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DIBENZOQUINAZOLINE DIONES AS ANTIHYPERTENSIVE CYCLIC GUANOSINE MONOPHOSPHATE PHOSPHODIESTERASE INHIBITORS

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Abstract—The discovery and structure–activity of a new class of renal artery phosphodiesterase inhibitors is reported, some of which are highly selective for the guanosine cyclic 3′,5′-monophosphate phosphodiesterase. One of these compounds, 5,6-dihydro-8,9,11,12-tetramethoxy-1,3-dioxo-1H-benz[*f*]-isoquino[8,1,2-*hij*]quinazoline-2(3H)-carboxylic acid, ethyl ester (**9**), is amongst the most potent and selective compounds of this class yet identified. Furthermore, this compound demonstrates an antihypertensive effect *in vivo* which is presumably mediated through vascular smooth muscle relaxation.

The nucleotides adenosine cyclic 3′,5′-monophosphate (cAMP) and guanosine cyclic 3′,5′-monophosphate (cGMP) are synthesised within most mammalian cells from their corresponding nucleoside triphosphates by the respective adenylate or guanylate cyclase. Degradation of the cyclic nucleotides operates by hydrolytic cleavage of the 3′-ribose-phosphate bond, catalysed by the appropriate cyclic nucleotide phosphodiesterases. Extensive evidence indicates a role for cAMP in the regulation of smooth muscle contraction, with an elevation of intracellular cAMP resulting in activation of cAMP-dependent protein kinase and subsequent protein phosphorylation associated with muscle relaxation [1–3]. Elevation of cAMP and relaxation may be elicited through direct or indirect stimulation of adenylate cyclase by agents such as β-agonists and prostacyclin, or by inhibition of cAMP phosphodiesterase.

The role of cGMP in the regulation of smooth muscle contraction is less clear, although recent evidence has demonstrated that a number of compounds which elevate intracellular cGMP levels via activation of guanylate cyclase induce smooth muscle relaxation. Included within this category are the nitrovasodilators such as nitroglycerin [4] and nitroprusside [5], atrial natriuretic peptides [6, 7] and possibly endothelium derived relaxation factor (EDRF) [8, 9]. While the activity of cAMP phosphodiesterase inhibitors in causing smooth muscle relaxation and consequent reduction of blood pressure in animals is well recognised [10–12], few reports demonstrate that inhibitors of cGMP phosphodiesterase may also be related to the relaxation of smooth muscle.

Trequinsin (**1**) is a non-selective inhibitor of both cAMP and cGMP phosphodiesterases, which in addition to being a potent inhibitor of platelet aggregation also has cardiotonic and vasodilator activity [13]. Although the antiplatelet activity of this compound is probably attributable to its inhibition of the platelet Type III cAMP dependent phosphodiesterase, its vasodilator activity could be due to inhibition of either the smooth muscle Type I cGMP or Type III cAMP dependent phosphodiesterase [14].

During the course of our work on phosphodiesterase inhibitors it was apparent to us that the structural requirements for phosphodiesterase inhibition by Trequinsin-like compounds were incorporated within several classes of papaverine-related isoquinoline alkaloids. Furthermore, in addition to papaverine [15] itself, a number of such alkaloids including the aporphine (**2**) [16] , the berberines (**3**, R = H, CH_3) [17] and stepharine [18] have been reported as being phosphodiesterase inhibitors or as having biological activity possibly attributable to phosphodiesterase inhibition. Thus Trequinsin (**1**), glaucine (**2**) and xylopinine (**3**; R = CH_3) are all 6,7-dimethoxytetrahydroisoquinolines bearing lipophilic substituted phenyl moieties. In view of these similarities we undertook to evaluate some Trequinsin related aporphine analogues [19] to help probe the conformational requirements for phosphodiesterase inhibition.

MATERIALS AND METHODS

Materials. 6,7-Dihydro-9,10-dimethoxy-2H-pyrimido[6,1-*a*]isoquinoline-2,4(3H)-dione (**4**) and 2,3,6,7-tetrahydro-9,10-dimethoxy-3-methyl-2-[2,4,6-(trimethylphenyl)imino]-4H-pyrimido[6,1-*a*]isoquinolin-4-one, hydrochloride (**1**), (Trequinsin) were synthesised according to the methods of Lal *et al.* [13]. 1-(3,4-Dimethoxyphenyl)-6,7-dihydro-9,10-dimethoxy-3-methyl-2H-pyrimido [6,1-*a*]isoquinoline-2,4(3H)-dione (**5**), 1-(3,4-dimethoxyphenyl)-6,7-dihydro-9,10-dimethoxy-2,4-dioxo-2H-pyrimido[6,1-*a*]quinazoline-3(4H)-carboxylic acid,

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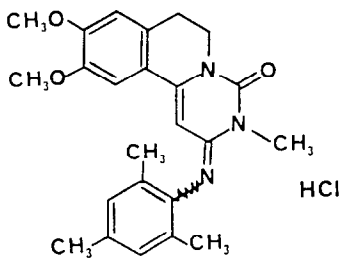

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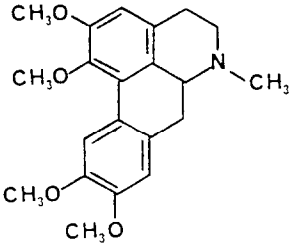

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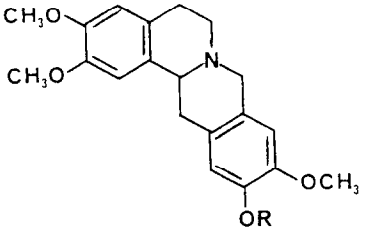

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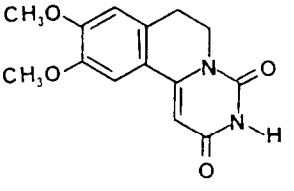

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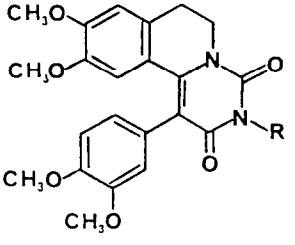
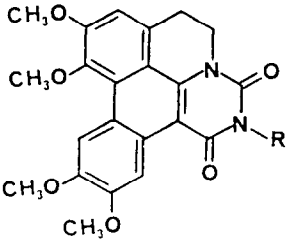


(5) R=CH_3
(6) R = $COOCH_2CH_3$



(7) R = H
(8) R = CH_3
(9) R = $COOCH_2CH_3$

ethyl ester (**6**), 5,6-dihydro-8,9,11,12-tetramethoxy-1H-benz[*f*]-isoquino [8,1,2-*hij*]quinazoline-1,3(2H)-dione (**7**), 5,6-dihydro-8,9,11,12-tetramethoxy- 2-methyl - 1H - benz[*f*]isoquino[8,1,2 - *hij*]quinazo - line-1,3(2H)-dione (**8**) and 5,6-dihydro-8,9,11,12-tetramethoxy - 1,3 - dioxo - 1H - benz[*f*]isoquino - [8,1,2-*hij*]quinazoline-2(3H)-carboxylic acid, ethyl ester (**9**) were obtained from G. D. Searle & Co. (Skokie, IL) and were synthesised by G. R. Lenz [19].

Phenylmethylsulphonyl fluoride, calmodulin and snake venom 5′-nucleotidase *(Ophiophagus hannah)* were obtained from Sigma (Poole, Dorset, U.K.). DEAE-Cellulose (Sephacel) was supplied by Pharmacia. DEAE anion exchange resin AG 1-X8, 200–400 mesh chloride form, was obtained from Bio-Rad Laboratories (Watford, U.K.). Inactin-Byk was obtained from Byk Gulden (Konstanz, F.R.G.). [^{3}H]-Adenosine cyclic 3′,5′-monophosphate, ammonium salt (29.4 Ci/mmol) and [^{3}H]-guanosine cyclic 3′,5′-monophosphate, ammonium salt (15 Ci/mmol) were obtained from Amersham International (Amersham, Bucks, U.K.).

Preparation of phosphodiesterase enzymes from bovine renal artery. Bovine renal arteries and their medullary branches obtained from slaughterhouse material were homogenised at 4° using an Ultraturrax disintegrator (10 × 10) and a glass homogeniser in a medium containing: 50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 3.75 mM mercaptoethanol, 0.2 mM phenylmethylsulphonyl fluoride and 35% ethylene glycol. The homogenate was sonicated in an MSE Soniprep for 1 min (4 × 15 sec, maximal setting) and then centrifuged at 100,000 *g* for 45 min. The supernatant (approx. 35 ml) was applied to a DEAE-cellulose (Sephacel) column (bed volume approximately 80 ml) and was eluted with a linear gradient of 0–1000 mM sodium acetate in a total gradient volume of 500 ml. Fractions (5 ml) were collected for subsequent phosphodiesterase assay.

Cyclic nucleotide phosphodiesterase assay. Column fractions from the chromatographed bovine renal artery were assayed to identify the peaks of enzyme activity. After assay, the separated peaks were pooled and individual compounds were assayed for inhibitory activity against the isolated phosphodiesterase enzymes. The enzyme assay measured the formation of 5′-[^{3}H]-AMP or 5′-[^{3}H]-GMP from the corresponding 3′,5′-cyclic nucleotides. The 5′-nucleotide was then converted by a 5′-nucleotidase from snake venom *(Ophiophagus hannah)* to the equivalent nucleoside which was then isolated by anion exchange chromatography [20]. Assays were performed at 1 μM substrate concentration at 30° for 30 min using enzyme dilutions which gave 20–60% hydrolysis of substrate. Isobutylmethylxanthine was included in each series of assays to ensure that the enzyme preparation was functioning in a standardised manner.

The enzyme incubation mixture contained 50 mM Tris–HCl, pH 8.0, 30 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 1 μM cyclic 3′,5′-[^{3}H]-AMP or cyclic 3′,5′-[^{3}H]-GMP (5×10^5 cpm) and an appropriate dilution of enzyme in a volume of 0.5 ml. In enzyme inhibition assays, compounds were dissolved in DMSO and always added to the assay to give a final concentration of 2% DMSO. Each assay was initated by addition of substrate and was terminated after 20 min by boiling for 1 min followed by a further 10 min incubation with 50 μl snake venom (2 mg/ml) at 30°. Methanol (1 ml) was then added and the mixture added to a small anion exchange column (AG1 X-8, 0.6 × 2 cm column). [^{3}H]-Adenosine or [^{3}H]-guanosine were eluted by the addition of a further 1 ml of methanol directly into scintillation vials. Both control and non-enzyme blank assays were measured in the presence of 2% DMSO. The enzyme assay was linear for at least 20 min and none of the compounds investigated affected the activity of the 5′-nucleotidase. The concentrations of compounds which inhibited by 50% the hydrolysis of 1 μM substrate (IC_{50}) were determined from concentration-percent curves, and the results (Table 1) are expressed as a mean ±SEM from at least three different enzyme preparations.

Table 1. Comparative activities of compounds versus bovine renal artery cGMP dependent (Type I) and cAMP dependent (Type III) phosphodiesterases

Compound	Inhibition of phosphodiesterase IC_{50} (μM) ± SEM	
	Type I	Type III
Trequinsin (1)	2.09 ± 0.22 (N = 10)	0.28 ± 0.02 (N = 10)
4	150 ± 5 (N = 3)	29.0 ± 6.9 (N = 3)
5	40.2 ± 21.2 (N = 3)	54.3 ± 2.6 (N = 3)
6	42.5 ± 19.9 (N = 3)	89.3 ± 2.3 (N = 3)
7	0.39 ± 0.11 (N = 3)	1.64 ± 0.85 (N = 3)
8	0.49 ± 0.11 (N = 3)	11.5 ± 6.5 (N = 3)
9	0.45 ± 0.12 (N = 3)	>200 (N = 3)

Antihypertensive activity in anaesthetized spontaneously hypertensive rats. Blood pressure effects were evaluated in anaesthetized male spontaneously hypertensive rats (SHR) (300–350 g, 14–16 weeks old) which were derived from the Wistar–Okamoto strain. Rats were anaesthetized with Inactin (160 mg/kg, i.p.). The mean arterial blood pressure was measured through the carotid artery using an Ormed Type 4-422-0001-1-B4M5 pressure transducer. Arterial blood pressure was amplified and registered on an Ormed MT6 multi-channel recorder. All injections of drug were made via a catheter placed in the jugular vein. Test compounds were administered i.v. in a volume of 1 ml in a mixture of PEG 400–ethanol–saline (4:1:5). Blood pressure was recorded for 60 min after injection of the drug. Injection of the vehicle alone had no effect on the blood pressure.

RESULTS AND DISCUSSION

As shown in Fig. 1, the cyclic nucleotide phosphodiesterase activity of bovine renal artery was succesfully resolved using anion exchange chromatography into three separate peaks of enzyme activity, which generally corresponded to those peaks obtained from bovine coronary arteries using a similar preparative procedure [21]. The first peak of enzyme activity (designated the Type I enzyme) only hydrolysed cGMP at the substrate concentrations used in the assay (1 μM) and this enzyme was stimulated 8–10-fold in the presence of calcium

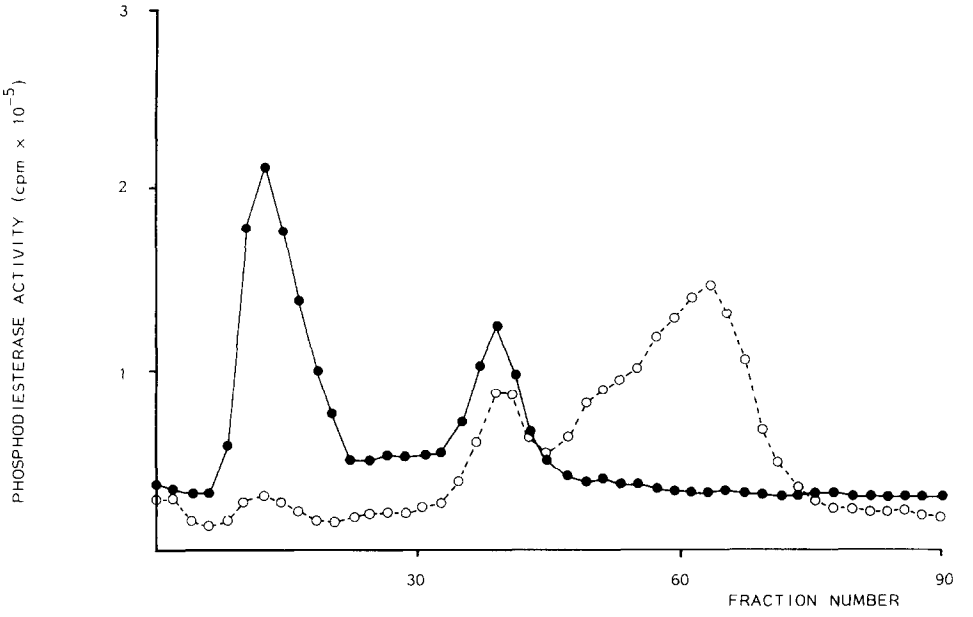


Fig. 1. Elution profile of bovine renal artery smooth muscle phosphodiesterase. Individual fractions were assayed at 1 μM cGMP (●—●) and at 1 μM cAMP (○—○) for phosphodiesterase activity.

(10 μM) and calmodulin (6 μg/ml). The presence or absence of calmodulin and/or calcium did not affect the inhibitory activity of any of the compounds studied, and it was therefore omitted from further screening assays. The second peak of enzyme activity, which eluted between column fractions 36 and 42, showed a lack of specificity for either cAMP or cGMP substrate and was not used in further studies. The third peak eluted in a broad homogeneous band between fractions 48 and 72 and showed a selectivity for the hydrolysis of cAMP at the substrate concentrations shown. This enzyme was designated the Type III enzyme and its activity was unaffected by the presence of calcium and/or calmodulin.

Table 1 shows the results obtained for the inhibitory activities of Trequinsin and related test-compounds against the bovine renal artery Type I and Type III enzymes. All of the compounds showing inhibitory activity were competitive inhibitors, but K_i values are not reported since (a) these determinations are, as previously reported [22], complicated by the negative cooperative behaviour of the enzyme, and (b) this study was designed simply to compare the relative potency of a series of compounds against the separated cGMP (Type I) and cAMP (Type III) enzymes.

The data in Table 1 demonstrate that the benz[*f*]isoquinoquinazoline diones (**7**, **8** and **9**) are potent inhibitors of the Type I cGMP dependent phosphodiesterase having IC_{50}s of 0.39, 0.49 and 0.45 μM respectively, and are therefore amongst the most potent compounds of this class reported [23–26]. Furthermore, whereas compounds **7** and **8** show inhibitory activity versus the Type III cAMP dependent enzyme, compound **9** is devoid ($IC_{50} > 200$ μM) of such activity and represents the most potent and selective cGMP phosphodiesterase inhibitor reported.

Whereas the structure–activity shown in Table 1 indicates that the nature of the quinazoline 2-substituent (R) is unimportant for cGMP inhibitory activity, the presence of the 1-(3,4-dimethoxyphenyl) substituent (as shown by the inactivity of **4**) and of its relative geometry to the pyrimidoisoquinoline moiety is critical. Thus compounds **5** and **6**, in which the interaction between protons on the two aromatic rings force the 1-(3,4-dimethoxyphenyl) group to adopt an orthogonal conformation to the pyrimidine ring, are one hundred-fold less active than compounds **7**, **8** and **9** in which this substituent is coplanar with the rest of the molecule.

In view of these findings compound **9** was evaluated in an anaesthetised spontaneously hypertensive rat (SHR) model to test the potential antihypertensive properties of a potent and selective cGMP phosphodiesterase inhibitor. Trequinsin was included in this study as a positive control, and in agreement with previous studies [13] was active in reducing mean arterial blood pressure (Fig. 2). After intravenous administration of **9** (3 mg/kg) the initial antihypertensive effect (31 ± 3 mm.Hg) was less than that of Trequinsin; however, by 60 min **9** had demonstrated a potent hypotensive effect, comparable to that of Trequinsin, lowering the mean arterial blood pressure by 50.3 ± 3.7 mm.Hg (18.6%, N = 4).

The hypotensive activity of compound **9** could conceivably be attributable to rapid metabolism to an active metabolite, such as **7** which has activity versus both the cGMP (Type I) and cAMP (Type III) phosphodiesterases. However, when tested in the SHR model, **7** (3 mg/kg i.v.) only reduced blood pressure by 12 ± 4 mm.Hg at 1 min and with a maximal effect of 21 ± 11 mm.Hg at 60 min. It is possible that the activity of **7** was limited by the rate at which it entered smooth muscle cells. However, the data

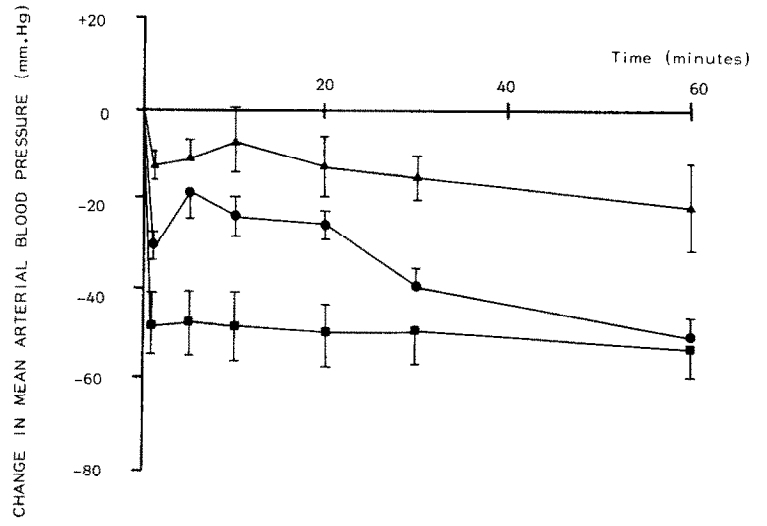


Fig. 2. Effects of Trequinsin (■; 3 mg/kg, i.v.), **7** (▲; 3 mg/kg i.v.) and **9** (●; 3 mg/kg i.v.) on mean arterial blood pressure in anaesthetised spontaneously hypertensive rats. Values represent the mean ±SEM of four animals.

are not supportive of the activity of **9** resulting solely from its metabolism to **7**, although a component of the activity at the latter time points could possibly be due to this potential metabolite.

The hypotensive activity of Trequinsin may be due to inhibition of either the cAMP (Type III) or the cGMP (Type I) phosphodiesterase, or to a combination of both of these effects. In contrast, at least the initial activity of **9** in reducing blood pressure is, in the absence of alternative unknown mechanisms, probably due to selective inhibition of the cGMP (Type I) phosphodiesterase, resulting in a sequence of cardiovascular changes leading to a hypotensive effect.

In conclusion, the benz[*f*]isoquinoquinazoline dione **9** is a potent and selective inhibitor of cGMP dependent phosphodiesterase and it is likely that this activity is involved in its mechanism of action in lowering systemic blood pressure in the spontaneously hypertensive rate. The profile of this class of compound would therefore indicate a potential in antihypertensive therapy, and its selectivity should make it a useful tool in the elucidation of the role of cGMP in a range of pharmacological processes.

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