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In vitro selection of RNA aptamers that block CCL1 chemokine function ☆

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Abstract

CCL1, the CCR8 ligand, is a CC chemokine secreted by activated monocytes and lymphocytes and is a potent chemoattractant for these cell types. The *in vivo* role of the CCL1/CCR8 axis in Th2-mediated inflammation is far from clear. Ligand neutralisation studies reported discrepancies in the effect of CCL1/CCR8 and CCR8 knockout studies showed very different insights into the functional role of the CCR8. To further study the biological function of CCL1, we focused on the generation and characterisation of RNA aptamers. We report here the *in vitro* isolation of the first nuclease resistant and selective RNA aptamer (T48) with high-binding affinity for human and mouse CCL1. The T48 aptamer but not a random control aptamer antagonises CCL1 function in a dose-dependent fashion in both heparin binding and chemotaxis assays. To our knowledge, the T48 aptamer constitutes one of the most potent CCL1 antagonists reported to date and is an excellent tool to dissect CCL1-specific function *in vivo*. The T48 aptamer may also have potential as new generation of therapeutic tools.

Keywords: Aptamer; Chemokine; CCL1; I-309; TCA-3; CCR8; GAGs; Chemotaxis; SELEX; Inflammation

Chemokines are small chemoattractant cytokines that control a wide variety of biological and pathological processes, ranging from immunosurveillance to inflammation and from viral infection to cancer. Unlike other cytokines, chemokines signal via seven-transmembrane GPCRs (G-protein-coupled receptors), which are favoured targets by the pharmaceutical industry. They are the first cytokines for which small-molecule-receptor antagonists have been developed [1].

CC chemokines are a family of related proteins characterised by two adjacent cysteine residues that function as leukocyte activators and chemoattractants in inflammatory reactions [2,3]. CCL1, the CCR8 ligand, is a CC chemokine secreted by activated monocytes and lymphocytes [4,5] and is a potent chemoattractant for both of these cell types [6,7]. CCL1 increases the metalloproteinase-2 production by human umbilical vein endothelial cells (HUVECs), an activity that enables these cells to remodel the vascular matrix. The induction of CCR8 and CCL1 under conditions associated with vascular smooth muscle cell (VSMC) proliferation and migration raises the possibility that CCR8 may play an important role in vessel wall pathology [8,9].

☆ *Abbreviations:* hCCL1, human CCL1 (also known as I-309); mCCL1, mouse CCL1 (also known as TCA-3); SELEX, Systematic Evolution of Ligands by Exponential enrichment; FCS, foetal calf serum; GAGs, glycosaminoglycans.

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CCL1 is constitutively expressed at strategic cutaneous locations, including dermal microvessels and epidermal antigen-presenting cells. These findings define a chemokine system for homeostatic T cell traffic in normal human skin [10]. The CCL1/CCR8 axis is also known to link adaptive and innate immune functions that play a role in the initiation and amplification of atopic skin inflammation [11].

The *in vivo* role of the CCL1/CCR8 chemokine axis in Th2-mediated inflammation is controversial. Ligand neutralisation studies reported discrepancies in the effect of CCL1/CCR8 on eosinophil recruitment [12,13]. Intriguingly, three CCR8 knockout studies have been reported that give very different insights into the functional role of CCR8 in allergic reactions *in vivo* [12,14,15]. All these data taken together, suggest that the role of CCL1 and its receptor in different disease states still requires clarification. Thus, new chemical, biochemical and biological approaches are still required to investigate the role of this target in human disease. The aim of these studies was to generate biological antagonists which could be used to better understand the biological function of CCL1.

In view of the advances in SELEX methodology [16,17] and reports demonstrating the successful generation of aptamers against proteins, this procedure was used to generate nuclease-resistant RNA antagonists to CCL1. RNA aptamers with very good affinity and specificity have been generated against a wide variety of targets [18,19], including chemokines, as described in our lab [20,21]. Moreover, a number of the reported aptamers to different targets have been shown to be biologically active both *in vitro* [20–22] and *in vivo* [23]. We report here the *in vitro* selection of T48, an anti-mCCL1 RNA aptamer that has also high-binding affinity for hCCL1 and acts to antagonise its function. This aptamer could be utilised as a powerful target validation tool in mouse models of disease and may also have therapeutic potential.

Materials and methods

Materials. Human and mouse CCL1 were purchased from R&D Systems, Europe Ltd. (Abingdon, Oxon, UK).

SELEX. Iterative rounds of selection and amplification were performed as described [20] with minor modifications. Human and mouse CCL1 (3 μg/μl) were diluted to the required concentration in SCHMK buffer (110 mM NaCl, 1 mM $MgCl_2$, 20 mM Hepes, pH 7.0, 1 mM $CaCl_2$, and 5 mM KCl) and incubated with 100 μl Dynal beads overnight at 4 °C to allow hydrophobic bead binding and SELEX stringency was applied as described [24]. For each SELEX round, RNA, diluted in 50 μl wash buffer, was added to beads in individual Eppendorf tubes. Tubes were incubated at 37 °C for 30 min after which individual samples were washed to remove unbound RNA. To elute specifically bound RNA, 20 μl of 3 N7 primer (5 pmol/μl) was added to individual tubes, the mix heated at 95 °C for 3 min and then left at room temperature (RT) for 5 min. Reverse transcription of the eluted RNA was then carried out in the presence of the beads and the supernatant was transferred to a fresh tube. PCR and *in vitro* transcription were then performed to generate RNA for the next round of SELEX as described previously [17]. 2′fluoro pyrimidines were used in the selection because they are known to impart a greater level of RNA stability, making the transcribed material stable enough to be used in a cell culture assay.

Aptamer-chemokine-binding assays. Binding assays were carried out by nitrocellulose filter partitioning as described [17].

Cloning and sequencing. Aptamer cDNA was cloned into the plasmid pCR2.1 using the TA cloning kit (Invitrogen) according to the manufacturer's instructions. Plasmid clones were purified and sequenced and the sequences derived the SELEX experiment were aligned using the Clustal W software package of the GCG suite of molecular biology programs.

Oligonucleotide synthesis. Synthesis of full-length RNAs was performed by *in vitro* transcription of synthetic DNA templates and purification by denaturing gel electrophoresis, as described [17]. 2′F pyrimidines (aimed to increase endonuclease resistance, Trilink BioTechnologies Inc, San Diego CA) and 2′OH purines (Roche Molecular Biochemicals, Mannheim, Germany) were used in each case.

Secondary structure prediction of RNA aptamers. Secondary structures of individual aptamers were predicted using the MFOLD programme [25].

Assay for CCL1 binding to heparin, measured by surface plasmon resonance. Surface plasmon resonance measurements were conducted using a BIAcore 2000 instrument as described [20]. First, a dose-dependent study was performed for both human and mouse CCL1. Then, the inhibitory effects of pre-incubating increasing concentration of aptamers with the chemokine (150 nM hCCL1 and 100 nM mCCL1) for 30 min at RT prior to injection were observed and the reduction in maximum-binding response calculated as a percentage of that of random control aptamer responses.

Generation of BaF3 transfectants expressing recombinant hCCR8. The BaF3-hCCR8 line was made by electroporation of BaF3 cells with linearized hCCR8 pCDNA3.1 construct. The hCCR8 transfected BaF3 cells were selected by limiting dilution culture under selection with 0.8 mg/ml G418 (Gibco Life Technologies) in RPMI 5% FCS + additives in 96-well plates. Wells containing single colonies were selected and half of the cells used in a chemotaxis assay as described below using 1 nM hCCL1. Wells containing migrating cells were moved up, sub cloned one more time and frozen down to form a cell bank for future experiments.

Chemotaxis assay. This assay was performed as described [20,26] with the following modifications. BaF3-hCCR8 cells were washed once in PBS and resuspended in chemotaxis buffer (RPMI 2% FCS 10 mM Hepes) at 5×10^6 cells/ml. 96-well chemotaxis chambers fitted with a 3 μm pore polycarbonate filter (Receptor Technologies Ltd, Adderbury, Oxon, UK) were used. Aptamer and chemoattractant (1 nM) were pre-incubated for 30 min at RT in chemotaxis buffer and then 30 μl was transferred to the bottom well of the chemotaxis plate. 50 μl of the cells, washed once in PBS and resuspended in chemotaxis buffer at 5×10^6 cells/ml, was placed on top of the filter above each well. After 1–2 h incubation, cells were transferred to a 96-well flat bottom plate. Then the wells were washed once with 60 μl of chemotaxis buffer and this was also transferred to the 96-well flat bottom plate. Ten microliters of Alamar Blue (Serotec Ltd, Kidlington, Oxford, UK) in PBS was finally added to the wells of the flat bottom plate. After 17–18 h, the plate was read on a Cytofluor II fluorescent plate reader (Applied Biosystems, Warrington, Cheshire, UK) at 530 nM (excitation), and 590 nM (emission). Each point represents the mean and standard error of three replicate wells.

Data analysis. Dissociation constants (K_d), EC_{50} and IC_{50} values were determined by fitting data using Prism 3.0 or Robosage Microsoft Excel add-in software.

Results

SELEX

In order to generate a target validation tool with therapeutic potential, our goal was the isolation of an aptamer capable of recognising and antagonising both human and mouse CCL1. Thus, nuclease resistant and high affinity RNA ligands to human and mouse CCL1 were generated. Five hCCL1 and six mCCL1 iterative rounds of SELEX

were carried out as described [21]. To ensure the isolation of high affinity aptamers, the stringency of the SELEX procedure was increased in later rounds by reducing the amount of protein and RNA, reducing the binding time and by increasing the stringency of post-binding washes in the experiment, as described [24]. As there was no significant change in the apparent affinity of the last RNA pools, the SELEX was assumed to have reached completion. The cDNA from the final pools were cloned and 59 individual aptamer clones derived from the hCCL1 SELEX and 61 clones from the mCCL1 SELEX experiment were sequenced. Aptamers in this study have a core variable region (40 bases) and 5′ and 3′ terminal regions of 15 and 16 bases, respectively. An alignment of the variable region of both human and mouse CCL1 pooled clones led to the identification of 16 sequence families along with 9 "orphan" sequences that were only represented once in the final pools yielding 25 distinct representative sequences (Fig. 1).

Binding of the CCL1 aptamers to the CCL1 chemokine

To characterise individual aptamers, RNA was made from each representative clone (A1 to A25, Fig. 1) and the affinity of each of the family-derived or orphan-derived RNAs to the both human and mouse CCL1 chemokine target was determined in an RNA-binding assay by using nitrocellulose filter partitioning. The highest affinity aptamers were screened in dose-dependent nitrocellulose-binding assays and were found to have affinities of 1–400 nM. T48 (A20 clone, Fig. 1) was one of a panel of aptamers able to bind both hCCL1 (Fig. 2) and mCCL1 (data not shown)

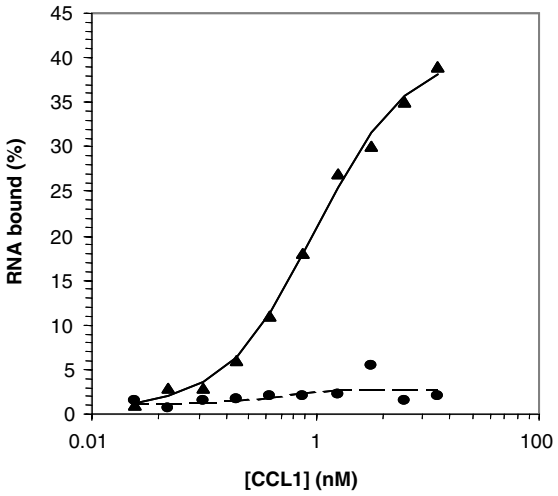

Fig. 2. Binding studies of the T48 aptamer to hCCL1. Nitrocellulose binding studies of the T48 aptamer (▲, solid line) and a random control aptamer (●, dashed line).

with high affinity. The random control aptamer showed no activity (Fig. 2). Given its ability to interact with both human and mouse CCL1, T48 was one of the aptamers chosen for further functional characterisation.

In vitro functional characterisation of CCL1 aptamers

Upon activation of the endothelium by pro-inflammatory cytokines, chemokines are secreted and presented on the endothelial cell surface by GAGs (glycosaminoglycans). In addition, chemokines are able to bind to GAGs that are

Clone	Sequence	#	h	m
A1	....CUCGAGAUCCUCGGUCAUAUC..UUCACCUACCAUUUUGCC.....	1	1	0
A2	....UCCUUCCCACUCCUCCACGU...UCGAUAUACCACACCUCCC....	1	1	0
A3	.......CGCCUCUUCUGACACUAC..UUAACUUUCUAUCACUCCUGUG.	3 (1, 1, 1)	3	0
A4	....GGAUUCCUCUGCUUAUGCUGU..UCCUUUAACGACCUCUCUG....	2 (1)	1	1
A5	........GCCUCCUCCACUCCUCCACGAUUAACUGCCAACACACUCGC..	1	1	0
A6	......ACGACUCCUCUGGCAUGCCUGAAAAAAU.CCAUCUCACCCCUG.	1	1	0
A7	....UGACUUG.UCUGGCCCUUUUGUACU.CCUCCCC.AAUUGCCC....	16 (1, 1, 1, 3, 3, 8, 1, 6, 5)	6	10
A8	....UGACUUCGUUCUGGCCCUCUGUAUU.UUCCAUA.CACUGCCC....	3 (1, 1)	1	2
A9	....GCCUCUCGAUCUGCCCAUUCGUAUC.GUAUUACGCACUGCC.....	3	0	3
A10	ACGACUCCUCUGCCCUACGC.UUUACAUCAUUGCCU....UUGCC.....	9 (3)	3	6
A11	..CAUUCAUAAGUUUUGCACAUCUCCGUUUCCACUC....UUGCCC....	2	0	2
A12	....AGUUCUCUGCCCAAAUACUACACCUCCUUAUG.UGUUUGCC.....	4 (1, 1, 1, 2)	4	0
A13	....ACGACUUUCCUCUGCCACUGACCAUCUUAAUGCUCCUUGC.....	1	1	0
A14	....UCUAGUCGAUAAUUCUGCCCAAGACAUCCUUC..UCUUGCC.....	28 (1, 9, 1, 1, 6)	16	12
A15	....UCGACUCGUAUUGCCCAUAGUCAUUCCUUUCC.CCUAUGCC.....	1	1	0
A16	..GGUCGAUUUGCCCAGAACACUUAAGUCCAUCCUU...UCUGCC.....	2 (1, 1, 1, 1)	2	0
A17	.....CACACUAGUCAAGAUGCCCACCUCUUCCCCU. AUCCGUCCU...	3 (1, 1, 1, 1)	3	0
A18	........AGUUCGCCACCCUCCAAGACUCGUCUA.AUAAUUACCUCCU.	2 (1)	0	2
A19	............UGUC.CCAACCAAUUCCCAGCUACAUUUUUACCCUGCCC	1	1	0
A20	**.....UGACUCCUCUGACAGCCUAAUUUCUCC..C..GAUUACCCUG..**	**25 (5, 4, 8, 1)**	**10**	**15**
A21	...GUCUAACUUGCCGCAAUUCCGGGAUCUGGUC.C..AAUCGU.....	1	1	0
A22	......CAACGUCUUUGUAUGUCUCAACUAGCAA.C.UAAACGGCCUG..	3 (1, 1, 1, 1)	0	3
A23	...ACGCGAUAAAAUCGAAUAACUUCC...ACAUCGA.AACCGACCA...	2 (1)	2	0
A24	..UAGUUAAACAGGUGAGAUAAACUCAC.AAUGUCCC.GAUUGG.....	4 (1, 1)	0	4
A25	.UGAAUGGCUCGCCUCCAAGACUCAAAUGGAUCAGC....CUAUC.....	1	0	1

Fig. 1. Sequence of aptamer clones from both hCCL1 and mCCL1 SELEX experiments. Sequences shown illustrate the 40-nucleotide variable region of each aptamer that were aligned resulting in 25 different clones (A1–A25). Numbers on the right hand-side indicate how many times a specific sequence of a clone occurred, in brackets are the number of clones that differed only by a single nucleotide sorted according to the order of the position underlined. "h" and "m" columns depict how many aptamers derived from the human or mouse CCL1 SELEX experiments. A20 family clone containing the T48 aptamer is in boldface.

present in the extracellular matrix. The resultant chemokine gradient provides a directional signal that the cells may use to navigate towards the site of inflammation, where they exert their effects [1]. The *in vivo* requirement for chemokine binding to GAGs in order to mediate cell recruitment has recently been demonstrated [27]. hCCL1, like many chemokines binds to heparin (one of most common GAGs) *in vitro*. In fact, affinity chromatography on a heparin–Sepharose matrix followed by reverse-phase HPLC was used to purify to homogeneity hCCL1 from culture supernatant [6]. It is known that different chemokines bind to GAGs with different affinities but no clear binding studies of either hCCL1 or mCCL1 to heparin have been ever reported in the literature. To determine if the high affinity aptamers generated in this study were capable of functionally antagonising CCL1, we first performed hCCL1 and mCCL1-heparin-binding studies *in vitro* using surface plasmon resonance (BIAcore). As shown in Fig. 3A, both hCCL1 and mCCL1 were able to bind to heparin in a dose-dependent fashion. Then, the ability of the CCL1 aptamers to block the binding of both the human and mouse chemokine to heparin was measured at a fixed CCL1 concentration (150 nM hCCL1 and 100 nM mCCL1). Several aptamers were able to functionally antagonise the binding of either human or mouse CCL1 to heparin in a dose-dependent fashion (data not shown). Interestingly, T48 was found to be the only aptamer able to completely block both hCCL1-heparin (IC50: 100–200 nM) and mCCL1-heparin (IC50: 25–50 nM) interactions. The random control aptamer showed no significant activity (Fig. 3B). The specificity of T48 function was also tested against human and mouse CXCL10 chemokine. No functional antagonism of these unrelated chemokines were noted (data not shown), in agreement with the usual high specificity of aptamers.

Cell-based functional characterisation of the T48 aptamer

The chemotaxis assay probably constitutes the most relevant assay when studying chemokine function. In order to test the ability of the T48 aptamer to antagonise CCL1-induced chemotaxis, BaF3-hCCR8 transfectants were generated. In agreement with the above studies, the T48 aptamer but not a random control aptamer blocked hCCL1-induced chemotaxis in a dose-dependent fashion (Fig. 4, IC50: 336 nM).

In summary, the *in vitro* selection of the T48 aptamer and its characterisation *in vitro* and in a cell-based assay

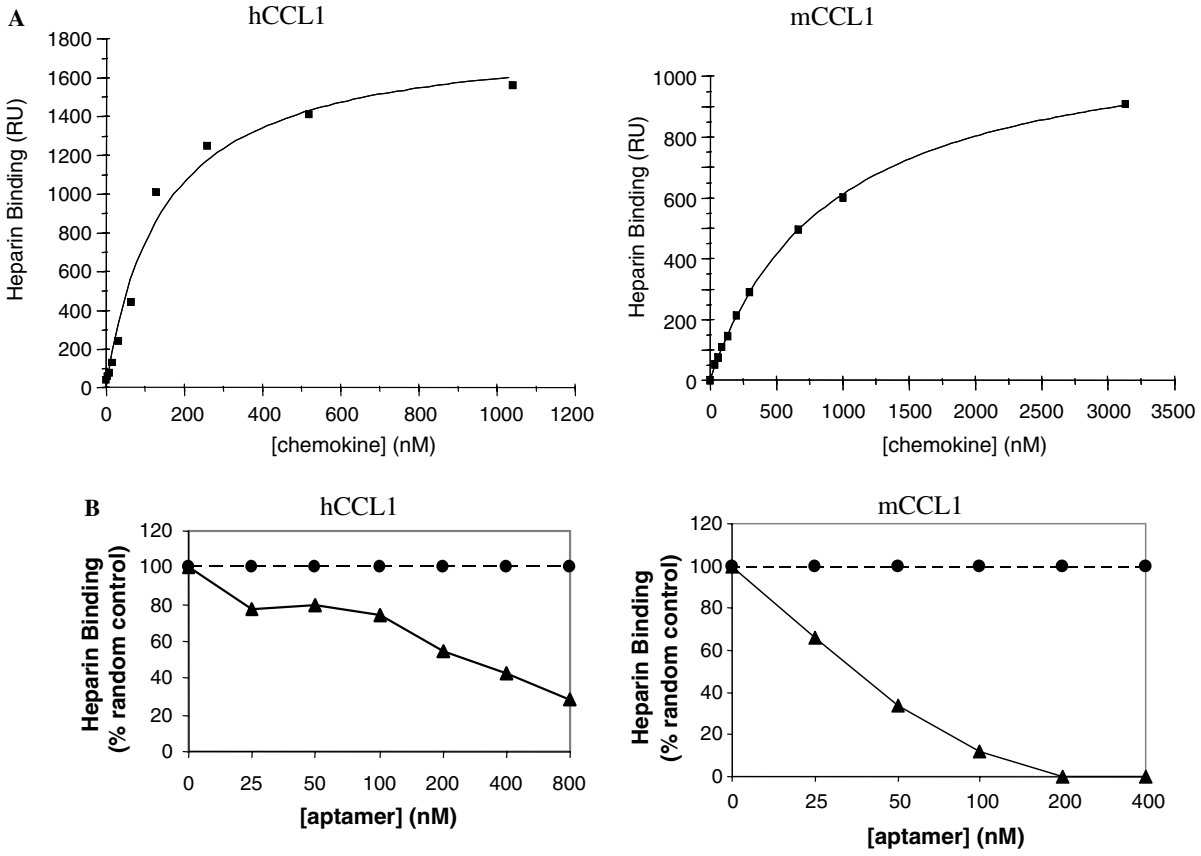

Fig. 3. Functional characterisation of the T48 aptamer *in vitro*. (A) Dose-dependent heparin binding of hCCL1 and mCCL1 chemokines (■) by using surface plasmon resonance (BIAcore). (B) Dose-dependent T48 aptamer (▲, solid line) blockade of heparin binding to hCCL1 and mCCL1 chemokines. Each point represents the sensorgram peak which is expressed as a percentage of the random control value, which is shown as (●, dashed line).

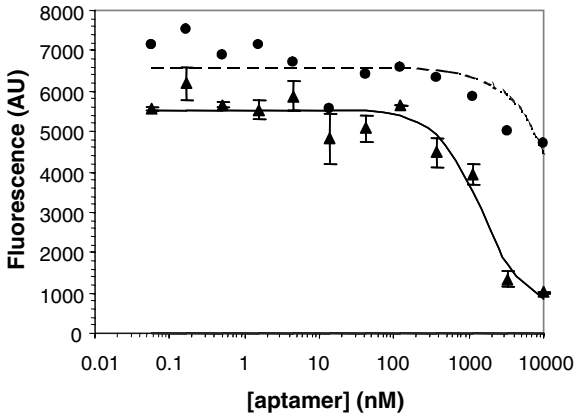

Fig. 4. Functional characterisation of the T48 aptamer in a cell-based assay. T48 aptamer (▲, solid line) antagonism of hCCL1-induced chemotaxis in BaF3 cells expressing hCCR8. Compared with the random aptamer control (●, dashed line). This experiment was carried out in duplicate. Error bars represent SE.

has resulted in the identification of an excellent candidate for future studies of human and mouse CCL1 function.

Discussion

The elucidation of the role of chemokines and their receptors in both normal physiology and in disease states has previously been approached in different ways. However, the range of activity in the chemokine network is highly complex and involves several levels of redundancy and as a consequence, there is a growing interest in unravelling the function of individual chemokine ligands.

The deletion of a gene encoding a protein of interest from the germ line provides a powerful tool for the understanding of protein function. However, the generation of knockout animals is technically complex and time-consuming and can lead to spurious observations due to genetic adaptation. The deletion of an inducible chemokine does not appear to generate an overt phenotype until subjected to conditions of stress, associated with models of disease [28]. In addition, unexpected results can be obtained with knockout models, in particular in the case of CCR8 knockouts [12,14,15]. As mentioned before, CCL1 neutralisation studies have also proven to be controversial.

To complement the technologies available to study the biological function of a chemokine, techniques have been developed that allow the generation of specific peptide or nucleotide aptamers to target proteins. In contrast to antibody generation, aptamers can be generated rapidly using an *in vitro* selection procedure. In this study, we have focused on the generation and characterisation of RNA aptamers to better understand the biological function of CCL1. Our aim was the generation of a single molecule that could be useful in a mouse model of disease but also with the possibility of extending its application in humans. To achieve this, two SELEX experiments were carried out in parallel using either hCCL1 or mCCL1 chemokines. RNA molecules capable of binding both orthologues in a specific way were then identified. Although several aptamers bound to their targets with high affinity in this study, we report here the isolation of T48, the only RNA aptamer with high affinity in a nitrocellulose-binding assay for both human and mouse CCL1 that acts to completely antagonise CCL1 function. Interestingly, this aptamer belongs to one of the family clones with the most frequent number sequences (Fig. 1). Although a number of these aptamers interacted with both human and mouse CCL1 in the nitrocellulose-binding assay, they were not able to completely antagonise CCL1 function in the assays used.

An important step in chemokine-mediated leukocyte recruitment is the immobilisation of the chemokines on cell surface GAGs [28]. In addition to the high-affinity receptor interaction, chemokines have an *in vivo* requirement to bind to GAGs in order to mediate directional cell migration [1]. To our knowledge, we have characterised for the first time, the dose-dependent binding of heparin to human and mouse CCL1. We then demonstrated that T48 was able to completely block the heparin binding to both human and mouse CCL1 with high potency but did not have any effect on the function of the unrelated chemokines, human and mouse CXCL10.

Additionally, T48 was able to antagonise hCCL1 function in a dose-dependent fashion in an hCCL1-induced chemotaxis system, which is the most important assay when characterising any chemokine antagonist (Fig. 4). This result is in agreement with the binding (Fig. 2) and BIAcore (Fig. 3) studies.

In summary, we report here the isolation of a nuclease resistant selective RNA aptamer (T48) with high-binding affinity for both human and mouse CCL1 that is able to completely antagonise CCL1 function in two assays. The T48 aptamer constitutes an excellent tool to dissect CCL1-specific function. In addition, the T48 aptamer could also be beneficial in situations where exaggerated CCL1 synthesis is harmful and may therefore have therapeutic potential. For instance, the T48 aptamer could be used in conjunction with the anti-CCL2 aptamer [20] and anti-CXCL10 aptamers [21], both developed in our lab, in order to dissect the role of different chemokines in atherosclerosis. Moreover, the T48 aptamer molecule might be useful in determining how CCR3, CCR4, and CCR8 interact to mediate the recruitment of Th2 cells and eosinophils to the allergic lung in a mouse model. Its ability to interact with human CCL1 could permit similar human studies. A secondary structure prediction of the T48 aptamer using the M-fold programme has generated a rod-like structure composed of four loops intercalated by three stems of varying lengths (Fig. 5). This structural information could assist a future truncation strategy for this aptamer that would be required before analysis of *in vivo* activity.

Considering the contradictory target validation studies reported in the literature concerning the CCL1/CCR8 axis, we strongly believe that the wider use of reagents in several

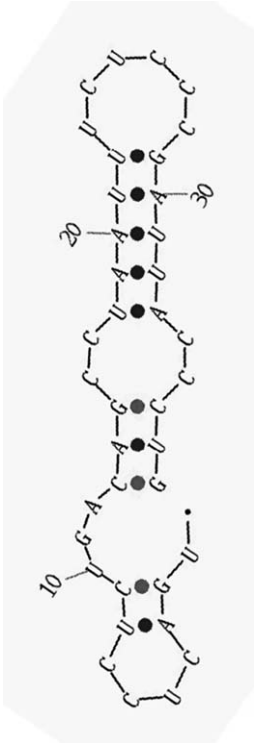

Fig. 5. Secondary structure prediction of the T48 aptamer core region. The putative structure was determined using the MFOLD programme.

models is likely to answer these apparent contradictions and the T48 aptamer could be an excellent candidate to achieve this.

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