The generation and characterisation of antagonist RNA aptamers to MCP-1

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Abstract Monocyte chemoattractant protein-1 (MCP-1) has been implicated as a powerful pro-inflammatory mediator and may represent a potentially important, therapeutic opportunity for treatment of inflammatory disease and atherosclerosis. To further investigate the role of MCP-1 in inflammatory disorders we have isolated a series of RNA aptamers that bind specifically to mouse MCP-1. The highest affinity aptamers, designated ADR7 and ADR22, have been functionally characterised in vitro and in cell based assays. ADR7 and ADR22 have an affinity of 180 pM and 370 pM respectively for mouse MCP-1, they can antagonise MCP-1 binding to heparin and specifically antagonise MCP-1 induced chemotaxis in a cell based assay. An interesting feature of ADR22 but not ADR7 is that it is capable of antagonising the function of human MCP-1, demonstrating the high level of specificity of these aptamers and that the aptamers recognise MCP-1 in different ways. The aptamers may be used as a tool to further investigate the role of MCP-1 in inflammatory disorders and may also have a role as a therapeutic agent.

Key words: Aptamer; Monocyte chemoattractant protein-1; Proteomics; CCR2; Inflammation

1. Introduction

Monocyte chemoattractant protein-1 (MCP-1), a β/CC chemokine, is responsible for the recruitment of monocytes, T-cells, eosinophils and NK cells. Chemokine family members are 8–10 kDa basic heparin binding proteins that are related in both primary structure and by the position of four cysteine residues. MCP-1 is suspected as being involved in a number of inflammatory disease states [1]. Inflammation in a number of animal models including lung granuloma [2], lipopolysaccharide induced death [3], glomerulonephritis [4], delayed type hypersensitivity in the skin [5] and arthritis in mice [6] can be reduced using neutralising antibodies. MCP-1 has been shown to be elevated in the inflamed synovium of rheumatoid arthritis patients and can be reduced upon treatment with anti-arthritic drugs [7,8]. MCP-1 is elevated in asthmatic patients and there is a correlation between severity of symptoms and level of MCP-1 [9,10]. High levels of MCP-1 are also found in atherosclerotic lesions of humans and cholesterol fed rabbits [11,12]. The above observations and mouse knockouts of either MCP-1 or its receptor CCR2 [13,14] demonstrate that a reduction of MCP-1 activity may be beneficial in treating many inflammatory diseases and atherosclerosis.

The systematic evolution of ligands by exponential enrichment (SELEX) procedure is a protocol in which single stranded oligonucleotides are selected from vast libraries of sequences, based on binding affinity at a target protein or other molecule [15–17]. The SELEX procedure is usually initiated with an RNA or DNA library consisting of some 10^{14}–10^{15} random oligonucleotide sequences. In a fully randomised oligonucleotide library, each molecule will exhibit a unique tertiary structure which will be dependent on the nucleotide sequence of that molecule. The binding affinity of the oligonucleotide for the target protein will be determined by the fit between moieties on the surface of the oligonucleotide and epitopes on the target protein. As a consequence of starting from a library of vast diversity it is often possible to identify aptamers of nM or sub-nM affinity for the target protein and with selectivity for that target protein over other proteins with a high degree of structural homology [18]. Using SELEX methodology RNA or DNA aptamers have been generated to many proteins, peptides and small molecules including dopamine [19], substance P [20], subtilisin [21], platelet derived growth factor [22], vascular endothelial growth factor [23], thrombin [24] and L-selectin [25].

Aptamers have been demonstrated to have biological activity, both in vitro and in vivo. For example an RNA aptamer to human oncostatin M (OSM) [26] was able to block OSM induced signalling in a variety of cell based assays and OSM, receptor binding in in vitro assays. In a second example high affinity DNA aptamers have been raised against platelet derived growth factor (PDGF) and used to investigate the role of PDGF in renal disease [27]. The modified aptamer was evaluated in a rat mesangioproliferative glomerulonephritis model. The aptamer lead to 95% reduction in proliferating mesangial cells and a 78% reduction in mitotic activity within the glomerular.

In view of the recent advances in SELEX methodology and numerous reports demonstrating the successful generation of aptamers against proteins we decided to use the SELEX procedure to generate RNA aptamers to MCP-1. We report the isolation of a series of RNA aptamers with high binding affinity for human and mouse MCP-1 that act to antagonise MCP-1 function in a number of in vitro and cell based assays.

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2. Materials and methods

2.1. SELEX

Iterative rounds of selection/amplification were performed as described [28] except that protein was bound to magnetic beads (Dynal) for the partition phase of the SELEX process. 2′-Fluoropyrimidine modified RNA containing a randomised region of 40 nucleotides was prepared by in vitro transcription from synthetic random DNA templates. Template DNA (5′-gggaggacgaugcgg-40N-ccgcatcgtcctccc-3′), designated 40N7, was prepared on an automated solid phase synthesiser (Applied Biosystems) according to the manufacturer's protocol.

For the SELEX experiment mouse MCP-1 (R&D Systems, Minneapolis, MN, USA) at 3 μg/μl was diluted to the required concentration in SCHMK buffer (110 mM NaCl, 1 mM $MgCl_2$, 20 mM HEPES pH 7.0, 1 mM $CaCl_2$, 5 mM KCl) and incubated with 100 μl M-450 Dynabeads (Dynal) overnight at 4°C to allow hydrophobic bead binding. Prior to incubation with MCP-1, the Dynabeads were washed three times (500 μl) with 100 mM KPO_4, pH 8.0, followed by one wash (500 μl) with SCHMK buffer. All washes were carried out using a Dynal MPC-E magnetic particle concentrator. Following overnight incubation, unbound MCP-1 was removed and beads were washed five times with 500 μl SHT buffer (1×SCHMK buffer, 0.001% HSA, 0.05% Tween 20). The beads were resuspended in 400 μl 1×SHT buffer and stored for up to 14 days at 4°C. For each SELEX round, RNA, diluted in 50 μl wash buffer, was added to beads in individual Eppendorf tubes at the concentrations shown in Table 1. Tubes were incubated at 37°C for 30 min after which individual tubes were washed six times (500 μl each) with 1×SHT buffer at 37°C to remove unbound RNA. To elute specifically bound RNA 20 μl water was added to individual tubes and the tube heated at 95°C for 10 min, the water containing eluted RNA was then removed from the magnetic beads to a fresh tube using the magnetic particle separator. Reverse transcription was then carried out, followed by PCR and transcription to generate RNA for the next round of SELEX as described previously [28].

2.2. Dissociation constant measurements

Binding assays were carried out by nitrocellulose filter partitioning as described [28].

2.3. Cloning and sequencing

Aptamer DNA was cloned using the TA cloning kit (Invitrogen) in the plasmid pCR2.1 according to the manufacturer's instructions. Plasmid clones were sequenced according to a PCR sequencing protocol [29].

2.4. Construction of the CCR2 G_{16} stable CHO cell lines

CCR2 was cloned at Glaxo using PCR and its sequence confirmed to be identical to the published cDNA sequence [30]. The CCR2 gene was cloned in a bi-cistronic expression vector pCIN [31] that conferred resistance to the antibiotic G418 and was introduced using standard methods [31] into a CHO host cell line that expressed the G_{16} G protein from a bi-cistronic vector that conferred resistance to the antibiotic hygromycin [32].

2.5. Assay for MCP-1 binding to heparin measured by surface plasmon resonance

Surface plasmon resonance measurements were conducted using a Biacore 2000 instrument at 25°C. SCHMK buffer (110 mM NaCl, 1 mM $MgCl_2$, 20 mM HEPES pH 7.0, 1 mM $CaCl_2$, 5 mM KCl) was used as a running buffer and diluent in the preparation of injection samples. Heparin was coupled to a sensor chip by injecting biotinylated heparin (prepared at Glaxo Wellcome, Stevenage, UK) across a streptavidin coated dextran surface (SA chip) at 2 μg/ml and a flow rate of 5 μl/min until an immobilisation level of 200 response units (RU) was achieved. The immobilised heparin surface and a control surface were then blocked by injecting a 50 μg/ml solution of biotin for 8 min. Sensograms for MCP-1 binding to heparin were obtained by injecting MCP-1 (Peprotech EC Ltd, London, UK) at 20 nM over both surfaces and subtracting the control channel data from the heparin channel data using Biaevaluation software (Biacore AB, Uppsala, Sweden). Flow rates of 10–20 μl/min and injection times of at least 4 min were used to obtain near equilibrium levels of binding. The inhibitory effects of adding aptamers to the MCP-1 prior to injection were observed and the reduction in maximum binding response calculated as a percentage of that of control responses obtained before and after the test injections. After each injection of MCP-1 the surfaces of the Biacore chip were regenerated with a 2 min pulse of NaCl at 1 M concentration.

2.6. Fluorometric imaging plate reader (FLIPR) assays

All experimental steps were conducted at 37°C unless stated otherwise. The stable Chinese hamster ovary (CHO) human CCR2 cell line was maintained in DMEM F-12 medium supplemented with 10% foetal calf serum, 2 mM L-glutamine and 1 mg/ml G418. A microtitre plate based Ca^{2+} mobilisation FLIPR assay [33,34] was used for the functional characterisation of the CHO hCCR2. Cells were plated at a density of 2×10^5 cells/ml into black 96-well viewplates (Packard Biosciences) 24 h prior to the assay. The following day the cells were washed once with 100 μl of FLIPR medium (10 mM HEPES, 10 mM glucose, 3 mM probenecid, 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$) and 200 μl of 1 μM Fluo 4AM (Molecular Probes) in FLIPR buffer was added to the cells. Following 1 h incubation (37°C, 5% CO_2), the cells were washed twice with the same buffer without dye and 180 μl of buffer added to each well. 10 min later, 20 μl of 30 nM MCP-1 or MCP-1 plus aptamer was added. Plates were read every 1 s for 1 min on a FLIPR (Molecular Devices, Sunnyvale, CA, USA), with the agonist added at 10 s. Concentration–response curves or single agonist responses were generated by calculating peak fluorescence counts above background. For agonist responses to MCP-1 in the presence/absence of aptamer, agonist was incubated with aptamer/buffer for 30 min prior to addition to the cells. pEC_{50} values (negative log of the concentration of agonist producing 50% of the maximal responses) were determined by fitting data using a four-parameter logistic equation.

2.7. THP-1 transwell migration assay

Cells of the myelomonocytic line THP-1 (European Collection of Cell Cultures, Wiltshire, UK) were maintained in RPMI 1640, supplemented with 10% foetal bovine serum, at a density between 2×10^5 and 1×10^6 per ml. The assays were carried out in 96-well chemotaxis chambers fitted with a 5 μm pore polycarbonate filter (PVP free ChemoTX, Neuroprobe). Aptamer and chemoattractant were pre-incubated for 20 min at room temperature in SCHMK buffer, 32 μl of either buffer alone, chemoattractant or both aptamer and chemoattractant was added to each well. The filter was aligned with the pegs on the plate and placed over the wells. 50 μl of THP-1 cells, resuspended in maintenance medium at a concentration of 2×10^6 cells/ml was placed on top of the filter above each well. The chemotaxis plate was then incubated for 2 h at 37°C in a humidified atmosphere of 5% CO_2.

After incubation, cells remaining on top of the filter were removed gently with a pipette and replaced with 50 μl of 20 mM EDTA in PBS and incubated for 10 min at room temperature. The filter was carefully removed and 4 μl of Calcein AM (Molecular Probes, Leiden, The Netherlands) at a concentration of 42 μg/ml in PBS was added to each well. The plate was incubated for a further 20 min at 37°C. Cell associated fluorescence was measured in a Wallac Victor 1420 Multilabel Counter (Perkin-Elmer, Cambridge, UK) using 485 nm excitation and 535 nm emission wavelengths. A chemotaxis index was calculated by dividing the fluorescence of cells migrating towards chemoattractant by the fluorescence of cells migrating towards buffer alone. Each point represents the mean and standard deviation of four replicate wells.

3. Results

Mouse MCP-1 was chosen for aptamer generation as it was hoped that generated aptamers could be applied in mouse models of disease. Mouse MCP-1 expressed and purified from *E. coli* was purchased from R&D Systems. To generate high affinity RNA ligands to mouse MCP-1 six iterative rounds of SELEX were performed using the conditions described in Table 1. To ensure the isolation of high affinity aptamers the stringency of the SELEX was increased in later rounds by reducing the amount of protein in the experiment [28]. The binding affinity of RNA pools for the starting pro-

Table 1
Protein/RNA amounts used during each round of the SELEX experiment

Round of SELEX	Coated bead volume (μl)	RNA (pmol)
1	50	1500
2	30	500
3	10	200
4	20	250
5	20	250
6	20	250

tein was assessed after five and six rounds of SELEX. The fully randomised starting pool of RNA did not bind significantly to mouse MCP-1 at any concentration tested up to 100 nM. The apparent affinity dissociation constant (K_d) of the RNA pool for mouse MCP-1 was 4 nM after five rounds of selection, and was 9 nM after six rounds of selection. As there was no significant change in affinity the SELEX was assumed to have reached completion. The round 6 pool was cloned and 60 individual aptamer clones were sequenced. The sequence from these clones was aligned using the Clustal software package of the GCG suite of molecular biology programmes (Fig. 1). Four large and four small sequence families were identified along with a number of orphan sequences that were only represented once in the final pool. To characterise individual aptamers, RNA was made from representative clones of each sequence family and each orphan by in vitro transcription and the binding affinity for mouse MCP-1 determined (Fig. 1). With the exception of one orphan (ADR45) all sequences bound mouse MCP-1 with an affinity of less than 20 nM. The binding curves for the two highest affinity aptamers (ADR7 and ADR22) and for random sequence RNA are

		K_d (nM)
ADR42	....AGGATC CACCTAGATG GCAATGAGGT CTATCACTTC TCTC......	
ADR50	....AGGATC CACCTAGATG GCAATGAGGT CTATCACTTC TCTC......	
ADR31	....AGGATC CACCTAGATG GCAATGAGGT CTATCACTTC TCTC......	
ADR1	....AGGATC CACCTAGATG GCAATGAGGT CTATCACTTC TCTC......	
ADR28	....AGGATC CACCTAGATG GCAATGAGGT CTATCACTTC TCTC......	
ADR6	....AGGATC CACCTAGATG GCAATGAGGT CTATCACTTC TCTC......	2
ADR51	....AGGATC CACCTAGATG GCAATGAGGT CTATCACTTC TCTC......	
ADR60	....AGGATC CACCTAGATG GCAATGAGGT CTATCACTTC TCTC......	
ADR14	....AGGATC CACCTAGATG GCAATGAGGT CTATCACTTC TCTC......	
ADR57	....AGGATC CACCTAGATG GCAATGAGGT CTATCACTTC TCTC......	
ADR9	....AGGATC CACCTAGATG GCAATGAGGT CTATCACTTC TCTC......	
ADR53	....AGGATC CACCTAGATG GCNATGAGGT NTATCACTTC TCTC......	
ADR30	....ANNACC CGCCNNTNTG CCNATNAGGT NNATCNGTTC TNTC......	2
ADR39	AGTGATCTTC CACCTAGTTG ACA..GAGGC TTAACACTAG AT........	2
ADR58	.....GATCT CGCGTAAGAG AGGGTCAC.C GCTCATCTGG TCTCGC....	
ADR17	.....GATCT CGCGTAAGAG AGGGTCAC.C GCTCATCTGG TCTCGC....	6
ADR27	......ATCG CAC.TACGCG AGAGTCACGC ATGCTTTGGG TCTCGCCCAGA	6
ADR21	..........CGAGTTAGCA ACG..TACCC CCACTT..AG CTCTCCTGCA ACGG	6
ADR12	......GCAA CATCTGCACA CCA..AGCTC AAAATTCGAG CCCTACCGC.	18
ADR33	.....CTTTC CGTCCCTGAC ACGT....AA CTATCACTTA CTCAACCCG.	
ADR62	.....CTTTC CGTCCCAGAC ACGT....AA CTATCACTTA CTCAACCCG.	
ADR4	.....CTTTC CGTCCCAGAC ACGT....AA CTATCACTTA CTCAACCCG.	
ADR38	.....CTTTC CGTCCCAGAC ACGT....AA CTATCACTTA CTCAACCCG.	0.4
ADR59	.....CTTTC CGTCCCAGAC ACGT....AA CTATCACTTA CTCAACCCG.	
ADR16	.....CTTTC CGTCCCAGAC ACGT....AA CTATCACTTA CTCAACCCG.	
ADR20	.....CTTTC CGTCCCAGAC ACGT....AA CTATCACTTA CTCAACCCG.	
ADR36	.....CTTTC CGTCCCAGAC ACGT....AA CTATCACTTA CTCAACCCG.	
ADR13	.....CTTTC CGTCCCAGAC ACGT....AA CTATCACTTA CTCAACCCG.	
ADR40	.....CTTTC CGTCCCAGAC ACGT....AA CTATCACTTA CTCAACCCG.	
ADR8	.....CTTTC CGTCCCAGAC ACGT....AA CTATCACTTA CTCAACCCG.	
ADR65	.....CTTTC CGTCCCAGAC ACGT....AA CTATCACTTA CTCAACCCG.	
ADR61	.....CTTNC CGTCCCAGAC ACGT....AA CTATCACTTA CTCAACCCG.	
ADR32	.....CTTTC CGTNCCAGAC ACGT....AA CTATCACTTA CTCAACCCN.	
ADR18	.....CTTTC CGTCCCAGAC ANGT....AA CTATCACTTA CTCAACCCG.	
ADR25	.......TAT AAATTCAGAT AAGTC..GAT CTATCGCGTA CGCGTCCGG.	18
ADR7	.....GAACT CACCGGGAAG AAGCCC.GTT CCGTCACAGA CATGTT....	0.2
ADR15	...GTCTTTC CGTCGCAGAC ATACCA...A TTGGTTCACA CTCCCG....	
ADR43	...GNCTTTC CGTCGCAGAC ATACCA...A TTGGTTCACA CTCCCG....	
ADR49	...GCCTTTC CGTCGCAGAC ATACCA...A TTGGTTCACA CTCCCG....	
ADR34	...GCCTTTC CGTCGCAGAC ATACCA...A TTGGTTCACA CTCCCG....	0.6
ADR11	...GCCTTTC CGTCGCAGAC ATACCA...A TTGGTTCACA CTCCCG....	
ADR41	...GCATTTC CGTCGCAGAC ATACCA...A TTGGTTCACA CTCCCG....	
ADR26	....CATTTC CGTCCCAGAC ATGCCATGAG TTGGCTCAGT GCCC......	0.6
ADR23	......GTTC AACCCTACCC CTGA.AGGTT CCGTCACTGA CATACCC...	2
ADR64	......GTTC CGTCACAGAA TATTAT..CC CCAGCGCGGG GTCAGCCC..	
ADR2	......GTTC CGTCACAGAA TATTAT..CC CCAGCGCGGG GTCAGCCC..	0.6
ADR5	.....ATGCA CATACCAAGG ATCT....AC CTATGACCCC CTACCACTG.	
ADR47	.....ATGCA CATACCAAGG ATCT....AC CTATGACCCC CTACCACTG.	1
ADR46	.......TCG GAGAACAATG CTCCGCAGAT CTGTCGCTAG TCCCCGC...	
ADR48	.......TCG GAGAACAATG CTCCGCAGAT CTGTCGCTAG TCCCCGC...	1
ADR35	......CACA TACTGGAAAG CTCCGTCG.T TTCTCAACGC CCAACGT...	0.6
ADR22	.AAAAATCTA GGCGCTGAAG CCCCGCTTCC TTACCTTCAC	0.2
ADR10	.AAAAATCTA GGCGCTGAAG CCCCGCTTCC TTACCTTCAC	
ADR44	.AAAAATCTA GGCGCTGAAG CCCCGNTTCC TTACCTTCAC	
ADR3	.TAAAATCTA GGCGCTGAAG CCCCGACTCT CTACTTCCCC C.........	1
ADR37	.TAAAATCTA GGCGCTGAAG CCCCGACTCT CTACTTCCCC	
ADR52	.......ACG CACTGTAAGG CTAC.AACCC GGGTCTCCGT TGCCTCCC..	4
ADR63	.......TGC TGCCACCAAC CTTATATGCC TGAGCTCACT CCGTCGC...	1
ADR19	.....ATACT TGC.TCGGGC CTGTGACCAC CTTTATCCGT AGCCCT....	18
ADR24	......AATT CCTCCTGCAG GTTTTAATAT AATTCTCCGT CGTGCT....	2
ADR45	......TGAG CGATTCTATA CTAC.AGGAT TGATCGAAAT ACCGGGC...	>100

Fig. 1. Sequence of aptamer clones. Only the 40N random region of each clone is illustrated. Sequences were aligned using the Clustal alignment programme from the GCG suit of molecular biology programmes. The equilibrium dissociation constant (K_d) of representative sequence groups is shown in the right hand column.

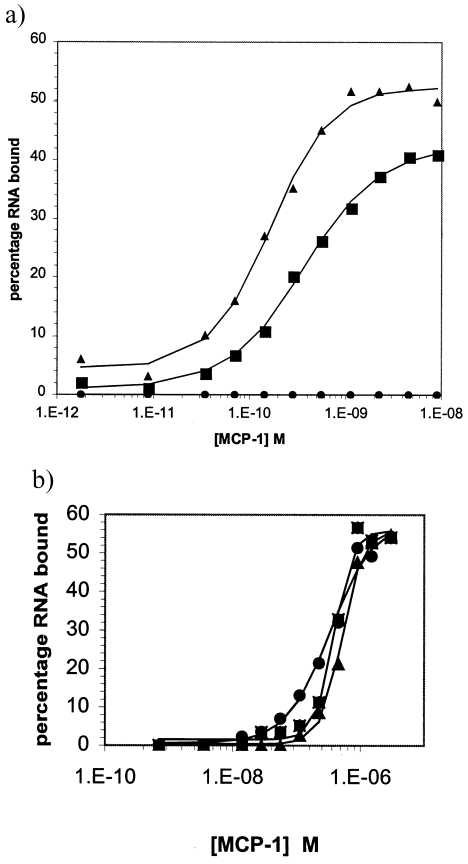

Fig. 2. Specificity of aptamer binding. Binding was carried out as described in Section 2. a: Binding of ADR7 (▲), ADR22 (■) and random RNA (●) to mouse MCP-1. b: Binding of ADR7 (▲), ADR22 (■) and random RNA (●) to human MCP-1.

shown in Fig. 2a. ADR7 and ADR22 bound with affinities of 180 pM and 370 pM respectively. This represents an improvement in affinity of at least 1000-fold over random RNA for the best aptamer clone. The affinity of ADR7 and ADR22 aptamer clones for human MCP-1 was then measured (Fig. 2b). The affinity of these aptamers for human MCP-1 is similar to that of random RNA for human MCP-1 (100 nM). To determine if the high affinity aptamers generated in this study were capable of functionally antagonising MCP-1, the ability of the aptamers to block the binding of MCP-1 to heparin was measured using a Biacore assay (Fig. 3). MCP-1 binding to heparin on the cell surface is thought to be an important initial step in the binding of MCP-1 to the receptor. It can be seen that both ADR22 and ADR7 functionally antagonise the binding of mouse MCP-1 to heparin down to a concentration equivalent to the concentration of MCP-1 injected (20 nM), indicating a high affinity interaction. ADR22 but not ADR7 also functionally antagonises the binding of human MCP-1 to heparin, however ADR22 shows a lower level of potency against human MCP-1 compared to mouse MCP-1, to which it was raised. The control aptamer (ADR147) is a sequence unrelated to the aptamer sequences evolved against MCP-1 and shows no activity in either assay. A common feature of RNA aptamers is the ability to distinguish between closely related proteins, thus the reduced potency against human MCP-1 is not surprising.

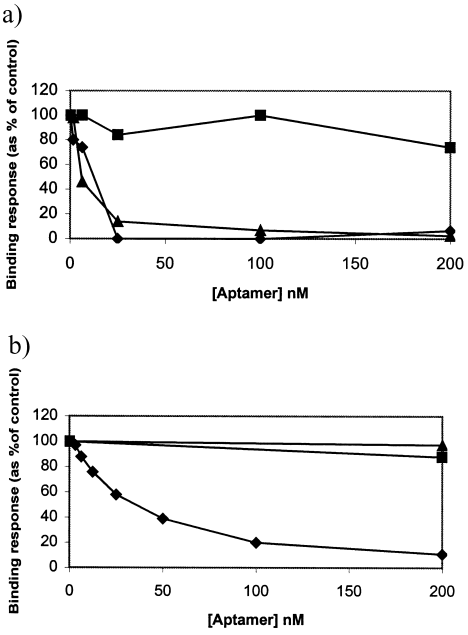

Fig. 3. Aptamer inhibition of heparin binding. Binding measurements were carried out by surface plasmon resonance using a Biacore 2000 instrument at 25°C. a: Inhibition of mouse MCP-1 binding to heparin by ADR7 (▲), ADR22 (♦) and random RNA (■). b: Inhibition of human MCP-1 binding to heparin by ADR7 (▲), ADR22 (♦) and random RNA (■).

The ability of ADR7 and ADR22 to antagonise human MCP-1 signalling in a CHO cell line was tested (Fig. 4). Calcium mobilisation within CHO cells, genetically engineered to express stably both human CCR2 and the human G protein G_{16}, was measured by fluorescence intensity plate reader (FLIPR) in response to human MCP-1. The results show that ADR22 but not ADR7 or ADR147 can block human MCP-1 signalling in a dose dependent fashion. The EC_{50} of ADR22 in this assay is low (164 nM) and this is consistent with the decreased efficacy of ADR22 in the human MCP-1 heparin binding assay. Unfortunately, a cell line expressing mouse CCR2 was not available for testing. It would however be predicted from the above results that both ADR7 and ADR22 would show efficacy in a FLIPR assay using such a cell line.

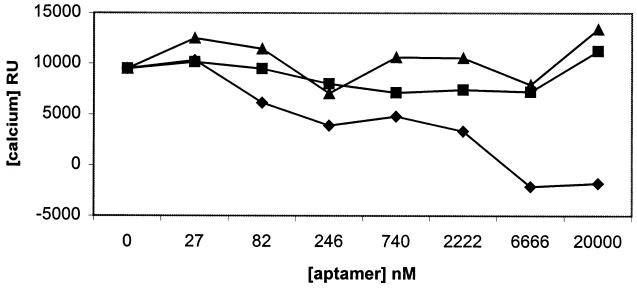

Fig. 4. Effect of aptamers on human MCP-1 signalling. Calcium mobilisation within CHO cells genetically engineered to stably express both human CCR2 and the human G protein G_{16}, was measured by fluorescence intensity plate reader (FLIPR) in response to human MCP-1 in the presence of ADR7 (▲), ADR22 (♦) and ADR147 control (■).

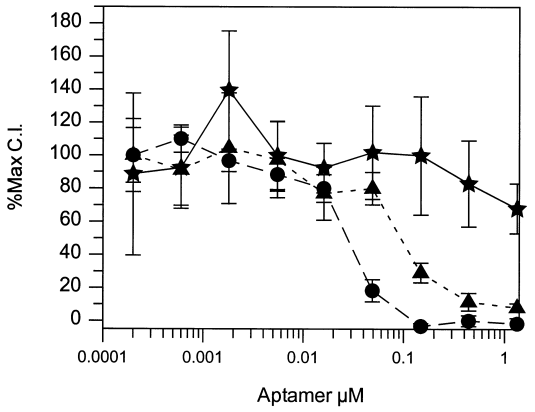

Fig. 5. Aptamer inhibition of chemotaxis. The assays were carried out in 96-well chemotaxis chambers fitted with a 5 μm pore polycarbonate filter using the myelomonocytic line THP-1. A chemotaxis index was calculated by dividing the fluorescence of cells migrating towards chemoattractant by the fluorescence of cells migrating towards buffer alone. Each point represents the mean and standard deviation of four replicate wells. ADR7 (♦), ADR22 (▲) and ADR147 control (★).

Finally the ability of ADR7 and ADR22 to antagonise mouse MCP-1 induced chemotaxis of the myelomonocytic cell line, THP-1, was tested. In this assay both ADR7 and ADR22, but not the control aptamer, ADR147, blocked mouse MCP-1 induced chemotaxis with a K_i of 3.5 nM and 14 nM respectively (Fig. 5). None of the aptamers were able to block human MCP-1 induced chemotaxis (results not shown), this is perhaps not surprising given the much reduced affinity and potency of the aptamers against human MCP-1.

4. Discussion

The elucidation of the role of a protein in both normal physiology and in disease states requires the generation of specific tools that are capable of regulating the activity of that target protein. A number of methods are available to do this including transgenic animals and antisense RNA. To complement these technologies, techniques have been developed that allow the generation of specific nucleotide aptamers to target proteins. In this manuscript we have used SELEX methodology to generate high affinity RNA aptamers to mouse MCP-1 that inhibit MCP-1 activity in vitro and in cell based assays. Following six rounds of SELEX an improvement in RNA affinity of over 1000-fold for mouse MCP-1 was achieved. As is often seen in a SELEX experiment a large variation in the affinities of aptamer clones selected from the round 6 SELEX pool was observed (180 pM to >100 nM). Surprisingly some clones that are very prevalent in the final RNA pool (e.g. ADR6) have amongst the lowest affinity for MCP-1. Although the primary force on aptamer selection is affinity for MCP-1, a number of other factors may explain this observation. Firstly the efficiency of transcription of a particular aptamer sequence by T7 RNA polymerase will influence its prevalence in the pool and secondly some clones may recognise epitopes on MCP-1 that change upon hydrophobic adsorption to the magnetic bead surface. The larger families of evolved aptamer sequence contain clones that differ in only one or two residues. It is likely that these have been generated through PCR mutation during the SELEX process resulting in a sequence that still binds MCP-1, rather than selection of highly homologous independent sequences from the starting library.

The specificity of the aptamer sequences is demonstrated by the observation that the affinity of ADR7 and ADR22 for human MCP-1 is about a 1000-fold lower than that for murine MCP-1. In fact there is little difference between the affinities of these aptamers and random RNA for human MCP-1. Murine and human MCP-1 have 56% amino acid sequence identity but mouse MCP-1 has an extra 53 amino acids in its C-terminal portion. Both ADR7 and ADR22 can antagonise murine MCP-1 function but only ADR22 can antagonise human MCP-1 function, although its potency is significantly lower than for murine MCP-1. The functional data are supported by the binding data and would suggest that ADR22 can bind specifically to human MCP-1 although its affinity is much lower than for murine MCP-1, it is likely that ADR7 binds to human MCP-1 in a non-specific manner possibly due to a charge effect. These results would also suggest that the MCP-1 binding site of ADR22 is different to that of ADR7.

It is likely that ADR7 and ADR22 can be truncated in length to facilitate solid phase synthesis [26] and that a substantial number of the purine residues could be substituted with 2′ *O*-methyl purine to further increase stability [26]. Once an aptamer has been truncated and maximally stabilised it is possible to make additions to the 5′ end to aid use of the aptamer in vivo. 5′ modifications could include addition of polyethylene glycol to decrease plasma clearance in vivo [35] or fluorescein or biotin for use in diagnostic applications [36]. The conjugation of a Tc99m chelating cage to ADR7 or ADR22 may enable the in vivo imaging of MCP-1 in inflammatory states [37].

ADR7 and ADR22 represent highly potent and selective functional antagonist of murine MCP-1. The aptamers generated in this study may be used to further probe the role of MCP-1 in normal physiology and diseased states such as rheumatoid arthritis. In addition to their use as validation tools, the aptamer can also be developed as a diagnostic agent and may be useful as a therapeutic molecule in its own right.

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