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Some Anti-allergic and Anti-inflammatory Actions of 2-*N*-Carboxamidinonormianserin (FCC5)

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Abstract

The aims of these studies were to examine the effects of FCC5 (2-carboxamidino-1,2,3,4,10,14b-hexahydrodibenzo (c,f) pyrazino (1,2,-a) azepine HCl), an analogue of mianserin, on immediate type hypersensitivity reactions in-vitro.

The actions of FCC5 were examined on the Schultz-Dale reaction of guinea-pig ileum and on histamine and leukotriene release from human- and guinea-pig-sensitized lung fragments. FCC5 (applied topically) was assessed for anti-inflammatory activity in-vivo against phorbol-12-myristate-13-acetate (PMA)-induced oedema in the mouse ear. FCC5 (IC50 = 0·17 μM) was a potent inhibitor of the Schultz-Dale reaction in-vitro, as assessed by a concentration-dependent attenuation of egg albumin-induced contractions of sensitized guinea-pig isolated ileum. Using human and guinea-pig isolated sensitized lung fragments, FCC5 (1–100 μM) attenuated antigen-induced release of sulphidopeptidoleukotrienes and histamine. FCC5 (50 μg topically) resembled mianserin and indomethacin in attenuating PMA-induced mouse ear inflammation.

These properties together with previously published evidence of long lasting antihistamine properties in-vivo, suggest that FCC5 has therapeutic potential as an anti-allergic agent, especially in pathological conditions where an inflammatory component is present.

Anaphylaxis of sensitized guinea-pig isolated ileum can be used in studying the actions of new anti-allergic drugs (Grupe & Pietzsch 1988). Exposure of isolated sensitized tissues to a specific antigen produces a rapid and maintained contraction. This is the basis of the Schultz-Dale reaction and these anaphylactic contractions have been demonstrated in a variety of tissues from different species (Chand & Eyre 1978). Release of biologically active substances from tissue mast cells in response to combination of the antigen to cell-fixed antibodies is generally considered to be the main initiating event for anaphylactic contraction of sensitized smooth muscle (Dale & Zilletti 1970).

In-vivo, given acutely, 2-*N*-carboxamidinonormianserin, FCC5, is an antagonist of both histamine (H_1) and 5-hydroxytryptamine (5-HT), and is also devoid of central nervous system activity (Leitch et al 1992b). As its parent analogue, mianserin, was originally developed as an anti-allergic agent (Van Der Burg et al 1970), it was decided to test the effectiveness of FCC5. This was initially done by examining its ability to inhibit antigen-induced anaphylactic contractions in-vitro using sensitized guinea-pig isolated ileum. Further experiments were then carried out to examine the effect of FCC5 on immunologically-induced histamine and sulphidopeptidoleukotriene release from human and guinea-pig sensitized chopped lung tissue in-vitro.

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Mianserin has also been shown to have anti-inflammatory activity (Vargaftig et al 1971; Van Riezen et al 1981). FCC5 was therefore examined for anti-inflammatory properties in-vivo using the phorbol-12-myristate-13-acetate (PMA) mouse ear inflammation test. The phorbol ester, PMA, activates protein kinase C both in-vivo and in-vitro (Castagna et al 1982) and causes inflammation when applied topically to the surface of the mouse ear (Van Arman 1974). The resultant swelling is reduced by anti-inflammatory drugs after topical administration.

Materials and Methods

Schultz-Dale reaction in-vitro: the effects of FCC5 on egg albumin-induced anaphylactic contractions of sensitized guinea-pig isolated ileum

Male guinea-pigs, 300–600 g, were sensitized three weeks before the experiment with 2 mL egg albumin (100 mg mL^{-1}, 1 mL administered intraperitoneally and 5 × 0·2 mL injections given subcutaneously at different sites) and then two weeks before experimentation with 0·4 mL egg albumin (25 mg mL^{-1}, 0·2 mL, i.p., and 0·2 mL, s.c.). Each was killed by stunning and exsanguination and the ileum removed. Segments of ileum (2 cm) were mounted in 20 mL organ baths containing Tyrode solution (bubbled with 95% O_2–5% CO_2) of the following composition (mM): NaCl 136·89, KCl 2·68, $CaCl_2$ 1·80, $MgCl_2$ 1·05, NaH_2PO_4 0·42, $NaHCO_3$ 11·90 and dextrose 5·55. Indomethacin (1 μM) was added to the bathing reservoir to prevent prostaglandin release from the ileum. Tissues were allowed to equilibrate for 30–60 min under 1 g resting tension and

contractions measured with Grass force displacement transducers (FTO3) recording on a Grass polygraph (79E).

Following addition of vehicle (as a control, 15 min contact), egg albumin (0·25–0·5 mL; 1%) was added to the bath and the magnitude of the contractile tension that developed (expressed as a % of maximum contraction, established with histamine 10–100 μM) was recorded. A fresh segment of ileum was then prepared and exposed to FCC5 for 15 min. Egg albumin (0·25–0·5 mL, 1%) was added and the response recorded. Each tissue was exposed to only one concentration of FCC5 or one of egg albumin. An IC50 value was calculated for FCC5, as the concentration causing 50% inhibition of egg albumin-induced contractions.

The effect of FCC5 on release of sulphidopeptidoleukotrienes and histamine from human and guinea-pig sensitized lung fragments

Passively sensitized human lung fragments. Samples of human lung were prepared as described previously (Hughes et al 1983). Briefly, macroscopically normal lung tissue from operative specimens was washed free of blood with Tyrode solution, cut into 2–3 mm^3 fragments and incubated overnight at room temperature (21°C) in serum from an atopic patient with a specific immunoglobulin E level (RAST titre) against *Dermatophagoides pteronyssinus* of 4+ relative to normal serum. The lung fragments were washed free of serum and divided into aliquots (250 mg).

Actively sensitized guinea-pig lung fragments. Guinea-pigs, 300–500 g, were injected intraperitoneally with 1 mL of solution containing 10 mg ovalbumin in a suspension of aluminium hydroxide (65 mg mL^{-1}). The animals were killed by cervical dislocation 3 to 6 weeks later, the lungs dissected out and perfused with oxygenated Tyrode solution at 37°C for 2 min until free of blood. Slices of lung 1 mm thick were chopped using a McIlwain tissue chopper into 1 mm^3 fragments, washed repeatedly with Tyrode solution and divided into aliquots (250 mg).

Release of mediators from lung fragments. Aliquots of sensitized lung fragments (250 mg) from either human or guinea-pig were suspended in centrifuge tubes containing oxygenated Tyrode solution at 37°C, final volume 3 mL, and challenged with antigen solution (3 μL) for 15 min to induce release of mediators. The antigen challenge solution for human lung was an extract of *D. pteronyssinus* (1 protein N unit mL^{-1}) and for guinea-pig, lung ovalbumin (50 $\mu g\,mL^{-1}$). FCC5 was added to the suspensions of lung fragments 15 min before antigen challenge to give concentrations of 1, 10 and 100 μM. Control replicates of lung were included to measure spontaneous release of mediators without antigen in the presence and absence of FCC5. Each treatment was carried out in duplicate. The antigen reaction was stopped by chilling the tubes in ice, centrifuging and decanting the supernatant solutions, which were assayed for histamine and leukotrienes.

Sulphidopeptidoleukotrienes were bioassayed, using a cascade system comprising four longitudinal strips of smooth muscle from guinea-pig ileum superfused with oxygenated Tyrode solution at 37°C. The superfusate contained mepyramine (1 μM) and hyoscine (1 μM) to block the effects of histamine and cholinergic compounds, respectively. Contractile responses to aliquots of the supernatant solutions were compared with responses to a standard solution of LTC_4, and both were shown to be blocked by the leukotriene antagonist FPL 55712. This bioassay has previously been shown in our laboratory to produce results that correlate significantly ($P < 0{\cdot}001$) with results obtained by radioimmunoassay (Tennant et al 1987). Histamine was assayed by an automated fluorimetric method (Evans et al 1973). Histamine released from lung into the supernatant was expressed as a percentage of the total tissue histamine liberated by boiling further aliquots of lung fragments for 10 min.

Results were corrected for any spontaneous and drug-induced effects on release, which were less than 10% of the antigen-induced release. The effect of each concentration of FCC5 was calculated in terms of the drug-free control release.

PMA mouse ear inflammation assay

Male Swiss albino mice, 25–30 g, were used. The method used was an adaptation of that of Van Arman (1974). Animals were divided into groups of five and placed in individual cages for the duration of the study. Each mouse was placed on a mesh floor inside a glass desiccator and anaesthetized with ether. A volume of 25 μL of the test solution (antagonist + PMA, 2 μg per ear) was topically applied to the inner pinnal surface of the left ear using a micropipette. An equivalent volume of the vehicle for the PMA and drugs was topically applied to the right ear to act as a control. Drugs investigated were FCC5, mianserin, desmethylimipramine (DMI) and indomethacin at doses of 10 and 50 μg per ear. Control mice received topically administered PMA solution only to the left ear and acetone vehicle to the right ear, to determine quantitatively the oedema produced by PMA alone. Ears were allowed to dry before the mice were placed back into their cage. During application care was taken to prevent solutions from running into the ear canal.

After a period of 3 h 20 min following topical administration of solutions the mice were killed by inhaled 100% CO_2 and cervical dislocation. Both ears were removed and uniform diameter cut sections (at the site of application of solutions) obtained by punching holes with a sharpened No. 3 (6 mm diam.) cork borer. The weight of the punch biopsy was recorded. The difference in weight between the samples from the two ears (one treated with solution containing PMA, the other treated with vehicle) of the same mouse was considered to be the amount of oedema caused by PMA. The weight difference between the two ears biopsy samples i.e. $L_{ear} - R_{ear}$ (drug treated–control) was also recorded. The mean weight difference (attributed to oedema) was calculated for each group of mice, as was its standard error. The anti-inflammatory action of each drug was expressed as the reduction in weight of oedema in ears exposed to the drug compared with the weight of oedema in ears treated with PMA only.

Drugs used

The following drugs and chemicals were used: acetone, diethyl ether (BDH, UK), azelastine hydrochloride (Wallace Laboratories, USA), desmethylimipramine, egg albu-

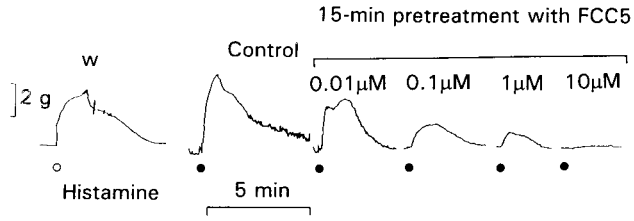

FIG. 1. Representative tracings of egg albumin-induced contraction (0·25 mL 0·1% egg albumin) of the guinea-pig isolated ileum, and the response to a concentration of histamine (100 μM) producing maximal effect. Also shown are tracings of the attenuation of egg albumin-induced contractions of guinea-pig isolated ileum by FCC5 (0·01–10 μM; 15 min contact time). Each tracing is from a separate preparation. w, Wash; ●, 0·25 mL 1% egg albumin administration; ○, histamine (100 μM) administration.

min grade II, histamine dihydrochloride, 5-hydroxytryptamine, indomethacin, phorbol-12-myristate-13-acetate (Sigma, USA), *D. pteronyssinus* extract (Beecham Research Laboratories, UK), FCC5 (Monash University, Australia), FPL 55712 (a gift from Fisons, UK), hyoscine hydrochloride (Burroughs Wellcome, UK), mepyramine maleate (May & Baker, UK), and mianserin (Research Biochemicals Inc., USA).

Histamine, egg albumin, FCC5 and azelastine were dissolved in distilled water. Indomethacin was dissolved in a small amount of 0·1 M Na_2CO_3 in 0·9% NaCl (saline) and the pH of the solution adjusted to 7·4 by the addition of 0·1 M HCl with vigorous stirring. For the PMA-induced ear inflammation experiments, phorbol-12-myristate-13-acetate, FCC5, desmethylimipramine, mianserin and indomethacin were made up in acetone.

FCC5 is 2-carboxamidino-1,2,3,4,10,14b-hexahydrodibenzo (c,f) pyrazino (1,2,-a) azepine hydrochloride of molecular formula $C_{18}H_{20}N_4$.HCl with a molecular weight of 328·701. It is a colourless microcrystalline solid with a melting point of 288–290°C (for more details on physicochemical and spectroscopic properties of FCC5 see Jackson et al (1992)).

Statistical analysis

All results are given as mean values ± standard error of the mean (s.e.m.). Student's paired and nonpaired *t*-tests were performed for comparison of two means when appropriate. Statistical significance was accepted for $P < 0·05$. The IC50 of FCC5 against anaphylaxis in-vitro was calculated using the LINUS program on an Apple IIE computer. Multiple comparisons were analysed by one way analysis of variance together with Tukey's test using the CLR ANOVA programme (Apple Macintosh). For experiments on human and guinea-pig lung fragments, a logarithmic transformation was used in the statistical treatment of the data, to normalize the distribution. The data in logarithmic form were analysed by 2-way analysis of variance and Duncan's multiple range test. IC50 values of FCC5 against histamine and leukotriene release from human and guinea-pig lung were calculated using linear regression analysis.

Results

The Schultz-Dale reaction: egg-albumin-induced anaphylactic contraction of the guinea-pig ileum

Following addition of egg albumin to the ileum of the actively sensitized guinea-pig, the maximum tension developed during the course of the complete contraction was 2·58 ± 0·15 g (n = 44). The form of the Schultz-Dale response of the guinea-pig ileum was relatively reproducible. In most instances there were three phases: an initial fast contraction followed by a partial relaxation, followed by a longer sustained contraction (Fig. 1). A delay of 16·4 ± 1·6 s (n = 33) lapsed before the start of the contraction which lasted 162·9 ± 13·2 s (n = 36). It was impossible to elicit more than one Schultz-Dale reaction in any one preparation. Pretreatment of the ileum with FCC5 (10 nM–10 μM) caused a concentration-dependent attenuation of the egg-albumin-induced contractions with an IC50 value of 0·17 μM (95% confidence limits = 0·05–0·27 μM). Azelastine (2·5 μM, n = 3) was also found to inhibit egg-albumin-induced contractions ($P < 0·01$) to a similar extent as 10 μM FCC5 (Table 1).

Antigen-induced release of sulphidopeptidoleukotrienes and histamine from human and guinea-pig sensitized lung fragments

In control experiments using human lung fragments from nine patients, the release of sulphidopeptidoleukotrienes was equivalent to 1·0 ± 0·1 nmol LTC_4 g wet weight^{-1} (mean ± s.e.m.), and from six guinea-pigs the corresponding release was equivalent to 0·8 ± 0·3 nmol g^{-1}. Corresponding figures for histamine, expressed as mean ± s.e.m. of percentage of total tissue histamine, were 10·8 ± 2·2% (human) and 30·8 ± 3.9% (guinea-pig). Table 2 shows that FCC5 (1–100 μM) caused a significant concentration-dependent inhibition of the release of leukotrienes and histamine

Table 1. The effects of FCC5 and azelastine on the contractile response (measured as the peak height on the tracing) to egg albumin.

	Contractile response (% maximum)
Control	85 ± 5
FCC5 (μM)	
0·01	75 ± 85
0·1	35 ± 11
1	30 ± 7*
10	4 ± 2**
Azelastine (μM)	
2·5	11 ± 4**

Results are means ± s.e.m. and are expressed as a % of the maximum response to histamine. *$P < 0·05$, **$P < 0·01$ compared with vehicle control.

Table 2. The inhibitory effect of FCC5 on antigen-induced histamine and leukotriene release (expressed as % inhibition release).

FCC5 (μM)	Guinea-pig		Human	
	Histamine	Leukotriene	Histamine	Leukotriene
1	15·4 ± 15	7 ± 6*	31 ± 13	21 ± 8
10	351 ± 9**	29 ± 7**	59 ± 6***	52 ± 5***
100	62 ± 5**	72 ± 8***	85 ± 3***	73 ± 5***

Values are mean ± s.e.m. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

from both human and guinea-pig lung fragments. Linear regression analysis showed that 50% inhibition of histamine and leukotriene release respectively was produced by 5·1 and 11·7 μM FCC5. Corresponding values for inhibition of histamine and leukotrienes from guinea-pig lung were 34·7 and 29·5 μM, respectively.

PMA mouse ear inflammation assay

Phorbol-12-myristate-13-acetate (2 μg) applied topically caused oedema of the mouse ear as measured by the resultant increase in weights from a control value of 11·28 ± 0·28 to 22·38 ± 0·59 mg (n = 15). This represents an increase in $L_{ear}-R_{ear}$ (control) of 11·05 ± 0·67 mg or 99·32 ± 7·29%. The effects of the drugs on the induced oedema are shown in Table 3.

Discussion

In most cases on exposure to egg albumin, ilea responded with immediate contractions followed by slower sustained ones lasting 1–3 min. FCC5 attenuated both phases of the antigen-induced contraction in a concentration-dependent manner with an IC50 value of 0·17 μM. Inhibition of the first phase by FCC5 may be expected to be due to its ability to block histamine H_1-receptors (Leitch et al 1992a,b). Inhibition of the complete anaphylactic contractile response suggested that FCC5 may not only interfere with the effects or release of histamine but also may inhibit the release or effects of secondary mediators.

Azelastine (2·5 μM) had similar actions to FCC5 on antigen-induced contractions of sensitized guinea-pig ileum. Azelastine which has a similar chemical structure, is an example of a more recently found non-sedating H_1-antihistamine that is an orally effective, long-acting anti-allergic drug useful in treatment of bronchial asthma and allergic rhinitis (Yin et al 1991). It has been shown to inhibit the release of histamine and other allergic mediators from mast cells (Chand et al 1988; Achterrath-Tuckermann et al 1988a) and to have 5-HT-ergic, leukotriene C_4, leukotriene D_4, and platelet-activating factor-receptor blocking effects (Zeckel et al 1981; Chand et al 1986; Achterrath-Tuckermann et al 1988b).

FCC5 (1–100 μM) was found to inhibit antigen-induced (house dust mite: *D. pteronyssinus*) release of sulphidopeptidoleukotrienes and histamine from human and guinea-pig sensitized lung fragments in a concentration-dependent manner. Its properties therefore resemble the histamine H_1 antagonist azatadine which also blocks leukotriene and histamine release from human lung. Daniels & Temple (1986) found azatadine inhibited histamine and leukotriene release with IC50 values of 13·8 and 2·7 μM, respectively. The corresponding IC50 values for FCC5 were 5·1 and 11·7 μM. The finding that FCC5 inhibits histamine and sulphidopeptidoleukotriene release from chopped human and guinea-pig lung suggests that FCC5 could show efficacy against both the early and late phases of the asthma reaction. FCC5 has been found previously (Leitch et al 1992b) to be a potent and long-lasting in-vivo inhibitor of bronchoconstrictor responses of guinea-pigs to histamine and 5-HT.

FCC5, and mianserin, applied topically were effective antagonists of PMA-induced mouse ear inflammation. PMA has structural similarities to diacylglycerol, and binds to and activates protein kinase C, a process that is Ca^{2+}-dependent (Nishizuka 1984). This results in release of arachidonic acid from phospholipids in membranes thus causing release of eicosanoids, which are believed to be mediators of the resultant inflammation (Carlson et al 1985; Opas et al 1985; Bouchlier et al 1990). The anti-inflammatory action of FCC5 in this test could therefore be due to block of one or more of the enzymes concerned in the release and metabolism of arachidonic acid. However, further contributing to its effect could be its ability to antagonize 5-HT and histamine plus its sympathomimetic actions (Leitch et al 1992b). 5-HT and histamine are major mediators of inflammation in mice (Amorim et al 1992; Oyanagui & Sato 1993). In addition, the sympathomimetic properties of FCC5 (Leitch et al 1992b), by causing vasoconstriction, would reduce local blood flow thus reducing oedema formation. However, high doses of desmethylimipramine, a drug causing sympathomimesis by a similar action to FCC5, had no effect. This suggests that it is more likely that FCC5 and mianserin block inflammation by the other actions mentioned above rather than by

Table 3. The effect of topical administration of indomethacin, FCC5, mianserin (10 and 50 μg per ear, n = 5) and desmethylimipramine (50 μg/ear) on PMA-induced (n = 15) oedema in the mouse ear.

Drug	μg/ear	Increase in ear weight (mg)
PMA		11·3 ± 0·3
Indomethacin	10	12·0 ± 1·0
	50	2·9 ± 1·6**
FCC5	10	11·0 ± 1·8
	50	5·2 ± 1·1**
Mianserin	10	10·7 ± 0·8
	50	5·9 ± 1·0*
Desmethylimipramine	50	11·5 ± 0·7

Results are means ± s.e.m. *$P < 0.05$, **$P < 0.01$ compared with the PMA control.

inhibition of neuronal uptake of noradrenaline which would be responsible for their vasoconstrictor effects.

The results of this acute study suggest the possible usefulness for FCC5 as an anti-allergic agent, perhaps when this is accompanied by inflammation. Two different studies demonstrated that FCC5 displayed anti-allergic properties in-vitro. Anti-inflammatory activity after topical administration in-vivo was also found, although this conclusion needs to be supported by studies of its effects after oral administration on chronic inflammatory conditions. Nevertheless, the properties of FCC5 demonstrated so far, taken together with previous findings that in-vivo it is a long-lasting non-competitive antagonist of histamine devoid of CNS activity (Leitch et al 1992b), indicate that it may be a useful anti-allergic/anti-inflammatory agent.

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