The peptide endothelin receptor antagonist, TAK-044, produces sustained inhibition of endothelin-1 mediated arteriolar vasoconstriction

C. J. Ferro, W. G. Haynes, N. R. Johnston, C. C. Lomax, D. E. Newby & D. J. Webb

Clinical Pharmacology Unit and Research Centre, University of Edinburgh, Western General Hospital, Edinburgh EH4 2XU

Aims Endothelin-1 (ET-1) has been implicated in the pathophysiology of a number of cardiovascular diseases for which endothelin receptor antagonists are currently under clinical development. We have previously reported that systemic administration of the combined endothelin A/B receptor antagonist, TAK-044, abolishes the forearm vasoconstriction caused by intrabrachial ET-1 infusion for at least 3 h. In this study we investigated whether TAK-044 can inhibit ET-1 mediated forearm vasoconstriction for longer periods.

Methods Eighteen subjects were recruited to a randomized, placebo-controlled, single-blind, three-way, crossover study. Subjects were divided into three groups of six. Groups received 25 mg, 50 mg or 100 mg TAK-044 on two separate occasions, 6 and 10 h before the start of a 2 h intrabrachial infusion of ET-1 (5 pmol min^{-1}). On a third occasion subjects received only placebo before intra-arterial ET-1 infusion. Forearm vasoconstriction to ET-1 was measured by venous occlusion plethysmography.

Results In the placebo phase, ET-1 caused significant, slowly-progressive local forearm vasoconstriction of ~30% ($P<0.01$) in all three groups. All three doses of TAK-044, administered at both timepoints, tended to blunt the vasoconstriction caused by ET-1. When the responses from all three groups were combined, TAK-044 significantly reduced ET-1 mediated vasoconstriction compared with placebo −9% (95% CI −15 to −3; $P=0.01$) at 8 h and by −9% (95% CI −17 to −2; $P=0.01$) 12 h after dosing.

Conclusions TAK-044 attenuated, but did not abolish, local ET-1 mediated vasoconstriction, for up to 12 h after administration. Vasoconstriction to local intra-arterial administration of ET-1 appears to represent a safe and reproducible pharmacodynamic index of systemic endothelin receptor antagonism in humans.

Keywords: endothelin, endothelin antagonists, pharmacodynamics, forearm blood flow, humans

Introduction

The endothelium derived vasoconstrictor peptide endothelin-1 (ET-1) has been implicated in the pathophysiology of a number of conditions associated with sustained elevation of vascular tone, such as hypertension and congestive cardiac failure, as well as in vasospastic disorders, such as subarachnoid haemorrhage and acute ischaemic renal failure [1]. Two endothelin receptor subtypes, ET_A [2] and ET_B [3], both of which have been identified and cloned in man, can mediate vasoconstriction [4]. Antagonists at these receptors may, therefore, have therapeutic potential in these conditions.

We have previously shown that systemic intravenous administration of the cyclic hexapeptide combined $ET_{A/B}$ receptor antagonist, TAK-044 [5], at doses of 10 to 1000 mg, is well tolerated and produces dose-dependent, long-lasting vasodilatation resulting in decreases of blood pressure and systemic vascular resistance persisting, at the highest dose, for at least 24 h [6]. In a separate study, systemic administration of TAK-044, at doses of 30, 250 and 750 mg abolished vasoconstriction to locally infused ET-1, as a model of ET-1 induced 'vasospasm', for up to 3 h [6].

Endothelin receptor antagonists are currently under clinical development [1]. Peptide antagonists such as TAK-044 requiring intravenous administration are better suited for the treatment of acute vasospastic conditions in which endothelin has been implicated in the pathophysiology. These conditions include subarachnoid haemorrhage, acute ischaemic renal failure and myocardial infarction. In these clinical situations intermittent dosing might have both practical and economic advantages over continuous infusion. We, therefore, studied the potential of TAK-044 to inhibit the forearm vasoconstriction produced by brachial artery infusion of ET-1 for up to 12 h after dosing.

Methods

Subjects

Eighteen healthy male subjects between 19 and 41 years of age participated in these studies, which were conducted

Correspondence: Professor D. J. Webb, Clinical Pharmacology Unit and Research Centre, University of Edinburgh, Western General Hospital, Edinburgh EH4 2XU.

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with the approval of the Lothian Research Ethics Committee and with the written informed consent of each subject. All had normal baseline results on routine biochemical and haematological screening tests. None of the subjects received vasoactive or non-steroidal anti-inflammatory drugs in the 2 weeks before the study or during the study period. All of the subjects abstained from alcohol for 48 h, from caffeine containing drinks and cigarettes for at least 24 h, and from food for 12 h before the start of the ET-1 infusion. All studies were performed in a quiet room maintained at a constant temperature of 24–26° C.

Drugs

TAK-044, cyclo[D-α-aspartyl-3-[(4-phenylpiperazin-1-yl) carbonyl]L-alanyl-L-α-aspartyl-D-2-(2-thienyl)glycyl-L-leucyl-D-tryptophyl] disodium salt (Takeda Chemical Industries Ltd, Japan), is a potent hexapeptide endothelin receptor antagonist that inhibits the binding of ET-1 to endothelin receptors on rabbit ventricular (mainly ET_A) and cerebellar membrane fractions (mainly ET_B) with IC_{50} values of 3.8 nM and 130 nM respectively *in vitro* [5]. TAK-044 inhibits, with equal efficacy, both ET_A and ET_B mediated responses *in vivo* [5, 7]. The initial dose of TAK-044 studied, 25 mg, was chosen on the basis that 30 mg TAK-044 completely inhibited ET-1 mediated vasoconstriction for up to 3 h after administration [6]. Depending on whether or not 25 mg TAK-044 abolished the vasoconstriction to ET-1, it was intended to study either lower (10 and 5 mg) or higher (50 and 100 mg) doses of TAK-044 respectively. TAK-044 and 50 mg sucrose placebo were dissolved in physiological saline (0.9%; Baxter Healthcare Ltd, Thetford, UK) and administered as a 50 ml intravenous infusion over 15 min using two Welmed P1000 syringe pumps (Welmed Clinical Care Systems, Bramley, Hampshire, UK) via a right antecubital fossa vein cannulated with an 18G intravenous cannula (Venflon; Viggo-Spectramed, Helsinborg, Sweden). This cannula was not used for blood sampling.

The left brachial artery was cannulated under local anaesthesia (1% lignocaine; Astra Pharmaceuticals, Kings Langley, UK) with a 27 standard wire gauge steel needle (Cooper's Needle Works, Birmingham, UK) attached to a 16G epidural catheter (Portex Ltd, Hythe, Kent, UK). Patency was maintained by infusion of physiological saline via a Welmed P1000 syringe pump. Pharmaceutical grade ET-1 (Clinalfa, Nottingham, UK) dissolved in physiological saline was infused at a rate of 5 pmol min^{-1}. The rate of intra-arterial infusion was maintained constant throughout all studies at 1 ml min^{-1}.

Measurements

Side-effect assessments The following assessments were performed to detect potential adverse effects: 12-lead electrocardiographs, repeated questioning for symptoms, urinalysis, clinical chemistry screen (liver enzymes, electrolytes, creatinine, blood urea, protein), and haematology screen (full blood count, white blood cell differential count).

Systemic haemodynamics A well-validated semi-automated non-invasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan) was used to make duplicate measurements of blood pressure and heart rate in the non-infused arm, which were then averaged [8].

Forearm blood flow Blood flow was measured in both arms by venous occlusion plethysmography [9] using mercury-in-Silastic strain gauges that were securely applied to the widest part of each forearm. The hands were excluded from the circulation during each measurement period by inflation of a wrist cuff to 220 mmHg. Upper arm cuffs were intermittently inflated to 40 mmHg for 10 s in every 15 s to temporarily prevent venous outflow from the forearm and, thus, obtain plethysmographic recordings. Recordings of forearm blood flow were made every 10 min over 3 min periods. Voltage output from two single channel Hokanson EC 4 strain gauge plethysmographs (DE Hokanson Inc, Bellevue, WA) was transferred to a Macintosh personal computer (Performa 475, Apple Computer Inc, Cupertino, CA) using a MacLab analogue digital converter and Chart software (v. 3.3.3; both from AD Instruments, Castle Hill, NSW, Australia). Calibration was achieved using the internal standard of the Hokanson plethysmography units.

Plasma assays Venous blood samples were taken from a right antecubital fossa vein cannulated with an 18G intravenous cannula attached to a manometer connecting tube (Portex Ltd, Hythe, Kent, UK). All assays were performed as single batches. Plasma endothelin was measured by radioimmunoassay (New England Nuclear Endothelin 1,2 kit) as previously described [10]. The sensitivity of this assay is 2.2 pg ml^{-1} immunoreactive ET. Cross-reactivity of this assay with ET-1, ET-2, ET-3 and big ET-1 is 100%, 53%, 4% and 70% respectively. The normal range for this assay is 12–28 pg ml^{-1}.

TAK-044 was extracted from buffered (Merck 9437; pH 5) plasma by methanol/buffer-preconditioned Bakerbond SPE cartridges and was measured by high performance liquid chromatography (h.p.l.c.). Eluate was evaporated to dryness under nitrogen at 40° C, and the residue reconstituted in 100 μl water. Chromatographic separation was achieved using two Merck LiChrospher columns with Hewlett Packard 1090 h.p.l.c. pumps. The first mobile phase comprised 40% acetonitrile and 60% 6 mmol l^{-1} KH_2PO_4/3 mmol l^{-1} tetrabutylammonium bromide. The second mobile phase comprised 52% acetonitrile/0.5% acetic acid/47.5% water. Detection was achieved by fluorimetry (excitation, 286 nm; emission 348 nm) with Jasco 821 fluorescence detectors. The limit of quantification of this assay, defined as the lowest quantifiable amount of compound at which the loss of precision was $\leq 15\%$ and the accuracy was $\pm 15\%$, was determined to be 2.1 ng ml^{-1}.

Study design

Eighteen subjects took part in a randomized, placebo-controlled, single-blind, three-way, crossover study. Subjects were studied on three occasions, each 1 week or more apart. They were admitted to the clinical research centre at 20.00 h on the day before the study and were discharged at

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13.00 h on the study day. On two occasions subjects received TAK-044 either at 24.00 h or 04.00 h. Sucrose placebo was substituted for TAK-044 at the other timepoint. On a third occasion subjects received only sucrose placebo at both timepoints. The first group of six subjects (group 1) was studied using 25 mg TAK-044. Because this dose did not appear to abolish ET-1 mediated vasoconstriction, groups 2 and 3 were studied using 50 and 100 mg TAK-044 respectively as previously determined. ET-1 was infused intra-arterially starting at 10.00 h and ending at 12.00 h, the end of the infusion corresponding to either 8 or 12 h after administration of TAK-044. Venous blood samples (10 ml) were obtained at 10.00 h and 12.00 h for assay of plasma endothelin and venous samples (5 ml) were obtained at 08.00 h, 10.00 h and 12.00 h for assay of serum TAK-044. Blood and urine samples were collected for safety assessments before the start of the study and at the end of the third study phase. Electrocardiographs were recorded before dosing, at 09.30 h and before discharge on every study day.

Data presentation and statistical analysis

From previous studies using intrabrachial ET-1 infusions done in our unit (data on file), studying six subjects at each dose would have 95% power to detect a 50% reduction in the vasoconstriction produced by ET-1 at the 5% level. However, studying six subjects would lack the power to detect smaller differences in ET-1 vasoconstriction. Therefore, the *a priori* decision to pool the data from all 18 subjects was taken. Combining the responses from all 18 subjects, would give 90% power of detecting a 25% decrease at the 5% level. Power calculations were done using Graphpad Instat software (GraphPad Software, San Diego, CA, USA).

Plethysmographic data were extracted from the Chart data files and forearm blood flows were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel 5.0; Microsoft). Recordings from the first 60 s after wrist cuff inflation were not used because of the reflex vasoconstriction this causes [11]. Usually, the last five flow recordings in each 3 min measurement period were calculated and averaged for each arm. To reduce the variability of blood flow data, the ratio of flows in the two arms was calculated for each time point: in effect using the non-infused arm as a contemporaneous control for the infused arm [9]. The analysis of all of the forearm blood flows was performed by one of the investigators (CJF) who was blinded to the study phases.

Data are described as mean $\pm$ s.e. mean, with 95% confidence intervals where appropriate. Mean arterial pressure was calculated as diastolic pressure plus one third of the pulse pressure. Haemodynamic data are presented as absolute values and as placebo-corrected changes from baseline (24.00 h). Data were examined by repeated measures analysis of variance (ANOVA) using Stat View 512+ software (Brainpower Inc, Calabasas, CA, USA) for the Apple Macintosh computer. Where the F value obtained by ANOVA was significant ($P<0.05$), the Fisher PLSD (protected least significant difference) multiple comparison test was used to compare pairs of mean values.

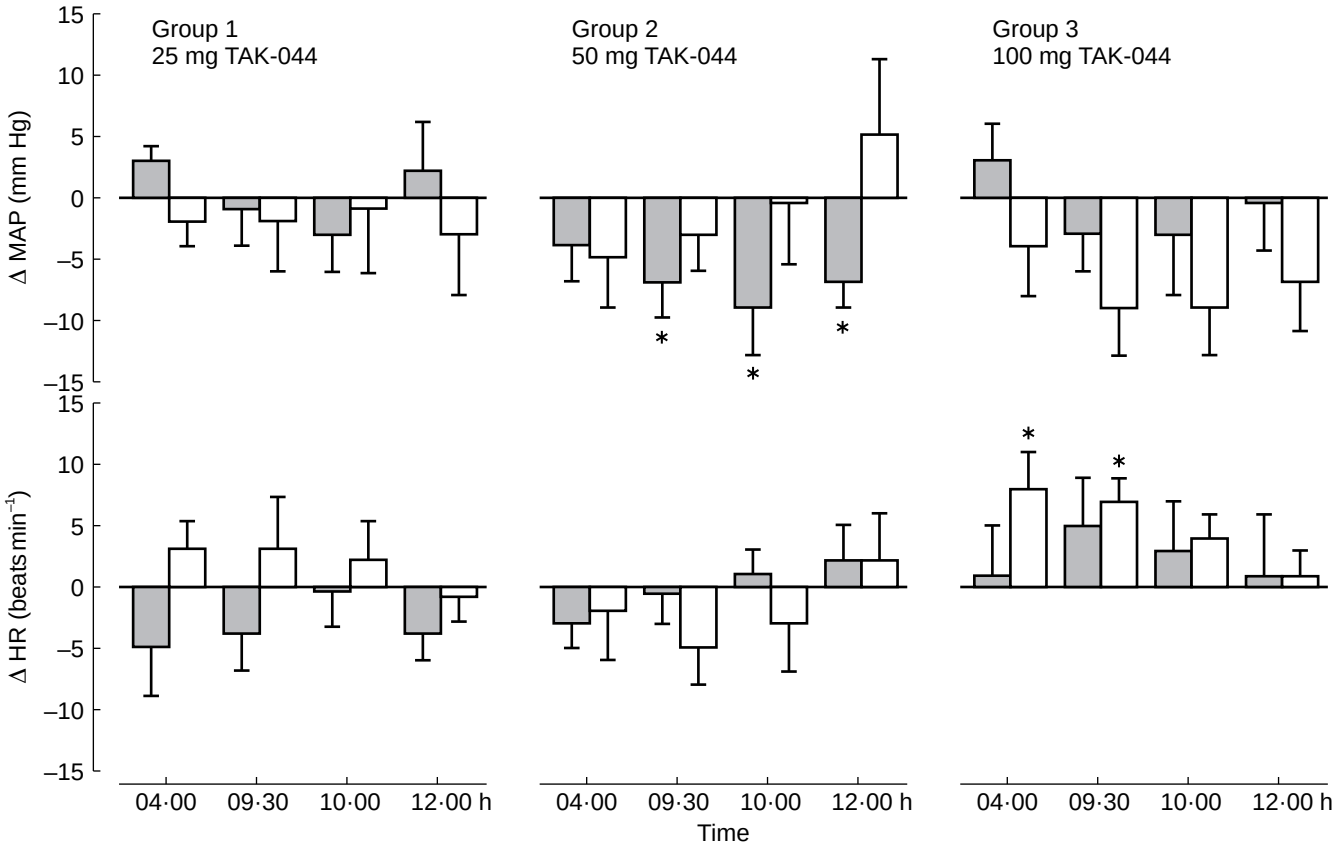

Figure 1 Placebo corrected changes (Δ) in mean arterial pressure (MAP) and heart rate (HR) from baseline (24.00 h) following intravenous administration of TAK-044 at 24.00 h (open bars) and 04.00 h (shaded bars) for group 1, 2 and 3. *$P \leq 0.05$ from baseline.

Results

TAK-044 was well tolerated, with no difference between placebo and TAK-044 phases in the prevalence of minor symptoms. There were no serious adverse events during the study, and no clinically significant abnormalities were detected on safety monitoring (urinalysis, haematology, clinical chemistry and electrocardiograph).

Mean arterial pressure showed a tendency to decrease after infusion of TAK-044 (Figure 1). However, this decrease was only significant in group 2 after 50 mg TAK-044 (maximum decrease: -10 ± 4 mm Hg; $P=0.005$). Similar trends were noted for systolic and diastolic pressures. Heart rate (Figure 1) increased only after dosing with 100 mg TAK-044 at 24.00 h (maximum increase: 8 ± 3 beats min^{-1}; $P=0.03$). There were no differences between phases in any of the haemodynamic parameters measured at the start of the intra-brachial ET-1 infusion at 10.00 h (Table 1). Mean arterial pressure and heart rate did not change during infusion of ET-1 (Figure 1). Administration of TAK-044 did not cause an increase in immunoreactive endothelin concentrations after 8 or 12 h compared with placebo (Table 2).

In group 1, TAK-044 plasma concentrations were not higher than the limit of quantification of the assay (2.1 ng ml^{-1}) at any time in any of the subjects. In group 2, TAK-044 was detectable in >50% of subjects only at 08.00 h and only after administration of TAK-044 at 04.00 h.

Table 1 Haemodynamics in groups 1, 2 and 3 before intra-arterial ET-1 infusion.

	Systolic blood pressure (mm Hg)	*Diastolic blood pressure (mm Hg)*	*Heart rate (beats min^{-1})*	*Forearm blood flow (ml min^{-1} dl^{-1})*
Group 1 (TAK-044 25 mg)				
Placebo	109 ± 6 (95 to 123)	62 ± 4 (52 to 72)	51 ± 2 (46 to 55)	2.8 ± 0.3 (2.0 to 3.6)
TAK-044 at 24.00 h	118 ± 6 (103 to 132)	58 ± 3 (51 to 66)	52 ± 1 (49 to 55)	2.8 ± 0.3 (2.0 to 3.5)
TAK-044 at 04.00 h	113 ± 2 (107 to 119)	58 ± 3 (49 to 66)	57 ± 3 (50 to 64)	4.0 ± 0.5 (2.7 to 5.3)
Group 2 (TAK-044 50 mg)				
Placebo	121 ± 6 (104 to 137)	64 ± 3 (55 to 72)	53 ± 4 (44 to 62)	3.5 ± 0.3 (2.7 to 4.3)
TAK-044 at 24.00 h	124 ± 7 (107 to 142)	63 ± 3 (55 to 71)	56 ± 1 (52 to 60)	3.4 ± 0.4 (2.3 to 4.5)
TAK-044 at 04.00 h	115 ± 4 (105 to 125)	57 ± 3 (51 to 63)	56 ± 3 (48 to 64)	4.0 ± 0.4 (3.0 to 5.0)
Group 3 (TAK-044 100 mg)				
Placebo	109 ± 6 (115 to 137)	68 ± 3 (60 to 75)	52 ± 2 (46 to 57)	3.4 ± 0.7 (1.6 to 5.1)
TAK-044 at 24.00 h	118 ± 6 (113 to 133)	59 ± 4 (49 to 69)	54 ± 2 (48 to 61)	3.7 ± 0.7 (2.0 to 5.4)
TAK-044 at 04.00 h	113 ± 3 (115 to 138)	62 ± 2 (56 to 67)	56 ± 3 (48 to 63)	4.0 ± 0.9 (1.7 to 6.3)

There were no significant differences between baseline values on the different study days for any of the three groups. 95% confidence intervals are shown in brackets.

Table 2 Plasma TAK-044 and plasma endothelin concentrations in groups 1, 2 and 3.

	Plasma TAK-044 concentration (ng ml^{-1})			*Plasma ET concentration (pg ml^{-1})*	
	08.00 h	*10.00 h*	*12.00 h*	*10.00 h*	*12.00 h*
Group 1 (TAK-044 25 mg)					
Placebo	<2.1	<2.1	<2.1	12.3 ± 1.1 (9.5 to 15.0)	12.9 ± 0.6 (11.3 to 14.5)
TAK-044 at 24.00 h	<2.1	<2.1	<2.1	16.1 ± 1.3 (12.9 to 19.4)	13.6 ± 0.7 (11.7 to 15.5)
TAK-044 at 04.00 h	<2.1	<2.1	<2.1	13.4 ± 1.0 (10.8 to 16.0)	14.1 ± 0.7 (12.4 to 15.8)
Group 2 (TAK-044 50 mg)					
Placebo	<2.1	<2.1	<2.1	11.6 ± 0.8 (9.5 to 13.7)	12.8 ± 0.5 (11.6 to 13.9)
TAK-044 at 24.00 h	<2.1	<2.1	<2.1	11.7 ± 0.5 (10.3 to 13.1)	13.1 ± 1.0 (10.5 to 15.6)
TAK-044 at 04.00 h	3.9 ± 0.9	<2.1	<2.1	10.9 ± 0.3 (10.3 to 11.6)	13.2 ± 1.1 (10.4 to 16.0)
Group 3 (TAK-044 100 mg)					
Placebo	<2.1	<2.1	<2.1	11.4 ± 0.8 (9.4 to 13.4)	11.6 ± 0.4 (10.7 to 12.6)
TAK-044 at 24.00 h	3.7 ± 1.3	4.5 ± 1.6	<2.1	11.7 ± 0.5 (10.4 to 13.0)	11.9 ± 0.6 (10.4 to 13.5)
TAK-044 at 04.00 h	8.9 ± 2.5	<2.1	<2.1	13.2 ± 0.5 (12.0 to 14.4)	13.7 ± 0.7 (11.9 to 15.4)

Descriptive statistics for plasma TAK-044 concentrations were calculated only for the timepoints where >50% of the subjects showed concentrations >2.1 ng ml^{-1}, the limit of quantification of the assay. There were no significant differences between plasma endothelin concentrations in any of the groups.

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In group 3, TAK-044 was detectable at 08.00 h after administration of TAK-044 at 24.00 h and 04.00 . TAK-044 was also detectable in plasma at 10.00 h after administration of TAK-044 at 24.00 h (Table 2). The inter-subject variation for this assay was high at $>50\%$.

There were no differences between any of the phases in absolute forearm blood flows at the start of the intra-brachial ET-1 infusions (Table 1). Blood flow in the non-infused arm did not change significantly during infusion of ET-1 in any of the phases. Brachial artery infusion of ET-1 caused significant slowly-progressive local forearm vasoconstriction. Infusion of ET-1 reduced forearm blood flow over 120 min by $30\pm5\%$ ($P=0.0001$ *vs* basal), $30\pm4\%$ ($P=0.0001$) and $28\pm5\%$ ($P=0.013$) after placebo groups 1, 2 and 3 respectively (Figure 2, Table 3). TAK-044 at all three doses and administered at both timepoints tended to blunt the vasoconstriction caused by ET-1 but these effects failed to achieve statistical significance (Figure 2, Table 3). However, when the responses from all three groups were combined, vasoconstriction to ET-1 was reduced by TAK-044 administered 8 and 12 h previously (Figure 3, Table 3), when compared with placebo.

Discussion

The combined endothelin $ET_{A/B}$ receptor antagonist, TAK-044, given as a 15 min intravenous infusion, attenuated peripheral vasoconstriction to exogenous ET-1 by $\sim30\%$ for up to 12 h after administration. This inhibition occurred at a time when plasma concentrations of TAK-044 were below the limit of quantification of the assay and plasma concentrations of endothelin were not elevated. These findings have important implications for the clinical development of endothelin receptor antagonists.

A simple and reliable pharmacodynamic index of endothelin receptor blockade would be useful for the clinical development of endothelin receptor antagonists. Forearm vasoconstriction to intra-brachial administration of ET-1 is highly reproducible [4, 6, 12] and this model may be safer than using systemic intravenous infusions of ET-1 to increase blood pressure, given the sustained and potent nature of vasocontriction to ET-1, especially in the coronary, renal and cerebral circulations [1, 13, 14]. It is possible that endothelin receptor antagonism may produce different effects in other blood vessels. However, responses in forearm resistance vessels are generally thought to be broadly representative of those in other vascular beds [9, 15].

The vasoconstriction produced by intra-arterial ET-1 in this study was consistent with other published reports [4, 6, 12]. The results demonstrate that bolus doses of TAK-044 up to 100 mg can still inhibit ET-1 mediated forearm vasoconstriction by $\sim30\%$ for up to 12 h after administration. However, this contrasts with complete inhibition for up to 3 h of ET-1 mediated vasoconstriction by TAK-044 30 mg [6] and suggests a marked time dependence for this inhibitory action. This was a small study with insufficient power to exclude a dose dependent effect and, therefore, studies with larger doses would be needed to show whether greater inhibition of ET-1 induced vasoconstriction could be achieved. However, the finding that 25 mg TAK-044 seemed to be as effective as 50 and 100 mg

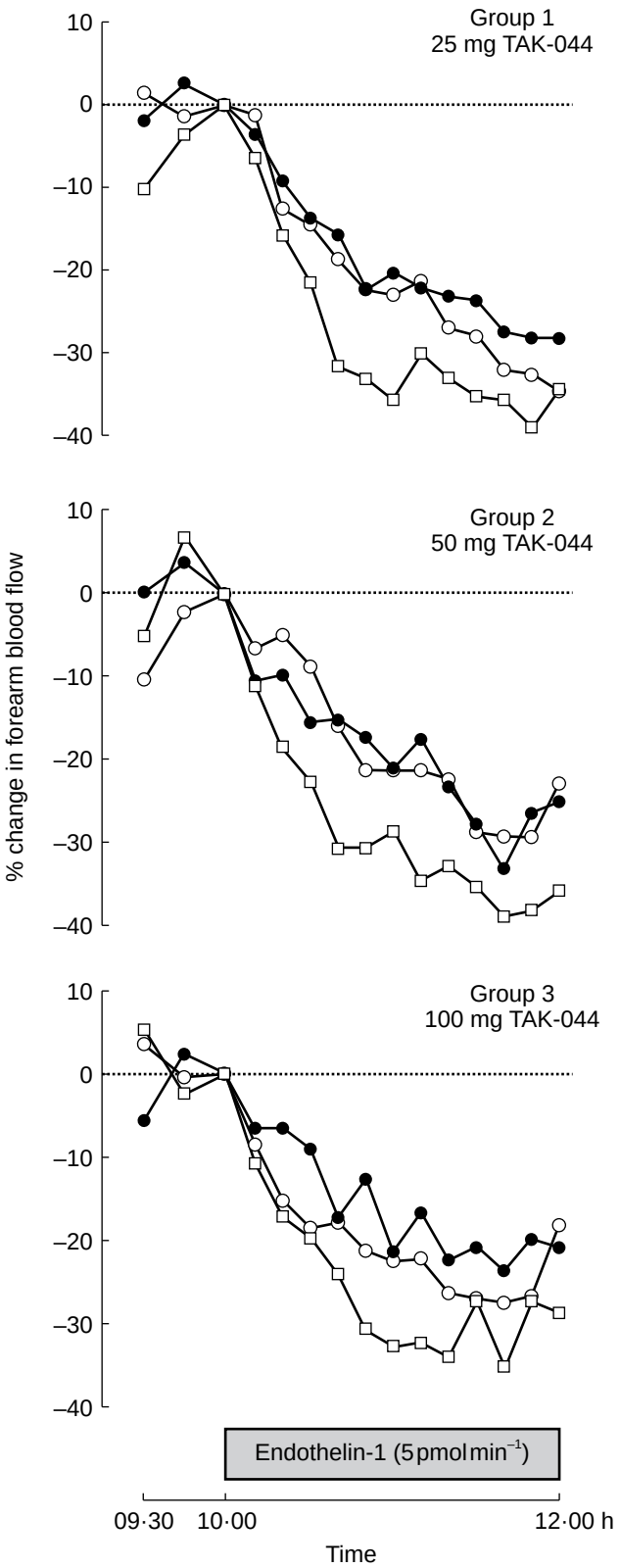

Figure 2 Percentage change in forearm blood flow produced by brachial artery infusion of endothelin-1 (5 pmol min^{-1} for 2 h) following intravenous administration of TAK-044 at 24.00 h (open circles) and at 04.00 h (closed circles) and during the placebo phase (open squares) for groups 1, 2 and 3. Standard errors have been omitted for sake of clarity.

TAK-044 suggests that 25 mg may achieve maximum inhibition at this late stage after systemic administration and that increasing the dose of TAK-044 further might not produce greater inhibition of ET-1 mediated vasoconstric-

Table 3 Mean percentage vasoconstriction to 120 min intra-arterial ET-1 infusion after placebo and TAK-044 8 and 12 h earlier.

	Placebo	*TAK-044 at 24.00 h*	*Difference from placebo*	*TAK-044 at 04.00 h*	*Difference from placebo*
Group 1 ($n=6$) (TAK-044 25 mg)	-30 ± 5	-20 ± 4	-10 (-20 to $+13$)	-22 ± 3	-8 (-22 to $+5$)
Group 2 ($n=6$) (TAK-044 50 mg)	-30 ± 4	-20 ± 3	-11 (-26 to $+5$)	-20 ± 7	-10 (-30 to $+10$)
Group 3 ($n=6$) (TAK-044 100 mg)	-28 ± 4	-21 ± 4	-7 (-22 to $+7$)	-19 ± 3	-9 (-26 to $+6$)
All ($n=18$)	-29 ± 2	-20 ± 2	-9 (-17 to -2)*	-20 ± 3	-9 (-15 to -3)*

*$P=0.01$. 95% confidence intervals for differences for placebo are shown in brackets.

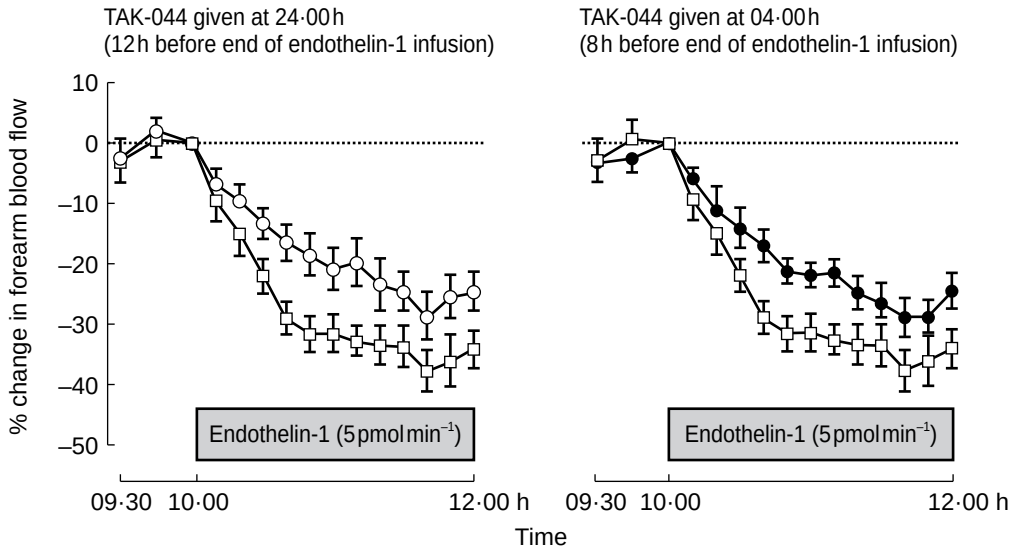

Figure 3 Percentage changes in forearm blood flow for all three groups of subjects combined, following brachial artery infusion of endothelin-1 (5 pmol min^{-1} for 2 h from 10.00 h to 12.00 h) following intravenous administration of placebo (open squares) and TAK-044 at 04.00 h (closed circles) and at 24.00 h (open circles). Endothelin-1 caused a slowly progressive forearm vasoconstriction during the placebo phase. TAK-044 (25–100 mg) administered at either 04.00 h and 24.00 h (corresponding to 8 and 12 h before the end of the endothelin-1 infusion) significantly inhibited this vasoconstriction ($P=0.01$).

tion. Therefore, if an inhibition $>30\%$ is required, more frequent dosing (3 to 6 hourly) or a continuous infusion, may be needed. Clinical trials with endothelin receptor antagonists, including TAK-044, are currently in progress. These should indicate the doses required for clinical effect and the forearm model can then be used to determine effective doses of other endothelin receptor antagonists. The effects of repeated dosing with TAK-044 on ET-1 mediated vasoconstriction are not yet known and it is possible that a cumulative inhibition might be achieved in this manner if the clearance mechanisms for TAK-044 were to become saturated. These issues remain to be addressed.

Although increases in plasma endothelin concentrations have been shown to correlate with some of the haemodynamic changes observed, these associations were relatively weak, with correlation coefficients of ~ 0.2 [6]. Furthermore, changes in circulating endothelin concentrations are likely to reflect only antagonism of the ET_B receptor [6, 16] which, in addition to its functional roles, appears to mediate clearance of circulating ET-1 [17, 18]. For a drug with ET_A and ET_B receptor blocking activity, such as TAK-044, effects on systemic haemodynamics may be apparent at concentrations that do not substantially increase circulating endothelin concentrations, as was the case in our previous study [6]. Similarly, here we found that plasma endothelin concentrations were not raised 12 h after administration of TAK-044 despite continuing inhibition of ET-1 mediated vasoconstriction.

TAK-044 was not detected in any of the volunteers 12 h after administration of any of the doses. These findings are in close agreement with our previously reported study in which the plasma half-life of TAK-044 was 30 to 60 min [6]. However, the IC_{50} for TAK-044 at ET_A receptors is 0.08 ng ml^{-1} [6] ~ 25 fold below the limit of quantification of the assay (2.1 ng ml^{-1}). It is, therefore, possible that circulating TAK-044 remains present in plasma at concentrations sufficient to inhibit the vasoconstriction produced by exogenous ET-1 at 12 h. It is also conceivable that TAK-044 binds tightly to endothelin receptors and remains bound for several hours in a similar manner to ET-1 [19]. Indeed, in intact cells ET-1 appears only to become dissociated from its receptors following receptor internalisation [20]. Thus, prolonged receptor binding may explain the sustained inhibition of ET-1 mediated vasoconstriction by TAK-044. Another possible explanation for the observed inhibition could be the entry of TAK-044 into another

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tissue compartment, probably within the vasculature. A similar situation arises with inhibitors of the renin-angiotensin system, where entry into and actions in other tissue compartments appear to explain the dissociation between actions and plasma concentrations observed [21, 22]. These possibilities require further investigation.

Our previous study showed that TAK-044 lowers systemic vascular resistance and blood pressure [6]. The current study was not designed primarily to assess these measures and factors such as diurnal variation of blood pressure, disturbed sleep and the measurement of forearm blood flow may all have interfered with their optimal assessment. Thus, although the study was placebo controlled, small changes in blood pressure and heart rate may have been obscured by these factors. Nevertheless, systolic, diastolic and mean arterial pressure tended to decrease after administration of TAK-044, to a similar extent to that reported previously [6], and heart rate was increased after administration of the highest dose of TAK-044. However, in this study we did not measure systemic vascular resistance, the most sensitive index of peripheral vasodilatation in our previous study [6].

In conclusion, the cyclic hexapeptide, combined $ET_{A/B}$ receptor antagonist, TAK-044, inhibited local ET-1 mediated vasoconstriction by $\sim$30% for up to 12 h after administration. This inhibition occurred at a time when plasma concentrations of TAK-044 were below the limit of quantification of the assay and plasma concentrations of endothelin were not elevated. Therefore, in this study, the most sensitive index of effect of endothelin receptor antagonism was inhibition of ET-1 mediated vasoconstriction. Although TAK-044 is a peptide, these features may be common to non-peptide endothelin receptor antagonists and this study sets up a marker against which other endothelin receptor antagonists can now be compared. The long lasting effects of the short lived peptide, TAK-044, are generally encouraging for the clinical development of endothelin receptor antagonists and emphasise the valuable contribution that the combination of local intra-arterial administration of ET-1 and forearm plethysmography can make to the early clinical evaluation of this novel class of vasoactive drugs.

This work was supported by a grant from Takeda Euro R&D Centre. CJF is supported by a grant from the British Heart Foundation (PG/94183). WGH is the recipient of a Wellcome Trust Advanced Training Fellowship (042145/114). DEN is the recipient of a British Heart Foundation Junior Fellowship (FS/95009). We wish to thank E. Stanley and Dr N. Lannigan of the Pharmacy Department at the Western General Hospital for preparing ampoules of ET-1 for parenteral use.

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(*Received 13 January 1997, accepted 10 June 1997*)