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1,3-Diacylaminopropan-2-ols and Corresponding 2-Acyl Derivatives as Drug Carriers: Unexpected Pharmacological Properties

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Abstract—The design of lipid vectors (pseudotriglycerides, PTGs), achieved by the amide isosteric substitution of the ester moieties of 1,3-diacylglycerols, is based on the structural similarity with natural triglycerides facilitating the passage of pharmacological agents across biological membranes. 2-*S*-Acetylthiorphan (hemiacetorphan) pseudotriglycerides, Z-glycine pseudotriglycerides and 1,3-diacylaminopropan-2-ols vector molecules differing by the nature of the acid side-chain are examined in acute toxicity, radioligand binding and guinea-pig ileum experiments. These evaluations have led us to distinguish two types of compounds. Linear derivatives, palmitoyl and decanoyl, are devoid of toxicity and intrinsic activity. Cyclic derivatives, which contain in the acyl chain a phenyl, cyclohexyl, cyclopentyl or adamantoyl ring, present additional properties. Cyclic derivatives of hemiacetorphan are lethal after intravenous administration. The mortality is governed by the 2-hemiacetorphan moiety in the cyclic vector molecules. Hemiacetorphan alone is also lethal. Cyclic vector molecules and related compounds inhibit the contractile response of the guinea-pig ileum induced by electrical stimulation, histamine or acetylcholine (noncompetitive antagonism) whereas linear entities and parent compounds are not active. In particular, the 2-hemiacetorphan 1,3-diadamantoylamide PTG presents pD'2 values 7·87±0·29 (vs histamine) and 7·97±0·12 (vs acetylcholine).

Diacylglycerols have been employed as carrier groups for a number of drugs in a variety of therapeutic approaches. In view of their structural similarity to natural triglycerides, diacylglycerols (and related compounds) esterified in the 2-position with a xenobiotic carboxylic acid are termed here pseudotriglycerides (PTGs). PTGs possess a high degree of membrane-like character, which facilitates the passage of pharmacological compounds across biological membranes.

PTG prodrugs of non-steroidal anti-inflammatory drugs (NSAIDs) were synthesized in order to diminish the ulcerogenicity of the NSAID (Sherlock 1972; Zaffaroni 1972; Kumar & Billimoria 1978; Paris et al 1979, 1980a,b; Jones 1980; Paris & Cimon 1982). Subsequently, it was demonstrated that L-dopa PTGs are partially resorbed from the intestinal lumen via the lymphatics. This lymphotropic effect is useful for drugs undergoing an important hepatic first-pass effect (L-dopa, Garzon-Aburbeh et al (1986)) and for compounds acting in the lymph itself such as chlorambucil (Garzon-Aburbeh et al 1983; Saraiva Goncalves et al 1989; Saraiva Goncalves 1990), and γ-aminobutyric acid (Deverre et al 1989, 1991). PTGs of γ-aminobutyric acid were also used to enhance brain penetration after parenteral injection (Shashoua et al 1984; Jacob et al 1985, 1987, 1990). This type of carrier group, however, suffers a major drawback in that a PTG's half-life generally does not exceed 20–30 min after parenteral administration, a figure similar to that encountered for the plasma half-life of natural triglycerides (Mead et al 1986; Saraiva Goncalves 1990).

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In an effort to overcome this limitation, we have previously synthesized 1,3-diacylaminopropan-2-ols as carrier groups (Fig. 1). The design of these compounds was based on the amide isosteric substitution of the ester moieties of 1,3-diacyglycerols to increase the metabolic resistance of PTGs (Mergen et al 1991a); amide PTGs are termed here aPTGs. The pharmacological evaluation was carried out after acyla-

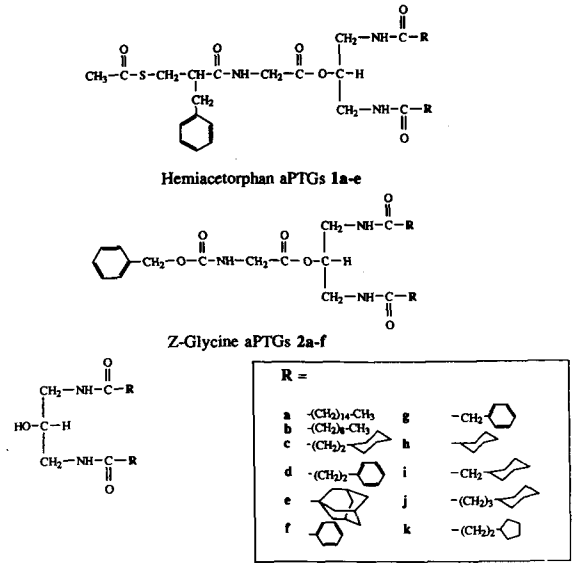

FIG. 1. Structures of hemiacetorphan amide pseudotriglycerides (aPTGs), Z-glycine amide pseudotriglycerides and vector entities.

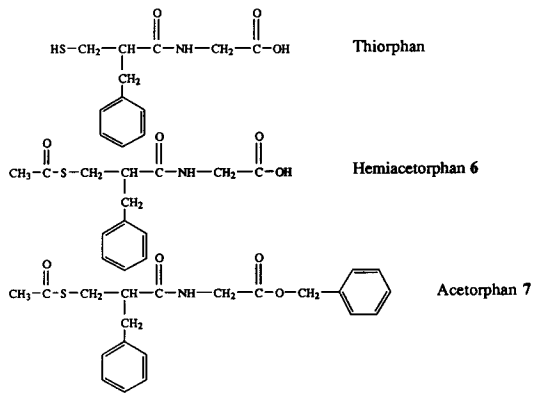

FIG. 2. Structures of neutral endopeptidase EC 3.4.24.11 (enkephalinase) inhibitors.

tion of the secondary alcohol function by valproic acid (Mergen et al 1991b) and by *S*-acetylthiorphan (hemiacetorphan, **6**, Fig. 2) (Mergen 1992). Hemiacetorphan is a precursor of thiorphan (Fig. 2), an inhibitor of the neutral endopeptidase EC 3.4.24.11 (enkephalinase (Roques et al 1980)). Thiorpan is not able to cross the blood-brain barrier whereas acetorphan (**7**, Fig. 2), a lipophilic derivative in which the thiol and the carboxylate groups are esterified by acetyl and benzyl residues, respectively, is parenterally active (Lecomte et al 1986). The influence of carrier acid chains was explored by means of five compounds which differ in the acid side-chains: two linear saturated moieties: palmitoyl (**1a**), decanoyl (**1b**), and three other acyl groups featuring a ring at the end of the chain: 3-cyclohexylpropanoyl (**1c**), 3-phenylpropanoyl (**1d**) and 1-adamantoyl (**1e**).

While **1a** and **1b** indeed exhibited, after intravenous administration (200 μmol kg^{-1}), an analgesic activity in the hot plate test, superior to the analgesic activity of acetorphan administered in the same conditions, **1c–e** surprisingly displayed a rather peculiar activity-time curve and acute toxicity after intravenous injection: **1c–e** demonstrated analgesic activity at 40 μmol kg^{-1} similarly superior to the activity of acetorphan, with a first maximum 1 h after the injection but were lethal at 200 μmol kg^{-1} (Mergen 1992).

This toxicity could be the expression of unexpected pharmacological properties. In this context, we have undertaken additional pharmacological and toxicological studies of these compounds and their precursors i.e. maximum tolerated dose (MTD) determinations, binding studies and experiments on inhibition of contractions of guinea-pig ileum.

Materials and Methods

Synthesis

Compounds **1a–e** were synthesized according to Mergen et al (1991a, b) in four steps: 1,3-diacylaminopropan-2-ols (**3a–k**) were obtained by acylation of diaminopropanol (Aldrich Chemical Company, 95%) with the corresponding acyl chlorides in diethylether or tetrahydrofuran. Subsequent acylation of the alcohol function of 1,3-diacylaminopropan-2-ols by *N*-benzyloxy-carbonylglycine (Z-glycine) was carried out via the symmetrical anhydride technique, giving an aPTG featuring a Z-protected glycine residue linked by an ester bond (**2a–f**). Cyclohexene in propan-2-ol in the presence of palladium on charcoal was used to remove the Z protecting group. The deprotected compounds, **2a–e**, were coupled in ethyl acetate with 3-acetylthio-2-benzylpropanoic acid using *N,N'*-dicyclohexylcarbodiimide as coupling agent to give **1a–e**.

Hemiacetorphan was obtained as described by Mergen (1992). Acetorphan was synthesized according to Roques et al (1981).

Determination of maximum tolerated dose (MTD)

Due to the poor solubility of our compounds in aqueous media, pure dimethyl sulphoxide (DMSO, 2 mL kg^{-1} 99%, Aldrich Chemical Company) was used as the injection vehicle. Compounds **1a–e**, **2a–e**, **3a–e**, free hemiacetorphan **6** and acetorphan **7** were injected into a tail vein of NMRI mice (male, 20–30 g) at 200 μmol kg^{-1}. Animals were observed during one week after the injection. Where some animals died, MTD determinations were carried out and approximated according to Homburger (1989).

For lethal compounds, controls were carried out with glycerolformal (2 mL kg^{-1}, Servon XGF, Servo, The Netherlands) as the injection vehicle.

Radioligand binding studies

Binding studies were performed on rat brain cortex. Samples were homogenized in 10 vol 50 mM Tris, pH 7·4 with a Potter-Elvejhem homogenizer. The whole homogenates were centrifuged twice at 20000 rev min^{-1} for 10 min at 4°C. The final pellets were suspended in 5 vol buffer. In a vial, 100 μL of radioligand and 100 μL of test compound were added to 1 mL of tissue preparation. After incubation, the homogenates were filtered through Whatman GF/B glass fibre filters and rinsed twice with 4 mL of ice-cold buffer. The filters were placed in plastic scintillation vials with 7 mL Aqualuma (Lumac, Schaesberg, The Netherlands) and, after vigorous agitation, counted in a Pharmacia Wallac 1410 scintillation counter. The assays were performed in triplicate.

All chemicals used for binding assays were purchased from Sigma Chemical Company (St Louis, USA).

Opiate receptor assay. [^{3}H]Naloxone (2242·2 GBq mmol^{-1}, 60·6 Ci mmol^{-1}, New England Nuclear, Dupont de Nemours) binding experiments were performed in 50 mM Tris buffer (pH 7·4) containing 154 mM NaCl as described by Pollack & Wooten (1987). The radioligand concentration in the assay was 2 nM. Unlabelled etorphine (1 μM) was used to determine non-specific binding.

μ- and δ-Receptor assay. [^{3}H]DAGO (Tyr-D-Ala-Gly-*N*-Methyl-Phe-Glycol, 2086·8 GBq mmol^{-1}, 56·4 Ci mmol^{-1}, New England Nuclear, Dupont de Nemours) and [^{3}H]-DADLE (Tyr-D-Ala-Gly-Phe-D-Leu, 1735·3 GBq mmol^{-1}, 46·9 Ci mmol^{-1}, New England Nuclear, Dupont de Nemours) binding experiments were performed in 50 mM Tris buffer (pH 7·7) without NaCl.

Non-specific binding was defined using 10 μM sufentanil and 10 μM unlabelled DADLE, respectively.

σ-Receptor assay. [^{3}H]PPP (3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine, 2120·1 GBq mmol^{-1} 57·3 Ci mmol^{-1}, New

Table 1. Results of binding studies.

Compounds	[^{3}H] naloxone KI(μM)	[^{3}H]DAGO μ IC50(μM)	[^{3}H]DADLE δ	[^{3}H]EKC κ IC50(μM)	[^{3}H]PPP σ	Other membrane receptors IC50(μM)	
1a	—	—	—	—	—		—
1b	—	—	—	—	—		—
1c	15	—	—	5·5	—	[^{125}I]Angiotensin	16
1d	37·1	10	—	—	—	CCK-A,[^{3}H]CCK8	1.6
1e	9·1	—	—	12	n.d.	M_2,[^{3}H]AFDX	6·7
						[^{3}H]Substance P	15
						[^{125}I]Angiotensin	25
2a	—	—	—	—	—		—
2b	—	—	—	—	—		—
2c	7·3	—	—	—	—	HT_2,[^{3}H]Spiperon	3·3
						[^{125}I]Angiotensin	11
6	—	—	—	—	—		—
7	—	—	—	n.d.	n.d.		n.d.

— = > 100 μM, n.d. = not determined.

England Nuclear, Dupont de Nemours) binding experiments were performed in 50 mM Tris buffer (pH 8·0) according to the method of de Costa et al (1990). Glass fibre filters were soaked in 0·5% poly(ethylenimine) for at least 30 min at 25°C before use. The radioligand concentration was 3 nM. Haloperidol (1 μM) was used to determine non-specific binding.

Distribution of guinea-pig ileum contraction

Male guinea-pigs, 400–500 g, were killed by decapitation. The ileum (except its 10 cm long terminal portion) was removed and 2·5–3 cm segments of the ileum were placed in an aerated organ bath containing 50 mL of Tyrode solution at 37°C. Stock solutions of lipid compounds were made up in 100% glycerolformal (Servon XGF, Servo, The Netherlands). Glycerolformal was preferred to DMSO due to its better tolerance for the guinea-pig preparation. The final concentration of glycerolformal in the bath was less than 0·1%. Measurements were started after a 30 min equilibration time.

Electrical stimulation. The ileum segment was threaded on a Pt electrode (Paton 1955); the stimulation parameters were 0·1 Hz, 10 ms and supramaximal currents (Jansen JSI ST stimulator).

All compounds were tested in triplicate on this preparation. Morphine HCl was used as standard. Additional assays in the presence of naloxone (Narcan, Dupont de Nemours) were carried out with some compounds.

Results are expressed as the ID50 (μM), i.e. the concentration of the drug which produces 50% inhibition of the contraction of the longitudinal muscle (Kosterlitz & Waterfield 1975).

Cholinergic stimulation. Guinea-pig ileum cholinergic-stimulated contraction was carried out with acetylcholine chloride (Merck, Darmstadt, Germany) and with bethanechol chloride (Muscaran, Christiaens, Belgium), a muscarinic agonist.

Dose-responses curves were constructed in the presence of acetylcholine alone and at least three concentrations of the tested compounds. Results are expressed as pD'_2 values.

Histaminergic stimulation. Dose-responses curves were constructed in the presence of histamine dihydrochloride (Merck, Darmstadt, Germany) alone and at at least three concentrations of the tested compounds. Results are expressed as pD'_2 values.

Results

Chemistry

Compounds synthesized are presented in Fig. 1.

MTD determinations

The administered DMSO was much less than the usually reported LD50 of 3·8–9·2 g kg^{-1} in mice (David 1972) and in our hands was found nontoxic for NMRI mice. In particular, no deaths occurred in control animals injected intravenously with 2 mL kg^{-1}. At 200 μmol kg^{-1} (NMRI mice), acyclic pseudotriglycerides of hemiacetorphan **1a–b** and acetorphan showed no toxic effects.

At the same dosage, cyclic derivatives **1c–e** caused death within 5 min after intravenous injection, with hind limb paralysis, respiratory disorders and occasionally seizures.

MTD values were 42 ± 6 (**1c**), 86 ± 10 (**1d**) and 77 ± 20 (**1e**) μmol kg^{-1}. Non-significant differences were found when glycerolformal was used as the injection vehicle.

2a–e and **3a–e** were found to be nontoxic below 400 μmol kg^{-1} after intravenous or intraperitoneal administrations.

Free hemiacetorphan was not devoid of toxicity: about 13% of animals died after the intravenous injection at 200 μmol kg^{-1}.

Radioligand binding studies

aPTGs **1a–b** of hemiacetorphan and their related compounds **2a–b** did not show any significant displacement of opiate receptors while cyclic aPTGs **1c–e** and precursors **2c–e** exhibited weak affinities (IC50 10–25 μM). Screening using the main membranes receptors was performed by Dr M. Fortin and Dr D. Philibert (Roussel Uclaf, Centre de Recherches, Romainville, France) on nine compounds: **1a–e**, **2a–c** and free hemiacetorphan **6**.

Table 2. Inhibition of contractile responses (ID50) to electrical stimulation of the guinea-pig ileum.

Compounds	ID50 (μM)
1a	>100
1b	>100
1c	0·25±0·08
1d	1·57±0·23
1e	0·74±0·08
2a	>100
2b	>100
2c	0·54±0·09
2e	0·66±0·03
2f	12·8±0·45
3a	>100
3b	>100
3c	3·35±0·19
3d	30% inhibition at 80 μM
3e	1·47±0·47
3f	26% inhibition at 80 μM
3g	56% inhibition at 80 μM
3h	28% inhibition at 80 μM
3i	46% inhibition at 80 μM
3j	1·23±0·16
3k	6·27±0·27
4c	2·72±0·10
5f	31% inhibition at 80 μM
6	>100
7	25% inhibition at 80 μM
Z-Glycine	>100
Thiorphan	>100
Morphine	0·026±0·002
Glycerolformal	Inactive

Mean±s.e.m. (μM, n=3)

Binding study results are summarized in Table 1. Cyclic aPTGs **1c–e** and precursors **2c–e** exhibited weak affinities for some receptors, mainly opioid and angiotensin. Free hemiacetorphan and acetorphan were found inactive in this screening.

Inhibition of guinea-pig ileum contractions

Electrical stimulation. Results are presented in Table 2 and expressed as ID50 (mean±s.e.m., n=3). Cyclic derivatives of hemiacetorphan **1c–e**, Z-glycine precursors **2c–f** and vector entities **3c–k** diminished the contractile response of intestinal smooth muscle after electrical stimulation. This inhibition is dose-dependent and reversible after washing-out.

Structure-activity relationships. The inhibition potency increases with the replacement of the phenyl ring by a saturated ring (**3d**<**3c**, **3f**<**3h**), with the size of the ring (**3k**<**3c**, **3h**<**3e**) and with the length of the chain between the amide function and the ring (**3h**<**3i**<**3c**<**3j**). The presence of the glycine residue in the 2-position (**4c**) enhances the effect compared with the carrier molecule (**3c**) while no statistical difference was found between the tribenzoyl (**5f**) compound and the corresponding carrier (**3f**).

Prior addition of naloxone at concentrations 1–10 times greater than the tested compound did not antagonize the relaxant activity of our compounds.

Cholinergic and histaminergic stimulations. Cyclic aPTGs are able to antagonize the contracile response to cholinergic and muscarinic stimulations (acetylcholine chloride and bethanechol chloride). Similar responses were obtained with the guinea-pig ileum longitudinal muscle preparation. Dose-response curves were constructed for **1c**, **1e**, **2e** and **3e** showing that this antagonism is non-competitive (Fig. 3a).

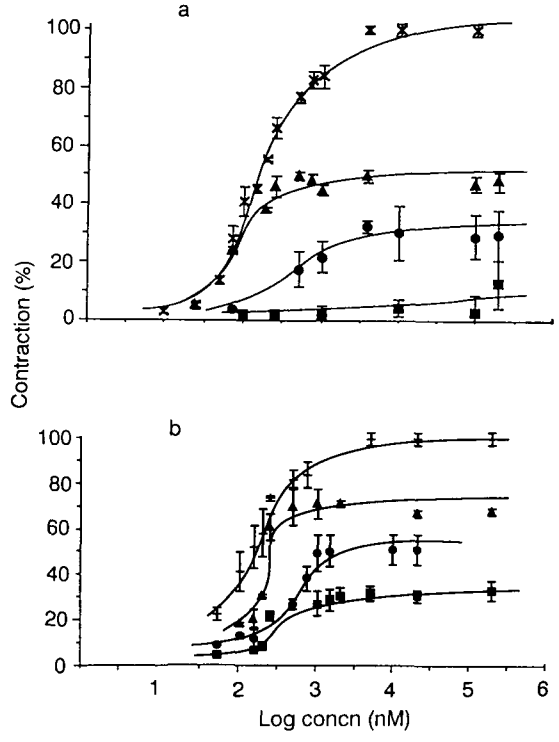

FIG. 3. Non-competitive antagonism of 2-hemiacetorphan 1,3-diadamantoyl aPTG (**1e**) after stimulation of the guinea-pig ileum. a. Acetylcholine (x) in the presence of **1e**: 10 (▲), 100 (●) and 1000 nM (■). b. Histamine (+) in the presence of **1e**: 1 (▲), 10 (●) and 100 nM (■). Each point is the average of three measurements.

Table 3. pD'_2 values of aPTGs after cholinergic and histaminergic stimulation in the guinea-pig ileum.

Compounds	Cholinergic stimulation pD'_2	Histaminergic stimulation pD'_2
1a	Inactive	Inactive
1c	7·10±0·36	6·96±0·42
1e	7·97±0·12	7·87±0·29
2e	6·88±0·31	6·36±0·22
3e	<6	<6

Mean±s.d. (n=3). For comparison, cinnarizine has shown an anti-histaminic activity (non-competitive antagonism) with a pD'_2 value of 5·5.

These compounds also antagonize the contraction of the ileum induced by histamine (non-competitive antagonism, Fig. 3b). Results are expressed as pD'_2 values in Table 3.

Discussion

Toxicity

The finding of a toxicity induced by **1c–e** could limit the usefulness of the cyclic analogues as carrier groups but their good bioavailability profile (Mergen 1992) compared with the acyclic compounds presents a real interest.

The symptomatology before death tends to indicate a central origin. It has been claimed that DMSO could

facilitate the passage of drugs into the brain (Broadwell et al 1982; Walters et al 1984; Iwen & Miller 1986). However, no effect on the blood-brain opening was found in our laboratory with the dose of DMSO used in the present experiments, as illustrated by the lack of analgesic activity for thiorphan (Mergen 1992). In addition, the finding of no significant differences on mortality of **1c–e** between DMSO and glycerolformal as injection vehicle favours the hypothesis of a specific toxic mechanism of the tested compounds.

It should be stressed that lethality occurs only when the side-chain in the 2-position is a hemiacetorphan moiety. However, we have not established whether the lethality of **1c–e** is caused by the release of free hemiacetorphan or by an intrinsic toxicity. Within this context, the absence of toxicity of acyclic derivatives **1a–b** could be explained by their poor bioavailability. Their accumulation by precipitation at the injection site and in the lungs drastically reduces the circulating fraction. As a consequence, the released hemiacetorphan may not reach a critical concentration in the biophase.

Binding characteristics

In binding evaluations cyclic compounds have shown a weak displacement and a lack of specificity. Both hemiacetorphan cyclic derivatives (**1c–e**) and Z-glycine derivatives (**2c–e**) were similarly able to bind to certain receptors while only cyclic hemiacetorphan aPTGs are lethal.

Two types of receptors are mainly represented—opioid and angiotensin. By analogy of the active site models of enkephalinase and angiotensin converting enzyme (Roques et al 1980), it could be anticipated that ester prodrugs of an enkephalinase inhibitor would display at least a weak displacement of [^{125}I]angiotensin. However, neither free hemiacetorphan nor **1a–b** interact with this enzyme. In contrast, **2c**, which is not an enkephalinase inhibitor, shows the best affinity for the angiotensin converting enzyme.

Only the binding to opioid receptors could explain a central origin of mortality of compounds **1c–e**. However, the absence of correlation between [^{3}H]naloxone displacement values and mortality and the lack of affinity of free hemiacetorphan do not support this hypothesis. The property to displace some labelled ligands is not only due to the enhancement of lipophilicity; in binding experiments, we could not establish a correlation between the ability to bind on some receptors and the lipophilicity of the tested compounds. Binding studies do not provide sufficient evidence to explain either the toxicity of free hemiacetorphan or the toxicity of cyclic derivatives **1c–e**.

Inhibition of contraction

There was an intrinsic pharmacological activity of the cyclic vector molecules, Z-glycine and hemiacetorphan cyclic derivatives whereas Z-glycine, hemiacetorphan or acyclic compounds have no activity. This pharmacological activity does not seem to be related to the lethality, or to the binding profile. Our results would seem to preclude a morphinomimetic activity of our compounds. Contrary to findings with narcotic analgesics (Kosterlitz & Waterfield 1975), no good correlation was found between affinity in brain homogenates and potency in the guinea-pig ileum after electrical stimulation, the prior addition of the opiate antagonist naloxone does not antagonize the relaxant activity of our compounds, and our compounds, unlike morphine, inhibit ileum contractions after cholinergic and histaminergic stimulation. We also observed that the inhibition of contractile responses by our compounds is constant, whatever the type of stimulation, which could indicate an effect of disturbance of the membranes or an activity on ion channels.

The ability to inhibit the contractile responses of intestinal smooth muscles seems to depend upon several structural requirements including the saturation of the ring, the ring size and the length of the chain which links the amide function to the ring.

In conclusion, the approach of drug delivery by means of modified pseudotriglycerides has led to two groups of vector molecules. The amide isosteric substitution of the ester moieties in linear aPTGs does not generate unexpected properties unlike the introduction of rings in the side-chains. We have pointed out a new pharmacological property of cyclic aPTGs, at least partially due to the vector entities themselves. The ability to inhibit the contractile response after stimulation of the guinea-pig ileum does not depend on the hemiacetorphan presence on the vector molecules: **1c–e**, **2c–e** and **3c–e** share this property. In particular, the 2-hemiacetorphan 1,3-diadamantoyl aPTG presents pD'_2 values $7{\cdot}87 \pm 0{\cdot}29$ (vs histamine, for comparison pD'_2 cinnarizine 5·5) and $7{\cdot}97 \pm 0{\cdot}12$ (vs acetylcholine).

The acute toxicity of cyclic aPTGs (which requires the hemiacetorphan presence) does not seem to be related to this pharmacological property or to the binding profile. At this stage, the acute lethality seems due to the intrinsic toxicity of hemiacetorphan.

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