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Application of N–C- or C–N-directed sequential native chemical ligation to the preparation of CXCL14 analogs and their biological evaluation

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ABSTRACT

CXCL14 is a chemokine that exhibits chemoattractant activity for activated macrophages, immature dendric cells, natural killer cells, and epithelial tumor cells. Its potential role as a metabolic regulator has recently been disclosed. However, a complete understanding of its physiological roles remains elusive. This is partly due to the lack of appropriate CXCL14-based molecular probes to explore the biological functions of CXCL14. In this context, we have developed synthetic protocols that provide access to a wide variety of CXCL14 analogs. Two sequential native chemical ligation (NCL) protocols, which proceed in opposite directions, have been used to assemble CXCL14 analogs from peptide fragments. The first involved a conventional C–N-directed sequential NCL, and afforded wild-type CXCL14. The other used peptide thioacids in N–C-directed elongation, and yielded CXCL14 analogs with molecular diversity at the C-terminal fragment. The CXCL14 analogs prepared showed biological activity on human monocytic leukemia-derived THP-1 cells that was comparable to that of wild-type CXCL14.

1. Introduction

1.1. Structure and biology

Chemokines that induce chemotactic activity in leukocytes and lymphocytes can be classified into two main groups, CC and CXC. Categorization into the groups CC and CXC is made on the basis of the spacing between two N-terminal cysteine residues (C) that are either adjacent to each other or separated by one amino acid residue (X), respectively. CXCL14 (originally designated BRAK, BMAC, or Mip-2g) belongs to the CXC chemokine family and was originally found via cloning of its corresponding gene, whose expression is down-regulated in human cancer cell lines and tumor specimens.[1–3] Human CXCL14 consists of 77 amino acid residues including four cysteines (Fig. 1).

Various vertebrate CXCL14 orthologs have been identified and their primary amino acid sequences are highly conserved, especially in mammals: the sequence differs by only one residue between human and bovine or rat proteins, and by two residues between human and porcine or mouse proteins.[4,5] Several reports have shown that CXCL14 has chemoattractant activity for activated macrophages,[6] immature dendric cells,[7–9] activated natural killer cells,[10] and breast or pancreatic cancer-derived cells.[11,12] However, a recent study indicated that there was no significant difference in the total number of macrophages and dendric cells in the epidermis of CXCL14-deficient mice and wild-type mice.[13] More recently, the anti-microbial activity of CXCL14 was disclosed.[14] We have demonstrated that CXCL14 functions as an important chemoattractant in obese mice and recruits macrophages to the white adipose tissue. This affects the regulation of glucose metabolism negatively in obese mice, due, in part, to the inhibition of insulin signaling in skeletal muscle.[15] While the biological roles of CXCL14 have been partially elucidated, as mentioned above, the physiological significance of CXCL14 remains to be fully revealed. Additionally, the receptor responsible for mediating CXCL14 activities has yet to be identified. In this context, uncovering the functions of CXCL14 at the molecular level requires both a sensitive method to monitor CXCL14 activity and molecular probes to evaluate its functions. The former requirement has been addressed by our discovery of the anti-CXCL14 monoclonal antibody MAB730, which robustly enhances CXCL14-mediated chemotaxis and chemokinesis in human monocytic leukemia-derived THP-1 cells.[16] To address the latter requirement, the construction of a molecular library consisting of a wide variety of CXCL14 derivatives has been undertaken.

1.2. Chemistry

Native chemical ligation (NCL) has shown great utility in the field of peptide/protein chemistry. Since the development of NCL

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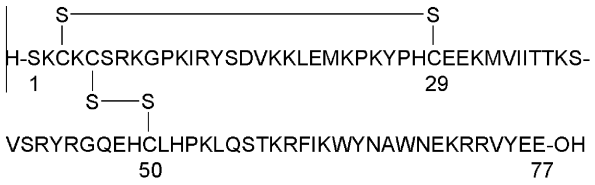
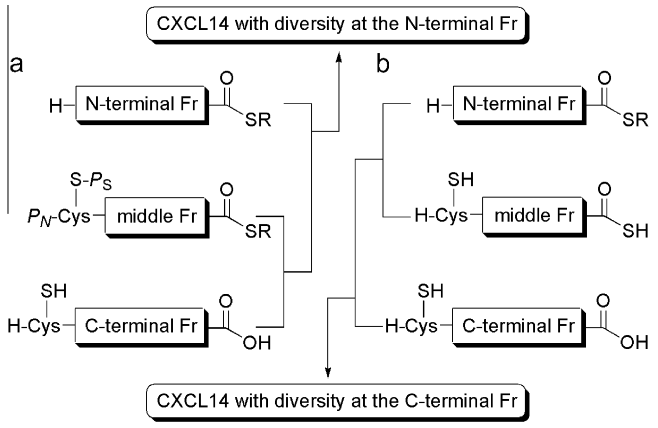
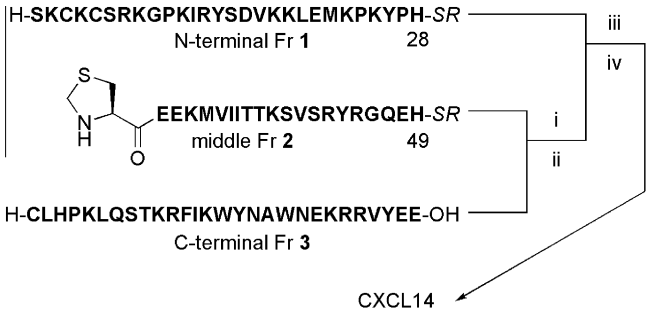


Figure 1. Primary amino acid sequence of human CXCL14.

by Kent and co-workers, the chemical synthesis of small proteins has become readily achievable.[17–19] The synthetic protocol features a chemoselective reaction of two unprotected peptide fragments, a peptide thioester and an N-terminal cysteinyl peptide, where intermolecular *S*–*S* and subsequent intramolecular *S*–*N* peptidyl transfer are involved. Extension of NCL protocol to the preparation of larger proteins requires a sequential NCL utilizing more than one thioester peptide.[20–22] Among sequential NCLs, a C–N-directed protocol using a thioester featuring a protected N-terminal cysteine residue has been widely used (Scheme 1a). Here, protection(s) of cysteine is essential to suppress undesirable intramolecular NCL. This protocol is favorable when the diversification of the N-terminal sequence is desired, as the N-terminal fragment is incorporated at a late stage in the fragment assembly. Conversely, diversification of the C-terminal sequence is more readily achieved by an N–C-directed sequential NCL (Scheme 1b). Recently, we developed an Fmoc-based synthetic procedure for the preparation of peptide thioacids.[23] The successful application of the resulting thioacid in N–C-directed sequential NCL is demonstrated here. The C-terminal sequence of CXCL14 (residues 56–73) is known to have an α-helical structure. Based on our speculation that the helical part should be involved in biological activity, we were interested in the rational structural manipulation of this α-helical part by the incorporation of intrahelical salt bridges between the amino acids pair (Glu–Lys).[24] The desire to generate analogs of CXCL14 that were modified in the α-helical portion prompted us to evaluate the applicability of the N–C-directed sequential NCL using peptide thioacid to the synthesis of CXCL14 analogs. Additionally, we attempted the C–N-directed protocol to generate wild-type CXCL14. In this article, we report the chemical synthesis of human CXCL14 and its analogs using N–C- and C–N-directed sequential NCL, and their biological evaluation.



Scheme 1. Two sequential native chemical ligations (NCLs). (a) C–N-directed NCL using N-terminal protected cysteinyl peptide thioester as the middle fragment (Fr) (P_N and P_S: protections for the amino group and the sulfanyl group, respectively). (b) N–C-directed NCL using N-terminal unprotected cysteinyl peptide thioacid as the middle Fr.



Scheme 2. Synthesis of CXCL14 using C–N-directed sequential NCL. (i) First NCL in 6 M Gn·HCl-0.2 M phosphate buffer (pH 6.8) in the presence of 1% thiophenol. (ii) 0.2 M HCl·H_2NOMe. (iii) Second NCL in 6 M Gn·HCl-0.2 M phosphate buffer (pH 6.8) in the presence of 1% thiophenol. (iv) Oxidation in 10% DMSO in 3 M Gn·HCl-0.1 M phosphate buffer (pH 7.7). R = $-(CH_2)_2-CO-Ala-NH_2$.

2. Results and discussion

2.1. Synthesis of CXCL14 using C–N-directed sequential NCL

As shown in Scheme 2, CXCL14 was assembled from the C-terminus by sequential NCL of peptide fragments **1**–**3**. The middle fragment, thioester peptide **2**, has an N-terminal thiazolidine carboxylic acid residue (Thz) as a protected cysteine,[25] which prevents the formation of undesired cyclic peptide. Protected peptide resins corresponding to thioester fragments **1** and **2** were prepared by Boc solid-phase peptide synthesis (SPPS) on sulfanylpropionic acid-incorporated 4-methylbenzhydrylamine (MBHA) resin.[26] For the incorporation of the Thz residue into the thioester, *N*-Boc protected material was used. Deprotection of each completed resin with 1 M TMSOTf/thioanisole (molar ratio 1:1) in TFA in the presence of *m*-cresol was followed by HPLC purification to yield thioesters **1** and **2**. Additionally, deprotection for **1** required Met-regenerating step, which was carried out by the addition of NH_4I and dimethyl disulfide.[27] The N-terminal cysteinyl fragment **3** was prepared by Fmoc SPPS-mediated peptide chain assembly followed by deprotection with a TFA-based reagent cocktail. With the three requisite fragments for construction of CXCL14 backbone in hand, we attempted the sequential C–N-directed NCL (Scheme 2 and Fig. 2). First, NCL of the Thz-containing thioester **2** with fragment **3** was carried out in 6 M guanidine hydrochloride (Gn·HCl)-0.2 M phosphate buffer (pH 6.8) in the presence of 1% thiophenol. The reaction was complete within 4 h and yielded ligated N-terminal Thz peptide **4**. Opening of the Thz ring of **4** was affected by the direct addition of methoxyamine hydrochloride ($MeONH_2$·HCl) to the ligation mixture to give N-terminal cysteinyl peptide **5**. After HPLC purification, **5** was subjected to the second NCL with thioester **1**, under the same conditions as those used in the first NCL. This yielded the crude, reduced form of CXCL14, **6**. After HPLC purification, the reduced form was subjected to a folding procedure in 3 M Gn·HCl-0.2 M phosphate buffer (pH 7.7)/DMSO (9:1, v/v) to afford folded CXCL14. After 6 h incubation at 37 °C, wild-type human CXCL14 was obtained directly from the reaction mixture by HPLC purification.

2.2. Synthesis of CXCL14 analogs using N–C-directed sequential NCL

The N–C-directed NCL for the synthesis of CXCL14 analogs is summarized in Scheme 3. The peptide fragments **1** and **3**, which were used in the C–N protocol, were also used for the N–C protocol. The requisite middle peptide, thioacid **7** with a ^{49}His/Gly mutation, was prepared by Fmoc SPPS, according to our recently reported method.[23]

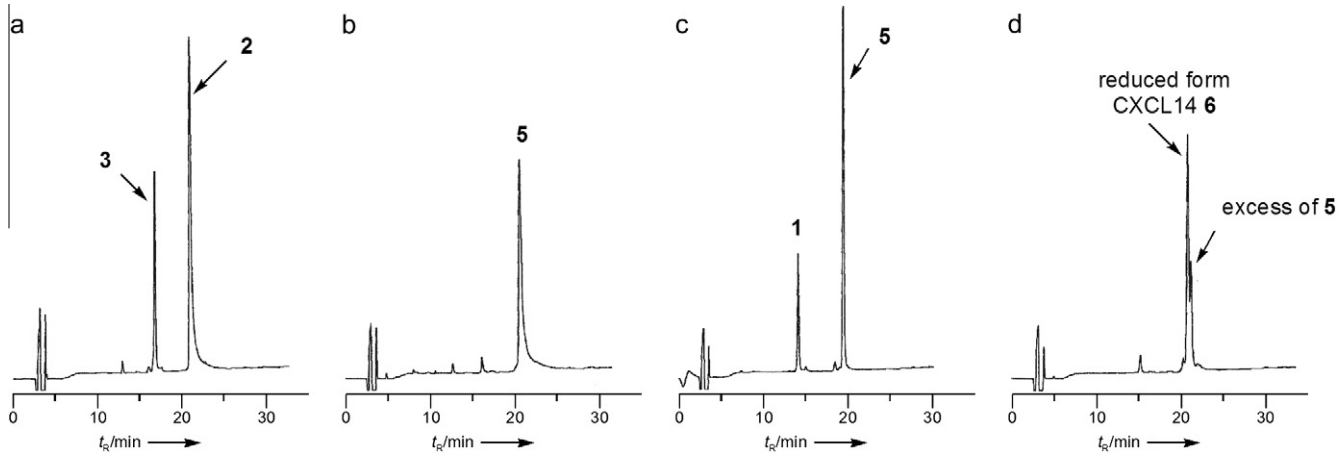
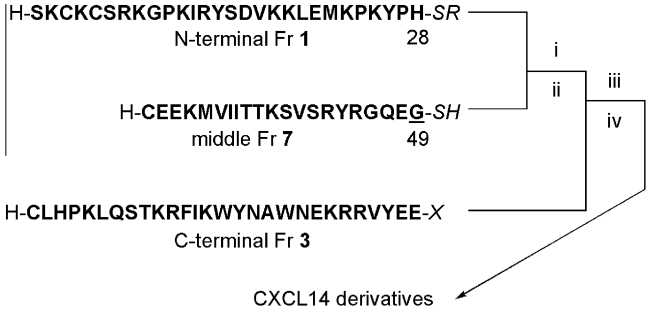
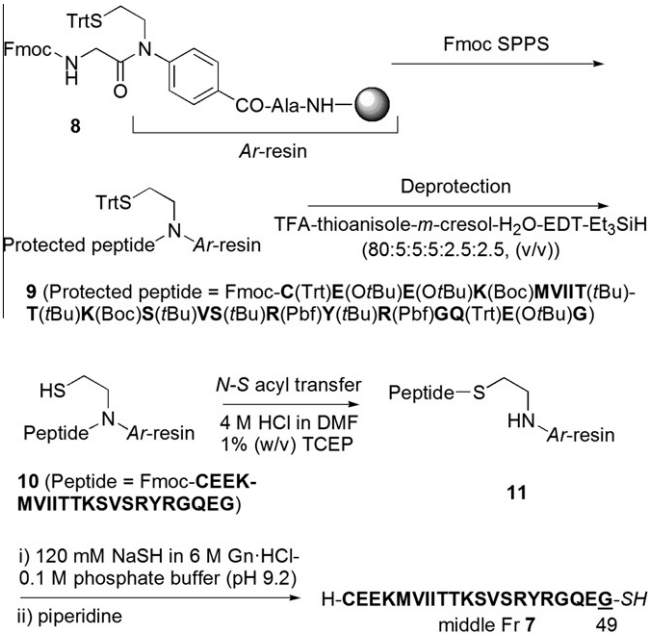


Figure 2. HPLC monioring of the C–N-directed sequential NCL for the synthesis of CXCL14. (a) First NCL (t <1 min). (b) First NCL (t = 4 h) followed by addition of $MeONH_2$·HCl. (c) Second NCL (t <1 min). (d) Second NCL (t = 6 h). HPLC conditions: cosmosil $5C_{18}$ AR-II column (4.6 × 250 mm) with a linear gradient of 0.1% TFA–MeCN/0.1% TFA aq (5:95–45:55 over 30 min) at a flow rate of 1.0 mL/min, detection at 220 nm.



Scheme 3. Synthesis of CXCL14 analogs using N–C-directed sequential NCL. (i) First NCL in 6 M Gn·HCl-0.2 M phosphate buffer (pH 6.8) in the presence of 1% thiophenol. (ii) Thioesterification with Ellman's reagent, $KHCO_3$ in DMF/H_2O (2:8), then TCEP. (iii) Second NCL in 6 M Gn·HCl-0.2 M phosphate buffer (pH 6.8) in the presence of 1% thiophenol. (iv) Oxidation in 10% DMSO in 3 M Gn·HCl-0.1 M phosphate buffer (pH 7.7). X = OH (**3**), X = Lys(FTC)–NH_2 (**3′**).



Scheme 4. Synthesis of N-terminal cysteinyl peptide thioacid **7** using (*N*-Fmoc–glycyl-*N*-sulfanylethyl)aminobenzoic acid linker-incorporated resin **8**.

In preliminary experiments, the rapid hydrolysis of the C-terminal histidyl thioester in the synthetic intermediate of CXCL14 (1–49) was observed. His was therefore replaced with Gly in subsequent syntheses (Scheme 4). Successive coupling of Fmoc–Ala-OH and 4-[Fmoc–glycyl(2-tritylsulfanylethyl)amino]-benzoic acid onto aminomethyl ChemMatrix resin gave the linker-incorporated resin **8**. This linker system allows facile synthesis of peptide thioesters by *N–S* acyl transfer.[28] Standard Fmoc SPPS on resin **8** followed by deprotection with a TFA-based reagent cocktail gave deprotected peptide anilide resin **10**. Subsequent treatment of **10** with 4 M HCl/DMF in the presence of 1% (w/v) tris(2-carboxyethyl)phosphine hydrochloride (TCEP) gave peptide thioester resin **11** via *N–S* acyl transfer on the resin. Hydrothiolitic release of the thioacid form of the peptide from resin **11** with 120 mM NaSH in 6 M Gn·HCl-0.1 M phosphate buffer (pH 9.2) was followed by addition of piperidine to remove the N-terminal Fmoc group. This yielded the middle peptide thioacid fragment **7**. Sequential N–C-directed NCL was then attempted (Fig. 3). The first NCL, using thioester **1** and thioacid **7**, was conducted in 6 M Gn·HCl-0.2 M phosphate buffer (pH 6.8) in the presence of 1% thiophenol, and afforded the condensed peptide thioacid **12**. After HPLC purification, **12** was converted to peptide thioester **13** by the action of Ellman's reagent in the presence of $KHCO_3$ in H_2O/DMF (8:2, v/v) at room temperature. After reduction of disulfides, including excess Ellman's reagent, with TCEP, the second NCL was initiated by addition of the C-terminal fragment **3** and thiophenol (1%) to the reaction mixture, followed by adjustment of the reaction pH to around 7.5 at 4 °C. The second NCL was complete in 2 h and yielded the ligated product **14**. HPLC purification of the crude material followed by folding in 3 M Gn·HCl-0.1 M phosphate buffer (pH 7.7)/DMSO (9:1, v/v) afforded CXCL14 (^{49}Gly).

For visualization of the putative CXCL14 receptor, a fluorescein-5-thiocarbonyl (FTC)-incorporated CXCL14 (^{49}Gly) analogs was also synthesized by N–C-directed sequential NCL, using modified C-terminal fragment **3′**, which features an FTC group on an additional C-terminal Lys ε-amino group. The FTC-incorporated fragment **3′** was synthesized by Fmoc chemistry utilizing Lys derivatives with the ivDde[29] (1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl) group that can be selectively removed by the action of 2% hydrazine in DMF. The synthetic protocols used to prepare the FTC CXCL14 (^{49}Gly) analogs were identical to those employed for the preparation of CXCL14 (^{49}Gly).

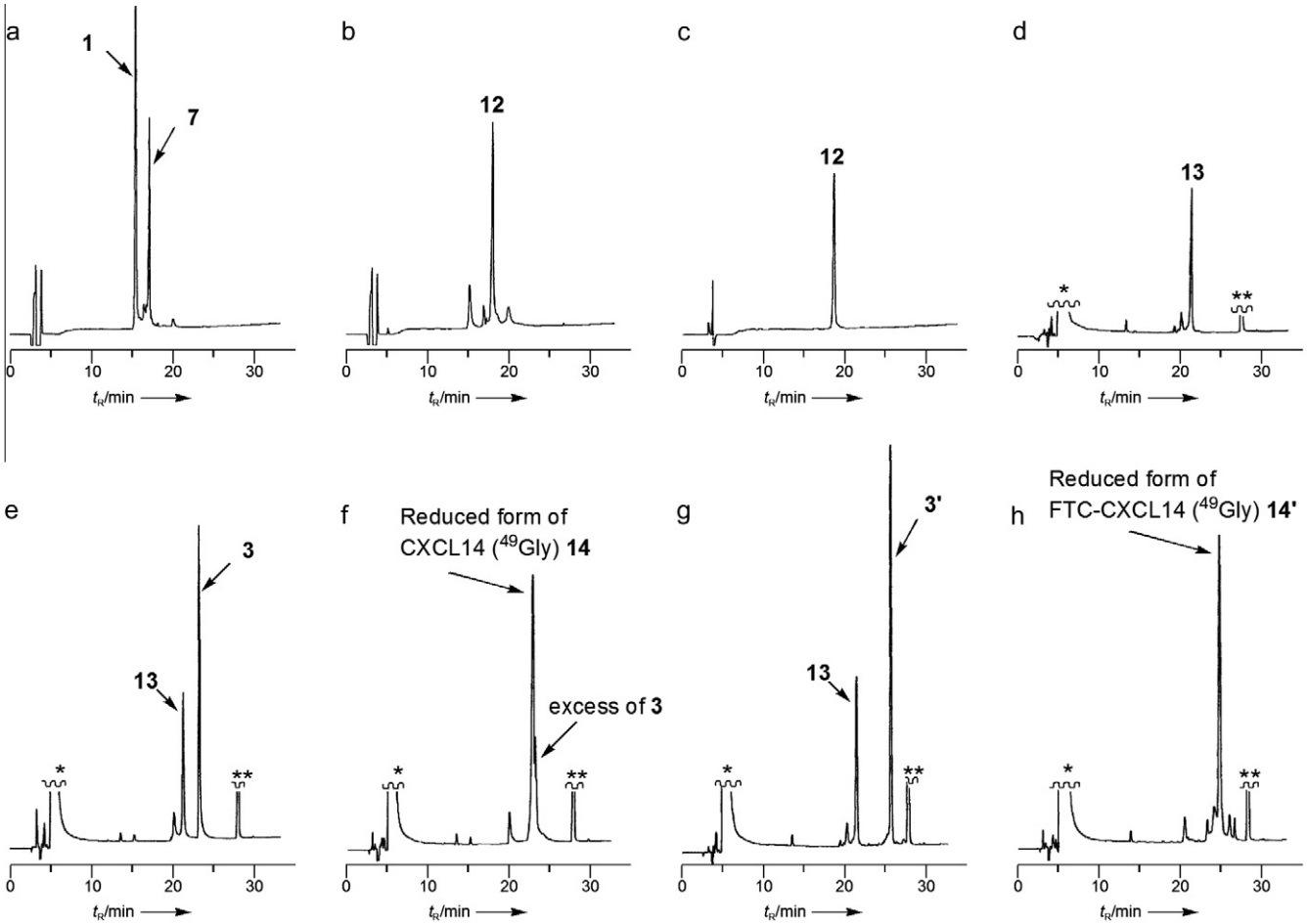


Figure 3. HPLC monitoring of the N–C-directed sequential NCL for the synthesis of CXCL14 (^{49}Gly) analogs. (a) First NCL (t <1 min). (b) First NCL (t = 4 h). (c) HPLC purified thioacid **12**. (d) Conversion of **12** to the corresponding Ar (= $-C_6H_4(NO_2)(CO_2H)$) thioester **13** by the reaction of **12** with Ellman's reagent in the presence of $KHCO_3$ in H_2O/DMF (8:2) at room temperature for 1 h followed by addition of TCEP. (e) Second NCL (t <1 min) to give **14**: to the crude reaction mixture containing **13** was added the C-terminal fragment **3** followed by adjustment of pH to around 7.5. (f) Second NCL (t = 2 h) to give **14**. (g) Second NCL (t <1 min) to give **14′** (protocol as in e above, but using fragment **3′**). To the crude reaction mixture containing **13** was added the FTC-incorporated C-terminal fragment **3′** followed by adjustment of pH around 7.5. (h) Second NCL (t = 2 h) to give **14′**. HPLC conditions: cosmosil $5C_{18}$ AR-II column (4.6 × 250 mm) with a linear gradient of 0.1% TFA–MeCN/0.1% TFA aq (5:95–45:55 over 30 min) at a flow rate of 1.0 mL/min, detection at 220 nm. *DMF. **5-mercapto-2-nitrobenzoic acid.

2.3. Biological evaluation of synthetic CXCL14 analogs

The biological evaluation of CXCL14 analogs was conducted using THP-1 cells. To evaluate the chemotactic activity of the synthetic CXCL14 analogs, we carried out a checkerboard analysis, during which the migration of THP-1 cells was quantified when the lower chambers were filled with CXCL14 and anti-CXCL14 monoclonal antibody (MAB730). The total number of THP-1 cells that migrated to the bottom surface of the membrane was quantified via acid-ethanol extraction of a cell-associated dye, followed by spectrometry. The presence of the MAB730 antibody is critical for the sensitive detection of CXCL14-mediated chemotactic activity in this assay. All synthetic proteins, including CXCL14 analogs, exhibited chemoattractant activity on THP-1 cells comparable to *E. coli*-expressed CXCL14 (Fig. 4).

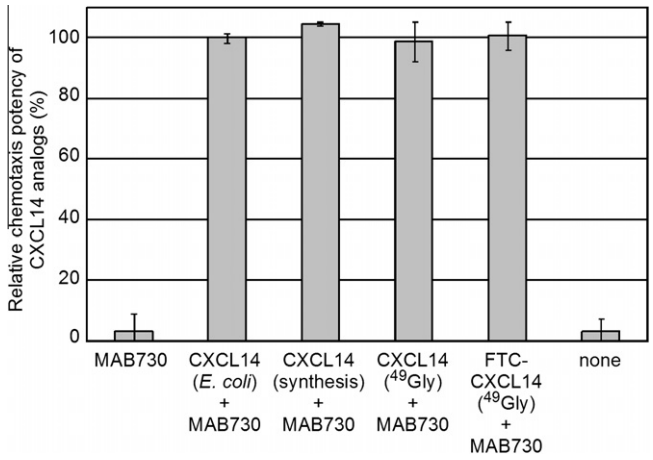


Figure 4. Chemoattractant activity of CXCL14 proteins on THP-1 cells.

3. Conclusion

CXCL14 proteins were efficiently synthesized by either N–C- or C–N-directed sequential NCL. In particular, the use of the N–C-directed protocol allowed the efficient incorporation of diversity at the C-terminus. The significant chemotactic activity on THP-1 cells of the CXCL14 (^{49}Gly) analogs indicates the potential for C-terminal modification of CXCL14. The synthesis of such analogs by an N–C-directed NCL method has been established. The FTC-incorporated analogs, which showed activity comparable to CXCL14, will serve as a molecular probe to elucidate the physiological role of CXCL14 and to locate receptors. A CXCL14 library with

diversity at the C-terminus is currently under construction. Uncovering the physiological roles of CXCL14 using a wide variety of CXCL14 analogs is also under investigation in our laboratory.

4. Experimental

4.1. General methods

Exact mass spectra were recorded on a Waters MICROMASS® LCT PREMIER. For HPLC separations, a Cosmosil $5C_{18}$-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1.0 mL/min), a $5C_{18}$-AR-II semi-preparative column (Nacalai Tesque, 10 × 250 mm, flow rate 3.0 mL/min) or a $5C_{18}$-AR-II preparative column (Nacalai Tesque, 20 × 250 mm, flow rate 10 mL/min) was employed, and eluting products were detected by UV at 220 nm. A solvent system consisting of 0.1% TFA aqueous solution (v/v, solvent A) and 0.1% TFA in MeCN (v/v, solvent B) was used for HPLC elution. The human monocytic leukemia-derived THP-1 cell line (JCRB0112) was obtained from the Human Science Research Resource Bank (Osaka, Japan) and maintained at 37 °C in RPMI1640 medium (Sigma, St. Louis, MO) containing 10% fetal calf serum (FCS) (Invitrogen, Carlsbad, CA), 0.5% penicillin–streptomycin (Sigma), and 50 μM of β-mercaptoethanol (Sigma). Rat anti-mouse CXCL14 monoclonal antibody (MAB730) was purchased from R&D Systems (Minneapolis, MN).

4.2. Preparation of peptide thioesters 1 and 2

On MBHA resin (0.70 mmol amine/g) was coupled Boc–Ala-OH (5.0 equiv) in the presence of DIPCDI (5.0 equiv) and HOBt·H_2O (5.5 equiv) in DMF at room temperature for 2 h followed by Boc removal by TFA/anisole/toluene (50:2:48, (v/v), 20 min). Next, *S*-Trt sulfanylpropionic acid was activated with HBTU (4.9 equiv) in the presence of DIPEA (7.0 equiv) and coupled for 1.5 h to H–Ala–MBHA resin followed by Trt removal by TFA/Et_3SiH/H_2O (95:2.5:2.5, (v/v), 5 min) to afford HS–CH_2CH_2CO–Ala–MBHA resin. On the resulting resin, standard in situ neutralization Boc SPPS (Boc amino acid (5.0 equiv), DIPEA (2.0 equiv), DIPCDI (5.0 equiv) and HOBt·H_2O (5.5 equiv) in DMF for acylation and TFA/anisole/toluene (50:2:48, (v/v), 20 min) for Boc removal) was performed for the chain elongation to give protected peptide resin Boc–Ser(Bzl)–Lys(Cl-Z)–Cys(MBzl)–Lys(Cl-Z)–Cys(MBzl)–Ser(Bzl)–Arg(Mts)–Lys(Cl-Z)–Gly–Pro–Lys(Cl-Z)–Ile–Arg(Mts)–Tyr(Br-Z)–Ser(Bzl)–Asp(OBzl)–Val–Lys(Cl-Z)–Lys(Cl-Z)–Leu–Glu(OBzl)–Met–Lys(Cl-Z)–Pro–Lys(Cl-Z)–Tyr(Br-Z)–Pro–His(Bom)–SCH_2CH_2CO-Ala-resin for **1** or Boc–Thz–Glu(OBzl)–Glu(OBzl)–Lys(Cl-Z)–Met–Val–Ile–Ile–Thr(Bzl)–Thr(Bzl)–Lys(Cl-Z)–Ser(Bzl)–Val–Ser(Bzl)–Arg(Mts)–Tyr(Br-Z)–Arg(Mts)–Gly–Gln–Glu(OBzl)–His(Bom)–SCH_2CH_2CO–Ala-resin for **2**. The resulting completed resin was treated with 1 M TMSOTf/thioanisole (molar ratio 1:1) in TFA, *m*-cresol (100:5, (v/v)) at 4 °C for 2 h. To obtain peptide **1**, addition of NH_4I (75 equiv) and Me_2S (75 equiv) to the reaction mixture was followed with additional stirring for 30 min at 4 °C. The reaction mixture was filtered into cooled Et_2O and the resulting precipitate was collected by centrifugation. The obtained precipitate was washed with Et_2O and purified by preparative HPLC to give desired peptide thioacid **1** or **2**, respectively.

Compound **1**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 15.5 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 13–23% over 30 min. MS (ESI–TOF) *m/z* calcd for ($[M+4H]^{4+}$) 874.0, found 873.5.

Compound **2**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5–45% over 30 min, retention time = 19.5 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 15–25% over 30 min. MS (ESI–TOF) *m/z* calcd for ($[M+3H]^{3+}$) 888.8, found 888.7.

4.3. Preparation of N-terminal cysteinyl fragment 3

The protected peptide resin was manually constructed on Fmoc–Glu(OtBu)–Alko–PEG resin (loading: 0.24 mmol amino acid/g) using standard Fmoc SPPS (Fmoc amino acid (5.0 equiv), DIPCDI (5.0 equiv) and HOBt·H_2O (5.5 equiv) in DMF for acylation and 20% piperidine in DMF (10 min) for Fmoc removal) was performed for the chain elongation to give protected peptide resin. The resulting completed resin was treated with TFA/thioanisole/*m*-cresol/H_2O/ethanedithiol (EDT) (80:5:5:5:5, (v/v)) at room temperature for 1.5 h. After the resin was filtered off, cooled Et_2O was added to the filtrate, and the resulting precipitate was collected by centrifugation. The obtained precipitate was washed with Et_2O and purified by preparative HPLC to give desired *N*-Cys peptide **3**.

Compound **3**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5–45% over 30 min, retention time = 23.8 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 23–33% over 30 min. MS (ESI–TOF) *m/z* calcd for ($[M+4H]^{4+}$) 906.5, found 906.5.

4.4. Preparation of N-terminal cysteinyl peptide thioacid 7

On-resin peptide Fmoc–Cys(Trt)–Glu(O*t*Bu)–Glu(O*t*Bu)–Lys(Boc)–Met–Val–Ile–Ile–Thr(*t*Bu)–Thr(*t*Bu)–Lys(Boc)–Ser(*t*Bu)–Val–Ser(*t*Bu)–Arg(Pbf)–Tyr(*t*Bu)–Arg(Pbf)–Gly–Gln(Trt)–Glu(O*t*Bu)–Gly-*N*-Ar-resin was prepared on Fmoc–Gly-*N*-Ar-resin **8**[23,28] using Fmoc protocol. Deprotection of the completed resin with TFA/thioanisole/*m*-cresol/H_2O/EDT/Et_3SiH (80:5:5:5:2.5:2.5, (v/v)) at room temperature for 1.5 h followed by the treatment with 4 M HCl/DMF in the presence of TCEP (1% (w/v)) at room temperature for 20 h gave deprotected on-resin peptide thioester (Fmoc–Cys–Glu–Glu–Lys–Met–Val–Ile–Ile–Thr–Thr–Lys–Ser–Val–Ser–Arg–Tyr–Arg–Gly–Gln–Glu–Gly–*S*-Ar-resin **11**). The on-resin peptide thioester was incubated with 120 mM NaSH in 6 M Gd·HCl-0.1 M sodium phosphate buffer (pH 9.2) at 37 °C for 30 min followed by addition of piperidine (5%, (v/v)) with additional 15 min treatment to yield the hydrothiolytically released peptide thioacid **7**. Crude material was then subjected to semi-preparative HPLC purification to afford the desired N-terminal cysteinyl peptide thioacid **7**.

Compound **7**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5–45% over 30 min, retention time = 17.8 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 12–27% over 30 min. MS (ESI–TOF) *m/z* calcd for ($[M+3H]^{3+}$) 810.7, found 810.7.

4.5. Preparation of N-terminal cysteinyl fragment possessing fluorescence dye 3′

On NovaSyn TGR® resin (0.25 mmol amine/g) was coupled Fmoc–Lys(ivDde)-OH (3.0 equiv) in the presence of DIPCDI (3.0 equiv) and HOBt·H_2O (3.3 equiv) in DMF at room temperature for 3 h followed by Fmoc removal by 20% piperidine in DMF. On the resulting resin standard Fmoc SPPS mentioned above was performed for the chain elongation to give protected peptide–Lys(ivDde)-resin. Next, the resulting resin was treated with 2% hydrazine/DMF at room temperature for two days for ivDde removal followed by coupling of fluoresceine-5-isothiocyanate (FITC) in the presence of DIPEA (2 equiv) in DMF at room temperature for 12 h to afford protected peptide–Lys(FTC)-resin. The resulting completed resin was treated with TFA/thioanisole/*m*-cresol/H_2O/EDT (80:5:5:5:5, (v/v)) at room temperature for 1.5 h. After the resin was filtered off, cooled Et_2O was added to the filtrate, and the resulting precipitate was collected by centrifugation. The obtained precipitate was washed with Et_2O and purified by semi-preparative HPLC to give desired peptide **3′**.

Compound **3′**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5–45% over 30 min, retention time = 21.7 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20–50% over 30 min. MS (ESI–TOF) *m/z* calcd for ($[M+4H]^{4+}$) 1035.5, found 1035.6.

4.6. Synthesis of human CXCL14 by the C–N-directed sequential NCL utilizing N-Thz thioester fragment 2

The First NCL: *N*-Thz peptide thioester **2** and N-terminal cysteinyl fragment **3** were dissolved at a final concentration of 1.0 mM in 6 M Gd·HCl-0.2 M sodium phosphate buffer (pH 6.8) in the presence of 1% (v/v) thiophenol. After incubation at 37 °C for 4 h, the reaction was completed. Then methoxyamine hydrochloride was added directly to the ligation reaction mixture at a final concentration of 0.2 M. The conversion of Thz to Cys residue was completed within 2 h and the crude material was purified by semi-preparative HPLC to afford the homogeneous ligated N-terminal cysteinyl peptide **5**.

The Second NCL: Peptide thioester **1** and N-terminal cysteinyl peptide **5** were dissolved at a final concentration of 1.0 mM in 6 M Gd·HCl-0.2 M sodium phosphate buffer (pH 6.8) in the presence of 1% (v/v) thiophenol. After 6 h, the starting materials were disappeared and the crude material was purified by semi-preparative HPLC to give reduced form CXCL14 **6**. Subsequent oxidation of the purified **6** (0.05 mM) was performed in 3 M Gd·HCl-0.1 M sodium phosphate buffer (pH 7.7)/DMSO (9:1). After incubation of the mixture at 37 °C for 6 h, wild-type human CXCL14 was isolated by semi-preparative HPLC.

Compound **5**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5–45% over 30 min, retention time = 23.6 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 15–45% over 30 min. MS (ESI–TOF) *m/z* calcd for ($[M+4H]^{4+}$) 1525.3, found 1525.2.

Compound **6**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5–45% over 30 min, retention time = 20.2 min. Semipreparative HPLC condition: linear gradient of solvent B in solvent A, 15–45% over 30 min. MS (ESI–TOF) *m/z* calcd for ($[M+11H]^{11+}$) 856.7, found 856.9.

Human CXCL14: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5–45% over 30 min, retention time = 19.7 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 15–45% over 30 min. MS (ESI–TOF) *m/z* calcd for ($[M+10H]^{10+}$) 941.9, found 941.9.

4.7. Synthesis of human CXCL14 analogs by N–C-directed sequential NCL utilizing peptide thioacid 7

The first NCL: Peptide thioester **1** and N-terminal cysteinyl peptide thioacid **7** were dissolved at a final concentration of 1.0 mM in 6 M Gd·HCl-0.2 M sodium phosphate buffer (pH 6.8) in the presence of 1% (v/v) thiophenol. After incubation at 37 °C for 2 h, the starting materials were disappeared and the crude material was purified by semi-preparative HPLC to give ligated peptide thioacid **12**.

The second NCL: The purified peptide thioacid **12** was dissolved at a final concentration of 1.0 mM in DMF/H_2O (2:8) containing 6.0 mM Ellman's reagent and 6.0 mM $KHCO_3$. The reaction mixture was shaken at room temperature for 1 h. Then 1% (w/v) TCEP was added to the reaction mixture to reduce an excess Ellman's reagent and the hetero disulfide generated from the cystein sulfhydryl group and 5-sulfanyl-2-nitrobenzoic acid. The conversion of thioacid **12** to the corresponding thioester **13** was confirmed by HPLC analysis. The N-terminal cysteinyl fragment **3** or **3′** and 1% (v/v) thiophenol were added to the reaction mixture and then the pH of the reaction was adjust to around 7.5 by addition of 10% K_2CO_3 aqueous solution at 4 °C. After incubation of the mixture at 37 °C for 2 h, the starting materials were disappeared and the crude material was purified by semi-preparative HPLC to give reduced form CXCL14 (^{49}Gly) **14** and FTC-CXCL14 (^{49}Gly) **14′**, respectively. Oxidation of the purified **14** or **14′** (0.05 mM) was performed in 3 M Gd·HCl-0.1 M sodium phosphate buffer (pH 7.7)/DMSO (9:1). After incubation of the mixture at 37 °C for 6 h, each human CXCL14 analogues was isolated by preparative or semi-preparative HPLC.

Compound **12**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5–45% over 30 min, retention time = 18.7 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 15–30% over 30 min. MS (ESI–TOF) *m/z* calcd for ($[M+5H]^{5+}$) 1150.0, found 1150.0.

Compound **13**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5–45% over 30 min, retention time = 21.3 min. MS (ESI–TOF) *m/z* calcd for ($[M+4H]^{4+}$) 1478.5, found 1478.6.

Compound **14**: Analytical HPLC condition: linear gradient of solvent B in solvent A, 5–45% over 30 min, retention time = 20.5 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 15–45% over 30 min. MS (ESI–TOF) *m/z* calcd for ($[M+9H]^{9+}$) 1037.9, found 1038.3.

Compound **14′**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5–45% over 30 min, retention time = 24.8 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20–50% over 30 min. MS (ESI–TOF) *m/z* calcd for ($[M+11H]^{11+}$) 896.4, found 896.5.

Human CXCL14 (^{49}Gly): Analytical HPLC condition: linear gradient of solvent B in solvent A, 5–45% over 30 min, retention time = 20.8 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 19–29% over 30 min. MS (ESI–TOF) *m/z* calcd for ($[M+7H]^{7+}$) 1333.7, found 1333.8.

FTC-human CXCL14 (^{49}Gly): Analytical HPLC condition: linear gradient of solvent B in solvent A, 5–45% over 30 min, retention time = 24.6 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 15–45% over 30 min. MS (ESI–TOF) *m/z* calcd for ($[M+9H]^{9+}$) 1094.9, found 1094.8.

4.8. Chemotaxis assay

THP-1 cells were washed and then resuspended at 10^6 cells/ml (THP-1) in RPMI 1640 containing 0.1% fatty acid-free BSA (Sigma) and 20 mM HEPES pH 7.5 (Invitrogen). Wells in a 24-well culture plate were filled with 550 μL of 100 nM each CXCL14 proteins in the presence of antibody (MAB730) (10 μg/mL). Chemotaxicell filters (5 μm pore size; Kurabo, Osaka, Japan) were placed in each well and THP-1 cells (200 μL) were added to the upper chamber. The plate was then incubated for 2 h at 37 °C. Remaining cells in the upper chamber were scraped off. Thereafter, cells that had migrated to the bottom surface of the membrane were stained with Diff–Quik (Kokusai Shiyaku, Kobe, Japan) and counted using a microscope. Alternatively, stained migrated cells were extracted with 0.6 mL 30% Ethanol-1% acetic acid in water and then cell number was quantified by measuring optical density (O.D.) at 580 nm with an Ultrospec 2000 spectrometer (Amersham Pharmacia Biotech, Piscataway, NJ).

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