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Acid-catalysed Hydrolysis and Benzodiazepine-like Properties of 5-(Dialkylamino)- and 5-(Alkylthio)-substituted 8-Chloro-6-phenyl-6*H*-[1,2,4]triazolo[4,3-*a*][1,5]benzodiazepines in Mice

SILVIO CACCIA, GIANCARLO GROSSI*, CLAUDIA FRACASSO, ANGELO NACCA, ALFREDO CAGNOTTO, TIZIANA MENNINI, MARCO GHIA† AND GIORGIO ROMA*

*Istituto di Ricerche Farmacologiche "Mario Negri", via Eritrea 62, 20157 Milan, *Dipartimento di Scienze Farmaceutiche and †Dipartimento di Medicina Interna, Sezione di Farmacologia Clinica, Università di Genova, viale Benedetto XV, 16132 Genoa, Italy*

Abstract

The in-vitro and in-vivo hydrolysis of two benzodiazepine compounds has been studied to evaluate their in-vivo activity in mice.

Compounds RL 218 and RL 236, selected as representative examples of *N,N*-dialkyl-8-chloro-6-phenyl-6*H*-[1,2,4]triazolo[4,3-*a*][1,5]benzodiazepin-5-amines (**1**) and of their 5-(alkylthio) substituted analogues (**2**), were rapidly hydrolysed to the corresponding 8-chloro-6-phenyl-4*H*-[1,2,4]triazolo[4,3-*a*][1,5]benzodiazepin-5(6*H*)-one **3** (RL 214) in aqueous acidic solution at pH 1·5. This reaction also occurred extensively in mice when compounds RL 218 and RL 236 were given orally but not intraperitoneally. Both compounds were active against pentylenetetrazole-induced lethal convulsions in mice only when administered orally. After administration of pharmacologically effective oral doses (ED50, the dose protecting 50% of mice), at the time of assessment of the anti-pentylenetetrazole activity, mean brain concentrations of RL 218 and RL 236 were below the limits of sensitivity of the analytical procedure whereas brain concentrations of their metabolite RL 214 were comparable with that present after an oral equiactive dose of this compound itself. RL 214 but not RL 218 or RL 236 had in-vitro affinity for brain benzodiazepine receptors.

These results indicate that the anticonvulsant activity of RL 218 and RL 236 in mice depends essentially on their in-vivo transformation into the common active metabolite RL 214 which most probably arises as a result of acid catalysed hydrolysis in the gastric juice.

We recently described the synthesis and the anti-inflammatory and analgesic properties of new 4*H*-[1,2,4]triazolo[4,3-*a*][1,5]benzodiazepine derivatives, characterized by a *N*-substituted amino group on the diazepine ring (Di Braccio et al 1990; Roma et al 1991). The introduction of 6-phenyl and 8-chloro substituents into this structural scheme endowed the resulting *N*-substituted 8-chloro-6-phenyl-6*H*-[1,2,4]triazolo[4,3-*a*][1,5]benzodiazepin-5-amines (**1**) with the expected anticonvulsant activity. Several of these compounds and their 5-(alkylthio)-substituted analogues (**2**; Figure 1) exerted clear-cut anti-pentylenetetrazole activity, with low acute toxicity, when administered orally to mice (Grossi et al 1993). The chemical novelty of structures **1** and **2** relates mainly to the presence of a 4,5-double bond together with a 5-(dialkylamino) or 5-(alkylthio) substituent. As far as we are aware this structural feature is never present in 1,5-benzodiazepine bicyclic or tricyclic derivatives with activity on the central nervous system (CNS).

In subsequent studies aimed at obtaining biologically interesting analogues of compounds **1** and **2**, it was noted that in strongly acidic aqueous media they were hydrolysed to the corresponding 8-chloro-6-phenyl-4*H*-[1,2,4]triazolo[4,3-*a*][1,5]-benzodiazepin-5(6*H*)-ones **3** (Figure 1). This suggested that enamines **1** and their analogues **2** could actually act in-vivo, partially or completely depending on the extent of their hydrolysis and their intrinsic activity, as latent forms of their derivatives **3** which are known to have anticonvulsant activity in animals (Bauer et al 1974;

Correspondence: G. Roma, Dipartimento di Scienze Farmaceutiche dell'Università di Genova, Viale Benedetto XV 3, 16132 Genoa, Italy.

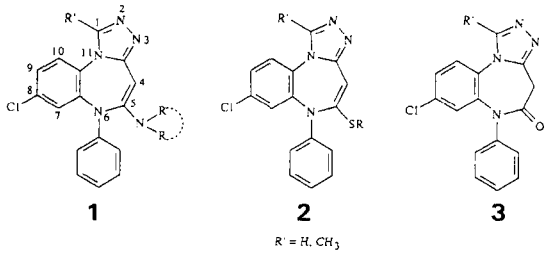

Figure 1. The chemical structures of 5-(dialkylamino)- (**1**) and 5-(alkylthio)-substituted (**2**) 8-chloro-6-phenyl-6*H*-[1,2,4]triazolo[4,3-*a*][1,5]benzodiazepines and their derivatives 8-chloro-6-phenyl-4*H*-[1,2,4]triazolo[4,3-*a*][1,5]benzodiazepin-5(6*H*)-ones (**3**).

Fryer et al 1978). This study, therefore, was designed to obtain basic information on the rate and extent of the in-vitro and in-vivo hydrolysis of compounds RL 218 and RL 236, representatives of **1** and **2**, to their derivative **3** (RL 214; Figure 2), and on the in-vitro affinity of these compounds for benzodiazepine receptors, to enable evaluation of the significance of these results to the in-vivo benzodiazepine-like activity of compounds **1** and **2** in mice. The chemical novelty of structures **1** and **2** has prompted us to evaluate their intrinsic activity, with the intention of determining the influence that an analogous structural feature could have on the CNS activity of other 1,5-benzodiazepine derivatives. In this connection, we have also studied the chemical stability of compounds **1** and **2** after in-vivo administration, in particular their possible hydrolysis to the corresponding active metabolites **3**.

Materials and Methods

Animals

Experiments were performed on male CD1 albino mice, 18–22 g, and male CRL : CD(SD)BR-COBS rats, 150–175 g, (Charles River, Italy). Procedures involving animals and their care were conducted in accordance with international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, 1987; NIH Guide for the Care and Use of Laboratory Animals, US National Research Council 1996).

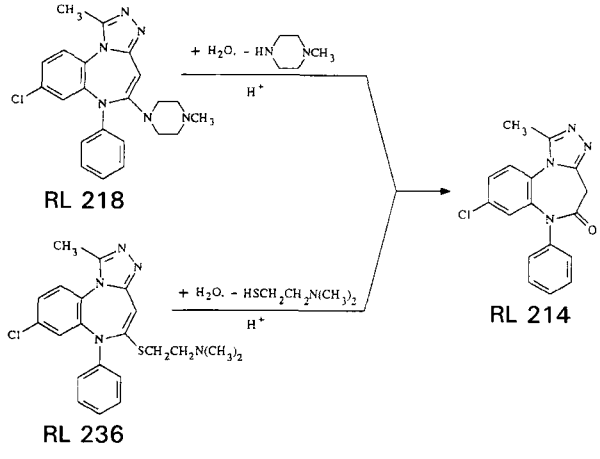

Figure 2. The chemical structures of compounds RL 218 and RL 236 and their hydrolysis to the triazolobenzodiazepinone RL 214.

Anti-pentylenetetrazole activity

The anti-pentylenetetrazole ED50 (i.e. the dose protecting 50% of mice from pentylenetetrazole-induced lethal convulsions) was determined as previously described (Grossi et al 1993). Compounds were administered orally in 1% carmellose suspension to groups of five mice, 1 h before intraperitoneal pentylenetetrazole (130 mg kg^{-1}) (Morpurgo 1971). The protection against lethal convulsions was evaluated for a 15-min observation period. At least four log-spaced dose levels were tested; ED50 and 95% confidence intervals were calculated by the method of Finney (1971).

In-vitro and in-vivo stability studies and drug analysis

For the in-vivo studies mice were given the triazolobenzodiazepines, suspended in 1% carmellose, either orally or intraperitoneally at their corresponding oral anti-pentylenetetrazole ED50, and were killed 1 h later, i.e. at the beginning of the pentylenetetrazole test. Blood samples were collected in heparinized tubes, centrifuged, and the plasma was stored at −20°C. Brains were removed immediately after exsanguination, blotted with paper to remove excess surface blood, quickly frozen in dry ice, and stored at −20°C until analysis.

Concentrations of triazolobenzodiazepines in aqueous solutions and in mouse plasma and brain were determined by high-performance liquid chromatography (HPLC) with UV detection at 229 nm. Briefly, aqueous acidic solution (0·25 mL, adjusted to approximately pH 7·4 with sodium hydroxide) and plasma were diluted to 1 mL with 0·1 M phosphate buffer, pH 7·4, and, after addition of *N*-ethyl-1-phenyl-4*H*-[1,2,4]triazolo[4,3-*a*][1,5]-benzodiazepin-5-amine (Roma et al 1991) as internal standard (I.S.), were extracted with chloroform (4 mL). After centrifugation the organic extract was evaporated to dryness under a stream of nitrogen and the residue was dissolved in mobile phase (0·15 mL) and analysed by HPLC.

Brain tissue was homogenized in CH_3OH–0·01 M KH_2PO_4 (20 : 80, v/v; 0·1 g mL^{-1}), centrifuged and 1–2 mL of the supernatant was processed as above.

Chromatography was performed on a reversed-phase column (Supelco LC 18 DB; 15 cm ×

4·6 mm i.d., 5 μm particle size) at room temperature. The mobile phase, KH_2PO_4 (0·01 M)–CH_3CN–CH_3OH (48 : 37 : 15, v/v) containing 0·01 M triethylamine, was delivered isocratically at a flow rate of 1 mL min^{-1}.

[^{3}H]RO 15-1788 binding

Rats were killed by decapitation and their cortex was rapidly removed and stored at −80°C until assay. The frozen tissues were homogenized in approximately 50 vols ice-cold phosphate buffer (50 mM, pH 7·4), by means of an Ultra Turrax TP 1810 homogenizer (2 × 20 s) and centrifuged at 50 000 *g* for 10 min (Beckman model J-21B refrigerated centrifuge). The pellet was washed four times by re-suspension in fresh buffer and centrifuged as before. The last pellet was resuspended just before the binding assay in 50 vols fresh buffer.

[^{3}H]RO 15-1788 binding was assessed in a final incubation volume of 260 μL consisting of membrane suspension (100 vols final dilution), [^{3}H]RO 15-1788 (S.A. 87 Ci $mmol^{-1}$, NEN, final concentration 1 nM) and displacing agents or solvent. Incubation (90 min at 4°C) was stopped by rapid filtration under vacuum (Brandell MR 48) through GF/B filters which were then washed with cold phosphate buffer (12 mL) and counted in a Wallac 1204 Betaplate BS liquid-scintillation counter, with a counting efficiency of 45%.

RL 214, RL 218 and RL 236 were tested at two concentrations, 10^{-5} and 10^{-7} M, in triplicate. The IC50 of diazepam (the dose having half the maximum effect), tested in the concentration range 10^{-5} to 10^{-10} M, was calculated using the Allfit program (De Lean et al 1978).

Results

Analytical procedure

Under the analytical conditions selected, RL 218, RL 236 and RL 214 were resolved adequately and there was no interference from endogenous plasma or brain constituents. Figure 3 shows typical chromatograms of extracts from drug-free brain homogenate and brain homogenate to which the three compounds had been added. Retention times were 6·3 min for RL 214, 13·2 min for RL 218 and 19·5 min for RL 236.

Recovery, determined by comparing the peak heights of RL 218, RL 236 and RL 214 after injection of non-extracted standard solutions and of extracts from plasma and brain homogenates containing respectively 0·1–2·5 μg mL^{-1} or 0·25–5·0 μg g^{-1} of all compounds, averaged 80 ± 9%, 85 ± 6%, 86 ± 9%, and 76 ± 3%, 75 ± 4%, 74 ± 9%, respectively. The lowest limits of quantification (i.e. the lowest concentrations on the standard curves that can be measured with acceptable accuracy and precision) were approximately 0·1 μg mL^{-1} or 0·25 μg g^{-1} (parent compounds) and 0·04 μg mL^{-1}0 or 0·1 μg g^{-1} (RL 214), using 0·25 mL plasma or approximately 0·1 g brain tissue.

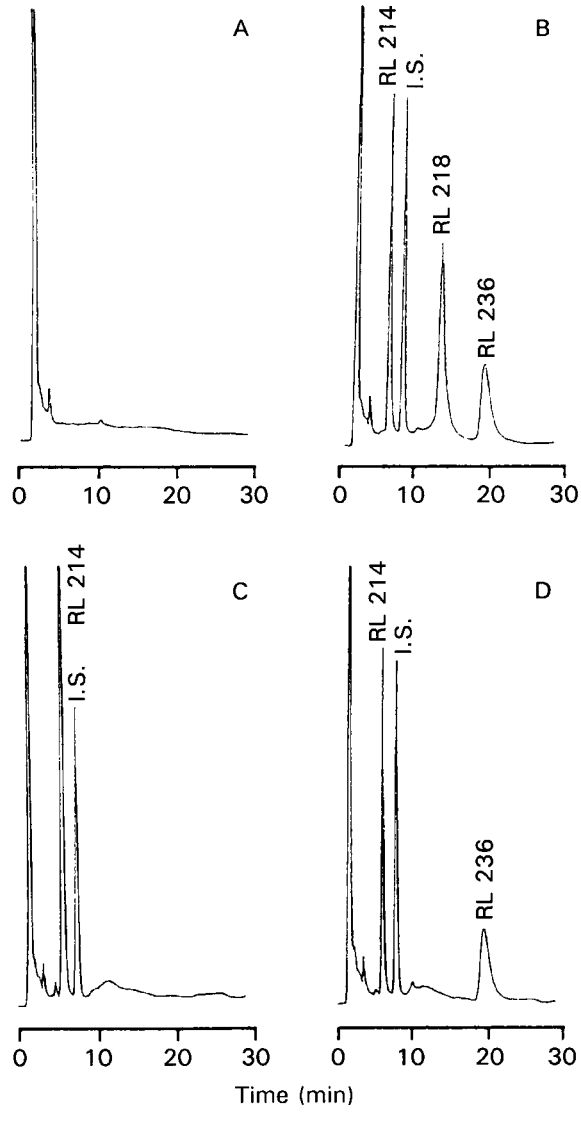

Figure 3. Chromatograms of extracts from drug-free brain homogenate (A) and brain homogenate (B) to which RL 214, RL 218 and RL 236 had been added. Chromatograms from brain homogenates of mice given compound RL 236 orally (C) and intraperitoneally (D) are also shown. I.S., internal standard.

Daily standard curves for known concentrations of the analytes were plotted concurrently with each set of unknown samples and quality control samples. The ratio of the peak height of compound to that of the I.S. was always linearly dependent on the amount of compound added, with correlation

Table 1. In-vitro stability of RL 218 and RL 236 under different conditions of pH, storage temperature and time.

Storage conditions	Recovery (%)	
	RL 218	RL 236
Aqueous solution, 1 h at 20°C		
pH 1·5[a]	< 5	< 5
pH 5·8	99 ± 4	102 ± 7
pH 7·4	104 ± 6	97 ± 5
Mobile phase[b], 24 h at 20°C	101 ± 2	100 ± 6
Mouse plasma, 1 h at 20°C	100 ± 5	100 ± 2
Mouse plasma, 1 h at 37°C	96 ± 7	93 ± 9
Mouse brain homogenate, 1 h at 20°C	97 ± 7	103 ± 4

Results are given as recovery in relation to freshly prepared samples (n = 5). [a]Based on a limit of quantification of 0·1 μg mL^{-1}. [b]KH_2PO_4(0·01 M)–CH_3CN–CH_3OH (48 : 37 : 15, v/v), containing 0·01 M triethylamine.

coefficients exceeding 0·99. For all compounds replicate analyses at two different concentrations yielded mean coefficients of variation of 5–8% in plasma and 10–15% in brain tissue.

In-vitro stability studies

Before in-vivo drug measurement in mice plasma and brain, the stability of RL 218 and RL 236 was investigated in-vitro under different conditions of storage, pH, temperature and time (Table 1). The analytical responses from these samples were compared with those from freshly prepared samples. Solutions in organic solvents such as chloroform, methanol and mobile phase were reasonably stable at room temperature for at least 24 h or in a refrigerator for one week. Similarly, concentrations did not drop appreciably when the compounds were stored in mouse plasma and brain homogenate for 1 h at room temperature or one month at −20°C, or in mouse plasma for 1 h at 37°C. As expected, however, both compounds had limited stability in strongly acidic aqueous solution (1 h at 20°C).

Figure 4 shows the time-course of disappearance of compounds RL 218 and RL 236 during incubation at 37°C in an aqueous solution at pH 1·5. On the basis of the chromatographic behaviour of authentic standards we established that the rapid disappearance of these compounds was associated with the concomitant appearance of compound RL 214, the reaction being slightly faster for RL 218 than for RL 236.

The formation of RL 214 was plainly a result of acid-catalysed hydrolysis of RL 218 and RL 236 (Figure 2). The pH of gastroenteric fluids varies between 1·5 in the stomach and 5–8 in the intestine. This suggested that after oral dosing compounds

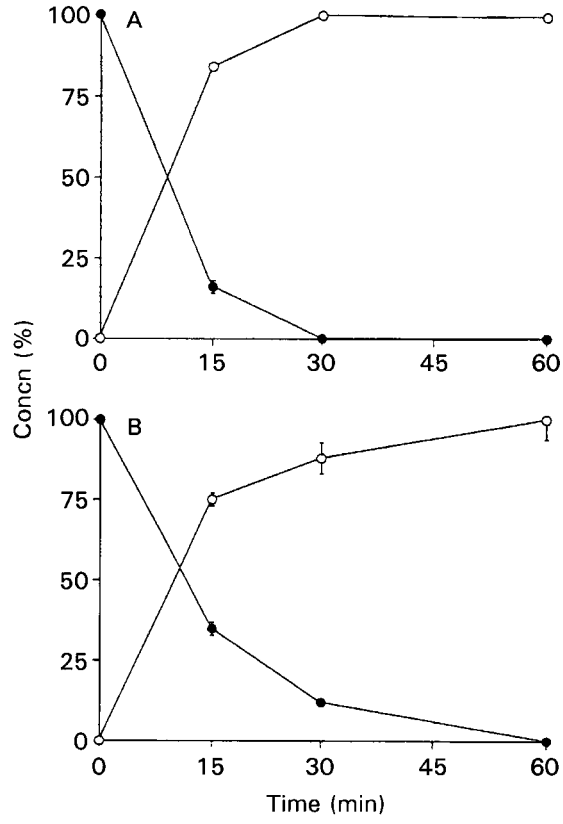

Figure 4. Time-course of the disappearance of RL 218 (A) and RL 236 (B) during incubation at 37°C in an aqueous solution at pH 1·5. Parent compound (●) and RL 214 (○).

RL 218 and RL 236 might be rapidly hydrolysed in the stomach to RL 214 which, after absorption, might reach the CNS, contributing to or accounting for the pharmacological effects of the parent compounds.

Anti-pentylenetetrazole activity and drug plasma and brain concentrations

To confirm this hypothesis and to evaluate the significance of the formation of RL 214 to the pharmacological effects of RL 218 and RL 236, we measured the plasma and brain concentrations of the three compounds in mice after administration

Table 2. Anti-pentylenetetrazole activity of compounds RL 218, RL 236 and RL 214 in the mouse.

Compound	ED50 (μmol kg^{-1})
RL 218	31·9 (14·7–63·9)
RL 236	60·7 (46·1–77·7)
RL 214	29·3 (15·4–52·3)

ED50 was at 1 h after oral dosing; the 95% confidence intervals are given in parentheses. The intraperitoneal dose of 130 mg kg^{-1} pentylenetetrazole was lethal to all the control mice treated only with the solvent.

Table 3. Anti-pentylenetetrazole activity and plasma and brain concentrations of parent compounds, RL 218 or RL 236, and their common metabolite RL 214 1 h after dosing in mice.

Compound	Dose (mg kg^{-1})	Route	Protection (%) (n = 5)	Plasma ($\mu g\ mL^{-1}$)		Brain ($\mu g\ g^{-1}$)	
				Parent	Metabolite	Parent	Metabolite
RL 218	13	Oral	40	< 0·10	1·34 ± 0·52	< 0·25	0·51 ± 0·11
		Intraperitoneal	0	< 0·10	0·07 ± 0·01	< 0·25	< 0·10
RL 236	25	Oral	40	< 0·10	2·83 ± 0·35	< 0·25	0·94 ± 0·20
		Intraperitoneal	0	0·25 ± 0·04	0·31 ± 0·01	0·29 ± 0·03	0·12 ± 0·01
RL 214	9·5	Oral	60		1·27 ± 0·34		0·50 ± 0·09

The dose corresponds to the oral anti-pentylenetetrazole ED50.

at their ED50 concentrations against pentylenetetrazole-induced lethal convulsions. In this connection we have also determined the oral ED50 of RL 214 and evaluated the anti-pentylenetetrazole activity of the three compounds after oral and intraperitoneal administration of these doses. Previous studies (Grossi et al 1993) have established that under these experimental conditions RL 236 is less effective in the mouse than is RL 218, which has now been shown to be almost equiactive with RL 214 in antagonizing pentylenetetrazole-induced convulsions (Table 2).

Plasma and brain concentrations of RL 218 and RL 236 1 h after oral dosing were below the limits of quantification of the analytical procedure. However, RL 214 was present in the brains of mice treated orally with RL 218 and RL 236 at concentrations approximately one-third those in plasma. These brain concentrations were comparable, within the limits of the pharmacological procedure, with that found at the same time after an equiactive oral dose of RL 214 (Table 3).

Interestingly, when these doses were given intraperitoneally RL 218 and RL 236 were inactive in the pentylenetetrazole test (Table 3). Mean brain concentrations of RL 218 and its active metabolite RL 214 were below the limits of quantification of the analytical procedure. The mean brain concentration of RL 236 was just above this limit, whereas that of the metabolite was markedly lower than after oral administration of the same dose of the parent compound. These results suggest that the concentrations of RL 214 in mouse brain after intraperitoneal injection of RL 218 and RL 236 do not reach the effective concentration necessary to ensure significant protection against pentylenetetrazole, thus possibly explaining their lack of activity.

In-vitro affinity for benzodiazepine receptors

RL 218 and RL 236 were not active at the concentrations tested (10^{-5} and 10^{-7} M) whereas RL 214 at 10^{-5} M inhibited [^{3}H]RO 15-1788-specific binding by approximately 94%. Its IC50 value was 639 ± 95 nM. In comparison, the IC50 value of diazepam is 22 ± 4 nM.

Discussion

These results provide evidence that 5-(dialkylamino)- and 5-(alkylthio)-substituted 8-chloro-6-phenyl-6*H*-[1,2,4]triazolo[4,3-*a*][1,5]benzodiazepines **1** and **2** undergo acid-catalysed hydrolysis in gastrointestinal fluids after oral dosing in mice. This reaction yields the corresponding pharmacologically active (Bauer et al 1974; Fryer et al 1978) 8-chloro-6-phenyl-4*H*-[1,2,4]triazolo[4,3-*a*][1,5]-benzodiazepin-5(6*H*)-ones **3** which reach the systemic circulation and then the CNS, as documented in this study for the representative compounds RL 218 and RL 236 and their active metabolite RL 214.

That trace amounts of RL 214 were also found in mouse plasma and brain after intraperitoneal administration of RL 236 (and RL 218 at higher doses, data not shown) suggests, however, that this reaction might occur in body fluids or tissues other than the gastrointestinal tract. In-vitro stability studies indicate that RL 218 and RL 236 are reasonably stable in mouse plasma and brain homogenate, and in aqueous solutions in the physiological pH range 5·8–7·4, but their possible in-vivo enzymatic hydrolysis to RL 214 in liver or other tissues remains to be established. It also remains to be clarified which metabolic processes other than hydrolysis to RL 214 these compounds can undergo. The plasma and brain levels of both are very low after intraperitoneal dosing, suggesting rapid and extensive biotransformation before excretion, possibly through the phase I and phase II reactions common to the lipophilic benzodiazepine derivatives in man and animals (Greenblatt et al 1983; Jochemsen & Breimer 1984; Caccia & Garattini 1985, 1990).

The metabolite RL 214 binds to benzodiazepine receptors with submicromolar affinity and

antagonizes pentylenetetrazole-induced lethal convulsions in the mouse, as expected. Although its in-vitro affinity for benzodiazepine receptors (IC50) is relatively weak compared with other known benzodiazepine derivatives (Mennini et al 1982, 1987), it comes close to the concentrations found in-vivo in mouse brain after a pharmacologically effective oral dose (ED50) of the compound. As shown in Table 3, the mean RL 214 brain concentration after administration of its oral ED50 against pentylenetetrazole-induced lethal convulsions in the mouse is $0{\cdot}50 \pm 0{\cdot}09$ $\mu g\ g^{-1}$ (i.e. approx. 1·54 μM, assuming tissue density close to unity). This means that the metabolite brain concentrations found after active doses are approximately in the same range as its IC50 value for benzodiazepine receptors (approx. 0·64 μM). Whether this compound can be converted to metabolites which retain its pharmacological activity, as can several benzodiazepine derivatives (Caccia & Garattini 1990), remains to be verified. However, 4*H*-[1,2,4]triazolo[4,3-*a*][1,4]benzodiazepine derivatives, like alprazolam and triazolam, were previously reported to yield hydroxylated metabolites with lower benzodiazepine receptor affinity and lower intrinsic biological activity than the parent drugs (Sethy & Harris 1982; Greenblatt et al 1983; Jochemsen & Breimer 1984; Greenblatt & Wright 1993).

The two parent compounds RL 218 and RL 236 also have anti-pentylenetetrazole activity in the mouse after oral dosing, but they were not found in appreciable amounts in brain and did not have significant affinity for benzodiazepine receptors in-vitro. This and the fact that concentrations of the metabolite were comparable in mouse brain after equiactive oral doses of RL 218, RL 236 and RL 214 indicate that the anti-pentylenetetrazole activity of RL 218 and RL 236 in the mouse depends on the metabolite rather than on the parent compounds, which act essentially as latent forms of their common active metabolite RL 214.

Finally, because RL 214 but not its parent compounds had significant in-vitro affinity for brain benzodiazepine receptors, it seems reasonable to suggest (Figure 1) that the presence of a 4,5-double bond, together with a 5-(dialkylamino) or 5-(alkylthio) substituent, in compounds **1** or **2** (as well as in possible analogous 1,5-benzodiazepine tricyclic derivatives) is negative for their binding to benzodiazepine receptors. However, the enamino group (compounds **1**), or an analogous group (compounds **2**), easily hydrolyses in-vivo to group $CH_2C{=}O$ (compounds **3**) after oral dosing (Figure 2). We think these conclusions can be extended to the corresponding bicyclic 1,5-benzodiazepine derivatives.

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