ANALYSIS AND SEPARATION OF RESIDUES IMPORTANT FOR THE CHEMOATTRACTANT AND ANTIMICROBIAL ACTIVITIES OF β-DEFENSIN 3

Karen Taylor 1, David J. Clarke2, Bryan McCullough3, Wutharath Chin2, Emily Seo2, De Yang3, Joost Oppenheim3, John R. W. Govan4, Dominic J. Campopiano2, Derek MacMillan5, Perdita E. Barran2 and Julia R. Dorin1

1MRC Human Genetics Unit, Edinburgh EH4 2XU, Scotland, U.K.
2School of Chemistry, University of Edinburgh, Edinburgh EH9 3JJ, UK
3Laboratory of Molecular Immunoregulation, Center for Cancer Research, Scientific Application and International Cooperation, Inc. (SAIC)-Frederick, National Cancer Institute at Frederick, Frederick, MD 21702, USA.
4Cystic Fibrosis Laboratory, Medical Microbiology, University of Edinburgh, 5Department of Chemistry, Christopher Ingold Laboratories, University College London, WC1H 0AJ, UK

Data deposition: Defb14 accession number is NM83026
Running title: Chemoattractant & antimicrobial studies on HBD3/Defb14

Address correspondence to Julia R. Dorin MRC Human Genetics Unit, Western General Hospital, Edinburgh EH4 2XU
Fax: (44) 131 467 8456. Tel: (44) 131 467 8411. E.mail: julia.dorin@hgu.mrc.ac.uk

Keywords: antimicrobial/β-defensins/chemotaxis/Defb14/HBD3

β-defensins are important in mammalian immunity displaying both antimicrobial and chemoattractant activities. Three canonical disulfide intramolecular bonds are believed to be dispensable for antimicrobial activity, but essential for chemoattractant ability. However, here we show that human β-defensin 3 (HBD3) alkylated with iodoacetamide, and devoid of any disulfide bonds, is still a potent chemoattractant. Furthermore, when the canonical six cysteine residues are replaced with alanine the peptide is no longer active as a chemoattractant. These findings are replicated by the murine ortholog Defb14. We restore the chemoattractant activity of Defb14 and HBD3 by introduction of a single cysteine in the fifth position (CysV) of the β-defensin six cysteine motif. In contrast, a peptide with a single cysteine at the first position (CysI) is inactive. Moreover, a range of overlapping linear fragments of Defb14 do not act as chemoattractants suggesting that the chemotactic activity of this peptide is not dependent solely on an epitope surrounding CysV.

Full length peptides either with alkylated cysteine residues or with cysteine residues replaced with alanine are still strongly antimicrobial. Defb14 peptide fragments were also tested for antimicrobial activity and peptides derived from the N-terminal region display potent antimicrobial activity. Thus, the chemoattractant and antimicrobial activities of β-defensins can be separated and both these functions are independent of intramolecular disulfide bonds. These findings are important for further understanding of the mechanism of action of defensins and for therapeutic design.

Human β-defensin 3 (HBD3, DEFB103) is a member of the β-defensin multigene family and is located in the major defensin locus on human chromosome 8 (1;2). β-defensins are small cationic antimicrobial peptides which constitute a major part of the innate immunity defence against pathogens(3). Several important in vivo experiments have shown that defensin molecules are important in antimicrobial defence(4-6). The peptide product from the DEFB103 gene, HBD3 was initially isolated from lesional psoriatic...
scales and has been cloned from both keratinocytes and lung epithelial cells although it is expressed in a variety of epithelial and non-epithelial tissues(1). HBD3 is a 45 residue mature peptide with potent antimicrobial activity against a broad spectrum of pathogens including multiresistant isolates and, unusually for a defensin, it appears to act in a salt insensitive manner(1). The antimicrobial action of HBD3 has been related to its high net charge (+11) coupled with its hydrophobicity (7).

Defensins have also been shown to have a potential role in adaptive immunity as well as innate immunity. Human β-defensin 1 (HBD1), HBD2, and HBD3 are chemotactic for CD4 memory T cells and immature (but not mature) dendritic cells (DCs) (8-10) by acting through the CCR6 chemokine receptor. Mouse β-defensin 2 (mBD2) is chemotactic for mouse immature DCs using CCR6 (11). It has also been shown that mBD29 acts as a chemottractant for the recruitment of DC precursors and immature DCs through interacting with mouse CCR6 in vivo (12). Thus CCR6 appears to be able to act as a receptor for β-defensins as well as the chemokine MIP-3α/CCL20. Although CCR6 has been shown to be the sole receptor for CCL20, HBD3 and HBD4 have been shown to be chemotactic for peripheral blood monocytes which do not express CCR6 and so must also act through another, as yet unidentified receptor (10;13).

Despite considerable variation between the sequences of β-defensin genes, the peptides share a striking similarity on the level of secondary and tertiary structure, suggesting that the fold is mainly stabilized by the presence of three disulfide bonds (14). Even orthologs such as HBD3 and the murine gene encoded peptide Defb14 are only 64% identical (see fig. 1) but the six cysteine motif is very highly conserved throughout evolution. β-defensins have an identifiable consensus sequence of \(X_2\alpha\alpha\alpha\alpha CX_5\alpha\alpha\alpha\alpha (G/A)XCX_5\alpha\alpha\alpha\alpha CX_9\alpha\alpha\alpha\alpha CX_5\alpha\alpha\alpha\alpha CCX_\alpha \) where \(X\) is any amino acid (15). The disulfide connectivities of synthetic or recombinant human β-defensin molecules, have been resolved for HBD1, HBD2, HBD3 and are C\(_1\)-C\(_5\), C\(_1\alpha\)-C\(_1\nu\), C\(_\alpha\\nu\)-C\(_\nu\\nu\) (10;16-18).

The antimicrobial activity of HBD3 has been shown to be independent of disulfide bonds (10). However, in the same study, it was also demonstrated that the chemotactic activity of HBD3 is influenced by the intramolecular disulfide connectivities. (10).

Previously we have reported a murine β-defensin peptide that does not have the first of the canonical six cysteines (19). The gene for this 5 cysteine peptide (Defr1) is a C57Bl/6 polymorphism of the 6 cysteine encoding Defb8 in other inbred strains of mice. The Defr1 peptide forms a covalent, disulfide-linked dimer with potent antimicrobial activity which is diminished upon reduction (20;21). This unique five cysteine defensin is also active as a chemoattractant (unpublished data). This interesting finding has prompted us to explore the minimum requirements for chemotaxis. Here we synthesise a range of peptide derivatives of HBD3 (see figure 1) and demonstrate that its chemoattractant activities are retained when the peptide is derivatized to prevent formation of disulfide bonds. Full length analogs of both human (HBD3) and mouse (Defb14) orthologs possessing only one of the six cysteines (cys \(\nu\)) retain comparable activity to the parental peptides. However linear fragments of HBD3 and Defb14 are not chemotactic. Peptide fragments of Defb14 were tested for antimicrobial activity and only those originating from the N-terminus are active.

Separation of the two principle activities of β-defensins has implications for therapeutic design.

**Experimental Procedures**

**Peptide synthesis**

All peptides were chemically synthesized by standard solid phase methodology. Defb14, Defb14-1c\^\* and HBD3 were obtained from Chemical Synthesis Services-Albachem Ltd (Gladsmuir, UK). Disulfide connectivities were determined by proteolysis and peptide mass mapping as previously published (10;20). Briefly, a 50 μM solution of the peptide was subjected to proteolytic cleavage with a combination of trypsin and chymotrypsin (Sigma) for HBD3. The digested peptides were analysed using LC-MS (Micromass Platform I) and by nano-electrospray using a Q-ToF. We also used a MALDI-TOF mass spectrometer (Voyager DE-STR (Applied Biosystems, Warrington, UK)), and the FT-ICR MS. Trypsin/chymotrypsin digestion of the CSS Albachem synthetic preparation of HBD3 allowed the N-terminus fragment (GIINTL, expected mass 629.37, measured mass 629.42) and the canonical C2-C4 peptide fragment.
([C2AVL][C3STR] expected mass 388.24 and measured mass 388.34) to be assigned and these are consistent with a canonical β-defensin fold. In addition the NMR signature of synthetic HBD3 (supplemental figure 2) is in agreement with that reported for the canonically folded HBD3 (22).

HBD3-1c\textsuperscript{5}, Defb14-1c\textsuperscript{5}, Defb14-0c, and the D14ip peptides were made “in-house” using automated peptide synthesis. This was carried out on an Applied Biosystems model 433A peptide synthesizer using Rink amide AM resin for peptide amides, pre-loaded NovaSyn\textsuperscript{®}TGT resin for peptide acids and Fmoc amino acids from Novabiochem. LC-Mass spectra confirming identity and purity were obtained on a Micromass Quattro LC mass spectrometer. Semi-preparative HPLC was performed using a Phenomenex Luna C18 column and a gradient of 5-95% acetonitrile (containing 0.1% TFA) over 45 minutes (flow rate of 3.0 mL/min). All other chemical reagents were obtained from Aldrich. Automated solid-phase peptide synthesis was carried out on a 0.05 mmol scale using 0.5 mmol of each Fmoc amino acid per coupling reaction and HBTU/HOBt as coupling reagents. Coupling time was 0.5 h. Peptide products were cleaved from the resin with 95 % TFA, 2.5 % ethanedithiol, 2.5 % water for 3 h, the resin filtered-off, washed with TFA, and filtrate poured into diethylether (10 volumes). Following centrifugation (3000 rpm, 15 mins) the precipitate was re-suspended in ether (5 volumes) and re-centrifuged (3000 rpm, 15 mins). The crude peptides were dissolved in water and loaded directly onto a semi-preparative HPLC column. Peptide fractions were identified by mass spectrometry and lyophilised.

Peptide oxidation
Peptides were dissolved in degassed Potassium Phosphate Buffer containing 10% DMSO, pH 8.1 in a sealed vial for 48 hrs to promote the oxidation of the cysteine to form an intermolecular disulfide bridge. Oxidation was monitored by LC-MS, and oxidised peptides were purified by HPLC, lyophilized and stored at -20 °C prior to use.

Peptide alkylation
HPLC purified peptides were treated with 1 molar equivalence of TCEP and left at room temperature for 30 minutes before treatment with 5 molar equivalents of iodoacetamide (IAA) and stirred at room temperature for 2 hours. The reaction was quenched by addition of 5 molar equivalents of DTT. LC-MS analysis of the peptides showed an increase in mass of 57 Daltons – consistent with thiol alkylation. Alkylated peptides were purified and stored in a similar way to the oxidised peptides above.

Mass Spectrometry Measurements
Accurate mass measurements were performed using a 9.4 T FT-ICR mass spectrometer (Bruker Daltonics, Bremen, Germany). The peptides were electrosprayed at a concentration of 20 µM from a solution of 50:49:1 water/methanol/acetic acid (v:v).

A Q-TOF mass spectrometer (Waters Micromass Technologies, Manchester, UK) was employed to obtain masses of all peptides and to assess the aggregation tendency of Defb14. All peptides were first prepared at 20 µM concentration in 10 mM ammonium acetate, pH 6.4 (Sigma Aldrich) and analysed by nano-electrospray MS. Defb14 was further analysed in varying buffer concentrations (1 mM, 10 mM, 25 mM and 50 mM – pH 6.4, 20 µM peptide conc.), pHs (pH 4, pH 6, pH 6.4, pH 7 and pH 9 – 10 mM ammonium acetate, 10µM peptide) and peptide concentrations (3.85 µM, 7.7 µM, 9.625 µM, 19.25 µM and 38.5 µM – 10 mM ammonium acetate, pH 6.4).

\textsuperscript{1}H NMR spectroscopy
1D \textsuperscript{1}H NMR spectra of peptides were measured using a Bruker 600 MHz Avance NMR spectrometer equipped with a 5 mm triple-resonance cryoprobe with z-gradients. Samples of 50 to 250 µg, were dissolved in 550 µl of a 9:1 mixture of H\textsubscript{2}O:D\textsubscript{2}O. The pH was adjusted to 3.5 - 4 and spectra were acquired at 298 K. A double pulse field gradient spin-echo (23) was used to suppress the water signal.

Chemotaxis Assay.
Mononuclear cells were isolated from human peripheral blood or bone marrow of normal donors by routine Ficoll–Paque density gradient centrifugation. The migration of monocytes, T cells, and CCR6-transfected human embryonic kidney (HEK)293 cells was assessed with a microchemotaxis chamber technique as described(8). Chemotactic activity was measured as the optimal concentration of test compound at which the highest chemotactic index value was obtained. Experiments were carried out a minimum of three times and medium alone was
used as a control or medium with 0.1-10 ng/ml N-acetyl carboxyamidomethyl cysteine. CCL20 was obtained from Peprotech EC (London UK).

**Defb14 expression by RT-PCR**

Total RNA was isolated from 14 tissues collected from both C57BL/6J and DBA mouse strains using RNAbee as described by the manufacturer (Biogenesis). cDNA synthesis was achieved using a 1st strand cDNA synthesis kit (Roche) and the resultant cDNA used as template in PCR reactions with the following primers. Forward primer: 5’TCTTGTTCTTGGTGCCCTGCT3’ Reverse primer: 5’TCTTCTTTGCCGACGATT3’ and internal oligonucleotide for hybridisation and product verification was 5’GGACGCATTCTACCAAAAA3’. The following conditions were used: denaturation at 94°C for 3 mins; 35 cycles of 94°C for 30 s: annealing at 53°C for 30 s and extension at 72°C for 1 min. The amplified products were analysed on 2% agarose gels by electrophoresis. To confirm RNA amplification, controls were included without reverse transcriptase. Template cDNA was amplified with primers for Hypoxanthine phosphoribosyl transferase (Hprt) as a positive control.

Southern blotting was performed using hybond-N membranes (Amersham) and hybridisation with radiolabelled oligonucleotide probes for 16 hours at 48°C. Filters were washed twice in 0.6M NaCl / 0.06M trisodium citrate (standard saline citrate) plus 0.5% SDS for 30 min before being exposed to photographic film.

**Antimicrobial assays**

These assays were carried out as previously described (20). Briefly, test organisms were grown to mid-logarithmic phase in Iso-Sensitest broth (Oxoid) growth media then diluted to 1.5 x 10^6 colony forming units (CFU)/ml in 10mM potassium phosphate containing 1% (v/v) Iso-Sensitest broth, pH 7.4. Different concentrations of test peptide were incubated in 100 μl of cells (1-5 x 10^8 CFU) at 37 °C for 3 h. Reduction of the peptides where performed, was done by adding 10mM dithiothreitol (DTT) and incubating at room temperature overnight. The oxidation state of each peptide was determined by mass spectrometry. 10-fold serial dilutions of the incubation mixture were spread on Iso-Sensitest plates, incubated at 37 °C, and the CFU determined the following day. The minimum bactericidal concentration (MBC) is the concentration of peptide where we observed >99.99% killing of the initial inoculum. All assays were performed in triplicate and repeated on two independent occasions. The MBC was obtained by taking the mean of all the results, and experimental errors were within one doubling dilution.

**Haemolytic assay**

Peptides in the concentration range 1–140 μM were incubated with washed human erythrocytes (2×10^7 cells) from a single donor in Dulbecco’s phosphate-buffered saline, pH 7.4 (100 μl) for 1 h at 37 °C. After centrifugation (12 000×g for 15 s), the absorbance at 541 nm of the supernatant was measured. A parallel incubation in the presence of 1% v/v Tween 20 was carried out to determine the absorbance associated with 100% hemolysis. The HC50 value was taken as the concentration of peptide producing 50% hemolysis.

**Results**

**HBD3 β-defensin derivatives as chemoattractants**

β-defensin peptides without disulfide bonds can chemoattract CCR6 expressing cells. HBD3 with canonical disulfide connectivity has previously been shown to chemoattract cells through the CCR6 receptor (10). We treated a full length reduced HBD3 peptide with iodoacetamide to prevent oxidation and disulfide bond formation. Iodoacetamide effectively ‘caps’ the thiol of the cysteine as a carboxyaminomethyl derivative. This full-length alkylated HBD3 peptide (HBD3-IAM) with six cysteines but without intramolecular disulfides could still chemoattract CCR6 expressing cells and had the same optimal concentration value as CCL20 (100 ng/ml) and the fully oxidised form (also 100ng/ml) (See Fig. 2). In order to investigate this intriguing result further, we synthesised an analog (HBD3-1cV) with five of the canonical six cysteines changed to alanines leaving only the fifth cysteine of the canonical cysteine motif (see Fig. 1). This HBD3-1cV peptide, like HBD3 (see supplemental figure 1) readily oxidises in DMSO to form a covalent dimer (see supplemental table 1) and when tested against Hek293 cells expressing the human chemokine receptor CCR6, its activity is equivalent to the activity of synthetic, oxidized
β-defensin HBD3 as shown in Fig. 2b and detailed in Table I.

To complement the experiments with HBD3, we then synthesised Defb14, its mouse ortholog, and the single cysteine analog Defb14-1cV. Defb14 is 64% identical to the HBD3 primary sequence (Fig. 1). Evolutionary analyses on the β-defensin superfamily have clearly demonstrated that Defb103 (the gene that encodes HBD3) and Defb14 are orthologs and there is a complex set of selective pressures acting on this gene family (24). Expression of Defb103 is localized to heart, skeletal muscle, placenta, oesophagus, trachea, oral mucosa, and skin tissues (1;25). We assessed the expression of Defb14 in various mouse tissues and a RT-PCR transcript is detected in the trachea, oesophagus, and strong expression is also evident in the testis and epididymis (see Fig. 3). Defb103 is subject to induced expression following exposure to microbe-derived molecules (26) and we find that a Defb14 transcript is weakly detectable in RNA samples taken from mouse skin after the addition of lipopolysaccharide (lps) (data not shown).

Table I compares the optimal concentration (ng/ml) and gives the migratory indices of HBD3, Defb14, the two single cysteine analogs (HBD3-1cV, Defb14-1cV) and a Defb14 control peptide with all the cysteines substituted by alanines (Defb14-0c). None of these peptides are chemoattractants for the control Hek293 cells but, the parent peptides Defb14, HBD3, and single cysteine analogues Defb14-1cV HBD3-1cV are equally chemotactic for the Hek293 cells that express the human CCR6 receptor. This activity is comparable to that which we observe with CCL20/MIP-3α (the only other chemokine known to interact with CCR6 (27)) with similar optimal concentrations. Each active peptide shows the classic bell shaped curve characteristic of a chemoattractant (Figs 2 and 4).

1D 1H 600 MHz NMR was used to analyse the structure of this set of related peptides (see Suppl. Fig.2). HBD3 displays resonances consistent with a folded protein and as reported for the canonically folded HBD3 (22). Moreover, the appearance of Hα resonances above the HOD signal indicates the presence of the β-sheet core in this protein characteristic of a β-defensin. In contrast, oxidized Defb14-1cV and Defb14-0c display NMR profiles suggesting that they are unstructured in solution. The cysteine-free peptide, Defb14-0c does not chemoattract the CCR6 expressing Hek293 cells (fig4b). The lack of chemoattractant activity of this cysteine-free peptide (Defb14-0c) agrees with the results of Wu et al (10) who showed that a HBD3 analog where all the cysteines were changed to α-amino butyric acid ([Abu-hBD3]) and without the ability to form intramolecular disulfides was also inactive as a chemoattractant. In summary, this series of peptides demonstrates that chemotaxis of cells expressing CCR6 is not dependent on the defensin adopting a discrete 3D structure.

Chemotaxis of Defb14 is dependent on CysteineV.

Having shown that β-defensin peptides with a single cysteine at position V in the six cysteine motif was active, to further explore the requirements for chemotaxis, we synthesised a peptide with a single cysteine at position I of the canonical β-defensin motif (Defb14-1cI (see Fig. 1 for sequence)). We chose CysI as it is the natural S-S partner for CysV in β-defensins. Interestingly, this peptide did not demonstrate chemotactic activity against CCR6 expressing cells (see Fig 4b). Since Deb14-0c and Defb14-1cI were inactive but Deb14-1cV was active in our assays it indicates that a peptide with a cysteine at this position in the canonical motif maybe sufficient for imparting chemotactic activity on a defensin.

Chemotaxis is not reliant upon covalent dimer structure of single cysteine analogs. The known ligand of CCR6, CCL20 (also known as MIP 3α) has recently been shown to be a non-covalent dimer in the crystalline form but the dependence of oligomerization state on chemotactic activity is unclear (28;29). To further investigate the role of oligomerisation of our peptides of interest, we assayed the active analogue of Defb14 (Defb14-1cV) and HBD3 (HBD3-1cV) in both monomeric form and as a S-S linked intermolecular dimer. Both peptides readily oxidised to form dimers with a single intermolecular disulfide-bond shown by mass spectrometry (supplemental table 1). Alternatively to ensure monomeric peptides that could not dimerise via a S-S bond, we modified each with iodo-acetamide (IAM). MS-MS techniques and high-resolution mass spectrometry of these purified derivatives confirmed efficient modification and no evidence of oligomerization (observation of a mass increased of +57 Da see supplemental Table 1)
Both the single cysteine peptides (HBD3-1cV and Defb14-1cV) displayed similar chemotactic properties in both monomeric and dimeric states (Figure 2b and 4a).

Defb14-1cV-derived peptide fragments do not act as chemoattractants. The discovery that the Defb14-1cV-IAM modified peptide still activated the CCR6 receptor led us to synthesise a range of peptide fragments (Defensin 14 inspired peptides; D14ips) inspired by this sequence (see figure 1). We found that the N-terminal half (D14ip1) and the C-terminal half of Defb14 (D14ip2), as well as a peptide which spans the two halves (D14ip7) did not act as chemoattractants (Table 1). Furthermore, the D14ip3 C-terminal fragment which contains the single cysteineV is inactive. We found that neither oxidation of D14ip3 to the S-S dimeric form, nor IAM treatment produced a peptide with chemotactic activity. Finally, we pooled D14ips 1 and 3 together but this equimolar preparation also does not act as an effective chemoattractant (Table 1). Taken together these data suggest that interaction of Defb14-1cV with CCR6 to stimulate chemotaxis requires residues from throughout the full length of the peptide. Single cysteineV analogs also chemoattract cells not expressing CCR6. HBD3 has been shown to be active against both the CCR6 chemokine receptor and another unidentified receptor present on monocytes (10). Defb14 and Defb14-1cV also chemoattract human monocytes (figure 5), and Defb14-1cV is as potent as that reported for the human β-defensin isoform of HBD3 (10). This implies that intramolecular S-S bonding is also not essential for the defensin-induced chemotactic response of monocytes which do not express the CCR6 receptor (10;30;31). The exact nature of the receptor mediating this reaction is currently under investigation.

HBD3 β-defensin derivatives as antimicrobial peptides

Linear peptides containing no disulfide bonds are potent antimicrobials. Assays against P. aeruginosa and S. aureus with Defb14 reveal that its antibacterial activity (MBCs of 1.5 and 3 μg/ml respectively, Table 2) is comparable with that reported for HBD3 (1;10;32). Unlike Defr1 (21), antimicrobial activity against both organisms is not altered upon reduction with DTT indicating that its antimicrobial action is independent of the disulfide oxidation state. Furthermore Defb14-0c, is as potent as oxidised HBD3, Defb14 and Defb14-1cV demonstrating unequivocally that the presence of disulfide bonds is not required for the antimicrobial action of HBD3 or its ortholog Defb14.

HBD3 has been demonstrated to retain its microbiocidal activity against S. aureus at physiological salt concentrations (1). The experiments described above were carried out under low salt conditions but we find that Defb14 and Defb14-1cV are also active against both pathogens at salt concentrations up to 250 mM (supplemental table 2).

The antimicrobial activity of Defb14 resides in the N-terminus. To further dissect the antimicrobial activity of Defb14-1cV we tested the peptide fragments (D14ips 1, 2, 3 and 7) described above in killing assays. The N terminal D14ip1 peptide displays the same potent activity as full length Defb14. In contrast, the C terminal peptides are inactive (MBCs > 50 μg/ml). Our findings are in contrast to previously published data on HBD3 and HBD3-derived fragments where only the entire peptide sequence retains activity against both Gram positive and negative organisms(33).

Haemolytic activity of Defb14 and Defb14-derived peptides The haemolytic activities of Defb14 and Defb14-1cV are very similar with a high HC50 of > 500 μg/ml. The D14ips also have low haemolytic activity with D14ip 1, 2 and 3 having an HC50 of 500 μg/ml (supplemental information figure 3).

Discussion

In this study we dissect the chemoattractant and antimicrobial abilities of HBD3 and its murine ortholog Defb14 using single cysteine derivatives and a series of peptide fragments.

Chemoattractant Function

Chemoattractant activity of HBD3/Defb14 is disulfide bond independent but cysteine V dependent. HBD3 and Defb14 have largely similar properties with both being able to chemoattract CD4+ T cells and monocytes. Both full-length, S-S oxidized peptides appear to act through CCR6 and an unidentified receptor on monocytes. We were surprised to discover that the full-length, single cysteine derivatives of both HBD3 and Defb14 that retained only the
fifth cysteine of the six canonical β-defensin cysteine residues, still displayed these chemoattractant properties.

Bauer et al (14) investigated the structure of two human and two mouse β-defensins by NMR and found that despite wide sequence variation they exhibit a virtually identical structure including a short aminoterminal α-helix starting at the first cysteine residue and a triple-stranded β-sheet. The length and relative arrangement of the three β-strands is highly similar. The presence of striking structural similarity despite high sequence variability suggested that the structural properties of the defensin fold are mainly stabilized by the three disulfide bonds.

Wu et al (10) have shown that intramolecular disulfide bonding is important for chemoattractant function of HBD3 and that altering the cysteine partners affects the chemotactic ability of this peptide. Indeed this probably explains why a recent report suggested that β-defensins did not act through CCR6 (34). The preparations used may not have had the precise, correct intramolecular disulfide bonding. In our hands HBD3 from the source in (34) does not chemoattract our Hek293 cells that express CCR6 to give a classic concentration dependent bell shaped curve (data not shown) although the HBD3 preparation we use here (see fig 2b) clearly does.

Wu et al also demonstrate that HBD3 without cysteines (hBD3-Abu) where the α-aminobutyric (Abu) acid residues that replace the cysteine residues cannot form disulfide bonds, does not chemoattract (10). The conclusion from this finding was that β-defensin disulfide bonding in HBD3 was required for binding and activation of receptors for chemotaxis. We show here that Defb14, the mouse ortholog of HBD3, with all the cysteines replaced by alanine (Defb14-0c) does not chemoattract through CCR6 which supports this conclusion. However, derivatisation of the parent hexa-thiol peptides with iodoacetamide to produce the carboxyaminomethyl-cysteine derivative (IAM, thus containing six capped cysteines unable to undergo disulfide bond formation) are still able to display chemoattractant activities against CCR6. These surprising results suggest that it is NOT the disulfide bond-constrained structure that is essential for chemotaxis, but the cysteine residues themselves.

We further demonstrate that cysteine V is sufficient to restore chemoattractant activity to the Defb14-0c peptide whereas cysteine I is not. Defb14-1cV has only the first cysteine retained and the remainder changed to alanine residues and is not active. In contrast, when the single cysteine retained is in position CysV (Defb14-1cV) then the peptide is as active as a chemoattractant as the parent molecule. Normally in β-defensin S-S connectivity, cysteine V (residue 40 of the mature peptide) would be a disulfide bond partner with the 1st cysteine (at residue 11) in the HBD3/Defb14 sequences. The presence of only cysteine V of the canonical six cysteine motif appears to allow a functional ligand receptor interaction. Either the residue itself interacts with the receptor and/or it allows the peptide to adopt a structural conformation that enables other residues in the peptide to interact functionally with the receptor. Alanine in place of cysteine V at position 40 does not replicate the chemotactic function of the Defb14-1cV peptide and neither does α-aminobutyric acid (10). The chemical nature of the more hydrophobic cysteine must confer a conformation upon the peptide that allows it to interact most favourably with the receptor. It is remarkable that this chemical difference is imparted by a single sulfur atom and activity is not lost when the residue is modified to carboxyaminomethyl-cysteine.

A recent comparison of the crystal structures of CCL20/MIP-3α (known ligand of CCR6) and HBD1 and 2 has revealed some structural similarities despite a lack of linear sequence similarity (29). An Asp4-Leu9 motif in HBD2, which resembles the Asp5-Leu8 motif of CCL20 is considered to be responsible for specific interaction with CCR6, providing a structural basis for the capacity of β-defensins and CCL20/MIP-3α to interact with the same receptor. This region flanks the first cysteine and yet HBD3-1cV and Defb14-1cV without cysteines at this first position are still active in attracting CCR6 expressing cells and Defb14-1cV, which retains that first cysteine, is not able to chemoattract.

Requirements for chemoattractant function for CCR6 are throughout the Defb14 peptide. The fact that the truncated D14ip peptide fragments do not have chemoattractant activity against CCR6 expressing cells implies that the ligand-receptor interaction necessary to allow chemotaxis is not mediated by a short continuous sequence. The lack of chemoattractant activity of
D14ip3 (even though it contains the fifth cysteine), demonstrates that the requirements for this are present throughout the peptide, whether this be specific residues or peptide length.

The chemokine MIP3-α and the defensins HBD1, 2 and 3, as well as Defb14 act through CCR6, a member of the 7 transmembrane GPCR superfamily (35). This receptor appears to display substantial promiscuity in the ligands which will stimulate a downstream chemoattract signal since we now show that a linear peptide, Defb14-1cV, is also active. Some receptor specificity is apparent; activity is dependent on residues along the entire length of defensins HBD3 and Defb14, which is also supported by recent elegant work by Pazgier et al (36) which analyzed the effect of single residue variants of HBD1 on CCR6-mediated chemotaxis properties. They show that residues involved in the interaction with CCR6 are distributed over most of the surface of the peptide although they did not subject the conserved cysteines to mutagenesis. It is also of interest that the single cysteine peptide is a strong chemoattractant for cells that express a different and as yet uncharacterised receptor. Exact details of molecular interactions of GPCRs and their respective ligands interaction are difficult to obtain since the structure of only two GPCRs structure has been determined.

Antimicrobial function

Our results show that the presence or absence of cysteine residues does not affect the antimicrobial properties of HBD3 or its ortholog Defb14. Both these peptides and their one cysteine analogs display equivalent activity against Gram positive and negative bacteria. Unlike the five cysteine peptide Defr1, the dimeric/monomeric status of the single cysteine peptides does not affect their antimicrobial activity (21).

The peptide fragments of Defb14 reveal that residues present only in the N-terminal half of the Defb14 peptide (fragment D14ip1) are active against both Gram positive and negative bacteria. This is in contrast to that observed with HBD3 where only the whole peptide sequence retains activity against both Gram positive and negative organisms (33).

Since the charge of the three peptide monomer fragments (D14ip1, 2, and 3) did not vary and all have a net charge of +6 we propose that the difference in activity between the N terminal and C terminal fragments may be due to the difference in hydrophobic properties of the fragments, as well as the fold presented by the amino terminus. The hydrophobicity scores of the inactive C-terminal fragments D14ip2 and 3 monomers (-12.35 and -11.94 respectively) are very similar to the parent molecule Defb14 (-11.51), and much lower for the inactive D14ip3 dimer (-23.88). However, the hydrophobicity of active peptide D14ip1 is markedly increased (-1.62).

Kluver et al (7) employ HBD3 to argue that increasing the net charge and hydrophobicity will increase the antimicrobial activity and additionally a greater hydrophobicity augments the cytotoxic effects. Thus less cationic HBD3 peptides with moderate hydrophobicity are virtually inactive, whereas peptides with a high net charge and significant hydrophobic character are active but cytotoxic. Our results are in partial agreement with this, however the haemolytic activity of the three peptides is very similar (with HC_{50} at greater than 500 μg/ml -see supplemental information figure 3) and none are cytotoxic.

An α-helix is often present in the N terminal stretch of full length oxidised β-defensins e.g. (HBD1-3). N-terminal regions of defensins are often more aliphatic, and since helices are a frequent structural motif in antimicrobial peptides it is likely that this is the region of the peptide which directs membrane insertion and disruption (37). It is also interesting to note that D14ip1 contains within it a stretch of eight amino acids (GGRAAVLN) which are identical to the XT4 peptide fragment of an antimicrobial peptide isolated from the skin secretions of the diploid frog (38). However this 22mer peptide is only active against Gram negative bacteria where D14ip1 is active against both Gram types.

The apparent irrelevance of the six cysteine motif and canonical disulfide bonding for both chemotaxis and antimicrobial function is is interesting since these six cysteines have been conserved throughout evolution and are a diagnostic of the defensin family (39). The S-S stabilised fold may be important either for some other as yet unknown function of these peptides but more probably to protect them from proteolysis in vivo as has been suggested for the cryptdins (40). Future studies will use the “minimum chemoattractant peptide” Defb14-1cV and its derivatives to further explore the interaction with CCR6.
In summary, we show here that HBD3 derivatives without the canonical six cysteine motif or disulfide bonding are active as chemoattractants. Derivatives of the murine ortholog Defb14 mirror this activity. A single cysteine residue at position five of the characteristic six β-defensin cysteine motif in both HBD3 and Defb14 is sufficient for their chemoattractant function but residues important in chemoattractant function may lie throughout the length of the peptide. In contrast, antimicrobial activity is located in the N-terminal region of the Defb14 peptide. Thus, we demonstrate here that for these peptides, the intramolecular disulfide bonds, although highly conserved in β-defensins are irrelevant for both chemoattractant and antibacterial function. In addition, the antimicrobial activity can be separated from the chemoattractant activity (see Figure 6). This has potential benefit for further understanding of the mechanism of action of defensins and design of therapeutic agents.

Reference List


Acknowledgements
This research was supported by the EPSRC, the Royal Society, Cystic Fibrosis Research Trust UK, MRC, The Royal Society of Edinburgh and the University of Edinburgh. We also thank Bob Bateman and Waters Micromass Technologies and the British Mass Spectrometry Society.
We thank both Professor Nick Hastie FRS for his enthusiasm for this project and Dr. Pat Langridge-Smith.

Figure 1: Sequence and properties of peptides.
Sequence, charge and hydrophobicity of human HBD3 and the mouse ortholog Defb14 β–defensin peptides and Defensin 14 inspired peptides.
Sequence alignment of human and mouse mature peptides are shown. * marks identical residues + indicates net charge of monomer and number in brackets indicates the charge of the dimer. ΔG the hydrophobicity score in water (kcal mol\(^{-1}\)) of monomeric peptides calculated using the scale of Wimley and White, with greater hydrophobicity indicated by a less negative value\(^{(41)}\) Figures in brackets indicate the values for molecules in their dimeric forms.

Figure 2:
**HBD3 derivatives without intramolecular disulfide bonds are effective chemoattractants for cells expressing CCR6.**
Graph to show the number of Hek293 cells expressing the human chemokine CCR6 cells that migrate towards the test peptides. The number of cells per high power field are given (No./HFP) and chemotaxis medium is given as a negative control. CCL20 is the chemokine ligand of CCR6. Fig2a- upper panel- shows that HBD3 when alkylated with iodoacetamide (HBD3-IAM) and unable to form disulfide bonds is still able to induce migration of the CCR6 expressing cells with the same optimal concentration as CCL20. N-acetyl cysteine-IAM is iodoacetamide treated N-acetyl cysteine and does not induce migration of these cells. Fig2b (lower panel) shows the migration of Hek293 cells expressing CCR6 exposed to oxidised HBD3 or the single cysteine derivative HBD3-1c\(^{V}\). The single cysteine peptide is either in its oxidised dimeric form (HBD3-1c\(^{V}\)Ox) or alkylated with iodoacetamide (HBD3-1c\(^{V}\)-IAM) to prevent the formation of disulfide dimers. Each assay was repeated three times and three fields of view taken for each experiment. Numerical data are means +/- standard deviation. Columns with an * indicate the peptide at that concentration is significantly better (p value less than 0.05) as a chemoattractant of the CCR6 expressing cells than culture medium.

Figure 3:
**Defb14 expression by RT-PCR**
Semi quantitative RT-PCR for Defb14 with Hprt as a loading control. Gels were blotted and hybridised with an internal oligonucleotide to verify the identity of the observed band. An RT-PCR transcript was detected in the trachea, oesophagus, and strong expression was evident in the testis and epididymis. The PCR gels were blotted and probed with an internal probe to verify the identity of the band. Band products were sequenced to also verify the clone identity.

Figure 4: **Defb14 derivative Defb14-1c\(^{V}\) is a strong chemoattractant for Hek293 cells expressing CCR6.**
The migration of Hek293 cells expressing CCR6 in response to peptides at different ng/ml concentrations. Shown is the average (mean ± SD) of three to five independent experiments, each of which was performed in triplicate and illustrated as the number of cells migrated per
high-power field (No./HPF). Peptides that induce migration of significantly (p value less than 0.05) more cells than control medium alone are indicated with an *.

**Figure 4a:** (upper panel) The Defb14-1cv peptide was oxidised with DMSO (Defb14-1cv-ox) or alkylated by treatment with iodoacetamide (Defb14-1cv-IAM) as described in experimental procedures. Both forms of Defb14-1cv have significantly greater chemoattraction activity than control medium alone for CCR6 expressing cells but Defb14-0c does not. **Figure 4b:** (lower panel) Defb14-1c (unlike Defb14-c and CCL20) does NOT significantly induce migration of CCR6 expressing cells.

**Figure 5:** Chemotaxis of human monocytes in response to Defb14 and Defb14-1cv

Graph to show the number of human monocytes that migrate towards the test peptides. The number of cells per high power field are given (No./HFP) and chemotaxis medium is given as a negative control and human fMLP a known chemotactic peptide for monocytes is the positive control. Defb14 and Defb14-1cv are oxidised monomer and dimer preparations respectively, synthesised by CSS Albachem. Each assay was repeated three times and six fields of view taken for each experiment. Numerical data are means +/- standard deviation. The optimal performance for Defb14-1cv is at 100ng/ml but it is 1000ng/ml for Defb14. All peptide concentrations except 0.1ng/ml of Defb14 show a statistically significant increase (p value less than 0.05) in chemoattractant ability compared to chemotaxis medium alone and this is indicated with an *.

**Figure 6:** Chemoattractant and antimicrobial activities of β-defensin 3 derived peptides

Summary of the activity of the various analogues and peptide fragments of HBD3 and its orthologue Defb14. The consensus structural components of HBD3 are given with the β-sheets indicated by arrows and the alpha helix by a wavy box. The position of the canonical cysteines are indicated by a C and replaced residues indicated by A for alanine, C* for iodoacetamide alkylated cysteine and Abu for α-amino-butyric acid.

**Table 1:** The chemotactic activity of HBD3 derivatives and related peptides.

The migration of various cells in response to various concentrations (0.1-10000 ng/ml in 10 fold dilutions) of HBD3, Defb14 and related peptides were tested on CD4+ T cells, and CCR6-expressing Hek293 cells, using a chemotaxis chamber assay as described in experimental procedures. The membranes used were: 5-μm uncoated for monocytes, 5-μm fibronectin-coated for T cells, and 10-μm collagen-coated for CCR6-expressing Hek293 cells. The incubation time for monocytes, T cells, and Hek293 cells was 1.5 h, 3 h, and 5 h respectively. Shown is the optimal concentration (ng/ml) that induced the maximal migration of a given target cell type. -, not tested. A minimum of three assays were done for each peptide and 3 fields of view counted. Dip1 and 3 is an equimolar mix of both peptides. Figures given in brackets are the migratory indices (ratio of migration in the presence peptide to migration in control medium alone).

**Table 2:** Bactericidal activity of Defb14 and related peptides against bacterial strains.

Values given are the minimum bactericidal concentrations (MBC), which are the lowest concentration of peptide by weight that is required for 99.99% of bacteria to die under the conditions described in the material and methods. Values in brackets for Defb14 and Defb141cv are those achieved after the peptide was treated with 10 or 50mM DTT and boiling. (–) indicates peptide not tested against that strain. A concentration of 1μg/ml equals 0.19 pM for Defb14 and 0.09pM for dimeric Defb14-1cv.
### Figure 1

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>charge</th>
<th>ΔG</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBD3-1c&lt;sup&gt;V&lt;/sup&gt;</td>
<td>GIINTLQYYARVRGGRAAVLSALPKEEQIGRASTRGRKCARRKK</td>
<td>+11 (+22)</td>
<td>-14.7 (-29.4)</td>
</tr>
<tr>
<td>HBD3</td>
<td>GIINTLQYYCRVRGGRCAVLSCPKEEQIGKCRSTRGRKCCRRKK</td>
<td>+11</td>
<td>-12.65</td>
</tr>
<tr>
<td>**</td>
<td>** ********************************** ** ** ** ** ** ** ** ** **</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Defb14</td>
<td>FLPKTLRKFCRIIRGGRCAVLCLGKEEQIGRCNSGRKCCRRKK</td>
<td>+12</td>
<td>-11.51</td>
</tr>
<tr>
<td>Defb14-1c&lt;sup&gt;V&lt;/sup&gt;</td>
<td>FLPKTLRKFFARIRGGRAAVNLGKEEQIGRASNNSGRKCARRK</td>
<td>+12  (+24)</td>
<td>-13.56 (-27.12)</td>
</tr>
<tr>
<td>Defb14-1c&lt;sup&gt;i&lt;/sup&gt;</td>
<td>FLPKTLRKFFCRIIRGGRCAVLCLGKEEQIGRASNNSGRKCCRRKK</td>
<td>+12  (+24)</td>
<td>-13.56 (-27.12)</td>
</tr>
<tr>
<td>Defb14-0c</td>
<td>FLPKTLRKFFARIRGGRAAVNLGKEEQIGRASNNSGRKCARRK</td>
<td>+12</td>
<td>-13.97</td>
</tr>
<tr>
<td>D14ip1</td>
<td>FLPKTLYRKFFARIRGGRAAVLNA</td>
<td>+6</td>
<td>-1.62</td>
</tr>
<tr>
<td>D14ip2</td>
<td>LGKEEQIGRASNNSGRKCAKKK</td>
<td>+6</td>
<td>-12.35</td>
</tr>
<tr>
<td>D14ip3</td>
<td>LGKEEQIGRASNNSGRKCAKKK</td>
<td>+6  (+12)</td>
<td>-11.94 (-23.88)</td>
</tr>
<tr>
<td>D14ip7</td>
<td>IRGGRAVNLGKEEQIGRAS</td>
<td>+2</td>
<td>-7.64</td>
</tr>
</tbody>
</table>
Figure 2
Figure 4

[Graph showing the effect of different concentrations of peptides on cell proliferation and cell migration.]
Figure 5
<table>
<thead>
<tr>
<th>Consensus</th>
<th>Chemotactant</th>
<th>AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBD3</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>HB3-IAM</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>HBD3-1c</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>HBD3-Abu</td>
<td>X</td>
<td>✓</td>
</tr>
<tr>
<td>Defb14</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Defb14-1c</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Defb14-1c-IAM</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Defb14-1c'</td>
<td>X</td>
<td>✓</td>
</tr>
<tr>
<td>Defb14-0c</td>
<td>X</td>
<td>✓</td>
</tr>
<tr>
<td>D14ip 1</td>
<td>X</td>
<td>✓</td>
</tr>
<tr>
<td>D14ip 2</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>D14ip 3</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>D14ip 7</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Table 1: Optimal chemotactic concentration (ng/ml) and migratory indices</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HBD3</td>
<td>HBD3-IAM</td>
</tr>
<tr>
<td>humanCD4⁺T cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mouseCD4⁺T cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hek293</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Hek293/CCR6</td>
<td>100(2.2)</td>
<td>100(2.2)</td>
</tr>
</tbody>
</table>
Table 2:

<table>
<thead>
<tr>
<th>Organism</th>
<th>HBD3</th>
<th>HBD3-1c</th>
<th>Defb14</th>
<th>Defb14-1c</th>
<th>Defb14-0c</th>
<th>D14ip 1</th>
<th>D14ip 2</th>
<th>D14ip 3</th>
<th>D14ip 7</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> PAO1</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5 (1.5)</td>
<td>1.5 (1.5)</td>
<td>1.5</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em> ATCC 25923</td>
<td>3.13</td>
<td>3.13</td>
<td>3.13</td>
<td>3.13</td>
<td>3.13</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td></td>
</tr>
</tbody>
</table>
Analysis and separation of residues important for the chemoattractant and antimicrobial activities of β-defensin 3


J. Biol. Chem. published online January 7, 2008

Access the most updated version of this article at doi: 10.1074/jbc.M709238200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2008/01/09/M709238200.DC1

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2008/01/07/jbc.M709238200.citation.full.html#ref-list-1