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Potent CCR4 antagonists: Synthesis, evaluation, and docking study of 2,4-diaminoquinazolines

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ABSTRACT

A series of CC chemokine receptor-4 (CCR4) antagonists were examined in a previous report in an attempt to improve metabolic stability in human liver microsomes. In this study, the cycloheptylamine moiety of *N*-cycloheptyl-6,7-dimethoxy-2-(4-pyrrolidin-1-ylpiperidin-1-yl)quinazolin-4-amine **1** was replaced with the *p*-chloroaniline moiety, and the resulting compound, *N*-(4-chlorophenyl)-6,7-dimethoxy-2-(4-pyrrolidin-1-ylpiperidin-1-yl)quinazolin-4-amine (**8c**), retained its potency ([^{35}S]GTPγS-binding inhibition and CCL22-induced chemotaxis in humans/mice). Based on the structure–activity relationships (SAR), a homology model was constructed for CCR4 to explain the binding mode of **8c**. Overall, there was good agreement between the docking pose of the CCR4 homology model and the human [^{35}S]GTPγS assay results. Administration of **8c** in a murine model of acute dermatitis showed anti-inflammatory activity (oxazolone-induced contact hypersensitivity test).

1. Introduction

Chemokines are relatively small (~8–14 kDa), mostly basic proteins that are involved in various physiological and pathological processes.[1] They are divided into two main subclasses, CXC (or α) and CC (or β);[2] this division is based on the position of the first two of four conserved cysteines. CC chemokine receptor 4 (CCR4) was originally cloned from T-lymphocyte and thymus cells, where it is highly expressed.[3–5] Both thymus and activation-regulated chemokine (TARC, CCL17) and macrophage-derived chemokine (MDC, CCL22) are CC chemokines that are highly specific biological ligands for CCR4.[6,7] In vivo studies of CCL17 and CCL22 antibodies have indicated their utility in preventing several immunological responses.[8–13] Therefore, the use of CCR4 antagonists would be a novel, therapeutic method of intervention for disease in which CCR4 participates.

In a previous manuscript,[14] a series of 2,4-diaminoquinazoline analogues were identified as potent CCR4 antagonists with moderate ability to functionally inhibit chemotaxis in vitro. To identify suitable small molecules for in vivo validation, subsequent efforts to optimize pharmacokinetic parameters were pursued. The in vitro oxidative metabolic stability of compounds **1** and **2**, which were identified as potent CCR4 antagonists in a previous report, was evaluated by measuring the rate of drug consumption in human liver microsomes (HML), thus providing intrinsic clearance values (CL_{int}). Compounds **1** and **2** exhibited relatively poor metabolic stability with CL_{int} values of 6600 and 17,377 mL/h/kg, respectively (Fig. 1). The compounds were postulated to be highly lipophilic, probably due to the cycloheptyl moiety and/or piperidinopiperidine/piperidinopyrrolidine moiety, which may be associated with poor metabolic stability. In this report, the results of an additional optimization attempt that led to the identification of a CCR4 antagonist with improved metabolic stability are reported.

2. Chemistry

The novel aminoalcohols **5** were prepared from the commercially available *tert*-butyl 4-oxopiperidine-1-carboxylate **3** in three

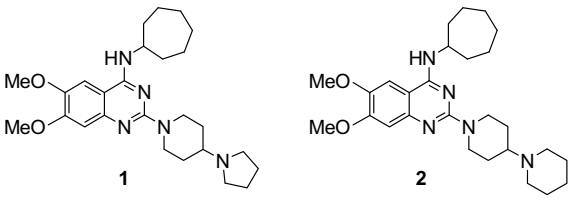

Figure 1. Oxidative metabolic stability of compounds **1** and **2**.

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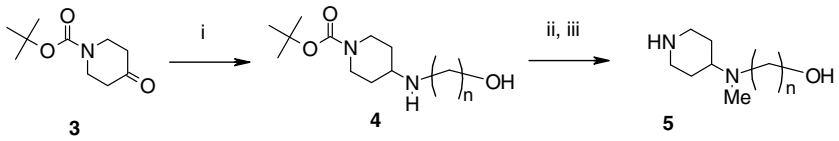
steps, as summarized in Scheme 1. The reductive amination of **3** with an appropriate primary aminoalcohol afforded a corresponding secondary amine, which was methylated with 37% formaldehyde aqueous solution and sodium triacetoxyborohydride to yield amine **4**. The Boc group was readily deprotected to yield the desired amine **5**, which was used for the next reaction without further purification.

The 2,4-diaminoquinazoline derivatives **8** was prepared as summarized in Scheme 2. The selective substitution of an aniline for the chlorine at the 4-position of quinazoline **6** proceeded in a mixture of aqueous hydrogen chloride and ethanol to yield an inseparable mixture of the desired 4-amino-2-chloro-quinazoline derivative (**7**) and 2,4-disubstituted quinazoline (ca. 9–10:1). This mixture was reacted with an appropriate amine to yield 2,4-diaminoquinazoline (**8a**–**j**). The monoprotected homopiperazine (**8j**), which was derived from mono-Boc homopiperazine and the appropriate 2-chloroquinazoline (**7**), was deprotected under acidic conditions, and then alkylated with an appropriate alkyl halide to yield compound **8k** or **8l**.

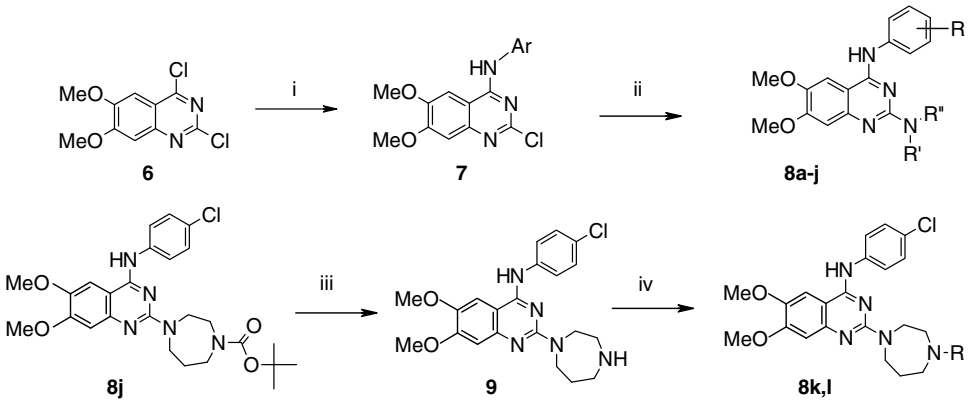
3. Results and discussion

The CCR4 antagonist activities of the synthesized compounds were determined by measuring the degree to which human CCL22-derived [^{35}S]GTPγS was prevented from binding to the receptor. The CCR4-antagonism of these compounds was also confirmed using the chemotaxis assay. The results are summarized in Tables 1–3.

The substituents at the 4-position of the quinazoline core were explored first. All aniline analogues (**8a**–**d**) exerted similarly potent activity levels in the [^{35}S]GTPγS-binding assays (Table 1). The 3,4-difluoro analogue (**8a**) was the most potent inhibitor of chemotaxis in humans among the aniline analogues, although its inhibition of chemotaxis in mice was only moderate. The 4-chloro analogue (**8c**)

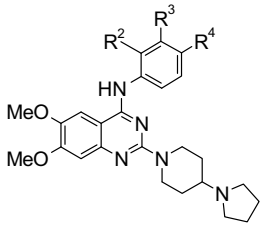


Scheme 1. Reagents and conditions: (i) amines, 10% Pd–C, H_2, EtOH; (ii) formaldehyde 37 wt. % in water, $NaBH(OAc)_3$, AcOH, CH_2Cl_2; (iii) 4M–HCl, dioxane.



Scheme 2. Reagents and conditions: (i) anilines, 1M–HCl aq., EtOH; (ii) amines, *n*-BuOH, Hunig's base or DBU; (iii) 4M–HCl, dioxane; (iv) alkylhalide, Na_2CO_3, NaI, DMF.

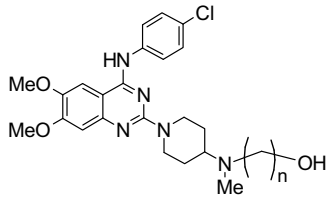
Table 1
CCR4 inhibitory activities of aniline derivatives



Compd.	R^2	R^3	R^4	[^{35}S]GTPγS-binding IC_{50} (μM)[a]	Chemotaxis IC_{50} (μM) human[a]	Chemotaxis IC_{50} (μM) mouse[a]	[^{121}I]CCL22-binding IC_{50} (μM)[a]	CL_{int} (mL/h/kg)
8a	H	F	F	0.025	0.069	0.82	NT[b]	NT[b]
8b	H	Br	H	0.22	36% at 1 μM	11% at 1 μM	0.51	NT[b]
8c	H	H	Cl	0.024	0.11	0.13	0.15	2532
8d	H	H	F	0.031	0.32	0.71	NT[b]	NT[b]
1				0.019	0.20	0.13	0.07	6600

[a] See Section 7.2, pharmacology.
[b] Not tested.

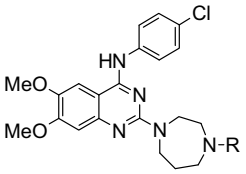
Table 2
CCR4 inhibitory activities of 4-substituted piperidine derivatives

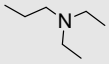
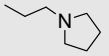
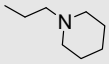


Compd.	*n*	[^{35}S]GTPγS-binding IC_{50} (μM)[a]	Chemotaxis IC_{50} (μM) human[a]	Chemotaxis IC_{50} (μM) mouse[a]	[^{121}I]CCL22–binding IC_{50} (μM)[a]
8e	2	0.098	47% at 1 μM	NT[b]	NT[b]
8f	3	0.043	0.28	32% at 1 μM	NT[b]
8g	4	0.061	0.36	42% at 1 μM	NT[b]
1		0.019	0.20	0.13	0.07

[a] See Section 7.2, pharmacology.
[b] Not tested.

Table 3
CCR4 inhibitory activities of homopiperazine derivatives



Compd	*R*	[^{35}S]GTPγS-binding IC_{50} (μM)[a]	Chemotaxis IC_{50} (μM) human[a]	Chemotaxis IC_{50} (μM) mouse[a]	[^{121}I]CCL22-binding IC_{50} (μM)[a]
8h	Cycohexyl	0.55	NT[b]	NT[b]	NT[b]
8i		0.092	15% at 1 μM	42% at 1 μM	NT[b]
8k		0.15	NT[b]	NT[b]	NT[b]
8l		0.13	NT[b]	NT[b]	NT[b]
1		0.019	0.20	0.13	0.07

[a] See Section 7.2, pharmacology.
[b] Not tested.

showed favorable results in the chemotaxis assays, which were the closest to those of the parent compound **1**. The metabolic stability of **8c** in human liver microsomes was further characterized and found to have a metabolic stability that was much better (CL_{int} = 2532 mL/h/kg) than that of **1**. This suggests that, in human liver microsomes, the 4-chloroaniline substituent was more stable than the cycloheptylamine. Encouraged by these data, further optimization was performed using the 4-(4-chloroaniline) analogues.

In an attempt to improve metabolic stability, the lipophilicity of the analogues was reduced by converting the pyrrolidine moiety in compound **8c** to hydroxyamine (Table 2). The methylaminoalcohol analogues (**8e–8g**) did not show any significant loss of potency in the [^{35}S]GTPγS-binding assays. This indicates that the hydroxyl group was well tolerated at this position. The [^{35}S]GTPγS-binding assay and chemotaxis (humans) assay results for compounds **8f** and **8g** were almost comparable to those of **8c**, even though these compounds inhibited chemotaxis in mice only weakly. The combinatorial studies conducted previously[14] indicated that compounds with a dimethylamine substituent at the 4-position of piperidine inhibit chemotaxis in humans only weakly (<50% inhibition at 3 μM). These findings indicate that having a hydroxyl group at this position would produce a favorable effect on the inhibition of chemotaxis in humans, and also indicate that the SAR trend seems to differ between humans and mice.

Finally, a homopiperazine analogue, one of the promising compounds found in an earlier combinatorial synthesis and the initial SAR studies (data not shown), was explored (Table 3). The *N*-cyclohexyl homopiperazine analogue (**8h**) showed moderate inhibitory activity in the [^{35}S]GTPγS-binding assays. This compound was 10-13-fold less active than the *N*-cyclohexyl piperazine analogue (IC_{50} = 55 nM) evaluated previously.[14] These results indicate that bulky substituents, like the cyclohexyl moiety, at this position in a homopiperazine analogue may lead to a loss of potency in [^{35}S]GTPγS assays. Other modifications performed in this series resulted in the discovery of a series of compounds that have additional amines connected to the homopiperazine moiety through the ethyl linker (**8i**, **8k**, **8l**). These compounds had potent activity in the [^{35}S]GTPγS assays. Compound **8i** was the most potent (IC_{50} = 92 nM) among these; however, its inhibition of chemotaxis in both humans and mice was weak.

4. Molecular modeling and docking study

A 3D model of the human CCR4-receptor was developed by using the homology modeling function in the Molecular Operating Environment (MOE 2006.08) program (Chemical Computing Group, Montreal, QC, Canada). The bovine rhodopsin crystal structure determined[15] [Protein Database (PDB) ID code: 1F88] was used as the template for CCR4-receptor homology modeling. Compound **8c** was docked into the receptor model by using GOLD software[16] [version 3.1.1 (Cambridge Crystallographic Data Center, Cambridge, UK)] with the standard default settings.

After examining a number of possible docking poses, the most favorable result was selected (Fig. 2). In this docking pose, the two methoxy groups at the 6,7-position of quinazoline were located at a deeper site on the receptor. Fig. 3 shows residues within 4 Å of the compound. The dimethoxyphenyl ring of the quinazoline, which was tightly packed with Phe121, Tyr122, Ser203, Ile206, Asn207, Leu211, Asn259, Leu262. Asn207 and Asn259 seemed to interact with the dimethoxy substituents. In addition, Ser203 formed a hydrogen bond with the nitrogen atom linked to quinazoline at the 4-position. Two basic amines of the substituent at the 2-position of the quinazoline formed an H bond with the residues of Tyr258 and Glu290. Both nitrogen atoms were required for potent activity, which indicates that Tyr258 and Glu290 are crucial elements in the protein. The area around the substituent

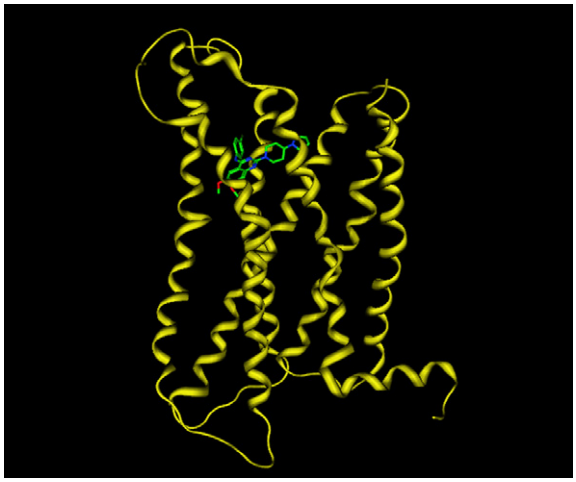


Figure 2. Binding mode for compound **8c**.

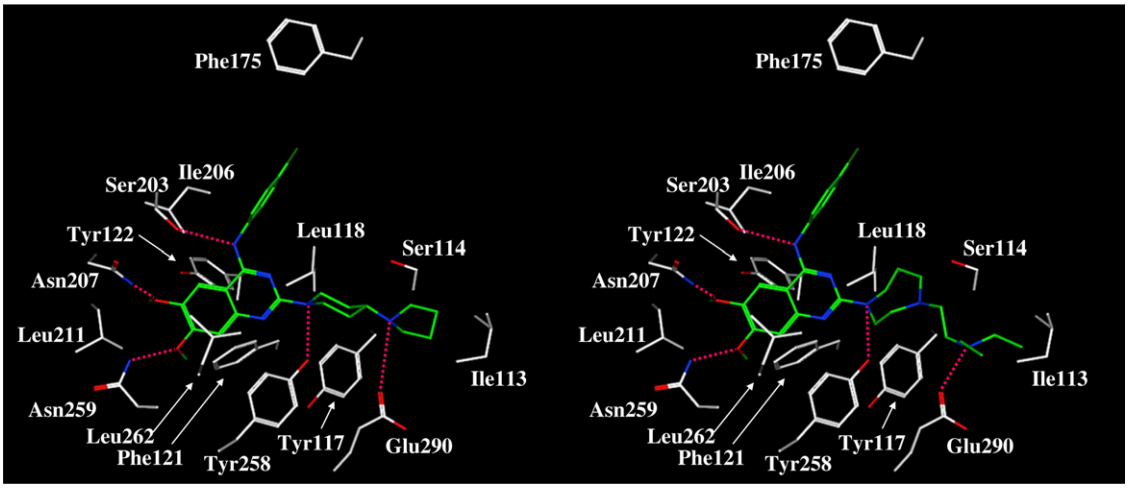


Figure 3. Binding interactions of compound **8c** and **8i**.

at the 2-position of the quinazoline was wide open, which correlates with the SAR study results and suggests steric tolerancy in this region. Compound **8i**, which is a more flexible and bulky compound than **8c**, was also docked in a similar manner (Fig. 3). In this complex, Glu290 seemed to interact with the nitrogen atom of the diethylamine moiety, and one of the nitrogens on the homopiperazine did not interact with any of the residues in this region. This suggests that the nitrogen would not affect its inhibitory activity.

5. In vivo pharmacology

Since compound **8c** was identified as the most promising CCR4 antagonist, its efficacy was evaluated in vivo (Table 4). The murine oxazolone-induced contact hypersensitivity test, known as a model of acute skin inflammation, was chosen to test the anti-inflammatory effect of the CCR4 antagonist. Compound **8c** was administered subcutaneously to mice 0.5 h before and 8 h after they were sensitized to oxazolone. A 42.5% inhibition of ear swelling was observed at the 30 mg/kg dose (administered twice). These results indicate that small molecular CCR4 antagonists have the potential to become treatments for skin inflammation disease.

6. Conclusion

Starting with CCR4 antagonists **1** and **2** (reported previously), compound **8c**, which was found to be more stable in human liver microsomes, was formed by converting the cycloheptylamine moiety of compound **1** with the 4-chloroaniline moiety. Compound **8c** retained the ability to inhibit [^{35}S]GTPγS-binding and chemotaxis in humans/mice. There was good agreement between the human [^{35}S]GTPγS assay results and the docking pose of the CCR4 homology model with the compounds. Subcutaneous administration of **8c** resulted in anti-inflammatory activity in a murine model of acute dermatitis. Further efforts to improve the potency of this series will be reported in due course.

Table 4
Oxazolone-induced contact hypersensitivity test

Compd	Oxazolone-induced contact hypersensitivity % inhibition of ear swelling[a]
8c	42.5% at 30 mg/kg s.c. × 2

[a] See Section 7.2, pharmacology.

7. Experimental

7.1. Chemistry

In general, reagents and solvents were used as purchased without further purification. Melting points were determined with a Yanaco MP-500D melting point apparatus and were left uncorrected. ^{1}H NMR spectra were recorded on a JEOL JNM-LA300 or on a JEOL JNM-EX400 spectrometer. Chemical shifts were expressed in δ (ppm) with tetramethylsilane as an internal standard (NMR description: s = singlet, d = doublet, t = triplet, m = multiplet, and br = broad peak). Mass spectra were recorded on a JEOL JMS-LX2000 spectrometer. The elemental analyses were performed with a Yanaco MT-5 microanalyzer (C,H,N) and Yokogawa IC-7000S ion chromatographic analyzer (halogens). All results were within ±0.4% of the theoretical values.

7.1.1. *tert*-Butyl 4-[(2-hydroxyethyl)amino]piperidine-1-carboxylate (4a)

A solution of *tert*-butyl 4-oxopiperidine-1-carboxylate (2.99 g, 15.0 mmol) in MeOH (50 mL) was added to 2-aminoethanol (2.75 g, 45 mmol) and 10% palladium on carbon (300 mg), and the resulting mixture was stirred at room temperature for 1 day under 1 atm of hydrogen gas. The reaction mixture was filtered through a pad of Celite. The filtrate was concentrated in vacuo, and the residue was purified using column chromatography (chloroform/MeOH/NH_4OH) to yield *tert*-butyl 4-[(2-hydroxyethyl)-amino] piperidine-1-carboxylate **4a** (4.48 g, quant.) as a colorless oil.

MS (FAB$^+$) *m*/*z* 245 [M+H]$^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.22–1.26 (2H, m), 1.45 (9H, s), 1.80–1.95 (2H, m), 2.57–2.65 (1H, m), 2.74–2.84 (4H, m), 3.63–3.66 (2H, m), 3.95–4.13 (2H, br).

Compounds **4b** and **4c** were prepared using procedures similar to those described for the synthesis of **4a**.

7.1.2. *tert*-Butyl 4-[(3-hydroxypropyl)amino]piperidine-1-carboxylate (4b)

tert-Butyl 4-[(3-hydroxypropyl)amino]piperidine-1-carboxylate **4b** (3.87 g, 99%) was obtained as a colorless syrup from *tert*-butyl 4-oxopiperidine-1-carboxylate (2.99 g, 15.0 mmol) and 3-amino-1-propanol (3.38 g, 45 mmol).

MS (FAB$^+$) *m*/*z* 245 [M+H]$^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.17–1.30 (2H, m), 1.45 (9H, s), 1.67–1.74 (2H, m), 1.80–1.92 (2H, m), 2.56–2.65 (1H, m), 2.72–2.85 (2H, m), 2.87–2.95 (2H, m), 3.78–3.83 (2H, m), 3.95–4.10 (2H, br).

7.1.3. *tert*-Butyl 4-[(4-hydroxybutyl)amino]piperidine-1-carboxylate (4c)

tert-Butyl 4-[(4-hydroxybutyl)amino]piperidine-1-carboxylate **4c** (10.9 g, 89%) was obtained as a pale yellow oil from *tert*-butyl 4-oxopiperidine-1-carboxylate (8.96 g, 45 mmol) and 4-amino-1-butanol (8.02 g, 90 mmol).

^{1}H NMR (400 MHz, $CDCl_3$) δ 1.20–1.31 (2H, m), 1.45 (9H, s), 1.61–1.71 (4H, m), 1.86–1.89 (2H, m), 2.57–2.65 (1H, m), 2.68–2.71 (2H, m), 2.74–2.80 (2H, m), 3.57–3.59 (2H, m), 4.04 (2H, br).

7.1.4. 2-[Methyl(piperidin-4-yl)amino]ethanol (5a)

Formaldehyde aqueous solution (37%, 6.1 mL) were added to a solution of *tert*-butyl 4-[(2-hydroxyethyl)amino]piperidine-1-carboxylate **4a** (15 mmol) in CH_2Cl_2 (100 mL), $NaBH(OAc)_3$ (9.54 g, 45 mmol), and acetic acid (20 mL), and the resulting mixture was stirred at room temperature for 1day. NaOH aqueous solution (15%) were added to the reaction mixture, and the mixture was extracted with ethyl acetate. The organic layer was washed with brine, dried over sodium sulfate, filtered, and evaporated in vacuo. HCl dioxane solution (4 M, 15 mL) was added to a solution of the residue in dioxane (45 mL), and the resulting mixture was stirred at room temperature for 12 h. The reaction mixture was concentrated in vacuo, and the residue was recrystallized from EtOH–Et_2O to yield 2-[methyl(piperidin-4-yl)amino]ethanol hydrochloride salt **5a** (2.94 g, 12.7 mmol, 85% from **4a**) as a colorless solid.

MS (FAB$^+$) *m*/*z* 159 [M+H]$^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.90–2.14 (2H, m), 2.10–2.19 (1H, m), 2.20–2.30 (1H, m), 2.73 (3H, d), 2.84-2.98 (2H, br), 3.05–3.14 (1H, m), 3.18–3.28 (3H, m), 3.52–3.64 (1H, m), 3.76–3.81 (2H, m), 9.19 (2H, br), 10.484 (1H, br).

Compounds **5b** and **5c** were prepared using procedures similar to those described for the synthesis of **5a**.

7.1.5. 3-[Methyl(piperidin-4-yl)amino]propan-1-ol (5b)

The hydrochloride salt **5b** (2.11 g, 57%, from **4b**) was obtained as a colorless solid from *tert*-butyl 4-[(3-hydroxypropyl)amino]-piperidine-1-carboxylate **4b** (15 mmol).

MS (FAB$^+$) *m*/*z* 159 [M+H]$^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.78–2.07 (4H, m), 2.11–2.31 (2H, m), 2.67 (3H, s), 2.83–2.99 (2H, m), 3.00–3.3.21 (2H, m), 3.38–3.42 (2H, m), 3.45–3.52 (2H, m), 3.53–3.58 (1H, m), 4.81 (1H, br), 9.13 (1H, br), 9.33 (1H, br), 11.02 (1H, br).

7.1.6. 4-[Methyl(piperidin-4-yl)amino]butan-1-ol (5c)

The hydrochloride salt **5c** (5.76 g, 57%) was obtained as a colorless solid from *tert*-butyl 4-[(4-hydroxybutyl)amino]piperidine-1-carboxylate **4c** (40 mmol).

^{1}H NMR (400 MHz, DMSO-d_6) δ 1.41–1.49 (2H, m), 1.69–1.78 (2H, m), 1.92–2.02 (2H, m), 2.15–2.26 (2H, m), 2.65–2.67 (3H, br), 2.93 (2H, br), 2.98–3.09 (2H, m), 3.37–3.59 (5H, m), 9.11 (1H, br), 9.27 (1H, br), 10.99 (1H, br).

7.1.7. *N*-(3,4-Difluorophenyl)-6,7-dimethoxy-2-(4-pyrrolidin-1-ylpiperidin-1-yl)quinazolin-4-amine (8a)

3,4-Difluoroaniline (0.39 mL, 3.9 mmol) and 1M–HCl aqueous solution (3 mL) was added to a solution of 2,4-dichloro-6,7-dimethoxyquinazoline **6** (1.0 g, 3.86 mmol) in EtOH (20 mL), and the resulting mixture was stirred at 60 °C for 16 h. The reaction mixture was cooled to room temperature, and the resulting precipitate was filtered to yield crude **7a** (1.25 g). 4-Pyrrolidin-1-ylpiperidine (0.6 mL, 3.5 mmol) and Hunig's base (0.60 mmol, 3.5 mmol) was added to a solution of the crude **7a** in *n*-BuOH (12 mL), and the resulting mixture was stirred at 100 °C for 18 h. The reaction mixture was cooled to room temperature and concentrated in vacuo, and the residue was purified using column chromatography (chloroform/MeOH/NH_4OH) to yield *N*-(3,4-difluorophenyl)-6,7-dimethoxy-2-(4-pyrrolidin-1-ylpiperidin-1- yl)-quinazolin-4-amine **8a** (1.06 g.) as a yellow amorphous. The residue was treated with 4M–HCl ethyl acetate solution (1.5 mL), and the mixture was concentrated in vacuo. The residue was recrystallized from methanol to yield the hydrochloride salt of **8a** (515 mg, 25% from **6**) as a colorless solid.

MS (FAB$^+$) *m*/*z* 470 [M+H]$^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.72–2.03 (7H, m), 2.14–2.26 (2H, m), 2.94–3.22 (6H, m), 3.89 (3H, s), 3.93 (3H, s), 4.64–4.78 (2H, m), 7.48–7.60 (2H, m), 7.73 (1H, s), 7.82–7.91 (1H, m), 8.20 (1H, s), 11.2 (1H, s), 11.3 (1H, s), 12.9 (1H, s).

Anal. Calcd for $C_{25}H_{29}F_2N_5O_2 \cdot 2HCl \cdot 3.\ 1H_2O$: C, 50.19; H, 6.27; N, 11.71; F, 6.35; Cl, 11.85. Found: C, 49.96; H, 6.17; N, 11.72; F, 6.54; Cl, 12.10.

Compounds **8b**–**g** were prepared using procedures similar to those described for the synthesis of **8a**.

7.1.8. *N*-(3-Bromophenyl)-6,7-dimethoxy-2-(4-pyrrolidin-1-ylpiperidin-1-yl)quinazolin-4-amine (8b)

Compound **7b** (900 mg, 46%) was obtained as a colorless solid from 2,4-dichloro-6,7-dimethoxyquinazoline **6** (1.3 g, 5 mmol) and 3-bromoaniline (950 mg). Compound **8b** (190 mg, 29%) was obtained as a colorless solid from **7b** (500 mg) and 4-pyrrolidin-1-ylpiperidine (390 mg).

Mp (dec.) 223–224 °C (EtOH); MS (FAB+) *m*/*z* 512 [M+H]$^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.28–1.41 (2H, m), 1.62–1.68 (4H, m), 1.83–1.92 (2H, m), 2.16–2.26 (1H, m), 2.47–2.52 (4H, m), 2.94–3.04 (2H, m), 3.88 (3H, s), 3.89 (3H, s), 4.49–4.58 (2H, m), 6.83 (1H, s), 7.22 (1H, d, *J* = 8.0 Hz), 7.33 (1H, t, *J* = 8.0 Hz), 7.65 (1H, s), 7.72 (1H, d, *J* = 8.0 Hz), 8.22–8.24 (1H, m), 9.34 (1H, s).

Anal. Calcd for $C_{25}H_{30}N_5O_2Br$: C, 58.60; H, 5.90; N, 13.67; Br, 15.59. Found: C, 58.40; H, 5.91; N, 13.49; Br, 15.29.

7.1.9. *N*-(4-Chlorophenyl)-6,7-dimethoxy-2-(4-pyrrolidin-1-ylpiperidin-1-yl)quinazolin-4-amine (8c)

Crude **7c** (6.92 g) was obtained from 2,4-dichloro-6,7-dimethoxyquinazoline **6** (5.18 g, 20 mmol) and 4-chloroaniline (2.55 g, 20 mmol). Compound **8c** (820 mg, 60%) was obtained as a yellowish solid from crude **7c** (1.05 g) and 4-pyrrolidin-1-ylpiperidine (930 mg). Compound **8c** (760 mg) was treated with 4M–HCl ethyl acetate, and the mixture was concentrated. The resulting residue was washed with ethyl acetate to yield the hydrochloride salt (790 mg, 90%) as a yellowish solid.

Mp (dec.) 210–213 °C (AcOEt); MS (FAB$^+$) *m*/*z* 468 [M+H]$^+$. ^{1}H NMR (400 MHz, DMSO-d_6, 80 °C) δ 1.76–2.05 (6H, m), 2.15–2.25 (2H, m), 3.00–3.35 (4H, m), 3.37–3.55 (3H, m), 3.92 (3H, s), 3.95 (3H, s), 4.64–4.76 (2H, m), 7.45–7.55 (2H, m), 7.70–7.80 (3H, m), 8.19 (1H, d, br), 10.92 (1H, s), 11.39 (1H, s).

Anal. Calcd for $C_{25}H_{30}N_5O_2Cl \cdot 2HCl$: C, 55.51; H, 5.96; N, 12.95; Cl, 19.66. Found: C, 51.51; H, 6.48; N, 11.94; Cl, 19.27.

7.1.10. *N*-(4-Fluorophenyl)-6,7-dimethoxy-2-(4-pyrrolidin-1-ylpiperidin-1-yl)quinazolin-4-amine dihydrochloride (8d)

Crude **7d** (6.58 g) was obtained as a colorless solid from 2,4-dichloro-6,7-dimethoxyquinazoline **6** (5.18 g, 20 mmol) and 4-fluoroaniline (2.22 g, 20 mmol). Compound **8d** (840 mg, 62%) was obtained as a colorless solid from crude **7d** (1.02 g) and 4-pyrrolidin-1-ylpiperidine (930 mg). Compound **8d** (800 mg) was treated with 4M–HCl ethyl acetate, and the mixture was concentrated. The resulting residue was washed with Et_2O to yield a hydrochloride salt (800 mg, 86%) as a colorless solid.

Mp (dec.) 226–228 °C (Et_2O); MS (FAB$^+$) *m*/*z* 452 [M+H]$^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.76–2.00 (6H, m), 2.14–2.22 (2H, m), 3.00–3.19 (4H, m), 3.35–3.55 (3H, m), 3.92 (3H, s), 3.94 (3H, s), 4.64–4.73 (2H, m), 7.23–7.30 (2H, m), 7.66–7.76 (3H, m), 8.16 (1H, s), 10.83 (1H, s), 11.35 (1H, s), 12.82 (1H, s, br).

Anal. Calcd for $C_{25}H_{30}N_5O_2Cl \cdot 2HCl \cdot 1.\ 5H_2O$: C, 54.45; H, 6.40; N, 12.70; F, 3.44; Cl, 12.86. Found: C, 54.33; H, 6.38; N, 12.67; F, 3.45; Cl, 112.96.

7.1.11. 2-[(1-{4-[(4-Chlorophenyl)amino]-6,7-dimethoxyquinazolin-2-yl}piperidin-4-yl)(methyl)amino] ethanol (8e)

The hydrochloride salt of **8e** (109 mg, 19%) was obtained as a pale yellow solid from crude **7c** (386 mg), 2-[methyl(piperidin-4-yl)amino]ethanol **5a** (347 mg) and DBU (0.45 mL) using procedures similar to those described for the synthesis of **8d**.

MS (FAB$^+$) *m*/*z* 472 [M+H]$^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.67–1.82 (2H, m), 2.05–2.24 (2H, m), 2.73 (3H, d, *J* = 4.8 Hz), 2.98–3.22 (3H, m), 3.23–3.42 (2H, m), 3.72–3.81 (2H, m), 3.92 (3H, s), 3.94 (3H, s), 4.66 (2H, br), 7.54 (2H, d, *J* = 8.8 Hz), 7.68–7.76 (3H, m), 8.14 (1H, br), 10.05 (1H, s), 10.96 (1H, s), 12.75 (1H, s).

Anal. Calcd for $C_{24}H_{30}ClN_5O_3 \cdot 2HCl \cdot 2.3H_2O$: C, 49.16; H, 6.29; N, 11.94; Cl, 18.14. Found: C, 49.04; H, 6.03; N, 11.98; Cl, 18.22.

7.1.12. 3-[(1-{4-[(4-Chlorophenyl)amino]-6,7-dimethoxyquinazolin-2-yl}piperidin-4-yl)(methyl)amino]propan-1-ol (8f)

The hydrochloride salt of **8f** (175 mg, 30%) was obtained as a pale yellow solid from crude **7c** (386 mg), 3-[methyl(piperidin-4-yl)amino]propan-1-ol **5b** (368 mg, 1.5 mmol) and DBU (0.45 mL) using procedures similar to those described for the synthesis of **8d**.

MS (ESI$^+$) *m*/*z* 486 [M+H]$^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.60–1.95 (4H, m), 2.08–2.25 (2H, m), 2.68 (3H, d, *J* = 4.8 Hz), 2.95–3.25 (3H, m), 3.30–3.52 (2H, m), 3.55–3.67 (2H, m), 3.91 (3H, s), 3.94 (3H, s), 4.68–4.84 (2H, m), 7.53 (2H, d, *J* = 8.8 Hz), 7.70–7.78 (3H, m), 8.18 (1H, br), 10.64 (1H, s), 11.04 (1H, s), 12.84 (1H, s).

Anal. Calcd for $C_{25}H_{32}ClN_5O_3 \cdot 2HCl \cdot 1.8H_2O$: C, 50.78; H, 6.41; N, 11.84; Cl, 17.99. Found: C, 50.87; H, 6.12; N, 11.96; Cl, 18.02.

7.1.13. 4-[(1-{4-[(4-Chlorophenyl)amino]-6,7-dimethoxyquinazolin-2-yl}piperidin-4-yl)(methyl)amino]butan-1-ol (8g)

The hydrochloride salt of **8g** (107 mg, 18%) was obtained as a pale yellow solid from crude **7c** (387 mg), 4-[methyl(piperidin-4-yl)amino]butan-1-ol **5c** (389 mg, 1.5 mmol) and DBU (0.45 mL) using procedures similar to those described for the synthesis of **8d**.

MS (FAB$^+$) *m*/*z* 500 [M+H]$^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.38–1.52 (2H, m), 1.68–1.84 (4H, m), 2.08–2.24 (2H, m), 2.66 (3H, d, *J* = 4.8 Hz), 2.90–3.04 (1H, m), 3.06–3.22 (2H, m), 3.28–3.64 (4H, m), 3.90 (3H, s), 3.94 (3H, s), 4.69–4.84 (2H, m), 7.53 (2H, d, *J* = 8.8 Hz), 7.72–7.82 (3H, m), 8.19 (1H, br), 10.65 (1H, s), 11.06 (1H, s), 12.87 (1H, s).

Anal. Calcd for $C_{26}H_{34}ClN_5O_3 \cdot 1.9HCl \cdot 0.4H_2O$: C, 52.44; H, 6.21; N, 11.76; Cl, 17.26. Found: C, 52.41; H, 6.52; N, 11.88; Cl, 17.42.

7.1.14. *N*-(4-Chlorophenyl)-2-(4-cyclohexyl-1,4-diazepan-1-yl)-6,7-dimethoxyquinazolin-4-amine (8h)

The hydrochloride salt of **8h** (44 mg, 7%) was obtained as a pale yellow solid from crude **7c** (386 mg), 1-cyclohexyl-1,4-diazepane (1.5 mmol) and DBU (0.45 mL) using procedures similar to those described for the synthesis of **8d.**

MS (ESI$^+$) *m*/*z* 496 [M+H]$^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.02–1.18 (2H, m), 1.20–1.50 (4H, m), 1.54–1.65 (1H, m), 1.74–1.84 (2H, m), 2.02–2.14 (3H, m), 3.14–3.34 (4H, m), 3.42–3.57 (2H, m), 3.68–3.85 (2H, m), 3.92 (3H, s), 3.94 (3H, s), 4.18–4.36 (1H, m), 7.46–7.60 (2H, m), 7.68–7.82 (3H, m), 8.18 (1H, s, br), 10.79 (1H, s), 10.99 (1H, s).

Anal. Calcd for $C_{27}H_{34}N_5ClO_2 \cdot 1.9HCl \cdot 3H_2O$: C, 52.36; H, 6.82; N, 11.31; Cl, 16.60. Found: C, 52.35; H, 6.73; N, 11.70; Cl, 16.63.

7.1.15. *N*-(4-Chlorophenyl)-2-{4-[2-(diethylamino)ethyl]-1,4-diazepan-1-yl}-6,7-dimethoxyquinazolin-4-amine (8i)

The hydrochloride salt of **8i** (170 mg, 24%) was obtained as a pale yellow solid from crude **7c** (386 mg), 2-(1,4-diazepan-1-yl)-*N*,*N*-diethylethanamine (463 mg, 1.5 mmol) and DBU (0.45 mL) using procedures similar to those described for the synthesis of **8d**.

MS (ESI$^+$) *m*/*z* 513 [M+H]$^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.20–1.28 (6H, m), 3.12–3.23 (4H, m), 3.34–3.39 (6H, m), 3.52–3.63 (6H, m), 3.93 (3H, s), 3.95 (3H, s), 7.51–7.62 (2H, m), 7.72–7.79 (3H, m), 8.16 (1H, br), 10.80 (1H, br), 10.96 (1H, br), 11.61 (1H, br).

Anal. Calcd for $C_{27}H_{37}N_6ClO_2 \cdot 3HCl \cdot 4H_2O$: C, 46.69; H, 6.97; N, 12.10; Cl, 20.42. Found: C, 46.64; H, 6.84; N, 11.95; Cl, 20.93.

7.1.16. *N*-(4-Chlorophenyl)-2-(1,4-diazepan-1-yl)-6,7-dimethoxyquinazolin-4-amine (9)

Compound **8j** (3.86 g, 70%) was obtained as a pale yellow solid from crude **7c** (3.77 g), *tert*-butyl 1,4-diazepane-1-carboxylate (2.38 g, 11.9 mmol) and DBU (3.5 mL) using procedures similar to those described for the synthesis of **8d**. 4 M-HCl dioxane solution (7.5 mL) was added to a solution of **8j** (3.86 g) in dioxane (50 mL), and the resulting mixture was stirred at room temperature overnight. The reaction mixture was concentrated *in vacuo*, and the residue was recrystallized from EtOH-Et_2O to yield *N*-(4-chlorophenyl)-2-(1,4-diazepan-1-yl)-6,7-dimethoxyquinazolin-4-amine **9** (3.76 g, quant.) as a pale yellow solid.

MS (ESI$^+$) m/z 496 [M+H]$^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.00–2.20 (2H, m), 3.50–3.65 (8H, m), 3.88 (3H, s), 3.91 (3H, s), 7.45–7.57 (2H, m), 7.74–7.86 (3H, m), 8.22 (1H, s).

7.1.17. *N*-(4-Chlorophenyl)-6,7-dimethoxy-2-[4-(2-pyrrolidin-1-ylethyl)-1,4-diazepan-1-yl]quinazolin-4-amine (8k)

1-(2-Chloroethyl)pyrrolidine hydrochloride (255 mg, 1.5 mmol) was added to a solution of **9** (487 mg, 1 mmol) in DMF (10 mL), sodium carbonate (318 mg), and sodium iodide (150 mg). The resulting mixture was stirred at room temperature for 16 h. The reaction mixture was concentrated in vacuo. Water was added to the residue, and the mixture was extracted with chloroform. The organic layer was washed with brine, and then dried over sodium sulfate, filtered, and evaporated in vacuo. The residue was purified using column chromatography (chloroform/MeOH) to yield **8k**. Compound **8k** was treated with 4M–HCl ethyl acetate solution, and the resulting precipitate was recrystallized from an EtOH–Et_2O-H_2O mixture to yield the hydrochloride salt of **8k** (248 mg, 36% from **9**) as a pale yellow solid.

MS (ESI$^+$) *m*/*z* 511 [M+H]$^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.03–1.12 (2H, m), 1.80–2.08 (4H, m), 2.97–3.12 (2H, m), 3.20–3.28 (2H, m), 3.38–3.50 (8H, m), 3.54–3.76 (4H, m), 3.91 (3H, s), 3.95 (3H, s), 7.49–7.60 (2H, m), 7.74–7.84 (3H, m), 8.20–8.30 (1H, m), 9.39 (1H, s), 11.03–11.25 (2H, m).

Anal. Calcd for $C_{27}H_{35}N_6ClO_2 \cdot 3HCl \cdot 4.1H_2O$: C, 46.71; H, 6.71; N, 12.10; Cl, 20.42. Found: C, 46.69; H, 6.24; N, 12.31; Cl, 20.21.

7.1.18. *N*-(4-Chlorophenyl)-6,7-dimethoxy-2-[4-(2-piperidin-1-ylethyl)-1,4-diazepan-1-yl]quinazolin-4-amine (8l)

The hydrochloride salt **8l** (252 mg, 35%) was obtained as a pale yellow solid from **9** (487 mg, 1 mmol), 1-(2-chloroethyl)piperidine hydrochloride (276 mg, 1.5 mmol) and sodium iodide (150 mg) using procedures similar to those described for the synthesis of **8k**.

MS (ESI$^+$) *m*/*z* 525 [M+H]$^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.35–1.46 (2H, m), 1.75–1.85 (4H, m), 2.25–2.35 (2H, m), 2.87–3.01 (2H, m), 3.30–3.49 (8H, m), 3.51–3.85 (6H, m), 3.91 (3H, s), 3.95 (3H, s), 7.50–7.62 (2H, m), 7.73–7.85 (3H, m), 8.26 (1H, s, br), 10.66 (1H, br), 11.15 (1H, br), 11.48 (1H, br).

Anal. Calcd for $C_{28}H_{37}N_6ClO_2 \cdot 3HCl \cdot 4.2H_2O$: C, 47.36; H, 6.87; N, 11.83; Cl, 19.97. Found: C, 47.20; H, 6.52; N, 11.79; Cl, 19.71.

7.2. Pharmacology

7.2.1. Human and murine CCR4-expressing cells

Cells from the mouse pre-B cell line B300-19 were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 50 μM 2-mercaptethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin. The expression vector pEF-BOS-Neo,[17] carrying full-length human CCR4 cDNA (X85740; GenBank) or mouse

CCR4 cDNA, (X90862; GenBank), was transfected into B300-19 cells via electroporation to isolate stable G418-resistant stable transformants.

7.2.2. [^{125}I]CCL22-binding assay

CCR4-receptor-binding assays were performed using a scintillation proximity assay (SPA). Human CCR4-expressing cells (2×10^5 cells/well) were incubated at 25 °C for 2 h with 25 pM [^{125}I]CCL22 (Perkin–Elmer Life Sciences) and 5 mg/mL wheat germ agglutinin SPA beads (GE Healthcare), with various concentrations of test compounds in 100 μL of binding buffer [50 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.1% (w/v) bovine serum albumin (BSA)]. Radioactivity was counted using a TopCount scintillation counter (Packard Biosciences). Control wells free from test compound (for total counts) or containing excess unlabeled CCL22 (10 nM, non-specific) were used to calculate the percent of total inhibition for each set of compounds. Assays were performed in duplicate at four different concentrations for each test compound, and the value represents the average of two (usually) determinations.

7.2.3. [^{35}S]GTPγS-binding assay

Human CCR4-expressing cell membranes (1 μg/well protein) were incubated at 25 °C for 1.5 h with 150 pM [^{35}S]GTPγS (GE Healthcare), 5 mg/mL wheat germ agglutinin SPA beads (GE Healthcare), 2 μM GDP, and 3 nM MDC with various concentrations of test compounds in 200 μL of binding buffer [20 mM HEPES–NaOH (pH 7.05), 100 mM NaCl, 5 mM $MgCl_2$, and 0.2% (w/v) BSA]. Radioactivity was counted using a TopCount scintillation counter. Control wells, in the absence of either test compound (for total counts) or CCL22 (non-specific), were used to calculate the percent of total inhibition for each set of compounds. Assays were performed in duplicate at four different concentrations for each test compound, and the value represents an average of usually two determinations.

In our studies, CCL22 was used as the main CCR4 ligand because it was shown to have higher affinity and efficacy than CCL17 for the [^{35}S]GTPγS-binding reaction and cell chemotaxis (Imai, T.; Chantry, D.; Raport, CJ.; Wood, CL.; Nishimura, M.; Godiska, R.; Yoshie, O.; Gray, PW. *J. Biol. Chem.*, **1998**, 273, 1764.). In addition, our test compounds were found to inhibit responses induced by CCL22 and CCL17 equally (data not shown).

7.2.4. Chemotaxis assay

Chemotaxis assays were performed using 96-well chemotaxis chambers (Neuro Probe)[18] that had 5-μm pores, polycarbonate filters, and were polyvinylpyrrolidone-free. The chambers were incubated for 3 h at 37 °C in a humidified 5% CO_2 atmosphere. Human or mouse CCR4-expressing cells were suspended at 5×10^6 cells/mL in RPMI 1640 supplemented with 0.1% (w/v) BSA and treated with various concentrations of test compounds. The cell suspension (200 μL) was placed in the upper wells, and 3 nM human or 1 nM mouse CCL22 in the lower wells. The number of cells migrating to the lower chambers was quantified using a bioluminescent assay (ATP-Lite; Perkin–Elmer). Control wells containing no test compound (for total migrating cells) or CCL22 (non-specific) were used to calculate the percent of total inhibition for each set of compounds. Assays were performed in triplicate at four different concentrations for each test compound.

7.2.5. Oxazolone-induced contact hypersensitivity (CHS)

Female BALB/c mice (7 weeks; $n = 5$; Charles River Laboratories, Japan) were sensitized with 150 μL of 3% oxazolone (Sigma) in ethanol by having it brushed onto their shaved abdomens. Mice brushed with ethanol only were used as the control (non-sensitized). Six days later, 10 μL of 1% oxazolone was applied to both sides of the right ear for immunological challenge. Ear thickness was measured using a dial thickness gauge (Mitsutoyo) 24 h after the challenge. Compound **8c** was administered 0.5 h before and 8 h after the challenge. The degree of ear swelling in each group was estimated from the difference in pre- and post-challenge ear thickness. The ear swelling in the control and vehicle groups was used to calculate the percent inhibition for compound **8c**.

7.3. Data analysis

The concentration causing 50% inhibition (IC_{50}) was determined by nonlinear curve fitting using the SAS system (SAS Institute, Cary, NC, USA).

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