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IDENTIFICATION OF A NON PEPTIDIC RANTES ANTAGONIST

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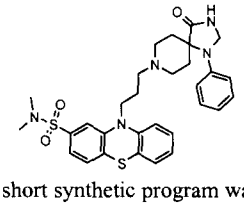
Abstract : A series of phenothiazines demonstrating inhibition of RANTES binding to THP-1 cell membranes has been identified. The lead compound RP23618 (IC_{50}=3μM) was found to inhibit specific binding of ^{125}I-RANTES, but not ^{125}I-MCP-1 to THP-1 cell membranes and furthermore to antagonise RANTES, but not MCP-1-induced chemotaxis of THP-1 cells.

Introduction. RANTES (Regulated on Activation Normal T-cell Expressed and Secreted) is a member of the CC superfamily of chemoattractant cytokines known as chemokines.[1] Chemokines are proinflammatory molecules which promote the recruitment and activation of leukocytes to sites of inflammation. *In vitro*, RANTES has been shown to be a potent chemoattractant for T-cells[2], monocytes[3,4], eosinophils[5,6,7] and basophils[7] and to bind to a number of chemokine receptors (CCR1, CCR3 and CCR5) with high affinity.[8] Increased RANTES expression has been documented in a number of inflammatory diseases including asthma, arthritis and atherosclerosis. Screening of a collection of compounds from the corporate database for inhibition of ^{125}I-RANTES binding to THP-1 cell membranes[9] has led to the identification of RP 23618 :



1 (RP23618) IC_{50} = 3 μM

Results and discussion. A short synthetic program was initiated to explore the SAR around this lead compound in terms of inhibition of RANTES binding to THP-1 cell membranes (see results in the Table below). Modification of the side chain showed that the spiropiperidine amide could be replaced with a benzylpiperidine **2**, a phenylpiperidine **3**, or phenylpropylmethylamine **4** without loss of activity. Reduction of the phenyl group of **3** gave the similarly active cyclohexyl derivative **5**, demonstrating that the aromatic interaction is not essential for activity. However, a hydrophobic group at this position does improve activity since the simple piperidine **6** showed a much reduced potency. Modification of the linker showed that elongation of the chain length as in **7**

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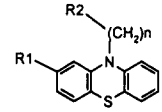
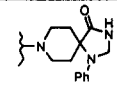
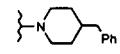
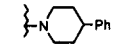
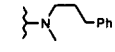
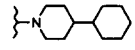
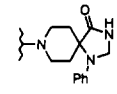
reduced activity slightly whereas shortening of the chain length as in **8** abolished activity. Replacement of the sulfonamide with: chlorine **9**, methyl ketone **10**, nitrile **11**, amide **12**, or methyl ester **13**, did not affect compound activity. However, the unsubstituted compound **14**, showed some reduced activity, indicating a substituent to be beneficial at this position. Compound **1** was also screened for inhibition of ^{125}I-MCP-1 receptor binding to THP-1 cell membranes. Compound **1** did not inhibit ^{125}I-MCP-1 receptor binding at concentrations up to 30 μM.

Table: Modification of **1**. Competition studies for inhibition of ^{125}I-RANTES binding to THP-1 cell membranes.



Cpd.	R1	R2	n	IC_{50} (μM)
1	SO_2NMe_2		3	3
2	SO_2NMe_2		3	7
3	SO_2NMe_2		3	4.5
4	SO_2NMe_2		3	4
5	SO_2NMe_2		3	2
6	SO_2NMe_2		3	38 % @ 30μM
7	SO_2NMe_2		4	10
8	SO_2NMe_2	"	2	< 20% @ 30 μM
9	Cl	"	3	7
10	COMe	"	3	3
11	CN	"	3	6
12	$CONMe_2$	"	3	5
13	CO_2Me	"	3	4
14	H	"	3	12

Scatchard analysis showed that RP23618 is not competitive with RANTES (results not shown) suggesting that the compound binds to an allosteric site. In addition, RP23618 was also shown to inhibit RANTES-induced chemotaxis of THP-1 cells in a concentration-dependent manner but failed to inhibit MCP-1-induced chemotaxis (see Figure below). These results are in contrast to a recent publication which report phenothiazines (related to chlorpromazine)[10] to be broad spectrum inhibitors of lymphocyte motility. More recently a patent from Takeda[11] has disclosed a series of potent RANTES and MIP-1α[12] antagonists acting at the CCR1 receptor with compounds sharing some structural features with RP23618.

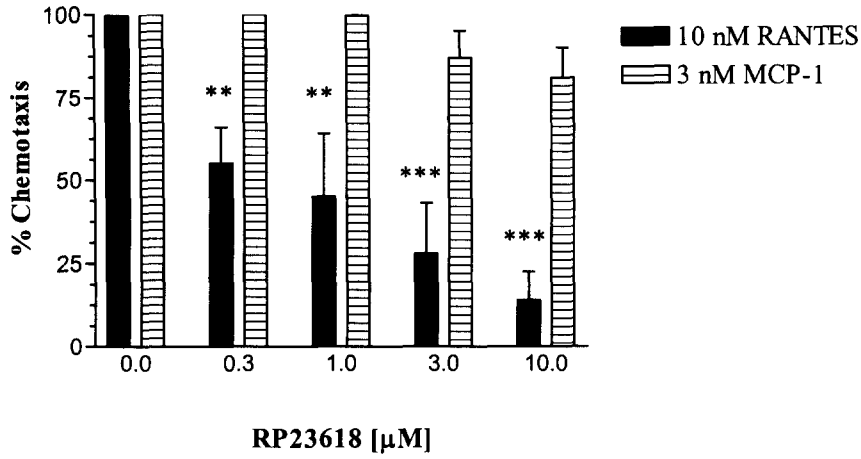
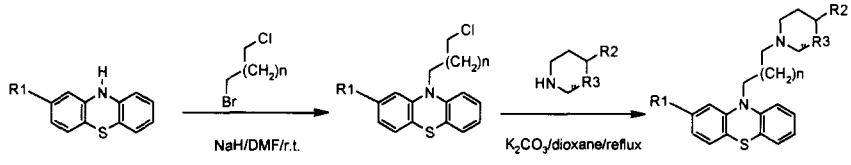


Figure: RANTES (10 nM) and MCP-1 (3 nM) induced THP-1 cell chemotaxis in the presence of compound **1** at 0.3, 1, 3 and 10 μM. Results are expressed as percentage of maximum chemokine-induced chemotaxis. Using ANOVA two-way analysis of variance and a modified 't-test' based on the error mean squared ** = $p \leq 0.01$, *** = $p \leq 0.001$ for treated compared with control groups.

Chemistry: The 2-chloro, 2-aceto and unsubstituted phenothiazines are commercially available, the 2-dimethylsulfonamide,[13] the 2-cyanophenothiazine[14] and the 2-carboxyphenothiazine[15] were made following published methods. The 2-methoxycarbonylphenothiazine and the 2-dimethylamidophenothiazine were prepared from the acid by standard procedures. The phenothiazines were then alkylated following the scheme below:



1-14 $R_3 = CH_2$ except for **4** n = 0, 1, 2

The amines used are all commercially available except for 4-cyclohexyl piperidine which was made by catalytic hydrogenation of 4-phenylpyridine in acetic acid containing sulfuric acid over Adam's catalyst (rt, H_2 =0.25bar, 48h, obtained in 78% yield).

Biology: Receptor Binding Assays: THP-1 cells were seeded at a density of 0.2 x 10^6 cells/ml in RPMI 1640 medium containing glutamax and supplemented with 10% foetal calf serum and 20 μM 2-mercaptoethanol three days prior to use. THP-1 cell homogenates were prepared using the BioNeb™ Cell and DNA Disruptor (Glas-Col, Thermometrics, U.K.). The homogenates were centrifuged at 400*g* for 10 min and the resultant supernatant centrifuged at 48,000*g* for 20 min at 4°C. The cell membrane pellet was resuspended in 10 mM HEPES, pH 7.2 at a protein concentration of 1 mg/ml and stored at -80°C until required. Receptor binding assays were carried out using 50 pM radiolabelled ligand (Amersham), 100 μg protein and 0.3 - 30 μM compound (2.5 % DMSO) in 96 well filtration plates (Millipore)and incubated for 90 min

at 37°C. Following incubation, samples were harvested onto 0.3% polyethyleneimime-treated Whatman GF/B filters using a Micromate 196 Cell Harvester (Canberra Packard Ltd.). ^{125}I-RANTES and ^{125}I-MCP-1 binding was quantified by liquid scintillation counting using a TopCount Microplate Counter (Canberra Packard Ltd.).

Chemotaxis Assay: The THP-1 cell chemotaxis assay was carried out using a 96 well chemotaxis chamber (NeuroProbe Inc.). Chemokines (10 nM RANTES or 3 nM MCP-1) were placed in the lower wells of the chemotaxis chamber, covered with a 5 μm pore-size polyvinylpyrrolidone-free polycarbonate filter (coated with fibronectin 20 μg/ml PBS) and overlaid with 200 μl of a THP-1 cell suspension (8 x 10^6 cells/ml in the presence or absence of compound **1** (0.3 - 10 μM in 0.2% DMSO). Following incubation at 37°C for 2 h, migrated THP-1 cells were stained with ethidium bromide (20μg/ml PBS) and the fluorescence of each well measured using a 96 well plate reader (Cytofluor 2300) at excitation of 530 nm and emission detection of 620 nm.

Conclusion: RP23618 has been identified as a RANTES antagonist, its structure has been simplified to give compound **5** with similar activity. Compound **1** has been shown to inhibit ^{125}I-RANTES binding but not ^{125}I MCP-1 specific binding assay to THP-1 cell membranes and to inhibit RANTES and not MCP-1-induced THP-1 cell chemotaxis.

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