_2PO_4 buffer, pH 3.9 (32:68 v/v) and was degassed under ultrasound for 30 min before use. Mobile phase was pumped at a flow rate of 1 mL min^{-1} with a run time of 30 min per sample.

6.7.5. Urinary excretion study

Urine samples were analysed using the same HPLC setup as described above except that mobile phase consisted of 30% v/v MeCN in 0.05 M NaH_2PO_4 buffer, pH 3.9. This assay method was validated for intra-day recovery of target compound **1e** and putative metabolite **3a** with the primary objective to characterise the extent of urinary excretion of target compound **1e**. As a secondary objective, the urine samples were subsequently analysed to obtain qualitative information regarding the metabolites on LC-MS/MS discussed in the later section. For chromatographic analysis utilizing UV detection, the urine sample (blank, spiked or test; 50 μL) was diluted with 1 mL of mobile phase, vortex mixed and centrifuged. The supernatant was transferred into the microvial and 50 μL was injected into the HPLC.

6.7.6. Identification of metabolites by LC-MS/MS

A pooled urine sample (2 mL) was applied onto a Bakerbond octadecyl solid phase extraction cartridge column (500 mg sorbent; 3 mL) previously conditioned with 3 mL of MeOH and 3 mL of water. The cartridge was washed twice with 1 mL of water and the retained components were eluted with 2 mL of MeOH. The MeOH eluent was evaporated to dryness under a gentle stream of nitrogen at 34 °C and the dried residue was reconstituted in 1 mL of mobile phase and analysed using a LC-MS as described. The LC-MS comprised of a Micromass Quattro triple quadrupole mass spectrometer coupled to a Waters 2695 separations module equipped with a Waters dual wavelength tunable absorbance detector in series. The separations were achieved at ambient temperature on a reverse phase C_{18} column (Waters XTERRA, 150 × 4.6 mm^2, i.d. 5 μ) preceded by a Supelguard™ guard cartridge column 20 mm, 5 μ. The mobile phase consisted of 40% v/v MeCN in CH_3COONH_4 buffer 10 mM, pH 3.9 and was delivered at a flow rate of 0.2 mL min^{-1} over a run time of 60 min. The analytes were monitored simultaneously on an UV detector and the mass spectrometer. The outlet flow from UV detector was directly introduced into the ESI probe of mass spectrometer. Positive ion electrospray mass spectra were obtained for target compound **1e** and its metabolites in urine. The mass spectrometer was operated using source temperature of 80 °C, desolvation temperature of 150 °C, capillary voltage of 3.2 kV, cone voltage of 40 V, extractor voltage of 2 V, RF lens voltage 0.5 V, cone gas flow 71 L h^{-1} and desolvation gas flow 513 L h^{-1}. Centroid mass spectra were acquired between m/z 100 and 800 for 1 s. In a pilot analysis, a full MS scan was obtained for all the components eluting at different retention times to determine their m/z values. Finally, the data was acquired in MS and MS/MS mode and TIC scan and daughter ion spectra were obtained for parent masses by operating the first quadrupole in static mode for parent ions with m/z 282, 236 and 308 as second function. Fragmentation of the analyte ions during the full acquisitions was achieved by using cone voltage and collision energy ramps. Degradation of selected analyte ion was performed by collision with Ar. System control and data acquisition was performed using MassLynx software Version 3.0.

6.7.7. Data analysis

Plasma and brain concentrations of target compound **1e** and the putative metabolite **3a** were calculated by internal standard method using the peak height ratios of analyte to carbamazepine (IS). The unknown concentrations of compounds **1e** and **3a** were read from the respective standard curves obtained by weighted linear regression analysis (Wt. $1/x^2$) of anlayte/IS peak height ratio versus concentration data on Microsoft Excel. The weighing scheme was selected on the basis of residuals and corresponding % error in the predicted concentrations at each level. Since the study involved destructive sampling, the trends in pharmacokinetic parameters were assessed from mean naïve-pooled plasma and brain concentration versus time data. Pharmacokinetic parameters were determined using non-compartmental analysis using WinNonlin professional software package (Pharsight Corporation, NC, USA). The values of C_{max} and T_{max} were calculated from the raw data as the maximum observed concentration in the naïve pooled data and the time at which it was observed, respectively. Brain uptake of target compound **1e** and its metabolite **3a** was determined both from the ratio of $AUC_{0-\infty}$ in brain to that of plasma and also from average of brain to plasma concentration ratio of each animal at different time points. Data for the analysis is found in Tables 3 and 4 and Figs. 3–5.

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