Synthesis and Evaluation of 6-Nitro-7-(1-piperazino)quinazolines: Dual-Acting Compounds with Inhibitory Activities toward Both Tumor Necrosis Factor-α (TNF-α) Production and T Cell Proliferation

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We investigated the chemical modifications of the nitroquinazoline derivative (1) through the replacement of the NH group at the C(4)-position with several *N*-alkyl groups to increase the lipophilicity at the C(4)-position. Among them, we found that the *N*-methyl analogue (5a) showed a 2-fold loss in the inhibitory activity toward tumor necrosis factor-α (TNF-α) production *in vitro* as compared with the NH analogue (1); however, 5a exhibited an oral inhibitory activity on TNF-α production with an ED_{50} value of 26 mg/kg, whereas 1 did not. Moreover, the oral bioavailability of 5a was higher than that of 1 (1, F=1%; 5a, F=21%), and the calculated ClogP value for 5a was higher than that for 1. These results suggest that the improved lipophilicity of 5a compared with that of 1 reflects its greater inhibitory activity on TNF-α production *in vivo* as well as oral bioavailability.

Key words tumor necrosis factor-α production; T cell proliferation; 6-nitroquinazoline; lipophilicity

Tumor necrosis factor-α (TNF-α) exerts a key role in the cytokine network with regard to the pathogenesis of autoimmune diseases such as rheumatoid arthritis (RA),[1,2] septic shock,[3] and Crohn's disease.[4] To date, two anti-TNF-α agents (Remicade™ and Enbrel™) are available for the treatment of patients with RA and Crohn's disease.[5—10] Additionally, autoimmune diseases are considered to be caused by abnormalities of T cell immune responses, and the activation of T cells has been shown to be a possible mechanism by which inflammation is enhanced in these diseases.[11—14] Based on the above information, we speculated that ideal anti-autoimmune diseases agents should possess the ability to block abnormal T cell-mediated immune responses as well as to inhibit TNF-α activity.

Recently, we have reported that a series of quinazoline derivatives showed inhibitory activities toward both TNF-α production and T cell proliferation.[15] Among them, compound **1** exerted potent inhibitory activities toward both parameters (Fig. 1). However, **1** did not show an inhibitory activity toward TNF-α production by oral administration.[15] Although **1** showed metabolic stability against rat liver S9 fraction *in vitro* (data not shown), its oral bioavailability was poor (F=1%). These data suggest that poor oral bioavailability of **1** reflects no inhibitory activity toward TNF-α production *in vivo*. To overcome the above mentioned problem, we considered the hypothesis that the enhancement of lipophilicity of **1** would lead to an orally-active compound, along with good oral bioavailability.

In the previous papers, we delineated the structure activity relationship (SAR) for the 6-nitroquinazolines.[15,16] The best framework at the C(7)-position of the quinazoline ring was the simple piperazine ring. The optimal length was 1 methylene unit as a spacer between the quinazoline core and the phenyl ring at the C(4)-position. The substitution with the 3,4-methylenedioxy group on the phenyl ring at the C(4)-position was suitable for the TNF-α inhibitory activity, whereas the hydrophilic substituents, such as the amide group and the carboxyl one, were unfavorable.

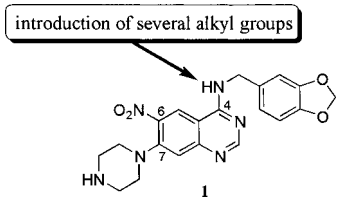

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Fig. 1. Structure of Compound **1**

Based on the above-mentioned SAR data, we concluded that the enhancement of the lipophilicity at the C(4)-position of the quinazoline ring would be desirable for potent inhibitory activity. In the present study, we examined further chemical modifications on the basis of the replacement of the NH group at the C(4)-position of **1** with several *N*-alkyl groups to increase the lipophilicity at the C(4)-position. Herein we wish to describe the discovery of a new series of 6-nitroquinazolines that show the inhibition of TNF-α production by oral administration.

Chemistry The synthetic pathway to the quinazoline derivatives is shown in Chart 1. The key intermediate 4,7-dichloro-6-nitroquinazoline (**3**) was prepared by the chlorination of 7-chloro-6-nitro-4-quinazolone (**2**)[15] with thionyl chloride. Compound **3** was treated with the corresponding amines to give 4-substituted-7-chloro-6-nitroquinazolines (**4a**—**e**). With the exception of **5e**, the target compounds **5a**—**d** were prepared by the reaction of **4a**—**d** with piperazine in the presence of Hunig's base, respectively. Reaction of **4e** with piperazine provided the mixture of the acid (**5e**) and its ester analogue, which was then hydrolyzed by treatment with 5 N NaOH to yield **5e** only.

Results and Discussion

The compounds listed in Table 1 were evaluated for their abilities to inhibit both TNF-α production and T cell proliferation as previously reported.[15] Also included in Table 1 are the results of cytotoxicity experiments using the MTS assay in human PBMCs.[17] As shown in Table 1, the replacement of the NH group at the C(4)-position with the *N*-methyl one (**5a**) led to a 2-fold loss in the TNF-α inhibitory activity as

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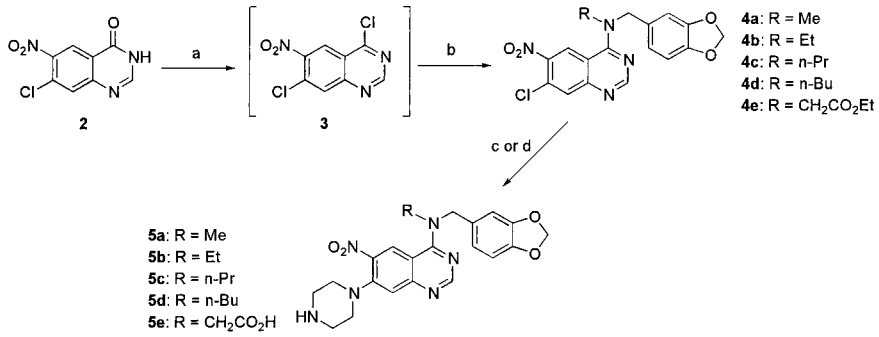
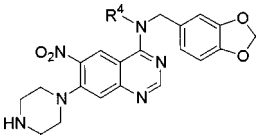


Reagents and conditions: (a) $SOCl_2$, DMF, reflux; (b) *N,N'*-(alkyl)piperonylamine, triethylamine, *i*-PrOH; (c) piperazine, *N,N'*-diisopropylethylamine, *n*-BuOH, 110°C and then 12 N HCl, EtOH; (d) pipearzine, *N,N'*-diisopropylethylamine, *n*-BuOH, 110°C and then 5 N NaOH, EtOH.

Chart 1. Synthesis of Compounds **5a**—**e**

Table 1. Inhibition of TNF-α Production and T Cell Proliferation by Compounds **5a**—**e**



Compound	R^4	IC_{50} (nM)			ClogP[d]
		TNF-α[a]	Con A[b]	MTS[c]	
1	H	78	2743	4240	2.21
5a	Me	158	2432	>30000	2.61
5b	Et	244	4242	>30000	3.14
5c	*n*-Pr	321	4136	8650	3.67
5d	*n*-Bu	535	4023	4427	4.21
5e	CH_2CO_2H	>10000	>10000	>30000	−0.24

a) IC_{50} for inhibition of TNF-α production from human PBMCs stimulated by LPS. *b*) IC_{50} for inhibition of Con A-induced proliferation of mice spleen cells. *c*) IC_{50} for the growth inhibition of human PBMCs stimulated by LPS. *d*) The ClogP values were calculated using the program ClogP for Windows, Version 4.0, a product of BioByte Corp.

Table 2. Effects of **1** and **5a** on LPS-Induced TNF-α Production in Mice, and Pharmacokinetic Profile in Rats

Compound	ED_{50} or % of TNF-α inhibition (mg/kg/*p.o.*)[a]	Pharmacokinetic profile[b]			
		C_{max} (nM)	T_{max} (h)	*AUC* (μM·min)	*F* (%)
1	18% @ 30	39	2	40	1
5a	26	462	2	414	21

a) ED_{50} value was determined from dose–response curve of TNF-α inhibition. Compounds were evaluated as their corresponding hydrochroride salts, and administered orally to mice 1 h prior to the LPS challenge. *b*) Compounds were examined for pharmacokinetics when orally administered to rats in water (30 mg/kg).

compared with the activity of **1**. However, **5a** improved against the cytotoxicity; *i.e.*, the IC_{50} value for the cell growth inhibition of **5a** was over 30 μM, whereas that of **1** was 4.2 μM. Elongation of the alkyl chain length (**5b**—**d**) resulted in the reduction of both inhibitory activities with increasing the cytotoxicity. On the other hand, compound **5e**, which was the hydrophilic analogue (ClogP=−0.24), led to almost complete loss of both inhibitory activities.

On the basis of the above-mentioned *in vitro* study, we selected compound **5a** and performed the comparative evaluation of **1** and **5a** toward the inhibitory effects on TNF-α production *in vivo*. As shown in Table 2 and Fig. 2, compound **5a** inhibited TNF-α production with an ED_{50} value of 26 mg/kg, whereas compound **1** showed no inhibitory activity in this model. Also included in Table 2 were the results of the pharmacokinetic studies on compounds **1** and **5a** conducted in rats, when orally administered at a dose of 30 mg/kg. Compound **5a** showed the C_{max} value of 462 nM and oral bioavailability of 21%, whereas **1** resulted in low C_{max} value (39 nM) and poor oral bioavailability (1%). Furthermore, as we expected, the lipophilicity of **5a** was higher than that of **1** (**5a**, ClogP=2.61; **1**, ClogP=2.21). These results suggest that the higher lipophilicity of **5a** compared with that of **1** reflects its greater oral inhibitory activity as well as oral bioavailability.

Conclusions

In order to find a novel quinazoline derivative possessing

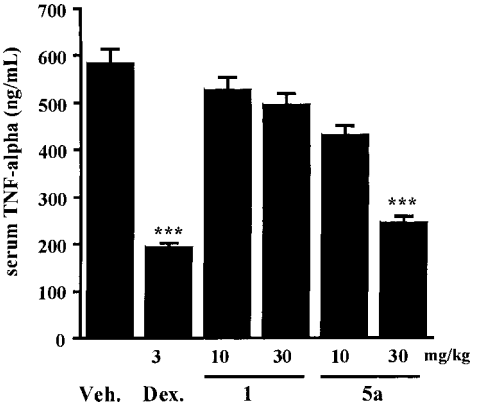


Fig. 2. Effects of **1** and **5a** on LPS-Induced TNF-α Production in Mice

Compounds **1** and **5a** were evaluated as their corresponding hydrochloride salts. The results are expressed as the means±S.E.M. of 4 mice per group. *** $p<0.001$ *versus* vehicle control (Dunnett's test). Veh., vehicle control; Dex., dexamethasone.

an oral inhibitory activity on TNF-α production, we examined further chemical modifications on the basis of the replacement of the NH group at the C(4)-position of **1** with several *N*-alkyl groups. Among them, we found that the *N*-methyl analogue (**5a**) showed a 2-fold loss in the inhibitory activity toward TNF-α production *in vitro* as compared with the NH analogue (**1**); however, **5a** exhibited an oral inhibitory activity on TNF-α production with an ED_{50} value of 26 mg/kg, whereas **1** did not. Moreover, the oral bioavailability of **5a** was higher than that of **1** (**1**, F=1%; **5a**, F=21%), and the calculated ClogP value for **5a** was higher than that for **1**. Therefore, we concluded that the improved lipophilicity of **5a** compared with that of **1** reflects its greater inhibitory activity on TNF-α production *in vivo* as well as oral bioavailability. Compound **5a** is currently being prepared for the pharmacological evaluation and safety as a candidate which may be used clinically as an anti-autoimmune disease agent.

Experimental

Chemistry All reagents and solvents were obtained from commercial suppliers and were used without further purification. Melting points were measured with a BÜCHI 535 melting point apparatus and were uncorrected. Proton NMR spectra were recorded on a JEOL GSX270 FT NMR spectrometer. Time-of-flight mass spectrometry (TOF-MS) was recorded on a KOMPACT MALDI III spectrometer. High-resolution mass spectra were obtained on a JEOL JMS-700 mass spectrometer. Elemental analyses were performed at the Toray Research Center. Monitoring of reactions was carried out using Merck 60 F_{254} silica gel, glass-supported TLC plates, and visualization with UV light (254, 365 nm). Following abbreviations are used for solvents: DMF (*N*,*N*-dimethylformamide), EtOH (ethanol), *i*-PrOH (2-propanol), *n*-BuOH (1-butanol), Et_2O (diethyl ether), *i*-Pr_2O (diisopropyl ether).

7-Chloro-4-(*N*,*N*-methylpiperonylamino)-6-nitroquinazoline (4a) To a suspension of **2**[15] (250 mg, 1.11 mmol) in thionyl chloride (6 ml) was added 1 drop of DMF at ambient temperature, and the mixture was refluxed for 2 h. After the reaction mixture was cooled, excess thionyl chloride was removed under reduced pressure to give crude 4,7-dichloro-6-nitroquinazoline (**3**), which was used directly. Subsequently, to a mixture of **3** and triethylamine (233 μl, 1.67 mmol) in *i*-PrOH (12 ml) was added *N*-methylpiperonylamine (367 mg, 2.22 mmol). The reaction mixture was stirred at ambient temperature for 1 h and evaporated *in vacuo*, and partitioned between CH_2Cl_2 and 5% citric acid aqueous solution; the organic layer was washed with 1 N NaOH, water, brine, and dried over Na_2SO_4. The solution was evaporated *in vacuo* and the residue was triturated with CH_2Cl_2–hexane (1 : 1, v/v). The precipitated solid was washed with *i*-Pr_2O, and was filtered to give **4a** (246 mg, 59% yield for 2 steps from **2**). mp 138—139 °C (*i*-Pr_2O). ^{1}H-NMR (DMSO-d_6) δ: 8.54 (1H, s), 8.13—8.05 (1H, m), 7.81—7.79 (1H, m), 6.94—6.82 (3H, m), 6.01 (2H, s), 4.90 (2H, s), 3.31 (3H, s). TOF-MS *m*/*z*: 373 (M^+). *Anal.* Calcd for $C_{17}H_{13}ClN_4O_4$: C, 54.78; H, 3.52; N, 15.03. Found: C, 54.71; H, 3.63; N, 14.93.

Similarly to the procedure described for **4a**, compounds **4b**—**e** were prepared from **2**, respectively.

7-Chloro-4-(*N*,*N*-ethylpiperonylamino)-6-nitroquinazoline (4b) Light yellow solid (67% yield for 2 steps from **2**). mp 123—125 °C (*i*-Pr_2O). ^{1}H-NMR (DMSO-d_6) δ: 8.65 (1H, s), 8.64 (1H, s), 8.01 (1H, s), 6.95—6.83 (3H, m), 6.02 (2H, s), 4.99 (2H, s), 3.78 (2H, q, J=7.0 Hz), 1.35 (3H, t, J=7.0 Hz). TOF-MS *m*/*z*: 387 (M^+). *Anal.* Calcd for $C_{18}H_{15}ClN_4O_4$: C, 55.89; H, 3.91; N, 14.49. Found: C, 55.86; H, 3.97; N, 14.52.

7-Chloro-4-(*N*,*N*-*n*-propylpiperonylamino)-6-nitroquinazoline (4c) Light yellow oil (58% yield for 2 steps from **2**). ^{1}H-NMR (DMSO-d_6) δ: 8.65 (1H, s), 8.64 (1H, s), 8.02 (1H, s), 6.93—6.82 (3H, m), 6.01 (2H, s), 5.01 (2H, s), 3.71—3.65 (2H, m), 1.91—1.76 (2H, m), 0.93 (3H, t, J=7.3 Hz). TOF-MS *m*/*z*: 401 (M^+). *Anal.* Calcd for $C_{19}H_{17}ClN_4O_4$: C, 56.93; H, 4.28; N, 13.98. Found: C, 56.73; H, 4.44; N, 13.92.

7-Chloro-4-(*N*,*N*-*n*-butylpiperonylamino)-6-nitroquinazoline (4d) Light yellow oil (40% yield for 2 steps from **2**). ^{1}H-NMR (DMSO-d_6) δ: 8.65 (2H, s), 8.02 (1H, s), 6.93—6.82 (3H, m), 6.01 (2H, s), 5.00 (2H, s), 3.74—3.68 (2H, m), 1.86—1.75 (2H, m), 1.42—1.29 (2H, m), 0.92 (3H, t, J=7.3 Hz). TOF-MS *m*/*z*: 415 (M^+). *Anal.* Calcd for $C_{20}H_{19}ClN_4O_4$: C, 57.90; H, 4.62; N, 13.51. Found: C, 58.15; H, 4.54; N, 13.42.

7-Chloro-4-[*N*,*N*-(ethoxycarbonylmethyl)piperonylamino]-6-nitroquinazoline (4e) Light yellow oil (46% yield for 2 steps from **2**). ^{1}H-NMR (DMSO-d_6) δ: 8.70 (1H, s), 8.58 (1H, s), 8.09 (1H, s), 7.04 (1H, s), 6.96 (2H, s), 6.04 (2H, s), 5.08 (2H, s), 4.50 (2H, s), 4.15 (2H, q, J=7.2 Hz), 1.20 (3H, t, J=7.2 Hz). TOF-MS *m*/*z*: 445 (M^+). *Anal.* Calcd for $C_{20}H_{17}ClN_4O_6$: C, 54.00; H, 3.85; N, 12.60. Found: C, 54.28; H, 3.90; N, 12.56.

4-(*N*,*N*′-Methylpiperonylamino)-6-nitro-7-(1-piperazino)quinazoline Hydrochloride (5a) To a suspension of **4a** (150 mg, 0.402 mmol) and *N*,*N*′-diisopropylethylamine (210 μl, 1.21 mmol) in *n*-BuOH (8 ml) was added piperazine (104 mg, 1.21 mmol). The reaction mixture was stirred at 110 °C for 10 h under a nitrogen atmosphere. After the mixture was cooled, the solvent was evaporated *in vacuo*, and then the residue was partitioned between CH_2Cl_2 and 5% citric acid aqueous solution. The aqueous layer was adjusted to pH 9 with 5 N NaOH, and extracted with CH_2Cl_2. The organic layer was washed with water and brine, dried over Na_2SO_4 and evaporated *in vacuo* to give crude **5a**. To a suspension of **5a** in EtOH (7 ml) was added 12 N HCl (84 μl). The mixture was stirred at ambient temperature for 3 h, and then evaporated *in vacuo*. The residue was triturated with Et_2O, and precipitated solid was collected by filtration. The obtained solid was dried *in vacuo* to give the hydrochloride salt as a light yellow powder (123 mg, 66% yield for 2 steps from **4a**). mp 263—265 °C (Et_2O). ^{1}H-NMR (DMSO-d_6) δ: 9.58 (2H, br s), 8.83 (2H, s), 7.57 (1H, s), 7.00—6.88 (3H, m), 6.02 (2H, s), 5.15 (2H, s), 3.57 (3H, s), 3.43—3.25 (8H, m). TOF-MS *m*/*z*: 423 (M^+). *Anal.* Calcd for $C_{21}H_{23}ClN_6O_4\cdot 2.0H_2O$: C, 50.96; H, 5.09; N, 16.98. Found: C, 50.75; H, 4.90; N, 16.65.

Similarly to the procedure described for **5a**, compounds **5b**—**d** were prepared from **4b**—**d**, respectively.

4-(*N*,*N*-Ethylpiperonylamino)-6-nitro-7-(1-piperazino)quinazoline Hydrochloride (5b) Light yellow solid (78% yield for 2 steps from **4b**). mp 86—88 °C (Et_2O). ^{1}H-NMR (DMSO-d_6) δ: 9.57 (2H, br s), 8.85 (1H, s), 8.57 (1H, s), 7.57 (1H, s), 6.99—6.87 (3H, m), 6.04 (2H, s), 5.15 (2H, s), 3.45 (2H, q, J=6.8 Hz), 3.40—3.24 (8H, m), 1.39 (3H, t, J=6.8 Hz). TOF-MS *m*/*z*: 437 (M^+). *Anal.* Calcd for $C_{22}H_{25}ClN_6O_4\cdot 3.5H_2O$: C, 49.30; H, 5.36; N, 15.68. Found: C, 49.55; H, 5.60; N, 15.31.

6-Nitro-4-(*N*,*N*-*n*-propylpiperonylamino)-7-(1-piperazino)quinazoline Hydrochloride (5c) Light yellow solid (76% yield for 2 steps from **4c**). mp 85—87 °C (Et_2O). ^{1}H-NMR (DMSO-d_6) δ: 9.48 (2H, br s), 8.83 (1H, s), 8.55 (1H, s), 7.53 (1H, s), 6.97—6.85 (3H, m), 6.03 (2H, s), 5.16 (2H, s), 3.81—3.76 (2H, m), 3.37—3.25 (8H, m), 1.91—1.86 (2H, m), 0.97 (3H, t, J=7.3 Hz). TOF-MS *m*/*z*: 451 (M^+). *Anal.* Calcd for $C_{23}H_{27}ClN_6O_4\cdot 4.5H_2O$: C, 48.63; H, 5.59; N, 14.80. Found: C, 48.94; H, 5.58; N, 14.82.

4-(*N*,*N*-*n*-Butylpiperonylamino)-6-nitro-7-(1-piperazino)quinazoline Hydrochloride (5d) Light yellow solid (80% yield for 2 steps from **4d**). mp 87—89 °C (Et_2O). ^{1}H-NMR (DMSO-d_6) δ: 9.56 (2H, br s), 8.84 (1H, s), 8.56 (1H, s), 7.56 (1H, s), 6.98—6.85 (3H, m), 6.03 (2H, s), 5.16 (2H, s), 3.85—3.79 (2H, m), 3.44—3.25 (8H, m), 1.84 (2H, br s), 1.44—1.36 (2H, m), 0.94 (3H, t, J=7.3 Hz). TOF-MS *m*/*z*: 465 (M^+). *Anal.* Calcd for $C_{24}H_{29}ClN_6O_4\cdot 3.5H_2O$: C, 51.11; H, 5.81; N, 14.90. Found: C, 51.36; H, 6.03; N, 14.60.

4-[*N*,*N*-(Carboxymethyl)piperonylamino]-6-nitro-7-(1-piperazino)-quinazoline (5e) To a suspension of **4e** (217 mg, 0.488 mmol) and *N*,*N*′-

diisopropylethylamine (261 μl, 1.46 mmol) in *n*-BuOH (10 ml) was added piperazine (126 mg, 1.46 mmol). The reaction mixture was stirred at 110 °C for 5 h under a nitrogen atmosphere. After the mixture was cooled, 5 N NaOH was added. The mixture was stirred at ambient temperature for 3 h, and then evaporated *in vacuo*. The resulting residue was poured into water (3 ml), and then was adjusted to pH 5 with 5% citric acid aqueous solution, and the precipitated solid was washed with EtOH. The light yellow powder was filtered to give **5e** (140 mg, 61% yield for 2 steps from **4e**). mp 217—219 °C (EtOH). ^{1}H-NMR (DMSO-d_6) δ: 12.02 (1H, br s), 8.54 (1H, s), 8.49 (1H, s), 7.21 (1H, s), 7.00 (1H, s), 6.92—6.85 (2H, m), 6.01 (2H, s), 4.97 (2H, s), 4.17 (2H, s), 3.08 (4H, br s), 2.98 (4H, br s). HR-MS (FAB) *m*/*z*: Calcd for $C_{22}H_{22}N_6O_6$ (M^+): 467.1679. Found: 467.1641.

Biology. LPS-Induced TNF-α Production in Human PBMCs Inhibition of TNF-α production was measured by ELISA using human PBMCs stimulated by LPS, as previously reported.[15)]

T Cell Proliferation Assay T cell proliferation was determined by the MTS assay using Con A-stimulated mice spleen cells according to a previous report.[15)]

LPS-Induced TNF-α Production in Mice Inhibitory effects of **1** and **5a** toward TNF-α production in LPS treated mice were evaluated according to a previously reported method.[15)] Briefly, BALB/c mice (Charles River Japan Inc.) were used at 10 weeks of age. LPS from *Escherichia coli* (serotype 026:B6) was purchased from Difco Laboratories (Detroit, U.S.A.). A solution of a test compound in water was orally administered to mice 1 h prior to i.v. injection of the LPS (25 μg/mouse). Blood samples were obtained 2 h after the LPS injection. Amounts of TNF-α in the blood were determined by a specific ELISA kit (Genzyme Techne, U.S.A.).

Pharmacokinetic Analysis The pharmacokinetic parameters of **1** and **5a** were studied in rats. Briefly, in the rat i.v. or *p.o.* administration studies, 3 male SD rats received either a 10 mg/kg intravenous dose or a 30 mg/kg oral dose. Blood samples were obtained after dosing at appropriate times and analyzed by reverse phase HPLC. The pharmacokinetic parameters for the compounds were estimated by a non-compartmental method.

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