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# UK-69,578, A NOVEL INHIBITOR OF EC 3.4.24.11 WHICH INCREASES ENDOGENOUS ANF LEVELS AND IS NATRIURETIC AND DIURETIC

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SUMMARY: A search for potent inhibitors of EC 3.4.24.11, an enzyme which is found most abundantly in the kidney and which degrades atrial natriuretic factor, has led to the identification of UK-69,578. Structure-activity studies starting from substituted N-carboxymethyl dipeptide inhibitors resulted in the introduction of a cyclo-alkane $P_1'$ residue and in the replacement of the aza-link between $P_1$ and $P_1'$ residues by a methylene group, with a net ten-fold potency gain. UK-69,578 increases endogenous ANF levels and produces natriuretic and diuretic responses intravenously in mice. 

INTRODUCTION: Atrial Natriuretic Factor is a cardiac hormone which is stored in atrial secretory granules. There is growing evidence that it may be involved in the regulation of electrolyte and fluid balance, since it is released into the circulation by atrial distension following plasma volume expansion (1). The main component of human ANF is a 28-residue peptide (2) infusion of which evokes diuretic, natriuretic and hypotensive responses, in addition to suppressing renin and aldosterone levels (3). This profile suggests that the peptide may be of use in the treatment of cardiovascular disorders such as hypertension and congestive heart failure. The therapeutic potential of ANF itself is, however, limited by its peptidic nature and rapid elimination (4). Thus, we considered the alternative approach of potentiating the activity of endogenous hormone by preventing its in vivo degradation. Following the structural elucidation of ANF we therefore began a search for the peptidases responsible for its inactivation. Focusing on the kidney as a major site of ANF action and metabolism (5,6), we found that synthetic ANF was rapidly degraded by a purified preparation (7) of EC 3.4.24.11 (neutral endopeptidase 24.11), a zinc dependent peptidase prominent in the brush border of the proximal convoluted tubule in the kidney. Similar observations have been reported by others (8). Consequently, we set out to find a potent inhibitor of this

ABBREVIATION: ANF, Atrial Natriuretic Factor.

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enzyme suitable for in vivo evaluation in order to establish the therapeutic potential of such an agent.

At the outset of our synthetic programme the close similarity between kidney and brain neutral endopeptidase 24.11, then commonly referred to as enkephalinase, had been established (9). It was appreciated that its proteolytic mechanism of action was probably similar to that of other zinc dependant peptidases such as angiotensin converting enzyme, carboxypeptidase-A and thermolysin. A closer similarity in active site topography to thermolysin was also apparent, as phosphoramidon inhibited both endopeptidases.

By studying dipeptide product-inhibitors of brain EC 3.4.24.11, Roques (10,11) demonstrated the importance of a $P_1'$ hydrophobic residue, preferably containing an aromatic system, in combination with a small $P_2'$ residue, and by analogy with captopril, synthesised thiorphan. Following the design principle which led to enalapril, Mumford et al (12) then replaced the thiol function by a carboxymethylamino system, and furthermore showed that the $P_2'$ residue could be extended, GABA being slightly superior to glycine. A similar approach was taken by Chipkin et al (13) and Almenoff and Orlowski (7), the latter workers incorporating a $P_2'$ 4-aminobenzoic acid residue. These initial studies resulted in relatively lipophilic compounds aimed at modulating brain enkephalin levels and hence analgesic activity (10, 13).

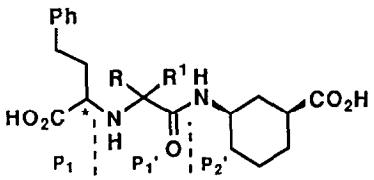
## MATERIALS AND METHODS

Synthesis of Inhibitors: Synthetic ANF refers to rat ANF (5-28) (atriopeptin III, Cambridge Research Biochemicals, U.K.). Compound **2** was prepared as follows: reaction of α-trifluoromethylsulphonyloxybenzene-butanoic acid benzyl ester with 1-aminocyclopentane carboxylic acid trimethylsilyl ester (prepared in situ by refluxing a suspension of the amino acid with trimethylsilyl diethylamine in carbon tetrachloride and evaporation) in the presence of triethylamine gave 1-[N-(1-benzyloxy-carbonyl-3-phenyl propyl)amino] cyclopentane carboxylic acid m.p. 164-6° in 72% yield. Coupling with cis-3-aminocyclohexane carboxylic acid benzyl ester under standard 1-hydroxybenzotriazole-diimide conditions followed by hydrogenolysis of the benzyl ester groups gave the required compound **2** as a pair of diastereoisomers. Compound **1** was similarly prepared starting from (S)-phenylalanine. Glutaramide analogues were prepared as described in European patent application 0 274 234 and British Patent application 88 11 873.

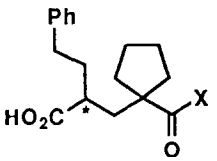
Measurement of EC 3.4.24.11. inhibition: Rat kidney enzyme was isolated as a brush border membrane fraction according to the method of Booth and Kenny (14), and activity was determined using [glycine 1-$^{14}$C] hippuryl phenylalanyl arginine as substrate. Inhibitory constants (Ki) were estimated by the method of Cheng and Prusoff (15) using a Km value of 3 mM for hippuryl-phenylalanyl arginine.

Measurement of urine output in conscious mice: Male CD-1 mice (Charles River, Manston, Kent), 22-25 g body weight were used throughout this work. Two days prior to study the mice were removed from their home cages and placed in metabowls in groups of ten. The animals were given free access to food (Diet 41B, Lab Sure, Cambridge) and water and kept under constant lighting conditions (12 hr light/12 hr dark). Following 24 hr acclimatisation the metabowls were cleaned and food withdrawn. After a further 18 hours either 600 µl 0.9% (w/v) saline or inhibitor dissolved in 600 µl of saline were administered by bolus injection via the tail vein. The mice were then returned to the metabowls and urine collected for 1 hr. Volumes were recorded and samples analysed for sodium using an ion selective electrode (Nova).

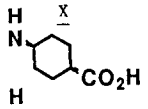
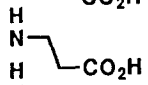
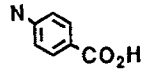
Measurement of plasma ANF levels in anaesthetised mice: Male mice, (CD-1 strain, Charles River) 25g body weight were anaesthetised with sodium pentobarbitone (Sagatal, 60 mg/kg i.p.). Groups of 10 mice were given either no treatment, 600 µl of saline, or UK-69,578 (3 mg/kg) dissolved in 600 µl of saline via the tail vein. At various times post treatment a mid line incision was made and 1 ml blood withdrawn from the vena cava into a syringe containing EDTA (1 mg/ml) and protease inhibitors soybean trypsin inhibitor (5 µg/ml), aprotinin (1.11 TIU/ml), pepstatin A (1 µg/ml) and phenylmethylsulphonyl fluoride (17.42 µg/ml). Blood from the 10 mice was pooled, centrifuged (1700 g, 4°C, 10 min), and the plasma removed and stored frozen until assayed for ANF content. This procedure was repeated three times at each time point. Plasma samples were processed using a Sep-Pack cartridge to extract ANF. Radioimmunoassay was carried out using a specific antiserum (Peninsula). See Scheme 1.

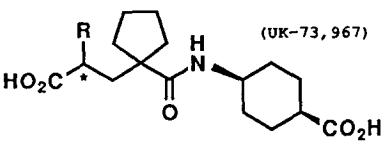



| | R | R1 | Ki |
|---|---|---|---|
| 1. | H | (s)$CH_2Ph$ | $2.6 \times 10^{-7}$M |
| 2. | $-(CH_2)_4-$ | | $1.5 \times 10^{-7}$M |




*(R,S)

| | X | Ki |
|---|---|---|
| 3. |  | $1.3 \times 10^{-8}$M |
| 4. |  | $1.0 \times 10^{-7}$M |
| 5. |  | $7.5 \times 10^{-8}$M |




*(R,S)
Except for 8 and 9.

| | | R | Ki |
|---|---|---|---|
| 6. | | $-(CH_2)_2Me$ | $2.2 \times 10^{-8}$M |
| 7. | (UK-69,578) | $-CH_2O(CH_2)_2OMe$ | $2.8 \times 10^{-8}$M |
| 8. | (UK-73,967) | (+)$-CH_2O(CH_2)_2OMe$ | $1.4 \times 10^{-8}$M |
| 9. | | (-)$-CH_2O(CH_2)_2OMe$ | $4.7 \times 10^{-7}$M |
| 10. | | $-(CH_2)_4NH_2$ | $7.0 \times 10^{-8}$M |
| 11. | | $-(CH_2)_2CO_2H$ | $1.0 \times 10^{-7}$M |
| 12. | | $-(CH_2)_4NHAc$ | $2.1 \times 10^{-8}$M |
| 13. | | $-(CH_2)_2CONH^iPr$ | $2.4 \times 10^{-8}$M |
| 14. | | H | (44% inhibition at $10^{-6}$M) |
| | | Thiorphan | $1.3 \times 10^{-7}$M |

Scheme 1

## RESULTS AND DISCUSSION

It had been shown that in substituted N-carboxymethyl dipeptide inhibitors it was advantageous to incorporate (S)-aromatic amino acid $P_1$ and $P_1'$ residues (7, 12), and when small aliphatic amino acids were introduced at the $P_2'$ position, the importance of a free C-terminal carboxyl group was emphasised (12). In our search for potentially more potent conformationally constrained systems, we found that cis-3-aminocyclohexane carboxylic acid, a constrained analogue of GABA, was well tolerated at the $S_2'$ site as in compound **1**. Using this system we then found that the $P_1'$ phenylalanine residue could be replaced by 'cycloleucine' as in **2**. In retrospect, that elimination of chirality at the $P_1'$ centre is allowable is perhaps not surprising, since the enantiomers of thiorphan only show a three-fold difference in enzyme inhibitory potency (16). Unexpectedly it was then observed that, quite unlike ACE inhibitors (17), replacement of the aza-link between $P_1$ and $P_1'$ residues by a methylene group significantly increased potency, as in glutaramide **3**. The cis-4-amino cyclohexanecarboxylic acid system was shown to confer similar inhibitory potency to the 3-isomer and was preferred as it removed a second chiral centre. In this new glutaramide series, β-alanine **4** and 4-aminobenzoic acid derivative **5** were now clearly inferior to **3**. Both carboxyl groups provided important active site interactions: esterification of either in UK-69,578 (ethyl esters), or removal of that on the cyclohexane ring, markedly reduced potency (Ki $>10^{-6}$M).

Unlike ACE, a hydrophobic interaction at the $S_1$ site was not especially beneficial and a range of groups such as in **3**, **6** and **7** was tolerated. Charged systems were slightly inferior, as in aminobutyl **10** and carboxyethyl **11** derivatives. However, full activity was restored with amides **12** and **13**. The $P_1$ side chain was clearly important, as evident by the lack of activity with compound **14**, but it is uncertain whether it provides some binding interaction or merely constrains the rotational freedom of the glutaramide backbone. The additional importance of stereochemistry was evident with UK-69,578, where activity resided largely in the (+)-enantiomer **8**. The (−)-enantiomer **9** was some thirty-fold less potent. Importantly, for the eventual unambiguous assessment of therapeutic potential, UK-69,578 lacked activity against ACE, and incidentally also against thermolysin. Of note is the finding that in our enzyme assay using [glycine 1-$^{14}$C] hippuryl-phenylalanyl arginine as substrate thiorphan appears thirty to one hundred-fold less potent than when $^{3}$H-Leu-enkephalin (10) or $^{3}$H-Met-enkephalin (13) are used as substrates. Comment has been made previously on the apparent dependence of in vitro inhibitor potency on assay method (18).

In the early phase of this work the similarity between endopeptidase 24.11 and thermolysin and the X-ray crystallographic studies of Matthews (19) on thermolysin-inhibitor complexes were conceptually very helpful. A schematic representation of the key binding interactions between inhibitor and endopeptidase 24.11, which we believe to be important, is shown in Fig 1. Indeed, the recent demonstration that there is in fact substantial active site homology between the two enzymes (20) has allowed us to construct a computer model of the active site, which may be useful in designing more potent inhibitors.

UK-69,578 was selected for more detailed in vivo evaluation. As can be seen in Table I, saline administration to conscious mice evoked a natriuretic and diuretic response which was significantly potentiated by UK-69,578 or its (+)-enantiomer, UK-73,967.

In a parallel experiment saline administration to anaesthetised mice gave a transient rise in plasma ANF, manifested as a doubling in plasma concentration five minutes after dosing, as shown in Table II. When UK-69,578 (3 mg/kg) was coadministered with saline a 2.5-fold elevation in plasma ANF was observed and most strikingly these elevated levels were maintained throughout the thirty minute duration of the experiment.

It has recently been reported that thiorphan potentiates the diuretic and natriuretic action of ANF in anaesthetised normotensive rats (21) and that an analogue, SQ-29072 prevents ANF degradation in conscious spontaneously hypertensive rats, diuretic and natriuretic as well as depressor responses to ANF being significantly enhanced by SQ-29072 (22). Haslanger et al (23) have also demonstrated antihypertensive activity with a substituted-N-

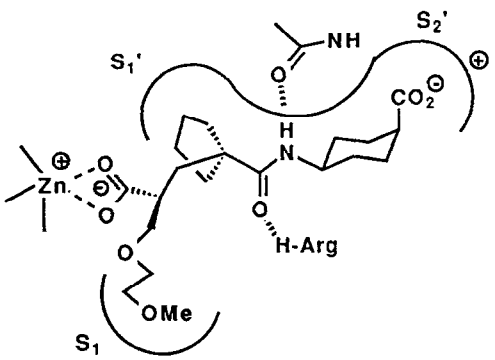



Fig 1. Schematic diagram showing proposed binding interactions of UK-73,967 and active site of EC 3.4.24.11.

Table I: Natriuretic and diuretic responses in conscious mice

| Treatment | Diuresis (µl/hr) | Natriuresis (µEq/hr) |
|---|---|---|
| None | 58 ± 6 | 4.3 ± 0.4 |
| Control (600 µl saline iv) | 136 ± 11 | 10.8 ± 1.1 |
| UK-69,578 3 mg/kg | 249 + 15* | 21.8 + 1.5* |
| UK-73,967 3 mg/kg | 219 + 28* | 21.7 + 2.6* |

* $P < 0.05$ compared to 600 µl saline control.
$n \geq 9$ metabowls per treatment group.

carboxymethyl dipeptide analogue (Sch 39370) subcutaneously in desoxycorticosterone acetate-salt hypertensive rats. We now extend these findings with our inhibitor, which is more potent than thiorphan, by demonstrating an increase in endogenous ANF coupled with a diuretic and natriuretic response in mice. Furthermore, UK-69,578 raises plasma ANF levels and has shown diuretic and natriuretic activity in a conscious dog model of cardiac insufficiency (24) and in patients with chronic heart failure (25). Clinical trials are also in progress with UK-79,300, an orally active 5-indanyl ester prodrug of its (+)-enantiomer.

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Table II: The effect of UK-69,578 on plasma ANF levels in anaesthetised mice following a saline load

| Time post saline load (min) | Plasma ANF (pg/ml) | |
|---|---|---|
| | Control | UK-69,578 (3mg/kg) |
| Pre-load | 41.8 ± 6 | 87.9 ± 1.3 |
| 5 | 91.4 ± 9.1* | 223.2 ± 13.7* |
| 10 | 52.0 ± 7.3 | 216.9 ± 12.8* |
| 30 | 55.5 ± 9.8 | 256.9 ± 15.8* |

The data in the table are the mean ± s.e.m. of plasma ANF values from 3 groups of mice at each time point. * indicates a significant difference from the respective pre-load value (Student's unpaired t-test).

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