Pergamon

Bioorganic & Medicinal Chemistry, Vol. 4, No. 2, pp 165–178, 1996

0968-0896/96 $15.00+0.00

0968-0896(95)00168-9

Triazolines—XXVII. Δ^2-1,2,3-Triazoline Anticonvulsants: Novel 'Built-in' Heterocyclic Prodrugs with a Unique 'Dual-Action' Mechanism for Impairing Excitatory Amino Acid L-Glutamate Neurotransmission[1]

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Abstract—The Δ^2-1,2,3-triazoline anticonvulsants (**1**) may be considered as representing a unique class of 'built-in' heterocyclic prodrugs where the active 'structure element' is an integral part of the ring system and can be identified only by a knowledge of their chemical reactivity and metabolism. Investigations on the metabolism and pharmacology of a lead triazoline, ADD17014 (**1a**), suggest that the triazolines function as 'prodrugs' and exert their anticonvulsant activity by impairing excitatory amino acid (EAA) L-glutamate (L-Glu) neurotransmission via a unique 'dual-action' mechanism. While an active β-amino alcohol metabolite, **2a**, from the parent prodrug acts as an *N*-methyl-D-aspartate (NMDA)/MK-801 receptor antagonist, the parent triazoline impairs the presynaptic release of L-Glu. Various pieces of theoretical reasoning and experimental evidence led to the elucidation of the dual-action mechanism. Based on the unique chemistry of the triazolines, the potential metabolic pathways and biotransformation products of **1a** were predicted to be the β-amino alcohols **2a** and **2a′**, the α-amino acid **3a**, the triazole **4a**, the aziridine **5a**, and the ketimine **6a**. In vivo and in vitro pharmacological studies of **1a** and potential metabolites, along with a full quantitative urinary metabolic profiling of **1a**, indicated the β-amino alcohol **2a** as the active species. It was the only compound that inhibited the specific binding of [^{3}H]MK-801 to the MK-801 site, 56% at 10 μM drug concentration, but itself had no anticonvulsant activity, suggesting **1a** acted as a prodrug. Three metabolites were identified; **2a** was the most predominant, with lesser amounts of **2a′**, and very minor amounts of aziridine **5a**. Since only **5a** can yield **2a′**, its formation indicated that the biotransformation of **1a** occurred, at least in part, through **5a**. No amino acid metabolite **3a** was detected, which implied that no in vivo oxidation of **2a** or oxidative biotransformation of **1a** or **5a** by hydroxylation at the methylene group occurred. While triazoline **1a** significantly decreased Ca^{2+}-dependent, K^+-evoked L-Glu release (83% at 100 μM drug concentration), triazolines **1a–1c** showed an augmentation of 50–63%, in the Cl^- channel activity, a useful membrane action that reduces the excessive L-Glu release that occurs during epileptic seizures. The high anticonvulsant activity of **1a** may be due to its unique dual-action mechanism whereby **1a** and **2a** together effectively impair both pre- and postsynaptic aspects of EAA neurotransmission, and has clinical potential in complex partial epilepsy which is refractory to currently available drugs.

Introduction

Studies in the authors' laboratories have led to the discovery of the Δ^2-1,2,3-triazoline heterocycles (4,5-dihydro-1*H*-1,2,3-triazoles) (**1**), as a potentially unique family of anticonvulsant agents hitherto unknown.[2–9] The triazolines afford a high degree of protection against seizure provocation by both chemical and electrical stimuli. They have high protective indices (TD_{50}/ED_{50}), are orally active with a good margin of safety, and compare very well with prototype antiepileptic drugs (phenytoin, ethosuximide, phenobarbital and valproate) in both mice and rats. ADD17014 [1-(4-chlorophenyl)-5-(4-pyridyl)-Δ^2-1,2,3-triazoline] (**1a**), a representative triazoline, has successfully advanced through all phases of preclinical testing, including 14- and 30-day dose-range finding studies, in the NINDS-sponsored Antiepileptic Drug Development (ADD) Program[10] and has emerged as a prime candidate for further development into a clinically useful antiepileptic drug. Unlike the prototype drugs, it offers complete protection against stimulus-induced electrographic after-discharge seizures and generalized convulsions when tested in both fully amygdalar- and entorhinal-kindled rats, at non-sedative, non-neurotoxic doses.[1,9]

Investigations on the metabolism and pharmacology, both in vivo and in vitro, of **1a** and its biotransformation products, suggest that the triazolines may be functioning as 'prodrugs' and exert their anticonvulsant

activity by impairing excitatory amino acid (EAA) neurotransmission via a unique 'dual-action' mechanism. While an active β-amino alcohol metabolite (**2a**), from the parent 'prodrug' acts as an NMDA/MK-801 receptor antagonist, the parent triazoline **1a** impairs the presynaptic release of L-glutamate (L-Glu), the transmitter at EAA synapses. This paper discusses the various pieces of theoretical reasoning and corroborative experimental evidence that have contributed to unravelling the mechanism of action of the 1,2,3-triazoline anticonvulsants.

Methods and Results

Chemistry

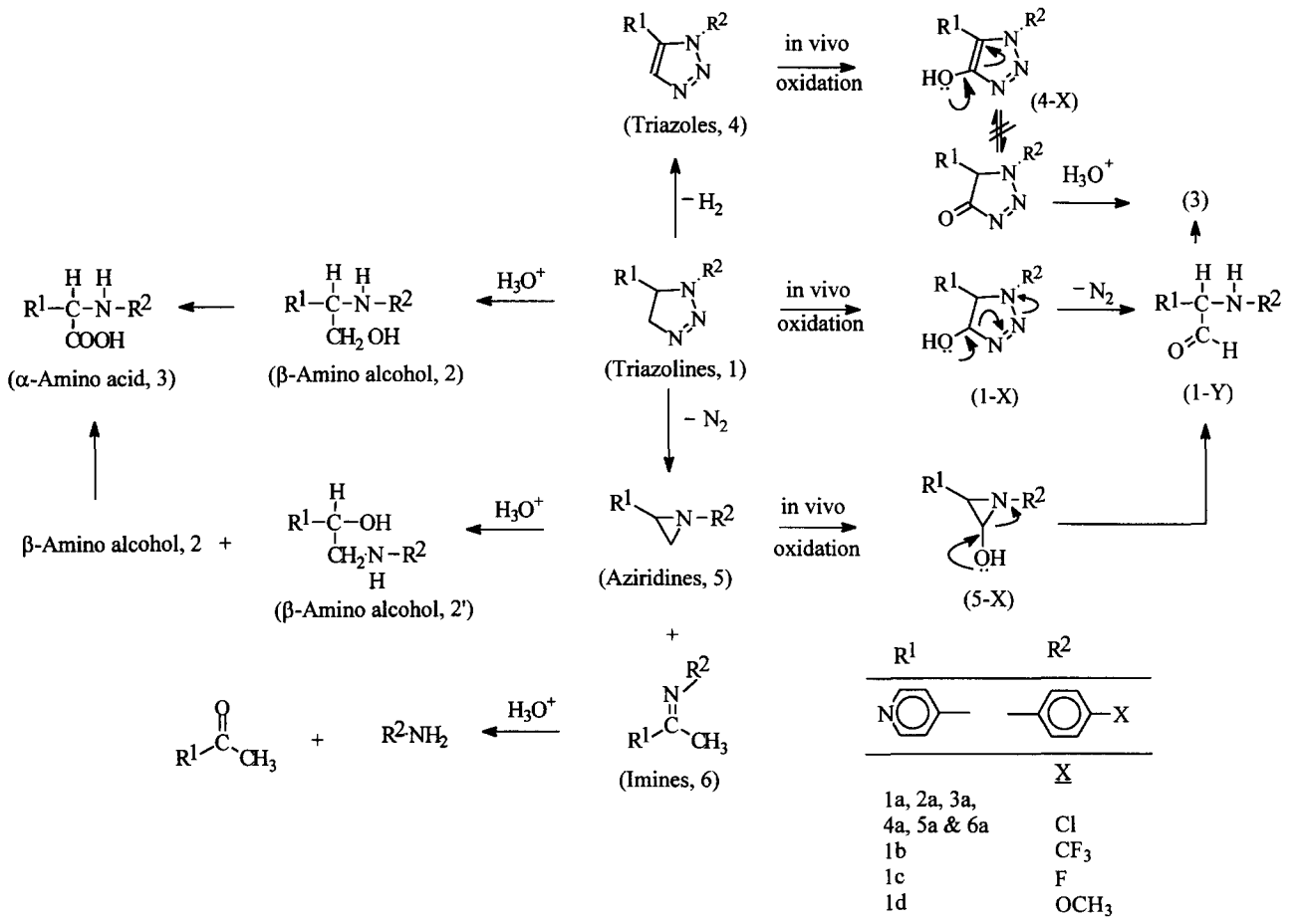
The potential metabolic pathways and biotransformation products of the 1,2,3-triazolines based on their unique chemistry,[11,12] and the inter-relationships of the various putative metabolites, all indicated that the triazolines may be exerting their anticonvulsant activity by functioning as prodrugs (Scheme 1).

Synthesis

The 1,2,3-triazolines (**1**) were prepared following the Kadaba procedure,[6,12] by the 1,3-cycloaddition of diazomethane to Schiff Bases in dioxane solution, in the presence of water as a catalyst. The triazoles **4** were obtained by NiO_2 oxidation[13] of the respective triazolines, while triazoline photolysis[11,12] yielded the aziridines **5**. The ketimine **6a** was obtained by thermolysis of **1a**.[12]

The aziridines were characterized by their typical ^1H NMR spectra. Since the methylene protons of the 3-CH_2 group are diastereotopic,[12] the NMR spectra gave two closely similar doublets for the 2-CH proton in the δ 3–4 ppm region and a multiplet of 8 peaks for the 3-CH_2 protons in the δ 2–3 ppm region resembling an ABX system. This is distinctly different from the NMR spectra of the parent 1,2,3-triazolines, where the 5-CH and 4-CH_2 protons appeared further downfield at δ 4–6 ppm, as an ABC multiplet of 12 peaks.[12]

From the NMR spectra, the aziridine samples were estimated to contain approximately 10–15% of the ketimine **6**, based on integration of the ketimine CH_3 peak which appears as a sharp singlet in the δ 2–2.5 ppm region along with the 3-CH_2 peaks of the aziridine ring. These samples were used as such for in vivo anticonvulsant testing, since only very small amounts of **6** were present and tests showed the ketimines had no anticonvulsant activity.



Scheme 1. Potential metabolic pathways and biotransformation products of triazolines.

The primary β-amino alcohol **2a** was prepared by acid hydrolysis of **1a** followed by sample purification by preparative thin-layer chromatography (TLC). The secondary β-amino alcohol **2a′** was isolated from rat urine following intraperitoneal (ip) administration of **1a**.

Pharmacology

The anticonvulsant activity of the different triazolines **1**, and aziridines **5**, and potential metabolites **2a**, **4a** and **6a**, was determined[10] after ip administration in the mouse, using the two standard animal tests, the maximal electroshock seizure test (MES) and the subcutaneous pentylenetetrazole or Metrazol (sc Met) seizure test. Central nervous system (CNS) toxicity was evaluated in the rotorod ataxia test. Testing was done at three doses of 30, 100 and 300 mg kg^{-1} of the compound at both 30 min and 4 h intervals, which provided a profile of the anticonvulsant activity, toxicity and potency of each compound, and minimized the likelihood of failing to identify slowly absorbed compounds or those with possible anticonvulsant activity residing in a metabolite.

Compounds demonstrating anticonvulsant activity in the sc Met and/or the MES test at a dose of 30 or 100 mg kg^{-1} and an estimated protective index of >1, were assigned an Anticonvulsant Screening Project (ASP) group classification of I and were deemed the most promising as anticonvulsants. Group II compounds showed activity at 300 mg kg^{-1} and those with no activity at this dose were classified inactive and placed in group III.

The ED_{50} values (the dose that elicits an anticonvulsant response in 50% of the animals) in the MES and sc Met tests were determined for class I compounds at the time of peak effect (TPE) in the MES test, except when preliminary testing indicated that sc Met activity occurred at another time. The rotorod ED_{50} or the TD_{50} (the dose that produces neurological deficit in 50% of animals) was determined at the time of peak neurological deficit. The ratio TD_{50}/ED_{50} defined the protective index (PI) value, which is a measure of the margin of safety, and the greater the PI value, the lesser the toxic side effects of the drug.

Additional antiepileptic drug differentiation was conducted in mice, ip, using four chemically induced seizure models, which included the subcutaneous bicuculline (sc Bic) and the subcutaneous picrotoxin (sc Pic) seizure threshold tests, the subcutaneous strychnine (sc Strych) seizure pattern test and the NMDA-induced seizure test. The CD_{97} (dose that produced convulsions in 97% of the animal population) of the convulsant agents was administered subcutaneously in all cases, except NMDA, which was injected intracerebroventricularly (icv). The sc Bic and sc Pic tests measured the ability of anticonvulsants to afford complete protection against seizures, while the sc Strych test measured the ability of the test substance to abolish all tonic components of seizures. In the NMDA test, the effect of drug on the forelimb tonic extension (FTE) and clonus was measured separately.

The results indicated that the aziridine metabolite **5a** had anticonvulsant activity and in general, the activity of parent triazolines almost paralleled that of the aziridines (Table 1). No anticonvulsant activity was present in the β-amino alcohol **2a**, the triazole **4a**, or the ketimine **6a**. In addition, **1a** and ADD95032 [1-(4-trifluoromethylphenyl)-5-(4-pyridyl)-Δ^2-1,2,3-triazoline] (**1b**), afforded protection against bicuculline- and picrotoxin-induced seizures and both compounds were far more effective in the sc Bic than in the sc Pic test, with an ED_{50} of 12.1 mg kg^{-1} for **1a** (Table 2). However, both triazolines failed to afford protection against strychnine-induced seizures. On the other hand, **1a** and ADD61060 [1-(4-fluorophenyl)-5-(4-pyridyl)-Δ^2-1,2,3-triazoline] (**1c**), abolished both NMDA-induced FTE and clonus, with lower ED_{50} values in the NMDA-FTE test.

Metabolic studies

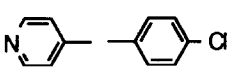
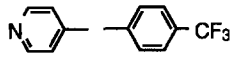
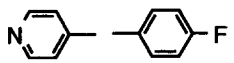
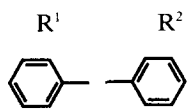
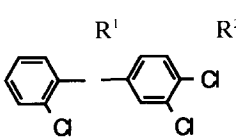
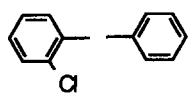
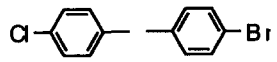
A full quantitative urinary metabolic profiling was performed following ip administration of **1a** to rats. Three drug related materials, along with unchanged **1a**, were isolated from rat urine, identified and quantitated. The two β-amino alcohols, **2a** and **2a′**, comprised the two major metabolites, with almost a 2.5-fold excess of the primary β-amino alcohol **2a** over the secondary β-amino alcohol **2a′**. The aziridine **5a** was present only in very minor amounts. No α-amino acid metabolite **3a** was detected. The per cent of administered dose of **1a** excreted as unchanged drug and the metabolites recovered in rat urine over a 48 h period are given in Table 3. None of the metabolites were sulfate or glucuronide conjugates.

All three metabolites and unchanged **1a** were purified using similar preparative TLC techniques and identified by comparison with authentic samples based on co-migration in TLC, co-elution upon high performance liquid chromatography (HPLC) (Table 4) and identical spectral results on mass spectral analysis. Quantitative determinations were based on methods developed for the complete recovery of triazolines and metabolites from biological fluids, and on fully validated HPLC assays by construction of calibration curves for **1a**, and metabolites **2a**, **2a′** and **5a**. TLC and HPLC chromatographic conditions were developed such that they did not lead to any ex vivo changes in the concentrations of drug and metabolites, and assured that the chemical integrity of the samples remained intact during analysis.

Radioligand binding studies

The interactions of triazolines **1a**, **1b** and **1c**, the β-amino alcohol **2a**, and the aziridine **5a**, at a variety of neurotransmitter, neuropeptide and ion-channel binding sites, were assayed in brain membranes at two

Table 1. Comparison of the anticonvulsant activity of triazolines **1** with the respective aziridines **5** and triazoles **4**, mouse, ip[a]

	TPE, h[b]	ED_{50}, mg kg^{-1}[c]		TD_{50}, mg kg^{-1}[c]	PI, TD_{50}/ED_{50}	
		MES	sc Met		MES	sc Met
R^1 R^2 						
Triazoline (ADD17014)	[0.5,0.5,6]	**257.4** (235.7–297.5) 12.9 ± 3.8	**43.5** (39.0–50.0) 8.7 ± 2.3	**1132.8** (970.4–1333.3) 7.9 ± 2.3	4.4	26
Aziridine	[2,2,4]	**57.2** (48.7–69.0) 8.9 ± 2.4	**96.7** (38.3–185.9) 2.0 ± 0.8	**330.0** (319.5–347.5) 39.2 ± 12.0	5.8	3.4
Triazole	Class III (inactive at doses up to 300 mg kg^{-1})					
 Triazoline (ADD95032)	[2,2,—] (461.8–1020.5)	**698.2** (332.2–491.2) 3.2 ± 1.0	**420.6** 7.0 ± 2.4	> 2000	> 2.9	> 4.8
	[1,0.5,1][d]	**51.3** (39.6–59.3) 8.2 ± 2.5	**51.3** (29.8–81.2) 2.9 ± 0.9	**116.6** (69.7–168.8) 2.9 ± 0.8	2.3	2.3
Aziridine	[2,2,0.5]	**139.0** (111.9–267.3) 5.9 ± 2.2	**254.3** (209.3–300.8) 8.3 ± 2.8	**258.4** (—) 12.4 ± 7.7	1.9	1.0
Triazole	Class I (active at 100 mg kg^{-1})					
 Triazoline (ADD61060)	[2,2,1]	**239.2** (192.1–296.8) 4.6 ± 1.2	**202.7** (110.6–296.9) 2.4 ± 0.7	**586.0** (546.4–626.3) 19.1 ± 5.3	2.4	2.9
Aziridine	[0.25,0.25,0.25]	**51.9** (44.8–62.5) 8.4 ± 2.1	**93.9** (66.7–118.2) 5.8 ± 1.6	**199.1** (159.9–220.9) 13.0 ± 4.8	3.8	2.1
Triazole	[0.5,0.5,0.25]	**91.6** (85.4–99.3) 18.9 ± 5.1	**119.3** (84.9–156.0) 2.8 ± 0.7	**181.1** (158.2–214.8) 10.1 ± 2.9	2.0	1.5
R^1 R^2 			R^1 R^2 			
Triazoline	Class III		Triazoline		Class III	
Aziridine	Class III		Aziricine		Class III	
Triazole	Class I (active at 100 mg kg^{-1})		Triazole		Class I	
						
Triazoline	Class III		Triazoline		Class III	
Aziridine	Class III		Aziridine		Class III	
Triazole	Class I					

[a]Administered in 30% PEG 400 (polyethylene glycol).
[b]The times of peak effect for the MES, sc Met and toxicity tests are shown in that order.
[c]Confidence limits are shown in ( ) and the values below are the slopes.
[d]Administered in 0.5% MC (methyl cellulose).

Table 2. The anticonvulsant profile of triazolines in chemically induced seizure tests, mouse ip

Compound [TPE, h]	ED_{50}, mg/kg[a]					TD_{50} mg/kg
	sc Bic	sc Pic	sc Strych	NMDA Clonus	NMDA FTE	
ADD17014	**12.1**[b]	**606.0**[b]	No protection	**107.2**[c]	**63.7**[c]	**1132.8**[b]
[0.25, 0.25, 0.25, 0.25, 6]	(7.6–16.9)	(281.3–1003.7)	up to	(56.9–165.4)	(31.6–112.4)	(970.4–1333.3)
	3.4 ± 1.1)	1.9 ± 0.7	1000 mg/kg[b]	2.9 ± 0.9	1.7 ± 0.5	7.9 ± 2.3
ADD95032[c,d]	**79.6**	**174.0**	**>500**	—[e]	—[e]	**116.6**[c]
[2,2,2,1]	(54.4–111.0)	(146.1–206.8)				(69.7–168.8)
	3.0 ± 0.8	8.3 ± 2.5				2.9 ± 0.8
ADD61060[c]	—[e]	—[e]	—[e]	**72.3**[c]	**10.5**[c]	**586.0**[b]
[2,1]				(48.2–104.8)	(2.9–26.7)	(546.4–626.3)
				2.6 ± 0.7	1.1 ± 0.4	19.1 ± 5.3
Phenytoin[f]	No protection	No protection	Maximum 50%	**8.6**	**0.6**	**65.5**
[2,2,2,1,2]	up to 100	up to 100	protection at	(6.6–14.1)	(0.3–0.9)	(52.5–72.1)
	mg/kg	mg/kg	55–100 mg/kg	8.4	2.1	15.1
Phenobarbital[f]	**37.7**	**27.5**	**95.3**	**2.7**	**3.1**	**69.0**
[1,1,1,2,0.5]	(26.5–47.4)	(20.9–34.8)	(91.3–99.5)	(0.2–4.9)	(1.4–4.7)	(62.8–72.9)
	4.1	4.8	18.5	1.7	2.4	24.7
Ethosuximide[f]	**459.0**	**242.7**	Maximum	**408.1**	**82.6**	**440.8**
[0.5,0.5,0.5,1,0.5]	(349.9–633.1)	(227.8–225.2)	62% protection	(341.7–488.0)	(30.9–131.9)	(383.1–485.3)
	3.2	26.4	at 1000 mg/kg	10.6	3.0	18.4
Valproate[f]	**359.9**	**387.2**	**293.0**	**146.2**	**82.8**	**425.8**
[0.25,0.25,0.25,0.25,0.25]	(294.1–438.5)	(341.4–444.4)	(261.1–323.4)	(120.9–161.8)	(57.4–112.9)	368.9–450.4)
	7.5	8.3	11.8	14.1	4.2	20.8

[a]ED_{50} values are followed by 95% confidence interval values in parenthesis and below that, the slope of the regression; line ± SE (standard error).
[b]Administered in 30% PEG.
[c]Administered in 0.5% MC.
[d]See Table 1 for ED_{50} values in the MES and sc Met tests and TD_{50} values, when administered in 30% PEG.
[e]Compound was not tested.
[f]Administered in 0.9% NaCl.

Table 3. Amounts of triazoline **1a** and its metabolites in rat urine over the periods 0–24 h and 24–48 h

Compound	% Administered drug recovered (±SD)[a,b,c]	
	0–24 h	24–48 h
β-Amino alcohol **2a**	45.7 ± 7.6	N.D.[d]
β-Amino alcohol **2a'**	17.3 ± 5.2	N.D.[d]
Triazoline **1a**	17.0 ± 4.6	~0.0
Aziridine **5a**	4.0 ± 0.02	~0.01

[a]Reference samples of these, necessary for construction of calibration curves, were obtained as described in experimental section.
[b]Animals were dosed 100 mg kg^{-1} of **1a**, by the ip route, n = 6.
[c]The relative molecular weights of metabolites were taken into consideration in these calculations.
[d]Not detectable or measurable.

Table 4. R_f values and retention times (t_R) of **1a** and its urinary metabolites (n = 3)

Compound	R_f values (TLC)[a]	Retention times (min) (HPLC)[a]
β-Amino alcohol **2a**	0.4	5.13
β-Amino alcohol **2a'**	0.58	5.88
Triazoline **1a**	0.63	7.35
Aziridine **5a**	0.81	10.7

[a]Conditions as outlined in Experimental.

different drug concentrations using appropriate radioligands. These studies included the various inhibitory [adenosine, benzodiazepine, γ-aminobutyric acid ($GABA_A$, $GABA_B$) and glycine] and excitatory [NMDA, kainate, quisqualate, dizocilpine (MK-801), phencyclidine (PCP), and strychnine-insensitive glycine] amino acid (IAA and EAA) receptor sites, and the chloride ion (Cl^-)-channel proteins that have been implicated in anticonvulsant action (Table 5).

All three triazolines and aziridine **5a** failed to show any degree of interaction at any of the IAA or EAA receptor sites even at 10 μM drug concentration. However, the triazolines evinced a significant degree of receptor activity at the Cl^--channel binding sites, inhibiting about 60% of the binding of *t*-[^{3}H]butylbicycloorthobenzoate (TBOB) to rat cortical membranes. The β-amino alcohol **2a**, on the other hand, inhibited [^{3}H]MK-801 binding at the MK-801 site on the NMDA receptor–ionophore complex, up to 56%, at 10 μM drug concentration, but had no effect on any of the other IAA or EAA receptor sites.

Presynaptic glutamate release

The effect of **1a** on Ca^{2+}-dependent K^+-evoked release of endogenous L-Glu was measured in guinea pig cortical slices. The results indicated a significant decrease of 54 and 83% in Glu release at 50 and 100 μM drug concentration, respectively (Fig. 1). An

Table 5. Radioligand binding studies

Receptor site[a]	Radioligand	Percent inhibition at 10^{-5} M concentration (n=2)				
		Triazolines			Aziridine, **5a**	β-Amino alcohol, **2a**
		ADD17014 (**1a**)	ADD9502 (**1b**)	ADD61060 (**1c**)		
Adenosine[b]	[^{3}H]NECA	−8.9	−2.0	−1.0	−20.2	1.3
Excitatory						
Glycine[c]	[^{3}H]glycine	−9.4	4.7	9.1	−18.7	−12.4
Kainate[d]	[^{3}H]Kainic acid	4.6	−0.1	1.9	−11.1	−13.7
MK-801[d]	[^{3}H]MK-801	29.9	−5.0	9.1	20.2	**55.9/40.9**[i]
NMDA[d]	[^{3}H]CGS19755	3.3	3.5	2.9	−2.4	19.1
PCP[c]	[^{3}H]TCP	12.5	12.3	−6.1	−14.3	11.6
Quisqualate[d]	[^{3}H]AMPA	−0.7	−5.0	−19.9	6.2	13.0
Inhibitory						
Benzodiazepine[f]	[^{3}H]Flunitrazepam	−1.4	−13.1	−19.4	0.4	0.5
$GABA_A$[g]	[^{3}H]GABA	−2.5	1.1	3.8	−5.2	−6.1
$GABA_B$[d]	[^{3}H]GABA in the presence of 40 μM isoguvacine to block $GABA_A$ sites	−11.7	−13.9	−10.6	−19.0	16.2
Glycine[b]	[^{3}H]Strychnine	−1.3	−6.7	−0.5	8.1	4.2
Channel proteins						
Chloride[c]	[^{3}H]TBOB	**63.4/55.9**[i]	**60.1/49.1**[i]	**56.6/56.9**[i]	26.0	4.6

[a]The receptor sources are: [b]rat striatal membranes; [c]rat cortical membranes; [d]rat forebrain membranes; [e]rat forebrain membrane fraction; [f]bovine cortical membranes; [g]bovine cerebellar membranes; [h]rat spinal cord membranes; [i]values from two different experiments.

approximate estimate of the relative effects of **1a**, aziridine **5a** and β-amino alcohol **2a** on Glu release from rat hippocampal tissue slices at 1 mM drug concentration was also obtained using a multiwell system; while the effect of aziridine **5a** was much less than that of **1a**, the β-amino alcohol **2a** had no effect.

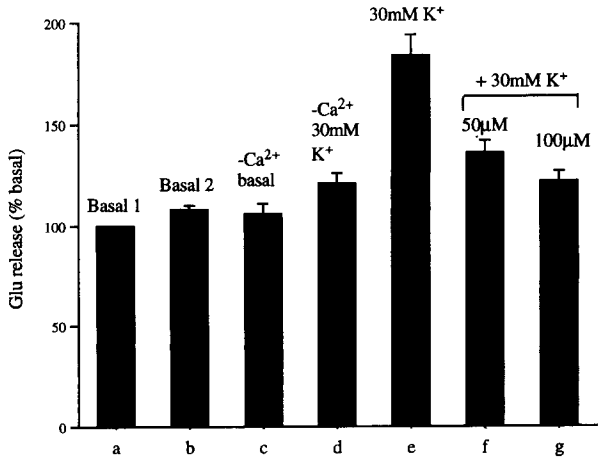


Figure 1. Effect of triazoline **1a** on K^+-evoked- Ca^{2+}-dependent endogenous L-glutamate release from guinea pig cortical slices. (a) Basal 1, spontaneous unstimulated release; (b) Basal 2, basal 1+drug; (c) Basal $1-Ca^{2+}$; (d) Basal $1-Ca^{2+}+30$ mM K^+ (c and d demonstrate that basal release is Ca^{2+}-independent and that K^+-stimulated release is primarily a Ca^{2+}-dependent process); (e) K^+-stimulated release; (f) K^+-stimulated release + 50 μM drug; (g) K^+-stimulated release + 100 μM drug.

Discussion

Triazolines as 'built-in' heterocyclic prodrugs: potential biotransformation products of 1a

Triazoline **1a** and congeners may be considered to represent a new class of heterocyclic 'prodrug' compounds in which the active 'structure element' is 'built in' as an integral part of the ring system. Thus, a knowledge of their chemical reactivity and metabolism is essential for identifying the 'hidden' pharmacophores (e.g. the β-amino alcohol **2a** or the α-amino acid **3a**) (Scheme 1) and provides a new approach to prodrug design.

In the last decade, several new families of anticonvulsants that do not have a dicarboximide (—CO—NH—CO—) and/or a ureide (—NH—CO—NH—) function have been described; these are integral parts of the traditional anticonvulsants and are also absent in the unique triazoline ring system (**1**). The triazolines are chemically related to triazoles **4**, aziridines **5**, and ketimines **6**, as shown in Scheme 1.[12] Both triazolines[11] and aziridines[14] are susceptible to acid hydrolysis. However, while the triazolines yield the primary β-amino alcohols **2** with loss of N_2, the aziridines yield two isomeric β-amino alcohols, a primary one (**2a**) from C(3)-N(1) bond opening, and a secondary one (**2a'**) from C(2)-N(1) bond breakage. Under in vivo conditions, the primary β-amino alcohols could be oxidized[15] to α-amino acids **3**, which are α-substituted glycines (Scheme 1). α-Amino acids can also result

from triazolines and aziridines, but not from triazoles, via the transient α-amino aldehyde intermediate **1-Y** arising from in vivo oxidation at the methylene group α- to the nitrogen[15] (**1-X**, **5-X**, Scheme 1). Thus, based on its unique structure, compounds **2a-6a** may be potential biotransformation products of **1a**, and the anticonvulsant activity of the triazolines may be mediated through one or more of these metabolites, with the triazolines functioning as prodrugs.

The triazoline ring system also bears a chiral center at C-5, which remains intact in metabolites **2**, **2′**, **3** and **5**, during biotransformation. However, in this study no attempts have been made to separate the two stereoisomeric forms or to determine their influence on anticonvulsant activity. All the chiral compounds described in this paper are racemate mixtures.

Anticonvulsant activity of the potential metabolites of 1a

Although triazolines can potentially be metabolized to triazoles and aziridines, only triazolines and aziridines can undergo hydrolysis to β-amino alcohols **2** or in vivo oxidation to α-amino acids **3** (Scheme 1). Biotransformation of 4-hydroxytriazolines **4-X** to α-amino acids requires keto⇌enol tautomerism, which cannot occur in the direction of the ketone because of the high energy barrier imposed by the aromaticity of the triazole ring system.[12] In fact, while the presence or absence of anticonvulsant activity in the aziridines paralleled that of the parent triazolines, such a relationship did not exist for the respective triazoles (Table 1). As a rule, triazoles such as **4a** derived from active triazolines were inactive and several triazoles derived from completely inactive triazolines evinced significant anticonvulsant activity.[6] Apparently, the triazoles work by a different mechanism of action. Similarly, the lack of anticonvulsant activity in ketimine **6a**, eliminated it as the active species. The ketimine, in spite of its lipophilicity, undergoes hydrolysis,[16,17] to yield the ketone and the aniline (Scheme 1), both of which showed no anticonvulsant activity in the standard MES and sc Met tests, mouse, ip. Thus it appears that the aziridine **5a**, the β-amino alcohol **2a** or **2a′**, and/or the α-amino acid **3a** may be the active metabolite species responsible for the anticonvulsant activity of the 'built-in heterocyclic prodrug', **1a**. However, the β-amino alcohol failed to afford seizure protection in both the sc Met and MES tests in the mouse, apparently because of its non-lipophilic character and inability to cross the blood brain barrier (BBB), thus providing further evidence for the prodrug hypothesis. The lipohilic triazoline prodrug enters the brain where it is biotransformed to the active β-amino alcohol **2a**.

Metabolite identification

Corroborative evidence for the triazoline prodrug hypothesis and the formation of the β-amino alcohol **2a** as the active species, was obtained from full quantitative urinary metabolic profiling studies (Table 3). Of the three metabolites isolated, the β-amino alcohols **2a** and **2a′** comprised the two major metabolites, with **2a** predominating over **2a′**, and the aziridine **5a**, the minor one. Metabolites and unchanged **1a** were identified unequivocally based on their chromatographic properties, i.e., R_f and t_R values of migration and retention times in TLC and HPLC, respectively (see Table 4), and their UV and mass spectra, all of which were identical to the authentic samples prepared by chemical synthesis or isolation from biological fluids as described in the Experimental. Calibration curves for drug and metabolite quantitation by HPLC techniques were obtained using procedures similar to those previously developed for **1a**.[18]

The mass spectral analysis of metabolites helped further to identify and distinguish between **2a** and **2a′** and also to positively discount the formation of triazole **4a** and ketimine **6a** as metabolites. The two isomeric β-amino alcohols showed characteristic fragmentation patterns in their mass spectra. The primary β-amino alcohol **2a** showed a molecular ion (15% relative abundance) at *m/z* 248 and a base peak at *m/z* 217 (C_5H_4N-CH-NH-C_6H_4Cl) due to loss of the -CH_2OH group, with other peaks at *m/z* 182 (5%) (C_5H_4N-CH-NH-C_6H_4), *m/z* 111 (5%) (C_6H_4Cl) and *m/z* 139 (3%) (CH-NH-C_6H_4Cl). On the other hand, the mass spectrum of the secondary β-amino alcohol **2a′** produced a base peak at *m/z* 108 due to the C_5H_4N-CH-OH species formed from loss of -CH_2-NH-C_6H_4Cl and a molecular ion peak occurring at *m/z* 248 (relative abundance 17%). Other peaks for **2a′** were found at *m/z* 140 (57%) (-CH_2-NH-C_6H_4Cl) and *m/z* 137 (5%) (C_5H_4N-CH(OH)-CH_2-NH).

In view of the fact that **1a** can be converted to the aziridine **5a** by simple chemical degradation,[17] the reaction occurring in vivo may be via enzymic or non-enzymic mechanisms. The predominance of the β-amino alcohol metabolite **2a** is important because it validates the proposed prodrug hypothesis. The identification of β-amino alcohol **2′** as a minor metabolite (Table 3) is also significant, because it can result only by the C(2)-N(1) ring opening of the aziridine **5a**, and provides evidence that the biotransformation of **1a** occurs, at least in part, via aziridine formation. This is further supported by the isolation and identification of β-amino alcohols, **2a** and **2a′**, in pharmacokinetic studies of both **1a** and **5a** (unpublished data). Thus the parallel activities (Table 1) of the aziridines and their parent triazolines appear to result from the production of the same metabolites and may also account for the action of **5a** as a prodrug similar to **1a**.

As the secondary β-amino alcohol **2a′** could not be obtained by synthesis, sufficient quantitites were not available for determining in vivo anticonvulsant activity. However, unlike **2a**, **2a′** does not seem to contribute to anticonvulsant activity, because of the striking differences between the metabolite pharmacokinetics of 1-(4-anisyl)-5-(4-pyridyl)-1,2,3-triazoline

(**1d**), which shows no anticonvulsant activity even up to 300 mg kg^{-1}, and that of the pharmacologically active **1a** (unpublished data). While the secondary β-amino alcohol **2d′** and the aziridine **5d**, both produced concentration–time curves similar to those obtained for the respective metabolites from the biologically active analogues **1a** and **1c**, the primary β-amino alcohol **2d** had an extremely low AUC (area under the curve), less than 6% of the AUC for the structurally related **2a**, over an 8 h period. In addition, while the concentration of **2a** was still increasing after 8 h, the **2d** metabolite was no longer detectable after 4 h. These unpublished pharmacokinetic data provide strong evidence that the primary β-amino alcohol **2** is the active species and the secondary β-amino alcohol **2′** is inactive. These results are obviously of great importance in explaining the differing antiepileptic activity of closely related triazoline analogues.

Although β-amino alcohol **2a** was found to be the predominant urinary metabolite, there was no evidence for the presence of the α-amino acid **3a**. Apparently, **2a** does not seem to undergo further in vivo oxidation to **3a** as proposed, and also no in vivo oxidation of triazoline **1a** or aziridine **5a** involving C-4 hydroxylation on the triazoline ring or C-3 hydroxylation on the aziridine ring appears to operate. On the other hand, the highly polar **3a** is nonextractable under the conditions utilized, or if it co-elutes with the solvent front, a more detailed study using radiolabeled **1a** is essential, to fully characterize the metabolic pathways of the triazoline.

The mean recovery of **1a** in rat urine over 48 h was 17%, and the total recovery of **1a** and metabolites together was 84%; this is in agreement with previous work[19] where the recovery of radioactivity in urine following dosing with radiolabeled **1a** was found to be 99%. Here, the lack of any long term residual radioactivity was interpreted to demonstrate either a lack of aziridine formation, or if formed, it lacked sufficient reactivity to alkylate tissue sites. From the present results indicating aziridine **5a** as a metabolite, it would appear that **5a** with no alkyl substitution, is not reactive enough to form a stable carbonium ion. This is also supported by the stability of **5a** at pH values of 7.4 and 7.0 with no resultant hydrolysis compared to **1a** which undergoes 22% and 30% hydrolysis at these respective pH values over 1 h at room temperature.

Neurochemical studies: amino acids in chemical neurotransmission

The importance of amino acids in chemical neurotransmission in the central nervous system (CNS) has been extensively reviewed.[20,21] While GABA and glycine serve as inhibitory neurotransmitters, L-Glu and L-Asp (L-aspartate) function as excitatory neurotransmitters in the CNS. EAAs may be critically involved in both epileptogenesis and as a focus for the mechanism of action of anticonvulsants. Because brain function in the normal state is a dynamic balance of excitatory and inhibitory processes, excessive neuronal activity leading to seizures can result from an increase in excitatory transmission, or, alternately, a decrease in inhibitory transmission. Consequently, effecting changes in the concentrations of either excitatory or inhibitory neurotransmitters at their synapses would represent potential mechanisms of anticonvulsant action and strategies for anticonvulsant drug design. There is mounting evidence that the excitatory neurotransmitter L-Glu plays a key role in the spread of epileptic activity from one brain region to another and may also be contributing to its initiation. Thus EAA agonists are convulsants and EAA antagonists show anticonvulsant activity in a variety of seizure models.

The postsynaptic actions of L-Glu are believed to be mediated by at least three receptor subtypes named after the prototypical agonists, NMDA, kainate (KA) and quisqualate (QA). Although NMDA itself is not present in the brain, endogenous L-Glu has the highest affinity for the NMDA receptor. The NMDA receptor is the best characterized of the three EAA receptor subtypes. NMDA receptors are associated with, and influenced by, three additional types of binding sites; a PCP (the anaesthetic and psychotomimetic drug phencyclidine) binding site that is located within the receptor-associated ion-channel, thereby blocking channel conductance; a strychnine-insensitive excitatory glycine binding site that is distinct from the strychnine-sensitive inhibitory glycine receptor of the spinal cord; and a polyamine recognition site. The PCP receptors, in turn, may constitute two subreceptor sites, each with a high degree of selectivity and specificity for the respective ligands, MK-801 and TCP (a PCP analogue with a thienyl group in place of the phenyl).

Receptor interaction profiles of triazolines 1a, 1b and 1c and metabolites 2a and 5a

The possible biotransformation of **1a** to yield the potential α-amino acid **3a**, a substituted glycine, had initially led us to suspect that the triazolines probably acted either by enhancing the neurotransmission of inhibitory glycine or by functioning as an NMDA/glycine site antagonist. However, radioligand binding studies of **1a** and metabolites **2a** and **5a**, as also of **1b** and **1c** at the various IAA and EAA receptor sites using appropriate radioligands, indicated no significant activity for any of the compounds at any of these receptor sites, except for the β-amino alcohol **2a**; it showed a noticeable 56% inhibition in the specific binding of [^{3}H]MK-801 to the MK-801 site at 10 μM test concentration, with no effect on the NMDA/glycine site or the KA and QA receptor sites (Table 5). These results are consonant with our earlier studies in which **2a** inhibited [^{3}H]Glu binding with an $IC_{50} = 24.5 \pm 3.7$ μM and $K_i = 14.3 \pm 2.2$.[8] The failure of **1a** to inhibit [^{3}H]-ligand binding at the IAA or EAA receptor sites, in contrast to the significant activity of the β-amino alcohol **2a** at the MK-801 site along with the lack of evidence for **3a** formation, further suggested that the postsynaptic action of **1a** may be as a

'prodrug', with the β-amino alcohol **2a** acting as an NMDA/MK-801 receptor antagonist. Indeed, this is supported by the high effectiveness of ip administered triazolines **1a** and **1c** against NMDA-induced FTE as well as clonus (Table 2) and also by observations in previous studies[1,9] on the complete protection afforded by **1a** in the kindling model of epilepsy.

The prodrug hypothesis

The absence of any postsynaptic activity in the aziridine metabolite **5a**, and the close similarity of its receptor binding profile with that of **1a** (Table 5), suggested that **5a** may also be functioning as a prodrug just as **1a**, apparently by undergoing transformation to the active **2a**. This is consistent with the chemical (Scheme 1) and in vivo pharmacological (Table 1) similarities of both compounds, and also the results obtained from urinary metabolic profiling and pharmacokinetic studies. Apparently, an 'open flexible' structure is a requirement for activity at the postsynaptic receptor sites; the closed ring structures in both the triazoline and the aziridine may impart a certain degree of rigidity, preventing them from attaining the proper substrate stereochemistry for receptor interaction. Computer-assisted analysis of the structures of several neuroprotective agents that are NMDA antagonists, indicates a common structural pattern associated with neuroprotective property which involves an aromatic moiety connected to another polarized moiety through a flexible short chain.[22]

The inability of the β-amino alcohol **2a** itself to afford any degree of protection in the standard sc Met and MES tests following ip administration in the mouse, provided additional support for the prodrug hypothesis. Apparently, the low lipophilicity and the pronounced ability of amino alcohols in general to undergo hydrogen bonding interactions with the physiological medium, prevent **2a** from penetrating across the BBB. In accordance with this, even at the high dose of 300 mg kg^{-1}, no neurotoxicity was observed; instead, the animals died from respiratory depression, suggesting there was no BBB penetration. On the other hand, **1a** is highly lipophilic; tissue distribution studies of radiolabel after ip administration of [^{14}C]-labeled **1a** have indicated rapid drug uptake, with brain attaining a maximum concentration of 0.1478% of administered dose g^{-1} of tissue in less than 30 min, and still retaining 0.1345% at 1 h.[19,23] These results are consistent with the time of peak effects of **1a** in the various seizure models (Tables 1 and 2) including the kindling model of human focal epilepsy.[9] Furthermore, earlier structure–activity relationship studies on the triazolines had also indicated an optimum anticonvulsant effect for an optimum π (lipophilicity) $+ \sigma$ (Hammett electronic constant) value.[6] Thus, unlike the β-amino alcohol **2a**, the highly lipophilic triazoline **1a** can rapidly cross the BBB in the prodrug form and then be transformed to **2a**. The triazoline clearly has a pharmacokinetic advantage over the highly polar NMDA antagonists, APB (α-amino-4-phosphonobutyrate), APV (α-amino-5-phosphonovalerate), and APH (α-amino-7-phosphonoheptanoate), which evoke anticonvulsant activity only when administered intracerebroventricularly, due to poor penetration of the BBB by the systemic route of administration.[20]

The inability of **2a** to displace [^{3}H]strychnine from the strychnine sensitive inhibitory glycine binding site in rat spinal cord membrane preparations, is consistent with the failure of triazoline **1a** and also **1b** to afford protection against strychnine-induced seizures in the mouse (Table 2). In addition, although **2a** did not show any activity at the strychnine-insensitive glycine binding site on the NMDA receptor complex (Table 5), the activity of **2a**, if any, at the NMDA/polyamine receptor site is not determined.

Presynaptic release studies

Measurement of the effect of triazoline **1a** on the Ca^{2+}-dependent K^+-evoked release of endogenous L-Glu from guinea pig cerebrocortical slices, indicated that the triazoline significantly decreased L-Glu release by 58 and 83% at 50 and 100 μM concentrations, respectively (Fig. 1). Also preliminary measurements in rat hippocampal slices had earlier indicated that while aziridine **5a** was much less effective than triazoline **1a**, **2a** had no effect at all.[9]

Excessive Glu release reaching neurotoxic levels is known to occur during epileptic seizures as a result of a runaway rise in extracellular K^+ concentration in the brain[24] and to lead to overstimulation of the NMDA receptor resulting in EAA-induced 'excitotoxicity' and chronic or acute cell death.[20,25] A rise in K^+ concentration will release more Glu into the extracellular space[24] which will cause the neurons to depolarize further and to release more K^+, in a 'positive feedback system', leading to a large rise in extracellular Glu concentration. The hippocampus, enriched in EAA receptors, suffers neuronal loss after an episode of status epilepticus or temporal lobe epilepsy, the most common form of focal (partial) epilepsy, probably by the release of EAAs in neurotoxic concentrations followed by Ca^{2+} influx through the stimulated NMDA receptor–ion-channel complex. The mitochondria in selectively vulnerable hippocampal neurons show massive overloading with Ca^{2+} during status epilepticus. Thus drugs that reduce Glu release may be strategically more important as anticonvulsants, because of their ability to impair the continued accelerated Glu release in the 'positive feedback cycle', in pathological conditions at the presynaptic level itself.

Activity at ion-channel proteins

Binding assays conducted for triazoline **1a** and metabolites **2a** and **5a** as well as triazolines **1b** and **1c** at the Ca^{2+} (L, N and T), Cl^- and K^+ channels, indicated significant activity for all three triazolines at the Cl^-

channels in the range of 50–63%, but none for metabolites **2a** and **5a** (Table 5).

Since Bic is a GABA receptor antagonist,[21] the activity of the triazolines at the Cl^- channel proteins along with the remarkable effectiveness of **1a** (ED_{50}, 12.1 mg kg^{-1}) and to a lesser extent that of **1b** (ED_{50}, 79.57 mg kg^{-1}) against sc Bic induced seizures (Table 2), would logically indicate that the anticonvulsant activity of the triazolines may be the result of an enhancement in inhibitory neurotransmission. However, the failure of all three triazolines to show any degree of activity at the GABA/benzodiazepine receptor sites or the adenosine uptake sites (Table 5), and the lack of evidence for **3a** formation, seemed to rule out this possibility.

Alternately, the significant augmentation in Cl^- channel activity could be associated with the ability of triazoline **1a** to inhibit L-Glu release. One of the mechanisms proposed for the control of presynaptic EAA release may involve the Bic-insensitive $GABA_B$ (baclofen) receptor; low doses of baclofen (4 μM) have been found to preferentially inhibit the stimulated release of ^{14}C-Glu and ^{14}C-Asp in preloaded slices of guinea pig cerebral cortex, in addition to causing an increase in glycine turnover.[26] It is suggested that this antispastic drug affects transmission not by modulating the release of the IAA (GABA), but by selectively suppressing the release of the EAAs (Glu and Asp), from nerve terminals and probably has an additional effect on inhibitory glycinergic interneurons. However, the lack of interaction of ADD17014 at the $GABA_B$ receptor, similar to that at the $GABA_A$ site, and also at the inhibitory glycine receptor, indicated that apparently, such a mechanism does not seem to be the prevailing one for impairment of Glu release by **1a**.

On the other hand, it has been proposed[25] that one straightforward method for reducing presynaptic L-Glu release might be to reduce neuronal firing at glutamatergic nerve terminals; augmentation in Cl^- influx is a useful membrane action that reduces membrane excitability or alters circuit behavior to favor inhibition, and thus might help suppress the firing of glutamatergic neurons, and hence L-Glu release. In line with this mechanistic pathway, is the remarkable observation that metabolites **2a** and **5a** that fail to inhibit Glu release[9] also lack activity at the Cl^- channels, particularly **5a**, in spite of its close similarities to the parent triazoline **1a**.

'Dual-action' mechanism and its significance

The foregoing results and discussions seem to suggest that the high anticonvulsant activity of triazoline **1a** may be due to its unique 'dual-action' mechanism whereby the parent **1a** attenuates presynaptic Glu release and the metabolite **2a** functions as a Glu antagonist by blocking the NMDA/MK-801 receptor site; together they effectively impair both pre- and postsynaptic aspects of EAA neurotransmission and hence the overstimulation of the NMDA receptor. The triazolines thus provide a unique class of EAA inhibitors that can afford protection against seizures in a variety of animal models[8] (Table 2), including the kindling model of human focal epilepsy.[1,9] Unlike MK-801 and AP5, which inhibit only kindling epileptogenesis but not epileptic seizures themselves,[27] the triazolines by virtue of their 'dual-action' mechanism for impairing EAA neurotransmission, may afford both prophylaxis as well as seizure protection particularly in complex partial epilepsy which is refractory to currently available drugs. Furthermore, drugs that reduce neuronal firing and hence Glu release by an augmentation in Cl^- influx may be most beneficial in the control of prolonged seizures such as in status epilepticus where excessive neuronal firing occurs.[25]

As the triazolines are capable of pharmacologically manipulating EAAs and are effective in a variety of animal seizure models, it is logical to expect that these compounds may provide new directions for the rational synthesis of therapeutic agents for other neurological disorders where EAAs play a key role. Overstimulation of the NMDA receptor by high levels of L-Glu has been implicated not only in epilepsy but in other neurological disorders such as stroke and trauma, and certain neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), Alzheimer's, Huntington's and Parkinson's diseases.[20,25] Since the triazolines can block the action of Glu and thus the overstimulation of the NMDA receptor, they may represent novel therapies as neuroprotective agents, for these disorders.

Experimental

Melting points were taken using a Thomas–Hoover capillary electric mp apparatus and are not corrected. UV spectra were determined on a multi-wavelength scanning detector, the Rapiscan Severn Analytical (Macclesfield, Cheshire, U.K.). Electron impact mass spectra were recorded by the peak matching technique on a VG16F mass spectrometer. NMR spectra were recorded on a 90-Hz Perkin Elmer R10 spectrometer in CD_3OD solutions with TMS as internal standard. Preparative TLC was performed on laboratory prepared TLC plates coated with silica gel 60AGF254 to a depth of 1 mm, and developed in a solvent system of ethyl acetate:butanol:ammonia (80:20:0.4, v/v). The HPLC apparatus consisted of a Constametric pump 3000, a variable wavelength UV Spectromonitor 3100 and a computing integrator CI 4000, (all from LDC Analytical, Stone, Staffs, U.K). The column was reversed phase Spherisorb 5-ODS column (25 × 0.46 cm) (HPLC Technology, Macclesfield, Cheshire, U.K.) with a pellicular ODS guard column (5 × 0.2 cm) (Whatman, Maidstone, Kent, U.K.) to protect the analytical column. Samples were introduced into the system via a Rheodyne injector fitted with a 20 μL loop. The mobile phase consisted of methanol:

acetonitrile:McIlvaine's citric acid:phosphate buffer (pH 8, 0.005 M) (30:30:40, v/v). Detection was by UV absorption at 254 nm and all chromatograms were recorded and peak areas and retention times calculated by electronic integration using the computing integrator CI 4000. For the photolysis reactions, a 275 Watt G. E. sunlamp served as the light source with wavelength in the 300 nm range. C, H and N elemental analyses were performed by Oneida Research Services, Inc., One Halsey Road, Whitesboro, NY 13492, U.S.A.

Chemistry

Synthesis of 1, 4 and 6a. Both the diaryl- (Table 1)[28] and the pyridyl-substituted triazolines (Scheme 1)[29] were prepared following previously published procedures.

The triazoles **4** were obtained by NiO_2 oxidation of triazolines **1**, and metabolite **6a**, by the permanganate catalyzed low temperature thermolysis[30] of **1a**.

Synthesis of aziridines 5. The aziridines were generated from the triazolines by photolysis.[11,31] In a typical experiment, an acetone solution of the triazoline, usually 2–3%, was irradiated at 20–25 °C, until N_2 gas evolution ceased.

Aziridine **5a**, used as a metabolite standard, was recrystallized twice from acetone:petroleum ether mixture, to yield a crystalline solid, mp 105–106 °C. Molecular formula, $C_{13}H_{11}N_2Cl$; ^1H NMR, δ, ppm (CD_3OD) 3.0 (q, 2-CH), 2.4 (m, 3-CH_2), 8.6 (d, 2, 6-Pyr H), 7.5 (s, 3, 5-Pyr H), 7.0 (s, 2, 6-Ph H), 7.3 (s, 3, 5-PhH); MS, *m/z* (M^+, %). 229 [(M-1)$^+$, 100], 111 (C_6H_4 Cl, 47), 125 (N-C_6H_4Cl, 57), 138 (CH-N-C_6H_4Cl, 43); R_f value, 0.81; t_R value, 10.7 min.

Preparation of β-amino alcohol 2a. This was prepared by the acid hydrolytic decomposition of **1a**. The triazoline (50 mg) was dissolved in ethanol (3.3 mL) to produce a solution containing 17.4 μmol of **1a**. To this was added an equivalent amount of HCl (174 μL of 0.1 M HCl). The mixture was then placed in a shaking water bath at 40 °C. Immediately upon addition of the acid, N_2 gas evolution occurred and the reaction was taken as finished when all gas evolution had ceased, usually about 15–20 min.

The reaction mixture was found to contain three products, as well as residual **1a**. It was separated and purified by preparative TLC. Six plates (each 20 × 20 cm) were coated to a thickness of 1 mm with silica gel GF254 and precleaned with toluene and methanol (both HPLC grade), to remove any impurities in the silica gel which would otherwise produce spurious peaks upon mass spectral analysis. The impure solution of **2a** was then applied as bands, 1.5 cm from the bottom of each plate. The plates were then developed in a solvent system of ethyl acetate: *n*-butanol (80:20 v/v) with 0.4% ammonia, an adaption of a method described previously.[16] Ascending TLC was performed in glass TLC tanks presaturated with the solvent system; after the solvent had risen to a height of 15 cm from the origin, the plates were removed and air-dried. After visualization under UV light at 254 nm, the bands of silica gel containing UV absorbing compounds were carefully removed and extracted into methanol (5 mL) by shaking (15 min). Each of the resulting extracts was analyzed by the Rapiscan UV detector and by HPLC using conditions previously developed.[16–18] Although the UV spectra obtained by the Rapiscan technique is not useful in the positive identification of metabolites, it allows for the determination of UV spectra directly on the HPLC eluants and indicates whether the HPLC peaks are the result of pure compounds, uncontaminated by any co-eluting substances. Those TLC isolates that corresponded to **2a** were evaporated to dryness. In order to confirm that the sample was totally dry and the final weight of **2a** did not include any residual methanol, a semi-gravimetric approach was used, which involved drying and weighing the sample to constant weight. This is important, since this sample would be used to construct calibration curves for the quantification of biological extracts. The pure, dry sample of **2a** thus obtained was 1.9 mg.

The identity of the sample as **2a** was confirmed by its chromatographic properties and by mass spectrometry. The electron-impact mass spectrometry (EIMS) was done by the peak-matching technique on a VG 16F mass spectrometer (electron energy, 70 eV; ion source temperature, 240 °C; trap current, 100 μA). Samples were introduced via the direct-insertion probe at an accelerating voltage of 4.0 kV and the spectra were recorded with the aid of VG Data System 2000, as rapidly as possible after sample introduction but without additional heating of the probe. MS, *m/z* (M^+, %); 248 (M^+, 15), 217 (C_5H_4N-CH-NH-C_6H_4Cl, 100), 182 (C_5H_4N-CH-NH-C_6H_4, 5), 111 (C_6H_4Cl, 5), 139 (CH-NH-C_6H_4Cl, 3); R_f value, 0.4; t_R value, 5.13 min.

Preparation of β-amino alcohol 2a′. This metabolite was isolated from rat urine, since unpublished in vitro metabolic studies of **1a** by rat hepatic microsomes indicated that **2a′** was readily formed in large amounts in vivo. It was purified by TLC and fully characterized by HPLC as described for **2a** and subsequently used as a chromatographic standard. Triazoline **1a** (100 mg kg^{-1}) was dosed to six rats and the urine collected as described below for metabolic profiling. The identity of **2′** was established by its chromatographic properties and its characteristic mass spectrum that has a different fragmentation pattern from that of **2a**. MS, *m/z* [M^+, %]; 248 (M^+, 17), 108 (C_5H_4N-CH-OH, 100), 140 (CH_2-NH-C_6H_4Cl, 57), 137 (C_5H_4N-CH(OH)-CH_2-NH, 5); R_f value, 0.58; t_R value, 5.88 min.

Pharmacology

The triazolines, triazoles and aziridines including metabolites **2a**, **4a**, **5a** and **6a**, were all tested by the Anticonvulsant Screening Project (ASP) under the

Antiepileptic Drug Development (ADD) program of the Epilepsy Branch of the National Institute of Neurological Disorders and Stroke (NINDS), following established standard procedures.[10]

The compounds were emulsified in 30% polyethylene glycol 400 (PEG) or 0.5% methyl cellulose (MC). The solvents were tested for anticonvulsant and toxic effects and found to introduce no significant bias into the testing of anticonvulsant activity. The compounds were administered ip in a volume of 0.01 mL g^{-1} body weight to male Carworth Farms No. 1 mice weighing ~20 g. Testing was done at three dose levels of 30, 100 and 300 mg kg^{-1}, and a total of 12 animals were used, four for each dose. After 30 min, each animal was examined for toxicity in the rotorod test. Immediately thereafter, anticonvulsant activity was evaluated by subjecting one mouse to the MES test and another to the sc Met test. The same tests were repeated 4 h later on the two remaining mice at each dose level. For compounds that afforded protection, the test was repeated at that dose level and time with four animals, and the results were expressed as number of animals protected per number of animals tested.

In the MES test, abolition of the hind limb tonic extensor component of maximal seizures was defined as protection. In the sc Met test, failure to observe even a threshold seizure (a single episode of clonic spasms of at least 5-s duration) was defined as protection.

Neurotoxicity was evaluated by placing the animal on a wooden rod of 2.8 cm diameter, rotating at 6 rpm. Normal mice remained on a rod rotating at this speed indefinitely, and failure of the animal to remain on the rod for 1 min was defined as neurological toxicity and expressed as number of animals exhibiting toxicity per number of animals tested.

To determine ED_{50} or TD_{50} values at TPE, five logarithmically spaced doses of the test compound were administered to animals in groups of 10, to cover 0–100% protection or toxicity. The dose required to produce the desired end point in 50% of the animals in each test (ED_{50}, TD_{50}), together with the 95% confidence limits and the slopes of the regression lines, were then determined.

The sc Bic, sc Pic and sc Strych tests were performed as described previously.[8] In the NMDA-induced seizure test, at the TPE of the test substance, the CD_{97} of NMDA (0.2 μg $5\mu L^{-1}$, for clonus and 3μg $5\mu L^{-1}$, for FTE) was injected into the lateral ventricle of mice. The animals were observed for 30 min for the presence or absence of a seizure. The animals were then sacrificed by cervical dislocation and the brains removed and dissected. The icv injection was scored positive only when the methylene blue dye was observed throughout the ventricular space. Absence of either FTE or clonus indicated that the test substance had the ability to interfere with Glu transmission (Table 2).

Metabolic profiling studies: animal treatment for urine collection

Animals (male Wistar rats, Kings College Animal Unit, 220–240 g, n = 6) were placed in individual perspex metabolism cages and control urine collected over a 24 h period. The animals were administered **1a**, ip at a dose of 100 mg kg^{-1} (dissolved in hydroxypropyl-β-cyclodextrin) and replaced in the metabolic cages.

In view of the chemical instability of **1a** at certain pH values,[16,17] and since the pH of rat urine can vary widely, the urine was immediately frozen by collecting it into tubes immersed in liquid nitrogen, thus ensuring no ex vivo alterations in concentration occurred. Under these conditions authentic compounds, when placed into control (blank) rat urine, did not undergo any degradation. Urine was collected for 0–24 h and 24–48 h. At the end of each period of urine collection, the funnels used to collect the urine were rinsed with 5.0 mL of distilled water to ensure all the urine produced was in the sample tube. Urine samples were stored at −20 °C until analysis.

Identification of metabolites in rat urine

The determination of **1a** and metabolites was performed using the same analytical techniques that were developed previously for the identification and quantitation of **1a** in rat blood.[18]

HPLC chromatograms obtained for pre- and post-dose urine extracts clearly showed four major peaks in the post-dose sample which were absent in the control (pre-dose) sample. Extraction was carried out using diethyl ether similar to that used for blood.[18]

Preparative TLC was performed as described above for the preparation of **2a** and the bands that fluoresced under UV light were carefully removed and eluted with methanol (5 mL) by mechanical shaking (15 min) and evaporated to dryness. The metabolites and unchanged **1a** corresponding to the four peaks in the HPLC chromatogram, were initially identified as **1a**, **2a**, **2a′** and **5a** by their comigration upon TLC and coelution upon HPLC with authentic samples (Table 4) and then confirmed by their mass spectra.

For quantitation of unchanged drug and urinary metabolites (Table 3), calibration curves for **1a**, **2a**, **2a′** and **5a** were constructed and the precision and accuracy of the assays by the HPLC technique used, were fully validated as described previously for **1a** in rat blood.[18] The calibration curves were produced by spiking known concentrations of the drug or metabolites into control rat urine (0.5 mL) together with the internal standard, dipyridamole (20 μL of 200 μg mL^{-1}), and extraction with ether as described.[18] The concentration ranges chosen were 0–10 μg mL^{-1} for

1a, 0–500 μg mL^{-1} for **2a**, 0–300 μg mL^{-1} for **2a'** and 0–100 μg mL^{-1} for **5a**. The concentration curves were linear with correlation coefficients >0.987 in all cases.

Receptor binding studies

Compounds **1a**, **1b** and **1c** and metabolites **2a** and **5a** were evaluated as inhibitors of the in vitro binding of several radioligands to their respective receptor sites. The studies were conducted by Nova Pharmaceuticals under the National Institute of Mental Health (NIMH) Psychotherapeutic Drug Discovery and Development Program, and included a variety of neurotransmitter, both IAA and EAA, neuropeptide and ion channel binding sites in brain membranes at two different drug concentrations, 10^{-7} and 10^{-5} M. No significant activity was observed at 10^{-7} M concentrations. At 10^{-5} M concentration, activity was observed only at the Cl^- channel binding sites and was confined to the three triazoline compounds. The β-amino alcohol **2a** was active only at the MK-801 site and aziridine **5a** showed no activity at any of the receptor sites studied. The results are presented in Table 5, along with the appropriate radioligands and brain membrane sources that were used.

The acid-sensitive triazolines and aziridine **5a** were solubilized by dissolving 0.005 mmol of test compound in 0.1 mL DMSO followed by thorough mixing of 0.1 mL cyclodextrin and then diluting the mixture with 4.8 mL water to obtain a 1 mM stock solution. The acid soluble compound **2a** was solubilized in 0.16 mL DMSO and 0.04 mL of 0.1 N HCl, and then 4.8 mL water added to yield the 1 mM stock solution.

Activity at the Cl^- channel was measured in rat cortical membranes using [^{3}H]TBOB according to the method of Lawrence et al.[32] TBPS (*t*-butylbicyclophosphorothionate) was used as the reference compound and GABA as the positive control.

Inhibition of [^{3}H]MK-801 binding was assayed in rat forebrain membranes as described by Javitt and Zukin.[33] MK-801 was used as both reference compound and positive control.

Presynaptic glutamate release, measured in guinea pig cerebrocortical slices

A 1 mM stock solution of **1a** was prepared as described earlier and the compound was tested at two concentrations, 50 and 100 μM, on both spontaneous release of Glu and that evoked by a depolarizing stimulus (30 mM K^+) (Fig. 1).

Cerebral cortices from Dunkin–Hartley guinea pigs (either sex) were rapidly dissected into 300 × 300 μm sections on a McIlwain chopper and dispersed in 250 mL oxygenated Krebs' bicarbonate medium. Slices were allowed to settle, the supernatant fluid aspirated and 0.8 mL aliquots of packed slices transferred on to nylon gauze rings in superfusion chambers and superfused with medium at a rate of 0.3 mL min^{-1}. After a 20 min stabilization period, samples were collected and assayed for their glutamate content using a fluorometric procedure,[34] and the results expressed as a percentage of the initial base release (basal 1) for each individual superfusion chamber.

Acknowledgements

The authors are grateful to Dr Harvey J. Kupferberg and Mr James P. Stables of NINDS, Epilepsy Branch, for the anticonvulsant tests, and Dr Jerry Cott of NIMH and Mr Charles W. Bauer, Jr. of Nova Pharmaceutical Corporation, for the receptor binding assays. Also acknowledged are the awards of Research Grants NS16843 and NS24750 to P.K.K. from the National Institute of Neurological Disorders and Stroke, an award of a Science and Engineering Research Council (United Kingdom) Ph.D. Studentship to P.J.S., the financial support from the Research Grants Council of Hong Kong, Direct Allocation No. 220403470, and a Chinese University of Hong Kong Faculty of Medicine Post-Doctoral Fellowship award to I.P.N., all of which made this work possible.

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1. This paper was presented in parts at the 196th and 200th National Meeting of the American Chemical Society, Abstrs. 1988, *MEDI 52*, and 1990, *MEDI 31*, and at the Fourth Chemical Congress of North America, Abstrs. 1991, *MEDI 144*.

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(*Received in U.S.A.* 30 *June* 1995; *accepted* 16 *October* 1995)