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The effect of SK&F 95654, a novel phosphodiesterase inhibitor, on cardiovascular, respiratory and platelet function

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1 SK&F 95654 inhibited the guanosine 3':5'-cyclic monophosphate (cyclic GMP)-inhibited phosphodiesterase (cGI-PDE) with an IC_{50} value of 0.7 μM. The IC_{50} values were greater than 100 μM for the other four phosphodiesterase isoenzymes tested. The **R**-enantiomer of SK&F 95654 (IC_{50} = 0.35 μM) was a more potent inhibitor of cGI-PDE than was the **S**-enantiomer (IC_{50} = 5.3 μM).

2 In the guinea-pig working heart, SK&F 95654 produced a positive inotropic response without altering heart rate.

3 Oral administration of SK&F 95654 to conscious dogs caused dose-dependent increases in left ventricular dp/dt_{max} in the range 10–50 μg kg^{-1}. These positive inotropic responses were maintained for 3 h without simultaneous changes in heart rate or blood pressure. The peak effects on left ventricular dp/dt_{max} were similar for orally and intravenously administered compound, indicating good oral bioavailability.

4 SK&F 95654 caused a potent inhibition of U46619-induced aggregation in both a human washed platelet suspension (WPS) (IC_{50} = 70 nM) and in human platelet-rich plasma (PRP) (IC_{50} = 60 nM), indicating that the compound shows negligible plasma binding.

5 The **R**-enantiomer of SK&F 95654 was twenty fold more potent as an inhibitor of platelet aggregation than was the **S**-enantiomer. The similarity of this ratio to that obtained on the cGI-PDE suggests that SK&F 95654 inhibits platelet aggregation via its effects on cGI-PDE. This was also indicated by studies which showed that SK&F 95654 increased adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels and activated cyclic AMP-dependent protein kinase in human platelets.

6 Collagen-induced aggregation of rat PRP was also inhibited by SK&F 95654 (IC_{50} = 65 nM). The effects of SK&F 95654, administered intravenously, on *ex vivo* platelet aggregation were studied in the conscious rat. At 1 mg kg^{-1}, SK&F 95654 inhibited aggregation for at least 4 h post dose and was more potent than the two other cGI-PDE inhibitors studied (siguazodan and SK&F 94120).

7 In contrast to its potent effects on heart and platelets, SK&F 95654 caused only a modest relaxation of histamine- or U46619-induced bronchoconstriction in the anaesthetized, ventilated guinea-pig.

8 Taken together, these results indicate that SK&F 95654 may be a suitable agent for the treatment of congestive heart failure.

Keywords: Phosphodiesterase; inhibitor; heart; platelet; SK&F 95654; cyclic AMP

Introduction

Cyclic nucleotide phosphodiesterases (PDEs) may be classified into five isoenzyme families according to their amino-acid sequence, kinetic properties and sensitivity to physiological and pharmacological modulators (Beavo & Reifsnyder, 1990). The guanosine 3':5'-cyclic monophosphate (cyclic GMP)-inhibited PDE (cGI-PDE), also known as PDE III (Weishaar *et al.*, 1986; Reeves *et al.*, 1987), forms one of these families. Inhibitors of cGI-PDE have been shown to be inotropes (Gristwood *et al.*, 1986; Muller *et al.*, 1990), relaxants of vascular (Silver *et al.*, 1988; Lindgren & Andersson, 1991) and airway smooth muscle (Torphy *et al.*, 1988; Heaslip *et al.*, 1991) and inhibitors of platelet aggregation (Simpson *et al.*, 1988; Murray *et al.*,1990a; Seiler *et al.*, 1987; 1991). In general, however, the effects of an agent on these various physiological parameters have not been systematically compared. In this manuscript we have investigated the effects of a SK&F 95654, a cGI-PDE inhibitor with a novel structure, on PDE isoenzymes and on cardiovascular, respiratory and platelet function. We have also performed studies in human platelets to investigate the mechanism of action of SK&F 95654, and the relationship between increases in adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels and effects on aggregation is discussed.

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Methods

Isolation and assay of phosphodiesterases

PDE isoenzymes were isolated by a mixture of anion-exchange and affinity chromatography as previously described (Murray *et al.*,1991). The Ca^{2+}/calmodulin-stimulated PDE (PDE I, see Beavo & Reifsnyder (1990) for nomenclature) was prepared from bovine cardiac ventricle. Cyclic GMP-stimulated PDE (PDE II), cyclic GMP-inhibited PDE (PDE III) and cyclic AMP-specific PDE (PDE IV) were all isolated from guinea-pig cardiac ventricle. Cyclic GMP-selective PDE (PDE V) was obtained from porcine lung. With the exception of PDE II, which displayed positive cooperativity, all the preparations showed simple Michaelis-Menten kinetics. The PDEs also responded to pharmacological and physiological modulators in a predictable

fashion. PDE activity was assayed by the boronate column method (Reeves *et al.*, 1987) with 1 μM cyclic GMP as a substrate for PDE I (in the absence of Ca^{2+} and calmodulin), PDE II and PDE V and with 1 μM cyclic AMP as a substrate for PDE III and PDE IV.

Guinea-pig isolated working heart

Male Dunkin-Hartley guinea-pigs (450–600 g) were killed by cervical dislocation 20 min after the administration of heparin (2,000 i.u., i.p). The heart and lungs were removed rapidly and placed in Krebs solution at 4°C. The lungs and extraneous pericardial tissue were then removed and the heart mounted on the working heart apparatus (Flynn *et al.*, 1978). Krebs solution, gassed with 5% CO_2 in O_2 at 37.5°C, entered the right atrium and was pumped by the left ventricle against a 70 cm column of Krebs solution. Both the coronary and aortic flow were normally recirculated. Aortic flow was measured by a flow meter (Gould Statham), by use of probe of 6.0 mm i.d. situated just after the aortic cannula. Left ventricular (LV) pressure was measured by a Bell & Howell pressure transducer connected by a saline (0.9%) filled polythene tubing to a 23 swg needle pushed into the left ventricle. The left ventricular pressure signal was amplified (Devices preamplifier 3552), differentiated (Devices differentiator 3640) and the derived signal, LV dp/dt_{max}, used as an index of left ventricular contractility. Instantaneous heart rate was measured by a rate meter triggered by the left ventricular pulse. All four parameters were recorded by a Devices M19 six channel recorder. Coronary flow, which dripped from the ventricle, was measured by collection into a volumetric measuring cylinder over 1 min periods and recorded on the chart. Total cardiac output was obtained from the sum of the coronary and aortic flows. Coronary flow and cardiac output were expressed per g dry weight heart tissue, determined after placing the hearts in an oven at 84°C for 24 h.

Cardiovascular studies in conscious dogs

The methods for measurement of cardiovascular and left ventricular function parameters have been described in detail elsewhere (Gristwood *et al.*, 1988). Briefly, unilateral carotid loops were produced in beagle dogs by aseptic techniques under general anaesthesia maintained with halothane in 50% nitrous oxide in oxygen. The carotid loop was approximately 7 cm long and could be used 6 weeks after surgery. When the carotid loops were healed a second surgical procedure to implant a left ventricular pressure transducer was performed. Using a left thoracotomy in the fifth intercostal space, a Konigsberg P-22 solid state pressure transducer was implanted into the left ventricle through a stab wound in the apex of the heart. The animals were allowed a minimum of six weeks to recover from the surgery before the first experiment was performed.

Carotid blood pressure was measured by introducing a teflon cannula (Surflo or Angiocath 20 gauge 2 inch) into the looped carotid artery and connecting it via a three-way tap to a Micron miniature pressure transducer, Model MP 15D (Micron Instruments Inc.) used in conjunction with a Lectromed 3552 preamplifier. The left ventricular pressure transducer was energized by a Lectromed 3559 preamplifier and the resulting signal electronically differentiated to give LV dp/dt_{max}. Lead II ECG signals were detected with subcutaneous titanium electrodes, implanted under general anaesthesia, one over the right shoulder and a second over the left hip. The signals were recorded on a Lectromed M19 polygraph and led to a CED 1401 interface connected to a Micro-Vax II computer (Digital).

Platelet studies

Human platelet-rich plasma (PRP) and a washed platelet suspension (WPS) were prepared from whole blood, freshly drawn from healthy volunteers who gave informed consent, as previously described (Murray *et al.*, 1990a; Merritt *et al.*, 1991). Aggregation was measured in a 4-channel PAP4 Biodata aggregometer. Aliquots, at a final count of 1.5×10^8 platelets ml^{-1}, were equilibrated to 37°C before being placed in the sample chamber and were then incubated for 5 min with various concentrations of SK&F 95654. After the addition of the agonist, aggregation was determined by the change in absorbance monitored for 4 min. Cyclic AMP levels and the cyclic AMP-dependent protein kinase activity ratio were measured in human WPS, that had been incubated with various concentrations of SK&F 95654 for 5 min, as previously described (Murray *et al.*, 1990a,b; Merritt *et al.*, 1991).

Blood obtained from the vena cava of anaesthetized male rats (Sprague-Dawley) was immediately mixed with 0.1 volume of 102 mM sodium citrate for the preparation of rat PRP. PRP was prepared by centrifugation (450 *g* for 5 min) and diluted to 4×10^8 cells ml^{-1} by the addition of autologous platelet-free plasma. Aggregation was determined as described above. For the studies of their effects on *ex vivo* aggregation, test compounds were dissolved in 40% (v/v) polyethylene glycol-400 and administered intravenously into the tail vein. Anaesthesia was induced by Sagatal (60 mg kg^{-1}, i.p.) 8 min before the collection of blood. PRP was prepared, and aggregation monitored, as described above.

Assessment of bronchodilator activity in guinea-pigs

Male Dunkin-Hartley guinea-pigs (450–600 g), were anaesthetized (Sagatal 50 mg kg^{-1}, i.p.) and during the experiment supplements of the anaesthetic (6 mg kg^{-1}, i.v.) were given as required. The trachea was cannulated and the animals ventilated by the use of a Palmer constant volume respiration pump at a frequency of 53 strokes min^{-1}. The stroke volume (6–10 ml) was adjusted to produce a basal airways inflation pressure (AIP) of 15 mmHg. Changes in AIP at a constant airflow were measured with a Bell & Howell 0–750 mmHg physiological pressure transducer connected to a side arm of the inflow circuit. Systemic blood pressure and heart rate were recorded from a cannula inserted into one carotid artery and the pulse pressure used to trigger an instantaneous rate meter to measure heart rate. Both jugular veins were cannulated for administration of drugs and supplementary anaesthetic. Heparin (100 i.u. kg^{-1}, i.v.) was also administered to maintain the patency of cannulae. The animals were maintained at 37°C and histamine (100 nmol kg^{-1}) or U46619 (10 nmol kg^{-1}), administered by i.v. injections, was used to increase bronchial tone at the times indicated.

For *in vitro* studies, sheets of guinea-pig tracheae containing four or five cartilage bands were dissected essentially as described by Hay *et al.* (1987). The tissues were mounted, under 2.5 g tension, in organ baths filled with gassed (95% O_2:5% CO_2) Krebs-Hensleit buffer (composition, mM: NaCl 118, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 1.18, $K_2H_2PO_4$ 1.18, (+)-glucose 5.5, Na pyruvate 2, $CaCl_2$ 2.5, pH 7.4) at 37°C. The sheets were primed three times with 1 μM carbachol and, after removal of the carbachol, the strips were left until a steady tone was produced. The effects of SK&F 95654 on this spontaneously generated tension were studied in a cumulative manner.

Drugs

SK&F 95654 (**R,S**-4,5-dihydro-6-[4-(1,4-dihydro-4-oxopyridin-1-yl)phenyl]-5-methyl-3(2H)-pyridazinone; see Figure 1), siguazodan (SK&F 94836, **R,S**-2-cyano-1-methyl-3-[4-(1, 4,5, 6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]guanidine) and SK&F 94120 (5-(4-acetamidophenyl)pyrazin-2-(1H)-one) were prepared in the laboratories of SmithKline Beecham Pharmaceuticals, Welwyn, as previously described (Coates *et al.*, 1983; Burpitt *et al.*, 1988). The **R**- and **S**-enantiomers of

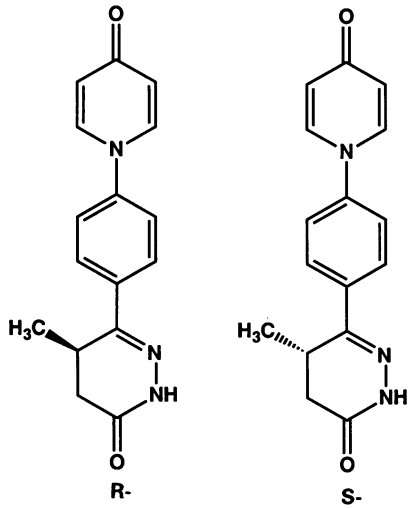

Figure 1 The structure of SK&F 95654 (4,5-dihydro-6-[4-(1,4-dihydro-4-oxopyridin-1-yl)phenyl]-5-methyl-3(2H)-pyridazinone).

SK&F 95654 were prepared from a chiral intermediate as described (Owings *et al.*, 1991). U46619 (15S-hydroxy-11α,9α-(epoxymethano)prosta-5Z,13E-dienoic acid) was obtained from Upjohn (Kalamazoo, U.S.A.) and collagen from Hormon-Chemie (Munich, Germany). Other drugs and chemicals were obtained from Sigma Chemical Company Ltd (Poole, Dorset) and radiochemicals were from Amersham International (Amersham, Bucks).

Analysis of data

All values are reported as means ± s.e.mean. Significant differences between two means were determined with Student's *t* test for unpaired observations and for paired observations (data in Figure 2). $P<0.05$ level was considered significant for all tests. Concentration-inhibition curves were fitted to the logistic equation by the programme, ALLFIT (De Lean *et al.*, 1978).

Results

Effects of SK&F 95654 on PDE isoenzymes

Table 1 shows the IC_{50} values for SK&F 95654 (racemate) and its enantiomers on cGI-PDE isolated from guinea-pig heart. SK&F 95654 potently inhibited cGI-PDE with the R-enantiomer being a more potent inhibitor than the S-enantiomer. The IC_{50} value was greater than 100 μM for the other four PDE isoenzymes tested demonstrating that SK&F 95654 is a potent, selective inhibitor of cGI-PDE. For comparison, the results obtained with previous SmithKline Beecham compounds and clinically used cGI-PDE inhibitors are shown. It can be seen that the potency and selectivity of SK&F 95654 are similar to those of siguazodan and that these compounds are more potent inhibitors of cGI-PDE than either enoximone or amrinone. Milrinone, while being a potent cGI-PDE inhibitor, shows decreased selectivity with respect to PDE IV (cyclic AMP-specific, rolipram inhibited PDE).

Effects of SK&F 95654 on guinea-pig working heart

The effects of SK&F 95654 on the guinea-pig working heart are shown in Figure 2. SK&F 95654 (0.1–10 μM) caused a positive inotropic response with maximal effect at 1 μM and with no significant effect on heart rate. Cardiac output and coronary flow remained constant over the same concentration-range but were reduced at 10 μM. SK&F 95654, therefore, shows selectivity as a positive inotropic agent *in vitro*.

Table 1 Inhibition of cyclic GMP-inhibited phosphodiesterase (cGI-PDE) and other PDE isoenzymes by SK&F 95654 and other selected cGI-PDE inhibitors

Inhibitor	*Isoenzyme* III	I	II	IV	V
	(IC_{50} μM or % inhibition at 100 μM)				
RS-SK&F 95654	**0.7**	14%	33%	31%	24%
R-SK&F 95654	**0.4**	0%	13%	14%	43%
S-SK&F 95654	**5.3**	0%	3%	10%	6%
Enoximone	**13**	11%	20%	25%	15%
Amrinone	**52**	0%	0%	10%	12%
Milrinone	**2**	25%	0%	**33**	27%
Siguazodan	**0.8**	7%	3%	4%	24%
SK&F 94120	**12**	21%	0%	13%	15%

cGI-PDE (PDE III) and other PDE isoenzymes were isolated from guinea-pig cardiac ventricle and other tissues and assayed for PDE activity with 1 μM cyclic AMP or 1 μM cyclic GMP as described under Methods. PDE I is the Ca^{2+}/calmodulin-stimulated PDE; PDE II is the cyclic GMP-stimulated PDE; PDE III is the cyclic GMP-inhibited PDE; PDE IV is the cyclic AMP-specific PDE; PDE IV is the cGMP-specific PDE; for further details see Beavo & Reifsnyder (1990). The IC_{50} (in μM) value is shown in bold, when this is greater than 100 μM the % inhibition obtained at 100 μM drug is shown.

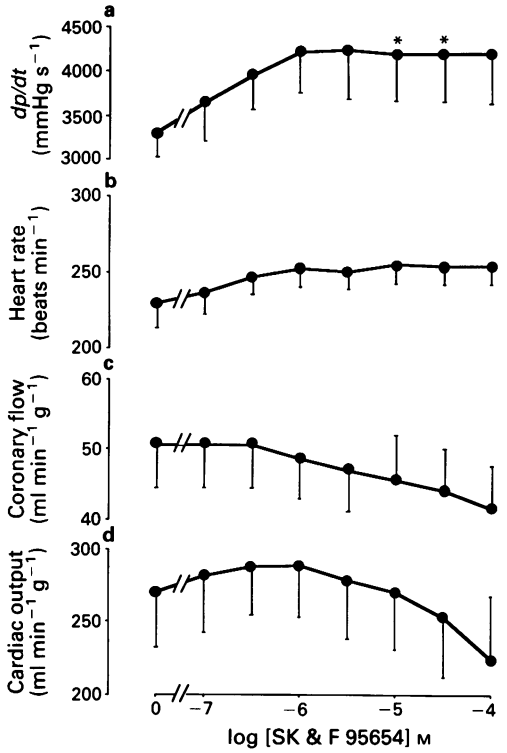

Figure 2 Effect of SK&F 95654 on guinea-pig isolated working heart. Guinea-pig hearts were set up and the various parameters measured as described under Methods. Cumulative dose-response curves to SK&F 95654 were constructed and the effects on (a) LV*dp/dt*, (b) heart rate, (c) coronary flow and (d) cardiac output were determined. Results are mean with s.e.mean shown by vertical bars ($n = 5$).

Results were analysed by Student's *t* test vs. zero SK&F 95654; $*0.05>P>0.01$.

Effects of SK&F 95654 on cardiovascular parameters in conscious dogs.

Single oral administration of SK&F 95654, 10, 25 or 50 $\mu g\,kg^{-1}$ caused a dose-related increase in myocardial contractility (measured as LV dp/dt_{max}), with no concomitant increase in heart rate or reduction of MABP (Figure 3). Parallel changes were seen when contractility was computed as dp/dt at 40 mmHg, an index independent of changes to afterload. Onset of the response was rapid, with a significant effect 20 min after administration at the highest dose. Contractility remained increased for the duration of the 3 h post dosing experimental period. Figure 4 compares the effects of SK&F 95654 administered as an intravenous bolus with those as a single oral dose. The similarity of the effects indicates excellent oral absorption of the compound, a lack of first pass metabolism and good bioavailability. Figure 5 compares the *in vivo* force/rate relationship of the compound with that of the β-adrenoceptor agonist, isoprenaline, which in contrast to SK&F 95654 caused a marked chronotropic as well as inotropic response. Thus SK&F 95654 shows force selectivity *in vivo*.

Effects of SK&F 95654 on human platelets

Table 2 shows the effects of SK&F 95654 and its enantiomers on U46619-stimulated aggregation in suspensions of washed human platelets and in human PRP. The similarity of the values in washed platelets and PRP suggest that SK&F 95654 has a low binding to plasma proteins. In both washed platelets and PRP, R-SK&F 95654 was a twenty fold more potent inhibitor of aggregation than the S-enantiomer. This ratio is very similar to that observed on the cGI-PDE and suggests that the effects of SK&F 95654 on platelet aggregation are due to cGI-PDE inhibition. SK&F 95654 increased the cyclic AMP content in a washed human platelet suspension in a dose-related manner (Figure 6). The activity of cyclic AMP-dependent protein kinase was increased by SK&F 95654 over the same concentration-range (Figure 6).

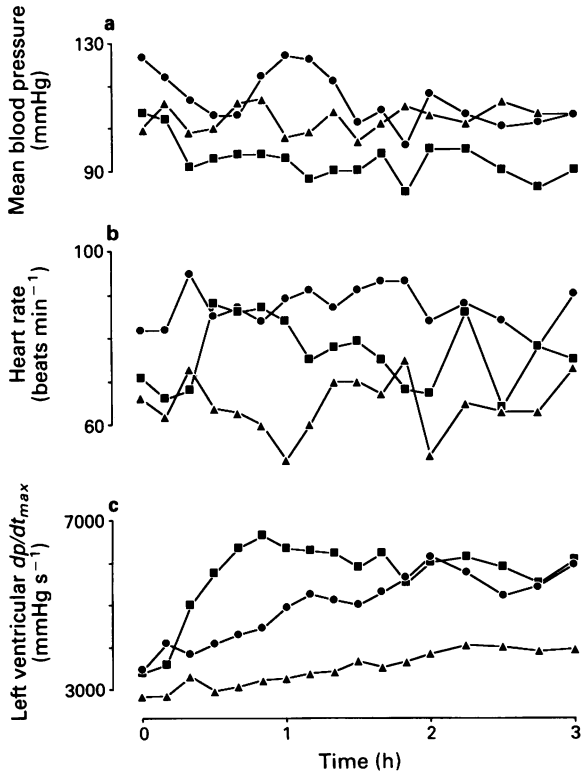

Figure 3 Haemodynamic changes following three doses of SK&F 95654 orally (in distilled water) to conscious instrumented dogs. The parameters measured were mean blood pressure (a), heart rate (b) and left ventricular dp/dt_{max} (c). The doses used were 10 $\mu g\,kg^{-1}$ (▲), 25 $\mu g\,kg^{-1}$ (●) and 50 $\mu g\,kg^{-1}$ (■). Points are means of $n = 3$; significant increases in LV dp/dt_{max} occurred at all dose levels.

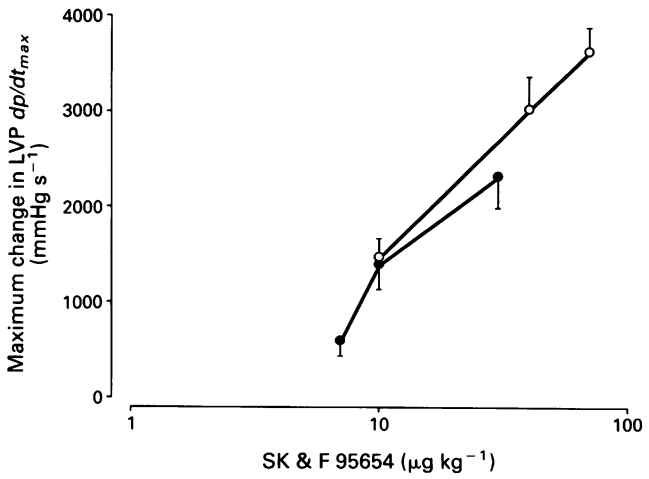

Figure 4 Comparison of the maximum inotropic response obtained from intravenously and orally administered SK&F 95654. SK&F 95654 was administered intravenously (●) or orally (○) to conscious instrumented dogs and the maximum increase in left ventricular dp/dt_{max} over the subsequent 3 h was recorded. Points are means with s.e.means shown by vertical bars ($n = 3$).

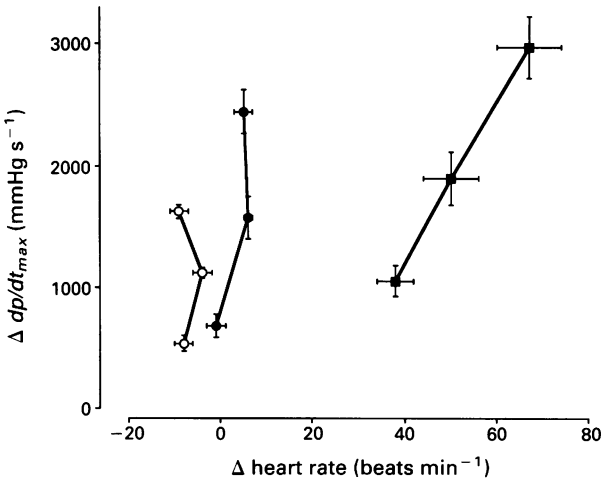

Figure 5 Comparison of the contractility/rate response relationship between SK&F 95654 and isoprenaline. SK&F 95654 was administered by oral (●) or by bolus intravenous injection (○), to conscious instrumented dogs. The points are the mean with s.e.mean (vertical and horizontal bars) ($n = 3$) of isochronous measurements taken at 5 min intervals. The doses used were 10, 25 and 50 $\mu g\,kg^{-1}$ (oral) and 5, 10 and 20 $\mu g\,kg^{-1}$ (i.v.). The values for isoprenaline (■) are taken from Gristwood *et al.* (1988).

Effects of SK&F 95654 on rat platelets

Collagen-induced aggregation of rat PRP was inhibited by SK&F 95654 (Table 2). SK&F 95654 and two other cGI-PDE inhibitors, siguazodan (Murray *et al.*, 1990a) and SK&F 94120 (Simpson *et al.*, 1988), were administered intravenously to rats and their effects on platelet aggregation in PRP obtained from blood taken 15 min after the dose studied. All three agents caused a dose-related inhibition of *ex-vivo* platelet aggregation, with SK&F 95654 being the

Table 2 Inhibition of platelet aggregation by SK&F 95654 and its enantiomers

Platelet preparation	*Stimulus*	**RS-*95654***	**R-*95654*** IC_{50} (nM)	**S-*95654***
Human WPS	U46619 (1 μM)	70 ± 24 (5)	30 ± 13 (3)	633 ± 76 (3)
Human PRP	U46619 (1 μM)	60 ± 27 (5)	31 ± 8 (3)	615 ± 209 (3)
Rat PRP	Collagen (2.5 μg ml^{-1})	65 ± 45 (2)	ND	ND
Rat PRP	Collagen (3.75 μg ml^{-1})	150 ± 40 (2)	ND	ND

Platelet-rich plasma (PRP) or a washed platelet suspension (WPS) was incubated with various concentrations of SK&F 95654 or its enantiomers for 5 min before the addition of U46619 or collagen, aggregation was monitored for the subsequent 4 min. The values shown are the calculated IC_{50} (in nM) for the inhibition of aggregation.
ND = not determined.

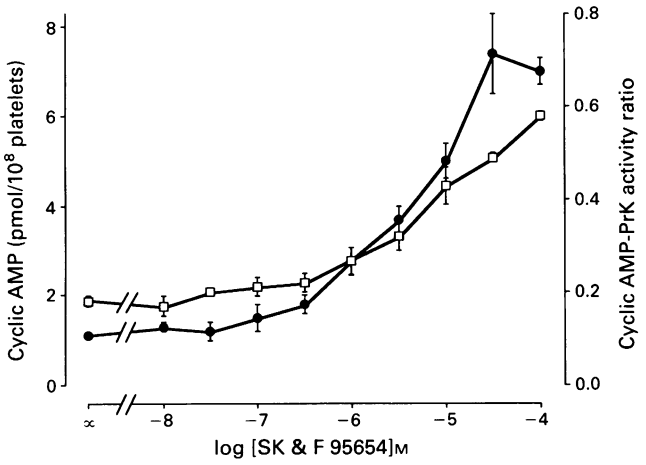

Figure 6 Effect of SK&F 95654 on cyclic AMP content and activity of cyclic AMP-dependent protein kinase (PrK) in washed human platelets. Washed platelet suspensions were incubated with SK&F 95654 and assayed for cyclic AMP (□) and cyclic AMP-dependent protein kinase (●) as described under Methods. Values are shown as means with s.e.mean (vertical bars) ($n = 4$); significant increases ($P < 0.05$) in both parameters were observed for concentrations of SK&F 95654 $> 10^{-6}$ M.

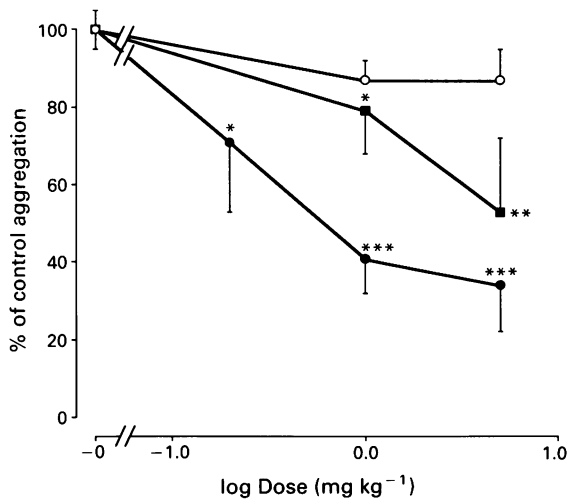

Figure 7 Comparison of the effects of SK&F 95654 (●), siguazodan (■) and SK&F 94120 (○) on platelet aggregation measured *ex vivo* in the rat. Compounds were administered as a bolus intravenous injection to rats and blood withdrawn 15 min later as described under Methods. Platelet aggregation was initiated with 3.75 μg ml^{-1} collagen. Values are means with s.e.mean (vertical bars) ($n = 6$–20) and were compared to vehicle-treated animals by use of Student's t test: *$0.05 > P > 0.01$; **$0.01 > P > 0.001$; ***$P > 0.001$.

most effective (Figure 7). The effects of SK&F 95654 (1 mg kg^{-1}; i.v.) on aggregation were still apparent 4 h after the dose when 2.5 μg ml^{-1} collagen was used as the challenge and for 2 h after the dose when 3.75 μg ml^{-1} collagen was used (Table 3).

Effects of SK&F 95654 on tracheal pressure in the anaesthetized guinea-pig and on isolated tracheal sheets

The effects of SK&F 95654 on U46619- and histamine-induced increases in airway inflation pressure in anaesthetized, ventilated guinea-pigs are shown in Table 4. The doses of U46619 (10 nmol kg^{-1}, i.v.) and histamine (100 nmol kg^{-1}, i.v.) caused an increase in airway inflation pressure of 26 ± 2 mmHg ($n = 12$) and 28 ± 3 mmHg ($n = 8$) respectively. SK&F 95654 caused a dose-dependent decrease in U46619-induced bronchoconstriction and was also effective, to a lesser extent, in reducing histamine-induced rises in airway inflation pressure. In these experiments SK&F 95654 (3 mg kg^{-1}, i.v.) caused a rapid fall in MABP from an initial value of 53 ± 4 mmHg to 29 ± 2 mmHg ($n = 6$); the decrease was evident for the 90 min duration of the experiment.

Racemic SK&F 95654 was a potent relaxant of spontaneous tone in guinea-pig isolated tracheal sheets (EC_{50} = 0.2 ± 0.02 μM) and some stereospecificity was observed (R-SK&F 95654 EC_{50} = 0.3 ± 0.09 μM; S-SK&F 95654 EC_{50} = 1.0 ± 0.07 μM) ($n = 3$, for all values). However, greater stereoselectivity was observed at lower levels of relaxation as indicated by the corresponding EC_{10} values (**RS-SK&F 95654**

Table 3 Effect of SK&F 95654 on *ex vivo* platelet aggregation in the rat

Collagen	*% of control aggregation at time after dose* 0 min	15 min	60 min	120 min	240 min
n =	(20)	(17)	(4)	(7)	(6)
2.5 μg ml^{-1}	100 ± 11	8 ± 3***	2 ± 2***	4 ± 4***	45 ± 27*
3.75 μg ml^{-1}	100 ± 5	41 ± 9***	32 ± 20***	47 ± 18*	95 ± 11

SK&F 95654 was administered as a 1 mg kg^{-1} bolus intravenous injection to rats and blood was withdrawn at the indicated times as described under Methods. Platelet aggregation was initiated by 2.5 or 3.75 μg ml^{-1} collagen. Values were compared by Student's t test to vehicle-treated controls: *$0.05 > P > 0.01$, *** > 0.001.

Table 4 Effect of SK&F 95654 on U46619- and histamine-induced increase in airway inflation pressure in anaesthetized, ventilated guinea-pigs

SK&F 95654 mg kg^{-1}	*Agonist*	*% of pre-dose increase in AIP* 5 min	15 min	30 min	60 min	90 min
0	U46619 ($n = 3$)	92 ± 6	102 ± 6	102 ± 13	119 ± 16	113 ± 4
3	U46619 ($n = 6$)	43 ± 8**	48 ± 7**	62 ± 6*	72 ± 5**	81 ± 7*
10	U46619 ($n = 3$)	18 ± 10**	15 ± 3***	21 ± 4**	40 ± 7**	ND
0	Histamine ($n = 4$)	107 ± 2	112 ± 6	110 ± 10	121 ± 7	124 ± 7
10	Histamine ($n = 4$)	46 ± 4***	73 ± 9*	97 ± 7	109 ± 14	130 ± 10

SK&F 95654 was administered as a bolus intravenous injection to anaethetized guinea-pigs and changes in airways inflation pressure (AIP) induced by U46619 or histamine measured as described under Methods. Values were compared by Student's t test to vehicle treated controls; *$0.05 > P > 0.01$, **$0.01 > P > 0.001$, ***$P > 0.001$.

$EC_{10} = 0.017\ \mu M$; **R**-SK&F 95654 $EC_{10} = 0.007\ \mu M$; **S**-SK&F 95654 $EC_{10} = 0.1\ \mu M$) perhaps indicating additional non cGI-PDE mediated effects at the higher concentrations of SK&F 95654.

Discussion

The results obtained with isolated PDE isoenzymes show that SK&F 95654 is a selective inhibitor of cGI-PDE. A range of dihydropyridazinones have been described in the literature as cGI-PDE inhibitors in which the activity is enhanced by the presence of a 5-methyl substituent in the dihydropyridazinone ring (e.g. Robertson *et al.*, 1986). Such compounds are chiral due to the asymmetric carbon at the 5-position and, for the first time, we present detailed results which show that the **R**-enantiomer is the active form for both biochemical and pharmacological action. The results are consistent with a model (Davis *et al.*, 1987) for the mimicry of cyclic AMP by dihydropyridazinone derivatives in which the **R**-5-methyl group is well accommodated in the region usually occupied by the ribose phosphate ring junction. The **S**-5-methyl would, less favourably, replace the hydroxylic function of the ribose phosphate. Similar results have previously been noted for SK&F 93505, the precursor of SK&F 95654 (Davis *et al.*, 1987).

In common with most other cGI-PDE inhibitors, SK&F 95654 had positive inotropic properties in both guinea-pig and dog with no effect on heart rate. In addition to increased cardiac contractility, it is thought that vasodilatation, resulting in reduction in both pre- and after-load, provides some of the therapeutic benefit of cGI-PDE inhibitors (Cargnelli *et al.*,1989). Although oral administration of SK&F 95654 caused an increase in LV dp/dt_{max} it had no effect on MABP in conscious dogs. However, similar results were obtained with the cGI-PDE inhibitor, siguazodan, which reduced MABP in anaesthetized, but not conscious, dogs (Gristwood *et al.*, 1988). Therefore, although not directly assessed in these studies, it is likely that SK&F 95654 also acts as a vasodilator. Indeed, in the anaesthetized guinea-pig SK&F 95654 caused a pronounced and long lasting fall in MABP, indicating it is probable that SK&F 95654 acts as an 'inodilator' (Cargnelli *et al.*, 1989). SK&F 95654 shows excellent bioavailability as demonstrated by the similarity of the doses required to obtain maximum changes in LV dp/dt_{max} when the compound is administered orally and intravenously. This, coupled with the sustained inotropic action observed after oral dosing, indicates that SK&F 95654 could be suitable for the treatment of congestive heart failure.

There is, at present, considerable debate as to benefit of cGI-PDE inhibitors in the treatment of congestive heart failure. Although clinical studies have shown that cGI-PDE inhibitors cause beneficial changes in the cardiovascular system (Murray & England, 1992), there is current concern regarding the increased mortality associated with two of these agents, milrinone (Packer *et al.*, 1991) and enoximone (Uretsky *et al.*, 1990). Failing human heart shows decreased production of cyclic AMP due to changes in both β-adrenoceptor density and subtype and also to increased levels of inhibitory G proteins (Brodde, 1991; Eschenhagen *et al.*, 1992); in contrast, PDE activity is unaffected (Movsesian *et al.*, 1991). If the reduction in cyclic AMP is regarded as a protective mechanism of the failing heart (Katz, 1990) then, as a class, cGI-PDE inhibitors by re-raising cyclic AMP levels could have undesirable effects.

However, it may be premature to judge all cGI-PDE inhibitors on the basis of current clinical results. Amrinone is usually regarded as the prototype of this class of compound although it is a very weak cGI-PDE inhibitor and almost certainly has other modes of action. Its successor, milrinone, is a potent cGI-PDE inhibitor although its selectivity with regard to inhibition of the cyclic AMP-specific PDE (PDE IV) is not great and it is now known that these two classes of PDE inhibitor interact in various cardiac preparations (Murray & England, 1992). Milrinone has also been reported to have various cyclic AMP-independent effects; for example, it has been reported to activate the calcium release channel of cardiac sarcoplasmic reticulum (Holmberg & Williams, 1991). It has also been postulated that other PDE inhibitors exert their cardiac stimulatory actions by alternate mechanisms, notably sensitization of the contractile proteins (Beier *et al.*, 1991). Therefore, clinical evaluation of novel cGI-PDE inhibitors may help in determining the benefit, or otherwise, of this class of compound.

Although the risk/benefit ratio of long term support with current cGI-PDE inhibitors and other inotropic agents is at present not acceptable, there is no doubt that such agents are suitable for the treatment of acute heart failure and as temporary support for patients waiting for heart transplants (Curfman, 1992). In this respect, the increased aqueous solubility of SK&F 95654 (430 mg l^{-1}) makes it a more suitable agent than siguazodan (51 mg l^{-1}) for acute intravenous treatment and its heightened bioavailability over siguazodan could be advantageous for oral medication. The enhanced anti-platelet action of SK&F 95654 over siguazodan (Figure 7) could also prove to be of benefit.

SK&F 95654 inhibited human and rat platelet aggregation in response to both U46619 and collagen. As previously observed with other cGI-PDE inhibitors, SK&F 95654 was apparently more potent when U46619 was the agonist (Simpson *et al.*, 1988; Murray *et al.*, 1990a). This difference in potency may reflect the ability of the cGI-PDE inhibitor to increase cyclic AMP levels in the presence of different agonists or could be due to differing functional antagonism of the signal transduction pathways used by the agonists (Murray *et al.*, 1990a). However, another explanation could simply be the concentration of agonist used as, when the concentration of collagen was lowered to 2.5 μg ml^{-1}, SK&F 95654 inhibited aggregation in rat PRP with an identical potency to that for inhibition of U46619-stimulated aggregation.

The addition of SK&F 95654 to a washed human platelet

suspension caused parallel increases in the cyclic AMP content and activity of cyclic AMP-dependent protein kinase over the concentration range 0.1–100 μM. These results, coupled with those of the enantiomers on platelet aggregation, indicate cGI-PDE inhibition as the mechanism, at least in part, by which the physiological effects of SK&F 95654 occur. There is an obvious discrepancy between the concentrations of SK&F 95654 required to increase cyclic AMP levels and cyclic AMP-PrK activity and those which inhibit platelet aggregation. A similar observation has been made with the imidazoquinolone cGI-PDE inhibitor, BMY-20844 (Seiler *et al.*, 1991) and with the adenylate cyclase stimulators, iloprost and octimabate (Merritt *et al.*, 1991). As discussed above, this discrepancy may, to some extent, be due to the conditions under which platelet aggregation is measured. By using an 'appropriate' agonist and by varying its concentration and the platelet count, it is possible that conditions will be found where the inhibition of aggregation and increase in cyclic AMP content occur over the same concentration-range. However, the small rises in cyclic AMP caused by cGI-PDE are suggestive of a pool or compartment of cyclic AMP within the platelet (Simpson *et al.*, 1988; Seiler *et al.*, 1987; 1991). When compared to receptor agonists, the compartment of cyclic AMP raised by cGI-PDE inhibition appears to be particularly effective in activating cyclic AMP-PrK. For example when 10 nM iloprost is added to human platelets, a cyclic AMP-dependent protein kinase activity ratio of 0.74 is observed and this is accompanied by a 20 fold increase in the cyclic AMP content (Merritt *et al.*,1990). However, 30 μM SK&F 95654 causes a similar increase in activity ratio with a less than three fold increase in cyclic AMP levels.

As well as inhibiting platelet aggregation *in vitro*, SK&F 95654 inhibited aggregation measured *ex vivo* when administered i.v. to the conscious rat. Aggregation was attenuated by 1 mg kg^{-1} SK&F 95654 for 2–4 h, depending on the concentration of collagen used to induce aggregation. The duration and potency of the effects of SK&F 95654 on platelet aggregation are similar to those on cardiac contraction. Therefore, it can be expected that anti-platelet action will be obtained at doses of SK&F 95654 that produce positive inotropic effects and this could well be of clinical benefit. Although SK&F 95654 and siguazodan have similar potencies with respect to inhibition of cGI-PDE and cardiovascular effects, SK&F 95654 is a more effective inhibitor of platelet aggregation in the rat (Figure 7). In this respect, it is worthy of note that the cGI-PDE inhibitor BMY-43351 has antithrombotic activity in anaesthetized dogs at doses that do not produce cardiovascular effects (Fleming *et al.*, 1991).

Intravenous administration of SK&F 95654 to anaesthetized guinea-pigs diminished bronchoconstriction caused by histamine or U46619. As with platelet aggregation, SK&F 95654 was more potent when U46619 was the agonist. The doses of histamine and U46619 used caused very similar levels of bronchoconstriction suggesting that the different efficacy of SK&F 95654 against these two agonists is not due either to relaxing from different absolute contractions. In this case, it may well be that the increased potency of SK&F 95654 against U46619-induced bronchoconstriction is due to it being a more effective functional antagonist of the particular biochemical changes invoked by this agonist although more detailed experiments are required to establish this. In this series of experiments it was noted that the effects on MABP were more pronounced and longer lasting than those on bronchodilatation and a non-uniform tissue distribution of the compound could explain these results, especially as SK&F 95654 was a potent relaxant of spontaneous tone in an isolated tracheal preparation. The apparently weaker bronchodilator effects observed *in vivo* could also be due to the fact that agonist induced tone was measured in this preparation.

Significant inotropic effects were observed in the dog at doses of SK&F 95654 of 10 μg kg^{-1} and inhibition of platelet aggregation in the rat was found at doses of 200 μg kg^{-1}, whereas 3 mg kg^{-1} SK&F 95654 was required to attenuate bronchoconstriction in the guinea-pig. It is possible that this apparent selectivity of SK&F 95654 for effects on heart and platelets over those on bronchodilatation is due to species variation. Although measurement of these effects in the same species is obviously required to address this, the magnitude of the different doses required for the cardiac and respiratory effects indicate that SK&F 95654 may show genuine tissue selectivity in its actions. In the anaesthetized dog, the cGI-PDE inhibitors CI-930 and imazodan have been reported to show no selectivity or bronchial selectivity, respectively. In these experiments preconstriction with 5-hydroxytryptamine was used to measure bronchodilatation and the dogs were treated with a β-adrenoceptor antagonist (Heaslip *et al.*, 1991). In all cases, methodology, species and the choice and concentration of the agonist could affect the apparent potencies and selectivities observed. As has already been noted above for BMY-43351, it would appear that individual cGI-PDE inhibitors have the potential to show tissue and/or specifies selectivity; therefore, it is inappropriate to extrapolate clinical benefit from the results of animal experiments.

In conclusion, the results obtained with SK&F 95654 show it to be a potent, selective inhibitor of cGI-PDE. The sustained and potent beneficial cardiovascular and platelet effects coupled with oral availability, low plasma binding and aqueous solubility indicate that SK&F 95654 could be a potential agent for the treatment of congestive heart failure. However, the recent clinical data with other cGI-PDE inhibitors coupled with observed differences between cGI-PDE inhibitors means that the therapeutic benefit of SK&F 95654 can only be determined in clinical trials specifically designed to address this point. The observations that the biological activity of SK&F 95654 is largely due to the **R**-enantiomer suggests that this, and not the racemate, may be the most appropriate compound to develop although, again, specific experimentation is required to establish this.

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