Full Paper

Synthesis and Immunomodulating Activity of New Analogues of Fingolimod

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Five new immunomodulators **1a–1e** by using a *trans*-4-alkyl-substituted cyclohexane to replace the flexible C8 alkyl chain of Fingolimod were synthesized. For *in-vitro* test, the compounds were dissolved in DMSO as a stock solution and diluted to a desired concentration with RPMI 1640 nutrient solution. For *in-vivo* test, the compounds were prepared in pathogen-free saline containing 0.5% DMSO. These new immunomodulators displayed more potent immunoinhibitory activities *in vitro* and moderate immunomodulating activities *in vivo*. They show therapeutic effects on DNFB-induced DTH reaction and inhibitory effects on the antigen-specific T-cell proliferation.

Keywords: Antigen-specific T-cell proliferation / Fingolimod / Immunomodulators

Received: March 10, 2011; Revised: May 24, 2011; Accepted: June 10, 2011

DOI 10.1002/ardp.201100092

Introduction

Immunomodulators play important clinical roles in organ transplantation and the treatment of autoimmune diseases [1]. To date, they have progressed through four generations. The first and second generations include the antiproliferative agents and the lymphocyte depletion agents, separately. The application of calcineurin antagonists was recognized as the third generation resulting in revolutionary changes in the development of immunomodulators. The fourth generation includes the cell proliferation signal inhibitors [2]. At present, both cyclosporin A (CsA) [3] and tacrolimus (FK506) [4, 5] are the established immunomodulators and widely used in organ transplantation.

Fingolimod (2-amino-2-(2-(4-octylphenyl)ethyl)propane-1,3-diol hydrochloride, FTY720), a novel synthetic analogue of sphingosine ISP-1 (myriocin, Fig. 1) currently in phase III clinical trials for the treatment of relapsing-remitting multiple sclerosis meanwhile approved in several countries including USA, has displayed favorable immunomodulating properties in numerous investigations [6–13]. It can form the active principle (*S*)-Fingolimod-phosphate by stereoselective phosphorylation *in vivo* to induce apoptosis of activated lymphocytes and inhibit lymphocyte egress out of lymphoid organs. As a consequence, the immunoreactive T-cells are depleted in the peripheral tissue [14, 15]. Only the phosphorylated molecule (*S*)-Fingolimod-phosphate, which binds to four of the five known sphingosine-1-phosphate receptors ($S1P_{1,3\text{-}5}$ but not $S1P_2$), acts as a S1P receptor agonist, while the enantiomer (*R*)-Fingolimod-phosphate shows weak or no agonistic activity on these receptors [16–18].

Additionally, Graeb *et al.* reported that Fingolimod was able to prevent tumor growth and metastasis. It showed a strong antiangiogenic effect by abrogating the stimulating effect of vascular endothelial growth factor (VEGF 20 ng/mL) in a human umbilical vein endothelial cell spheroid model [19].

Structure-activity relationship (SAR) studies of Fingolimod analogues showed that the 2-aminopropane-1,3-diol headgroup was critical for their immunomodulating activities. The phenyl ring was also important for the potent activities of these analogues by modifying their pharmacological properties [1, 20–22].

In order to develop new immunomodulators, we decided to design and synthesize Fingolimod analogues. To design new Fingolimod analogues, we decided to keep the 2-aminopropane-1,3-diol moiety and the phenyl ring, and plan to increase the conformational rigidity and sterical restriction of the flexible C8 alkyl chain of Fingolimod when conformational flexibility

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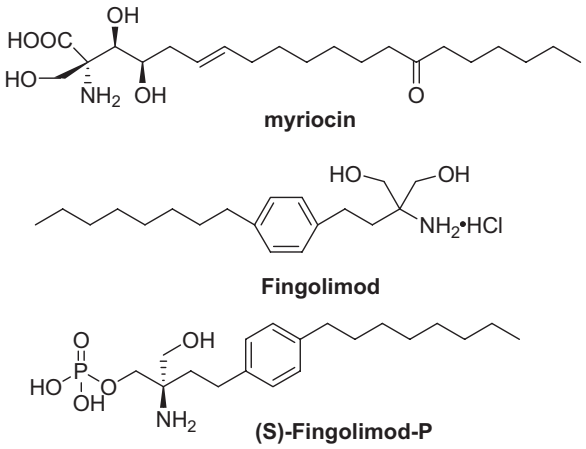
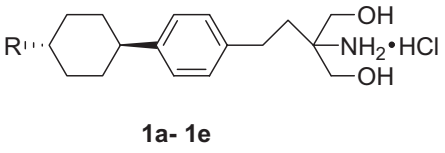


Figure 1. Myriocin, Fingolimod and (*S*)-Fingolimod-phosphate.

could be a complicating factor in drug development [23]. As can be seen in Fig. 2, we selected *trans*-4-alkyl-substituted cyclohexane to achieve effective rigidity and sterical restriction.

In this paper, we describe the design and synthesis of five new Fingolimod analogues with restricted conformational flexibility. We also report the immunomodulating activities of these novel molecules.



1a- 1e

1a: R = H; **1b**: R = Et; **1c**: R = *n*-Pr
1d: R = *n*-Bu; **1e**: R = *n*-Pen

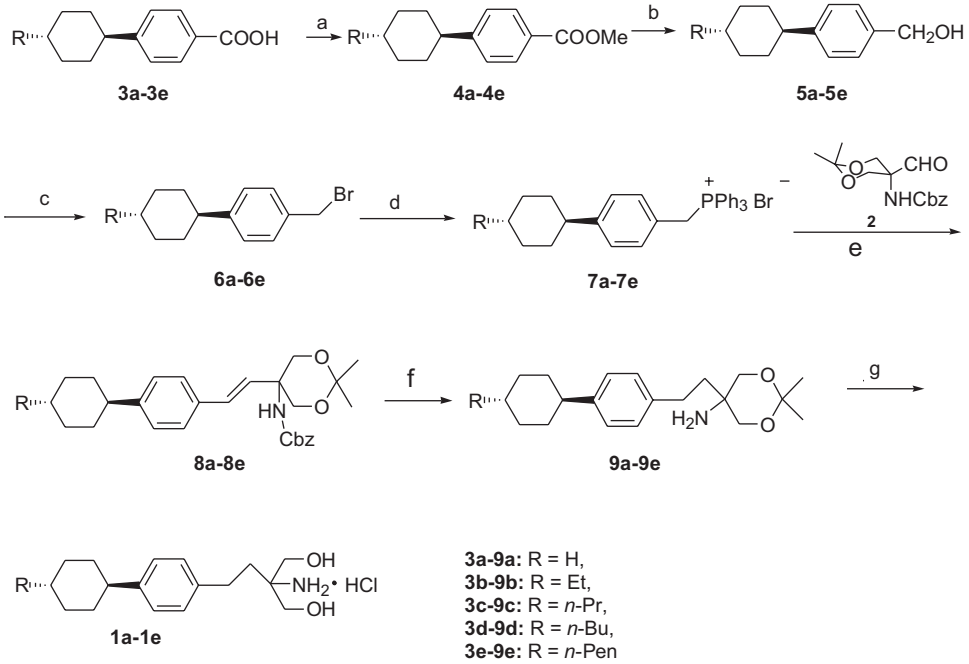
Figure 2. The structures of new immunomodulators.

Results and discussion

Chemistry

The synthetic effort towards these new immunomodulators was carried out as shown in Scheme 1. First, the hydrophilic headgroup and the lipophilic hydrocarbon chain were synthesized separately. The aldehyde moiety (**2**) was obtained from tris-(hydroxymethyl)aminomethane (TRIS) in three steps according to our earlier described method [24].

The lipophilic hydrocarbon chains can be synthesized from their corresponding acids **3a–3e** which are easily available starting materials. Reduction of ester which can be obtained from corresponding acids by conventional esterification, with KBH_4/LiCl gave corresponding alcohols **5a–5e**. These alcohols were treated with PBr_3 in toluene to afford the corresponding



Reagents and conditions: (a) MeOH, H_2SO_4, reflux, 10 h; (b) KBH_4, LiCl, THF, reflux, 8 h; (c) PBr_3, toluene, r.t., 4 h; (d) PPh_3, toluene, reflux, 6 h; (e) compound **2**, K_2CO_3, THF/DMF (3:1), reflux, 12 h; (f) H_2, Pd/C (10 wt%), MeOH, r.t., 3 h; (g) aq. HCl (1 M), MeOH, 50°C, 3 h.

Scheme 1. Synthesis of compound **1a–1e.**

Table 1. Inhibitory activities of Fingolimod and compounds **1a-1e** on ConA- and alloantigen-induced splenocyte proliferation.

	IC_{50} (μM)	
	ConA	MLR
Fingolimod	0.67 ± 0.46	0.64 ± 0.27
1a	1.42 ± 0.39	0.15 ± 0.01
1b	0.81 ± 0.15	0.31 ± 0.17
1c	0.68 ± 0.10	0.19 ± 0.01
1d	0.51 ± 0.11	0.24 ± 0.01
1e	0.57 ± 0.08	0.39 ± 0.18

ConA- and alloantigen-induced proliferation assays were carried out for 48 h and 96 h, respectively. The results were expressed as mean + standard error.

bromides **6a–6e**. Reaction of bromide with PPh_3 provided corresponding Wittig salts **7a–7e** in excellent yields.

Then, heating of these Wittig salts **7a–7e** with the aldehyde **2** in the presence of K_2CO_3 furnished the desired alkenes **8a–8e** as an *E*/*Z* mixture. Simple hydrogenation removed Cbz group and reduced the double bond in one step to give corresponding alkanes **9a–9e**. Removal of the acetonide groups of alkanes afforded compounds **1a–1e**.

In-vitro and *in-vivo* studies

For *in-vitro* test, the compounds were dissolved in DMSO as a stock solution and diluted to a desired concentration with RPMI 1640 nutrient solution. For *in-vivo* test, the compounds were prepared in pathogen-free saline containing 0.5% DMSO.

Inhibitory effect on murine splenocyte proliferation

The activities of compounds **1a–1e** are evaluated on splenocyte proliferation induced by Concanavalin A (ConA) and mixed lymphocyte culture reaction (MLR) *in vitro*. The results of these compounds are summarized in Table 1. The immunosuppressive activity of each compound was expressed as the concentration of compound that reduced cell proliferation to 50% (IC_{50}) of the control value. The data showed that all the new analogues displayed potent immunosuppressive activities *in vitro* and their activities were basically the same as that of Fingolimod.

Inhibitory effect on DNFB-induced DTH response

To test the immunosuppressive activities of these compounds *in vivo*, we used the DNFB-induced DTH model which is based on a cell-mediated pathologic response involved with CD4+ T cell activation. All the compounds, by oral administration, could inhibit DTH reaction, reduced the ear swelling and ear weight. As shown in Fig. 3, compounds **1a** and **1b** displayed slightly worse activities, but the inhibitory activities of compounds **1c–1e** were basic agreement with Fingolimod.

Inhibitory effect on OVA-specific T-cell response

Since compounds **1c** and **1e** showed more potent activities, they were further tested and confirmed for the immunosuppressive properties on antigen-specific lymphocyte proliferative response in OVA-immunized mice. As shown in Fig. 4, compared with lymphocytes from naÿve mice, lymphocytes from OVA-immunized mice exhibited a prominent antigen-specific recall response to OVA. Oral treatment with Fingolimod, compounds **1c** and **1e** (3 mg/(kg day)) significantly suppressed the OVA-specific lymphocyte proliferation. Both compounds **1c** and **1e** displayed basically same potent inhibitory activities with Fingolimod *in vivo*.

In summary, five new immunomodulators **1a–1e** by using a *trans*-4-alkyl-substituted cyclohexane to replace the flexible C8 alkyl chain of Fingolimod were synthesized. They show therapeutic effects on DNFB-induced DTH reaction and inhibitory effects on the antigen-specific T-cell proliferation.

Experimental

Chemistry

^{1}H NMR spectra were recorded on a Bruker DRX-500 (500 MHz). ^{13}C-NMR spectra were obtained on a JNM-EX400 (100 MHz). MS spectra (MS) were determined on a Finnigan MAT-95 mass spectrometer. Chemical shifts (δ) are reported in parts per million

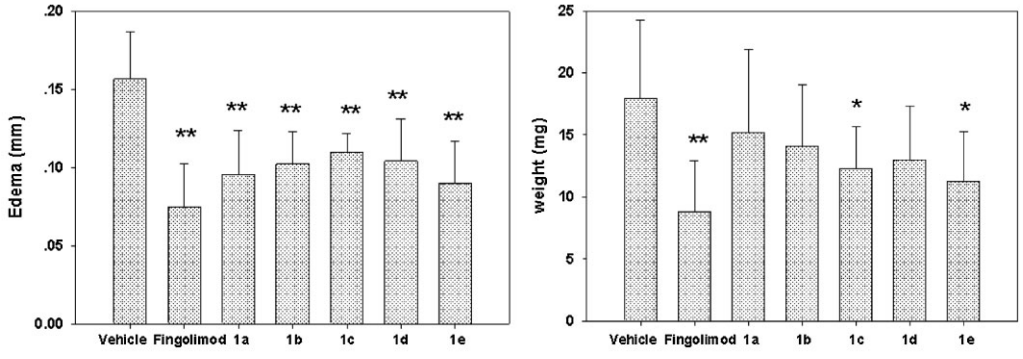


Figure 3. Compounds 1a–1e inhibit DNFB-induced DTH reaction with oral administration.
Data are expressed as mean ± S.D. Three independent experiments were performed with similar results. $^*p < 0.05$, $^{**}p < 0.01$, versus vehicle group; $n = 10$ mice/group.

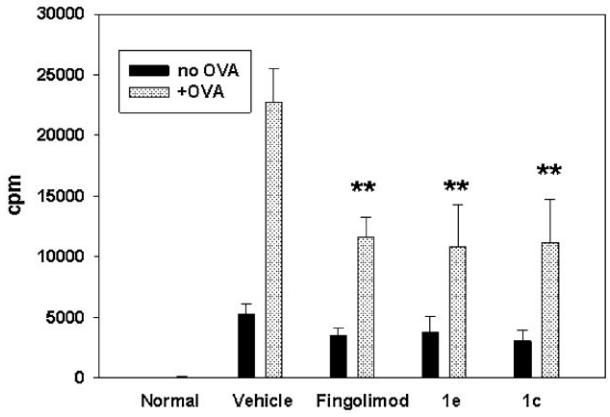


Figure 4. Compounds **1c** and **1e** suppressed OVA-specific T-cell response.
The results were expressed as mean ± S.E.M. of three samples. * $P < 0.05$, ** $P < 0.01$, versus vehicle group. Two independent experiments were performed that gave similar results.

(ppm). Data are reported as follows: chemical shift, multiplicity (br s = broad singlet, d = doublet, dd = doublet of doublet, dt = doublet of triplet, m = multiple, s = singlet and t = triplet), coupling constants (Hz), integration. All reagents were used directly as obtained commercially, unless otherwise noted.

*General procedure for the synthesis of compounds **4a–4e***

A mixture of **3a–3e** (0.1 mol), anhydrous MeOH (200 mL), and conc. H_2SO_4 (0.5 mL) was refluxed for 10 h and cooled to r.t. After removal of MeOH under reduced pressure, the residue was diluted with H_2O and extracted with ethyl acetate. The combined organic extract was washed with saturated Na_2CO_3 solution and brine, then dried over Na_2SO_4 and evaporated to give **4a–4e**, respectively.

*4-Cyclohexylbenzoic acid methyl ester **4a***

White solid, yield: 90%, m.p.: 41–43°C; ^{1}H-NMR ($CDCl_3$) δ: 7.94 (d, J = 8 Hz, 2 H), 7.25 (d, J = 8 Hz, 2 H), 3.88 (s, 3 H), 2.54–2.55 (m, 1 H), 1.84–1.88 (m, 4 H), 1.74–1.76 (m, 1 H), 1.34–1.45 (m, 4 H), 1.24–1.29 (m, 1 H).

*4-((1r,4r)-4-Ethylcyclohexyl) benzoic acid methyl ester **4b***

White solid, yield: 95%, m.p.: 43–44°C; ^{1}H-NMR ($CDCl_3$) δ: 7.95 (d, J = 9 Hz, 2 H), 7.27 (d, J = 9 Hz, 2 H), 3.90 (s, 3 H), 2.50–2.55 (m, 1 H), 1.89–1.91 (m, 4 H), 1.43–1.50 (m, 2 H), 1.25–1.90 (m, 2 H), 1.19–1.21 (m, 1 H), 1.04–1.08 (m, 2 H), 0.91 (t, 3 H).

*4-((1r,4r)-4-Propylcyclohexyl) benzoic acid methyl ester **4c***

White solid, yield: 84%, m.p.: 42–43°C; ^{1}H-NMR ($CDCl_3$) δ: 0.90 (t, 3 H), 1.06–1.08 (m, 2 H), 1.21–1.24 (m, 2 H), 1.33–1.36 (m, 3 H), 1.47–1.48 (m, 2 H), 1.87–1.90 (m, 4 H), 2.51–2.53 (m, 1 H), 3.90 (s, 3 H), 7.27 (d, J = 9 Hz, 2 H), 7.95 (d, J = 9 Hz, 2 H).

*4-((1r,4r)-4-Butylcyclohexyl) benzoic acid methyl ester **4d***

White solid, yield: 88%, m.p.: 39–40°C; ^{1}H-NMR ($CDCl_3$) δ: 7.95 (d, J = 8 Hz, 2 H), 7.27 (d, J = 8 Hz, 2 H), 3.90 (s, 3 H), 2.50–2.53 (m, 1 H), 1.87–1.90 (m, 4 H), 1.44–1.47 (m, 2 H), 1.30–1.31 (m, 5 H), 1.23–1.25 (m, 2 H), 1.04–1.08 (m, 2 H), 0.90 (t, 3 H).

*4-((1r,4r)-4-Amylcyclohexyl) benzoic acid methyl ester **4e***

White solid, yield: 86%, m.p.: 49–51°C; ^{1}H-NMR ($CDCl_3$) δ: 7.95 (d, J = 7 Hz, 2 H), 7.27 (d, J = 7 Hz, 2 H), 3.90 (s, 3 H), 2.50–2.52 (m, 1 H), 1.88–1.90 (m, 4 H), 1.45–1.47 (m, 2 H), 1.22–1.33 (m, 9 H), 1.04–1.08 (m, 2 H), 0.90 (t, 3 H).

*General procedure for the synthesis of compounds **5a–5e***

A mixture of **4a–4e** (0.09 mol), KBH_4 (0.11 mol) and LiCl (0.11 mol) in THF (100 mL) was refluxed for 8 h, then cooled to r. t. and quenched with saturated NH_4Cl solution (60 mL). After stirring for 2 h, the mixture was extracted with ethyl acetate; the organic phase was washed with brine, dried with Na_2SO_4, and concentrated to give crude **5a–5e**, respectively.

*4-Cyclohexylbenzyl alcohol **5a***

White solid, yield: 89%, m.p.: 35–37°C; ^{1}H-NMR ($CDCl_3$) δ: 7.28 (d, J = 8 Hz, 2 H), 7.20 (d, J = 8 Hz, 2 H), 4.64 (s, 2 H), 2.50–2.52 (m, 1 H), 1.83–1.88 (m, 4 H), 1.76–1.77 (m, 1 H), 1.38–1.43 (m, 4 H), 1.26–1.27 (m, 1 H); ^{13}C-NMR ($CDCl_3$) δ: 26.1, 26.8, 34.4, 44.3, 65.2, 127.0, 127.1, 138.3, 147.6. EI-MS: (*m*/*z*) 190 $[M]^+$.

*4-((1r,4r)-4-Ethylcyclohexyl) benzyl alcohol **5b***

White solid, yield: 98%, m.p.: 38–39°C; ^{1}H-NMR ($CDCl_3$) δ: 7.29 (d, J = 8 Hz, 2 H), 7.21 (d, J = 8 Hz, 2 H), 4.65 (s, 2 H), 2.44–2.49 (m, 1 H), 1.87–1.89 (m, 4 H), 1.57 (s, 1 H), 1.41–1.46 (m, 2 H), 1.20–1.28 (m, 3 H), 1.03–1.08 (m, 2 H), 0.90 (t, 3 H); ^{13}C-NMR ($CDCl_3$) δ: 11.5, 29.9, 33.2, 34.3, 39.1, 44.4, 65.3, 127.0, 127.1, 138.3, 147.5. EI-MS (*m*/*z*): 218 $[M]^+$.

*4-((1r,4r)-4-Propylcyclohexyl) benzyl alcohol **5c***

White solid, yield: 92%, m.p.: 48–49°C; ^{1}H-NMR ($CDCl_3$) δ: 7.31 (d, J = 8 Hz, 2 H), 7.23 (d, J = 8 Hz, 2 H), 4.67 (s, 2 H), 2.48–2.51 (m, 1 H), 1.89–1.91 (m, 4 H), 1.46–1.48 (m, 2 H), 1.34–1.37 (m, 3 H), 1.22–1.25 (m, 2 H), 1.07–1.08 (m, 2 H), 0.92 (t, 3 H); ^{13}C-NMR ($CDCl_3$) δ: 14.4, 20.0, 33.6, 34.4, 37.0, 39.7, 44.4, 65.3, 127.0, 127.1, 138.3, 147.5. EI-MS (*m*/*z*): 232 $[M]^+$.

*4-((1r,4r)-4-Butylcyclohexyl) benzyl alcohol **5d***

White solid, yield: 90%, m.p.: 47–48°C; ^{1}H-NMR ($CDCl_3$) δ: 7.29 (d, J = 9 Hz, 2 H), 7.21 (d, J = 9 Hz, 2 H), 4.66 (s, 2 H), 2.48–2.50 (m, 1 H), 1.86–1.90 (m, 4 H), 1.46–1.48 (m, 2 H), 1.24–1.32 (m, 7 H), 1.07–1.08 (m, 2 H), 0.92 (t, 3 H); ^{13}C-NMR ($CDCl_3$) δ: 14.1, 23.0, 29.2, 33.6, 34.4, 37.1, 37.3, 44.4, 65.2, 127.0, 127.1, 138.3, 147.4. EI-MS (*m*/*z*): 246 $[M]^+$.

*4-((1r,4r)-4-Amylcyclohexyl) benzyl alcohol **5e***

White solid, yield: 86%, m.p.: 48–50°C; ^{1}H-NMR ($CDCl_3$) δ: 7.29 (d, J = 7 Hz, 2 H), 7.21 (d, J = 7 Hz, 2 H), 4.66 (s, 2 H), 2.45–2.47 (m, 1 H), 1.87–1.88 (m, 4 H), 1.46–1.48 (m, 2 H), 1.23–1.33 (m, 9 H), 1.07–1.08 (m, 2 H), 0.90 (t, 3 H); ^{13}C-NMR ($CDCl_3$) δ: 14.1, 22.7, 26.6, 32.2, 33.6, 34.3, 37.3, 37.4, 44.4, 65.2, 127.0, 127.1, 138.3, 147.5. EI-MS (*m*/*z*): 260 $[M]^+$.

*General procedure for the synthesis of compounds **6a–6e***

To a solution of compound **5a–5e** (0.07 mol) in toluene (60 mL) at −10°C was added PBr_3 (0.028 mol) dropwise over a period of 20 min. Then, the mixture was stirred for 4 h at room temperature and poured into ice water. The solution was extracted with

ethyl acetate, washed with brine, dried over Na_2SO_4, and concentrated to give **6a–6e**, respectively.

1-Bromomethyl-4-cyclohexylbenzene ***6a***

Colorless oil, yield: 98%; ^{1}H-NMR ($CDCl_3$) δ: 7.31 (d, J = 8 Hz, 2 H), 7.20 (d, J = 8 Hz, 2 H), 4.51 (s, 2 H), 2.50–2.53 (m, 1 H), 1.85–1.89 (m, 4 H), 1.75–1.78 (m, 1 H), 1.36–1.46 (m, 4 H), 1.25–1.28 (m, 1 H); ^{13}C-NMR ($CDCl_3$) δ: 26.0, 26.8, 33.7, 44.3, 44.2, 127.2, 128.9, 135.0, 148.4. EI-MS (*m/z*): 252 $[M]^+$.

1-Bromomethyl-4-((1r,4r)-4-ethylcyclohexyl)benzene ***6b***

Colorless oil, yield: 74%; ^{1}H-NMR ($CDCl_3$) δ: 7.34 (d, J = 8 Hz, 2 H), 7.22 (d, J = 8 Hz, 2 H), 4.52 (s, 2 H), 2.48–2.53 (m, 1 H), 1.91–1.94 (m, 4 H), 1.44–1.51 (m, 2 H), 1.25–1.33 (m, 3 H), 1.08–1.12 (m, 2 H), 0.96 (t, 3 H); ^{13}C-NMR ($CDCl_3$) δ: 11.5, 29.9, 33.1, 33.7, 34.2, 39.0, 44.4, 127.2, 129.0, 148.3. EI-MS (*m/z*): 280 $[M]^+$.

1-Bromomethyl-4-((1r,4r)-4-propylcyclohexyl)benzene ***6c***

White solid, yield: 79%, m.p.: 35–37°C; ^{1}H-NMR ($CDCl_3$) δ: 7.29 (d, J = 8 Hz, 2 H), 7.16 (d, J = 8 Hz, 2 H), 4.47 (s, 2 H), 2.44–2.45 (m, 1 H), 1.84–1.86 (m, 4 H), 1.40–1.44 (m, 2 H), 1.31–1.34 (m, 3 H), 1.18–1.22 (m, 2 H), 1.02–1.04 (m, 2 H), 0.88 (t, 3 H); ^{13}C-NMR ($CDCl_3$) δ: 14.4, 20.0, 33.5, 33.8, 34.2, 37.0, 39.7, 44.4, 127.3, 129.0, 135.1, 148.3. EI-MS (*m/z*): 294 $[M]^+$.

1-Bromomethyl-4-((1r,4r)-4-butylcyclohexyl)benzene ***6d***

White solid, yield: 96%, m.p.: 33–35°C; ^{1}H-NMR ($CDCl_3$) δ: 7.33 (d, J = 9 Hz, 2 H), 7.20 (d, J = 9 Hz, 2 H), 4.51 (s, 2 H), 2.48–2.49 (m, 1 H), 1.88–1.90 (m, 4 H), 1.44–1.46 (m, 2 H), 1.25–1.34 (m, 7 H), 1.05–1.08 (m, 2 H), 0.92 (t, 3 H); ^{13}C-NMR ($CDCl_3$) δ: 14.1, 23.0, 29.2, 33.6, 33.7, 34.2, 37.1, 37.3, 44.4, 127.3, 129.0, 135.1, 148.3. EI-MS (*m/z*): 308 $[M]^+$.

1-Bromomethyl-4-((1r,4r)-4-amylcyclohexyl)benzene ***6e***

White solid, yield: 81%, m.p.: 31–32°C; ^{1}H-NMR ($CDCl_3$) δ: 7.28 (d, J = 7 Hz, 2 H), 7.15 (d, J = 7 Hz, 2 H), 4.45 (s, 2 H), 2.42–2.45 (m, 1 H), 1.85–1.87 (m, 4 H), 1.42–1.45 (m, 2 H), 1.21–1.32 (m, 9 H), 1.03–1.05 (m, 2 H), 0.89 (t, 3 H); ^{13}C-NMR ($CDCl_3$) δ: 14.1, 22.7, 26.6, 32.2, 33.5, 34.2, 37.2, 37.3, 44.3, 127.2, 128.9, 135.1, 148.2. EI-MS (*m/z*): 322 $[M]^+$.

General procedure for the synthesis of compounds ***7a–7e***

A mixture of **6a–6e** (67 mmol) and PPh_3 (67 mmol) in toluene (100 mL) was refluxed under a nitrogen atmosphere for 6 h. Then, the reaction mixture was cooled. The white solid which precipitated, was collected and washed with toluene (30 mL) and dried to give pure **7a–7e**, respectively.

4-Cyclohexylbenzyl (triphenyl) phosphonium bromide ***7a***

White solid, yield: 97%, m.p.: 237–239°C; ^{1}H-NMR ($CDCl_3$) δ: 7.68–7.77 (m, 9 H), 7.60–7.63 (m, 6 H), 6.94–6.98 (m, 4 H), 5.29 (d, J = 11 Hz, 2 H), 2.35–2.41 (m, 1 H), 1.70–1.81 (m, 5 H), 1.18–1.36 (m, 5 H).

4-((1r,4r)-4-Ethylcyclohexyl)benzyl(triphenyl)phosphonium bromide ***7b***

White solid, yield: 96%, m.p.: 243–245°C; ^{1}H-NMR ($CDCl_3$) δ: 7.69–7.78 (m, 9 H), 7.60–7.64 (m, 6 H), 6.94–6.97 (m, 4 H), 5.31 (d, J = 9 Hz, 2 H), 2.34–2.39 (m, 1 H), 1.80–1.85 (m, 4 H), 1.32–1.35 (m, 2 H), 1.20–1.26 (m, 3 H), 0.98–1.01 (m, 2 H), 0.90 (t, 3 H).

4-((1r,4r)-4-Propylcyclohexyl)benzyl(triphenyl)-phosphonium bromide ***7c***

White solid, yield: 96%, m.p.: 238–239°C; ^{1}H-NMR ($CDCl_3$) δ: 7.69–7.78 (m, 9 H), 0.89 (t, 3 H), 7.60–7.64 (m, 6 H), 6.94–6.99 (m, 4 H), 5.32 (d, J = 9 Hz, 2 H), 2.35–2.37 (m, 1 H), 1.77–1.84 (m, 4 H), 1.29–1.35 (m, 4 H), 1.18–1.21 (m, 3 H), 0.99–1.01 (m, 2 H).

4-((1r,4r)-4-Butylcyclohexyl)benzyl(triphenyl)phosphonium bromide ***7d***

White solid, yield: 97%, m.p.: 240–241°C; ^{1}H-NMR ($CDCl_3$) δ: 7.66–7.77 (m, 9 H), 7.59–7.63 (m, 6 H), 6.93–6.97 (m, 4 H), 4.26 (d, J = 11 Hz, 2 H), 2.32–2.37 (m, 1 H), 1.76–1.83 (m, 4 H), 1.20–1.33 (m, 9 H), 0.98–1.02 (m, 2 H), 0.88 (t, 3 H).

4-((1r,4r)-4-Amylcyclohexyl)benzyl(triphenyl)phosphonium bromide ***7e***

White solid, yield: 94%, m.p.: 242–243°C; ^{1}H-NMR ($CDCl_3$) δ: 7.70–7.78 (m, 9 H), 7.61–7.65 (m, 6 H), 6.95–6.99 (m, 4 H), 5.33 (d, J = 9 Hz, 2 H), 2.34–2.39 (m, 1 H), 1.68–1.85 (m, 4 H), 1.19–1.35 (m, 11 H), 1.00–1.03 (m, 2 H), 0.89 (t, 3 H).

General procedure for the synthesis of compounds ***9a–9e***

A suspension of aldehyde **2** (0.01 mol), Wittig salt **7a–7e** (0.01 mol), and K_2CO_3 (0.03 mol) in a mixture of THF (60 mL) and DMF (20 mL) was refluxed for 12 h. After completion of the reaction, THF was evaporated and the reaction mixture was quenched with water and then extracted with ethyl acetate. The combined organic layers were washed with brine, dried with Na_2SO_4, and concentrated to give a residue, which was purified by silica gel flash chromatography to give alkenes **8a** – **8e** in *E*/*Z* mixture. To a solution of the mixture **8a** – **8e** in methanol (30 mL) was added 10% Pd/C (10%). The reaction mixture was hydrogenated for 3 h at room temperature. Then the mixture was filtered. The filtration was concentrated to give compound **9a–9e**, respectively.

5-Amino-5-[2-4-cyclohexylbenzene)ethyl]-2,2-dimethyl-1,3-dioxane ***9a***

White solid, yield: 73% from **7a**, m.p.: 60–63°C; ^{1}H-NMR ($CDCl_3$) δ: 7.10–7.14 (m, 4 H), 3.79 (d, J = 11 Hz, 2 H), 3.48 (d, J = 11 Hz, 2 H), 2.61–2.65 (m, 2 H), 2.46 (s, 1 H), 1.82–1.84 (m, 7 H), 1.72–1.75 (m, 1 H), 1.60–1.63 (m, 2 H), 1.36–1.45 (m, 10 H); ^{13}C-NMR ($CDCl_3$) δ: 26.1, 26.9, 27.4, 28.2, 29.7, 34.5, 37.6, 44.1, 48.7, 70.1, 98.2, 126.9, 128.1, 139.2, 146.8. HRMS (EI): Calcd. for $C_{20}H_{31}NO_2$ $[M]^+$ 317.2355, found: 317.2355.

5-Amino-5-{2-[4-((1r,4r)-4-ethylcyclohexyl)benzene]-ethyl}-2,2-dimethyl-1,3-dioxane ***9b***

White solid, yield: 71% from **7b**, m.p.: 78–79°C; ^{1}H-NMR ($CDCl_3$) δ: 7.01–7.14 (m, 4 H), 3.78 (d, J = 10 Hz, 2 H), 3.47 (d, J = 10 Hz, 2 H), 2.61–2.65 (m, 2 H), 2.48–2.53 (m, 1 H), 1.86–1.88 (m, 4 H), 1.60–1.63 (m, 5 H), 1.42–1.44 (m, 8 H), 1.25–1.28 (m, 2 H), 1.02–1.05 (m, 2 H), 0.90 (t, 3 H); ^{13}C-NMR ($CDCl_3$) δ: 11.5, 19.8, 27.4, 28.2, 30.0, 33.2, 34.3, 37.6, 39.1, 44.2, 48.6, 70.1, 98.2, 127.0, 128.1, 139.2, 145.6. HRMS (EI): Calcd. for $C_{22}H_{35}NO_2$ $[M]^+$ 345.2668, found: 345.2667.

5-Amino-5-{2-[4-((1r,4r)-4-propylcyclohexyl)benzene]-ethyl}-2,2-dimethyl-1,3-dioxane ***9c***

White solid, yield: 67% from **7c**, m.p.: 74–76°C; ^{1}H-NMR ($CDCl_3$) δ: 7.09–7.13 (m, 4 H), 3.79 (d, J = 10 Hz, 2 H), 3.48 (d, J = 10 Hz, 2 H), 2.61–2.64 (m, 2 H), 2.44–2.45 (m, 1 H), 1.84–1.86 (m, 8 H), 1.60–1.63 (m, 2 H), 1.42–1.44 (m, 7 H), 1.32–1.35 (m, 2 H), 1.19–1.21 (m, 2 H), 1.02–1.04 (m, 2 H), 0.89 (t, 3 H); ^{13}C-NMR ($CDCl_3$) δ: 14.4, 19.9, 27.5, 28.2, 33.6, 34.4, 37.0, 37.5, 39.7, 44.2, 48.7, 70.0, 98.2, 127.0, 128.1, 139.2, 145.6. HRMS (EI): Calcd. for $C_{23}H_{37}NO_2$ $[M]^+$ 359.2824, found: 359.2819.

5-Amino-5-{2-[4-((1r,4r)-4-butylcyclohexyl)benzene]-ethyl}-2,2-dimethyl-1,3-dioxane ***9d***

White solid, yield: 69% from **7d**, m.p.: 78–80°C; ^{1}H-NMR ($CDCl_3$) δ: 7.09–7.13 (m, 4 H), 3.79 (d, J = 10 Hz, 2 H), 3.47 (d, J = 10 Hz, 2 H), 2.61–2.65 (m, 2 H), 2.44–2.45 (m, 1 H), 1.85–1.86 (m, 4 H), 1.60–1.63 (m, 2 H), 1.41–1.44 (m, 8 H), 1.23–1.31 (m, 7 H), 1.05–1.08 (m, 2 H), 0.90 (t, 3 H); ^{13}C-NMR ($CDCl_3$) δ: 14.1, 19.9, 23.0, 27.3, 28.3, 29.2, 33.6, 34.4, 37.1, 37.3, 37.6, 44.2, 48.7, 70.1, 98.2, 126.9, 128.1, 139.2, 145.6. HRMS (EI): Calcd. for $C_{24}H_{39}NO_2$ $[M]^+$ 373.2981, found: 373.2982.

5-Amino-5-{2-[4-((1r,4r)-4-amylcyclohexyl)benzene]-ethyl}-2,2-dimethyl-1,3-dioxane ***9e***

White solid, yield: 67% from **7e**, m.p.: 76–77°C; ^{1}H-NMR ($CDCl_3$) δ: 7.09–7.13 (m, 4 H), 3.78 (d, J = 10 Hz, 2 H), 3.47 (d, J = 10 Hz, 2 H), 2.61–2.64 (m, 2 H), 2.44–2.45 (m, 1 H), 1.84–1.86 (m, 4 H), 1.60–1.64 (m, 2 H), 1.42–1.44 (m, 8 H), 1.22–1.32 (m, 9 H), 1.05–1.08 (m, 2 H), 0.89 (t, 3 H); ^{13}C-NMR ($CDCl_3$) δ: 14.1, 19.7, 26.6, 27.5, 28.2, 32.2, 33.6, 34.4, 37.3, 37.4, 37.6, 44.2, 48.7, 70.0, 98.2, 126.9, 128.1, 139.2, 145.6. HRMS (EI): Calcd. for $C_{25}H_{41}NO_2$ $[M]^+$ 387.3137, found: 387.3139.

General procedure for the synthesis of compounds ***1a–1e***

A solution of **9a–9e** (3.1 mmol) in methanol (2 mL) and aqueous HCl (4.0 mL) was heated at 50°C for 3 h. Then, the solvent was evaporated to dryness to give crude compound **1a–1e**, which was crystallized from CH_2Cl_2/MeOH (15:1).

2-Amino-2-[2-(4-cyclohexylphenyl)ethyl]propane-1,3-diol hydrochloride ***1a***

White solid, yield: 81%, m.p.: 191–193°C; ^{1}H-NMR ($CDCl_3$) δ: 7.90 (s, 3 H), 7.10–7.14 (m, 4 H), 5.36–5.38 (t, 2 H), 3.50–3.54 (m, 4 H), 2.55–2.58 (m, 4 H), 2.43–2.45 (m, 1 H), 1.74–1.79 (m, 4 H), 1.68–1.70 (m, 1 H), 1.33–1.38 (m, 4 H), 1.21–1.24 (m, 1 H); ^{13}C-NMR ($CDCl_3$) δ: 25.6, 26.4, 27.9, 33.3, 34.1, 43.4, 60.2, 61.0, 126.6, 128.1, 139.0, 145.1; anal. calcd. ($C_{17}H_{28}ClNO_2$): C, 65.05; H, 8.99; N, 4.46. Found: C, 64.78; H, 8.70; N, 4.59.

NMR for the free base of **1a**: white solid, m.p.: 163–165°C. ^{1}H-NMR ($CDCl_3$) δ: 7.08 (m, 4 H), 4.53 (d, 2 H), 3.20–3.30 (m, 4 H), 2.50–2.55 (m, 4 H), 2.43 (s, 1 H), 1.68–1.76 (m, 4 H), 1.60–1.65 (m, 1 H), 1.35–1.41 (m, 2 H), 1.29–1.31 (m, 4 H), 1.21–1.22 (m, 1 H); ^{13}C-NMR ($CDCl_3$) δ: 25.6, 26.4, 28.5, 34.1, 36.9, 55.4, 65.3, 126.4, 128.0, 140.7, 144.5. HRMS (ESI): Calcd. for $C_{17}H_{27}NO_2$ $[M]^+$: 277.2042, found: 278.2112 (M^++H).

2-Amino-2-{2-[4-((1r,4r)-4-ethylcyclohexyl)phenyl]-ethyl}propane-1,3-diol hydrochloride ***1b***

White solid, yield: 78%, m.p.: 184–186°C; ^{1}H-NMR ($CDCl_3$) δ: 7.83 (s, 3 H), 7.09–7.14 (m, 4 H), 3.36–3.38 (t, 2 H), 3.32–3.52 (m, 4 H), 2.54–2.57 (m, 2 H), 2.40–2.43 (m, 1 H), 1.75–1.83 (m, 6 H), 1.38–1.42 (m, 2 H), 1.22–1.25 (m, 3 H), 1.00–1.03 (m, 2 H), 0.88 (t, 3 H); ^{13}C-NMR ($CDCl_3$) δ: 11.3, 27.9, 29.4, 32.6, 33.2, 33.8, 38.4, 38.9, 60.2, 60.9, 126.5, 127.9, 140.0, 144.8; anal. calcd. ($C_{19}H_{32}ClNO_2$): C, 66.74; H, 9.43; N, 4.10. Found: C, 66.49; H, 9.18; N, 3.98.

NMR for the free base of **1b**: white solid, m.p.: 166–168°C. ^{1}H-NMR ($CDCl_3$) δ: 7.06–7.10 (m, 4 H), 4.42 (s, 2 H), 3.31–3.24 (m, 4 H), 2.50–2.54 (m, 2 H), 2.42–2.47 (m, 1 H), 1.76–1.82 (m, 4 H), 1.44–1.48 (m, 2 H), 1.40–1.41 (m, 2 H), 1.22–1.26 (m, 3 H), 1.01–1.04 (m, 2 H), 0.88 (t, 3 H); ^{13}C-NMR ($CDCl_3$) δ: 11.3, 28.4, 29.4, 32.6, 33.9, 36.9, 38.4, 43.4, 55.3, 65.4, 126.4, 127.9, 140.7, 144.2. HRMS (ESI): Calcd. for $C_{19}H_{31}NO_2$ $[M]^+$: 305.2355, found: 306.2440 (M^++H).

2-Amino-2-{2-[4-((1r,4r)-4-propylcyclohexyl)phenyl]-ethyl}propane-1,3-diol hydrochloride ***1c***

White solid, yield: 76%, m.p.: 186–188°C; ^{1}H-NMR ($CDCl_3$) δ: 7.85 (s, 3 H), 7.09–7.14 (m, 4 H), 5.37 (t, 2 H), 3.51–3.52 (m, 4 H), 2.54–2.57 (m, 2 H), 2.49–2.50 (m, 1 H), 1.75–1.81 (m, 6 H), 1.39–1.42 (m, 2 H), 1.30–1.33 (m, 3 H), 1.18–1.19 (m, 2 H), 1.01–1.04 (m, 2 H), 0.87 (t, 3 H); ^{13}C-NMR ($CDCl_3$) δ: 14.2, 19.4, 27.9, 28.4, 33.0, 33.2, 33.8, 36.3, 39.2, 43.4, 60.2, 60.9, 126.5, 127.9, 140.0, 144.8; anal. calcd. ($C_{20}H_{34}ClNO_2$): C, 67.49; H, 9.63; N, 3.94. Found: C, 67.37; H, 9.66; N, 4.13.

NMR for the free base of **1c**: white solid, m.p.: 173–174°C. ^{1}H-NMR ($CDCl_3$) δ: 7.06–7.10 (m, 4 H), 4.44 (s, 2 H), 3.20–3.32 (m, 4 H), 2.50–2.54 (m, 2 H), 2.44–2.46 (m, 1 H), 1.75–1.81 (m, 4 H), 1.45–1.48 (m, 2 H), 1.38–1.41 (m, 2 H), 1.30–1.33 (m, 3 H), 1.17–1.19 (m, 2 H), 1.00–1.02 (m, 2 H), 0.87 (t, 3 H); ^{13}C-NMR ($CDCl_3$) δ: 14.2, 19.4, 27.9, 28.4, 33.0, 33.2, 33.9, 36.3, 36.9, 43.4, 55.3, 65.4, 126.4, 127.9, 140.7, 144.2. HRMS (ESI): Calcd. for $C_{20}H_{33}NO_2$ $[M]^+$: 319.2511, found: 320.2585 (M^++H).

2-Amino-2-{2-[4-((1r,4r)-4-butylcyclohexyl)phenyl]-ethyl}propane-1,3-diol hydrochloride ***1d***

White solid, yield: 80%, m.p.: 185–187°C; ^{1}H-NMR ($CDCl_3$) δ: 7.92 (s, 3H), 7.09–7.13 (m,4H), 5.39 (t, 3H), 3.51–3.54 (m, 4H), 2.55–2.58 (m, 2H), 2.48–2.52 (m, 1H), 1.75–1.81 (m, 6H), 1.38–1.41 (m, 2H), 1.27–1.28 (m, 5H), 1.21–1.22 (m, 2H), 1.00–1.04 (m, 2H), 0.87 (t, 3H). ^{13}C-NMR ($CDCl_3$) δ: 13.9, 22.4, 27.9, 28.6, 33.1, 33.2, 33.9, 36.5, 36.6, 43.4, 60.3, 60.9, 126.5, 127.9, 139.0, 144.7; anal. calcd. ($C_{21}H_{36}ClNO_2$): C, 68.17; H, 9.81; N, 3.79. Found: C, 68.20; H, 9.61; N, 3.93.

NMR for the free base of **1d**: white solid, m.p.: 174–175°C. ^{1}H-NMR ($CDCl_3$) δ: 7.06–7.10 (m, 4 H), 4.44 (s, 2 H), 3.21–3.24 (m, 4 H), 2.50–2.54 (m, 2 H), 2.44–2.46 (m, 1 H), 1.75–1.81 (m, 4 H), 1.46–1.49 (m, 2 H), 1.38–1.41 (m, 2 H), 1.27–1.28 (m, 5 H), 1.21–1.22 (m, 2 H), 1.00–1.04 (m, 2 H), 0.88 (t, 3 H); ^{13}C-NMR ($CDCl_3$) δ: 13.9, 22.4, 28.4, 28.6, 33.1, 33.9, 36.5, 36.6, 36.9, 39.3, 43.4, 55.3, 65.4, 126.4, 127.9, 140.8, 144.2. HRMS (ESI): Calcd. for $C_{21}H_{35}NO_2$ $[M]^+$: 333.2668, found: 334.2763 (M^++H).

2-Amino-2-{2-[4-((1r,4r)-4-amylcyclohexyl)phenyl]-ethyl}propane-1,3-diol hydrochloride ***1e***

White solid, yield: 75%, m.p.: 180–182°C; ^{1}H-NMR ($CDCl_3$) δ: 7.87 (s, 3 H), 7.09–7.14 (m, 4 H), 5.34 (t, 3 H), 3.51–3.53 (m, 4 H), 2.53–2.56 (m, 2 H), 2.45–2.47 (m, 1 H), 1.76–1.82 (m, 4 H), 1.41–1.44 (m, 2 H), 1.19–1.30 (m, 9 H), 1.05–1.06 (m, 2 H), 0.87 (t, 3 H); ^{13}C-NMR ($CDCl_3$) δ: 13.9, 22.1, 26.0, 27.9, 31.6, 33.1, 33.3, 33.9, 36.6, 36.9, 44.5, 60.3, 60.9, 126.6, 128.0, 139.1, 144.8; anal. calcd. ($C_{22}H_{38}ClNO_2$): C, 68.81; H, 9.97; N, 3.65. Found: C, 68.97; H, 9.85; N, 3.79.

NMR for the free base of **1e**: white solid, m.p.: 174–176°C. ^{1}H-NMR ($CDCl_3$) δ: 7.06–7.10 (m, 4 H), 4.45 (s, 2 H), 3.20–3.26 (m, 4 H), 2.50–2.54 (m, 2 H), 2.45–2.47 (m, 1 H), 1.75–1.81 (m, 4 H), 1.47–1.48 (m, 2 H), 1.40–1.43 (m, 2 H), 1.20–1.29 (m, 9 H), 1.05–1.06 (m, 2 H), 0.87 (t, 3 H); ^{13}C-NMR ($CDCl_3$) δ: 13.9, 22.1, 26.0, 28.5, 31.6, 33.1, 33.3, 33.9, 36.6, 36.8, 44.4, 55.5, 65.2, 126.4, 128.0, 138.3, 139.0, 144.3. HRMS (ESI): Calcd. for $C_{22}H_{37}NO_2$ $[M]^+$: 347.2824, found: 348.2830 (M^++H).

Biological assays

Female BALB/c and C57BL/6 mice (6- to 8-week-old) were obtained from Shanghai Laboratory Animal Center of the Chinese Academy of Sciences. The mice were housed under specific pathogen-free conditions. The environment was maintained at 22 ± 1°C with a 12 h light and dark cycle. All mice were allowed to acclimatize in our facility for 1 week before any experiments were started. Experiments were carried out according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Bioethics Committee of the Shanghai Institute of Materia Medica.

Splenocyte preparation

Mice were sacrificed, and spleens were removed aseptically. A single cell suspension was prepared after cell debris, and clumps were removed. Erythrocytes were depleted with ammonium chloride buffer solution. Lymphocytes were washed and resuspended in RPMI 1640 medium containing 10% FBS.

ConA-induced proliferation assay

Splenocytes were stimulated by 5 μg/mL ConA in a 96-well plate in triplicate for 48 h in the presence of compounds. Cells were pulsed with 0.5 μCi/well [^{3}H]thymidine for 8 h and harvested onto glass fiber filters. The incorporated radioactivity was then counted using a Beta Scintillation Counter (MicroBeta Trilux; PerkinElmer Life and Analytical Sciences, Boston, MA).

Mixed lymphocyte reaction assay

BALB/c splenocytes (3×10^5 cells/well) were γ-irradiated by 30 Gy (Gammacell 3000, Ottawa, ON, Canada) and then cocultured with C57BL/6 splenocytes (3×10^5 cells/well) for 96 h in the presence of compounds. Cells were pulsed with 1 μCi/well [^{3}H]thymidine for 8 h before harvested onto glass fiber filters.

DNFB-induced DTH reaction

Mice were initially sensitized with 0.7% DNFB dissolved in acetone/olive (4:1) on each hind foot on day 0 and 1. On day 9, mice were challenged with 0.6% DNFB on both sides of their left ear. Vehicle and compounds (0.3 mg/kg) were administered once daily from day 0 to the end of the experiment. Ear swelling was expressed as the increase in ear thickness and ear patch weight between left (DNFB treated) and right (untreated) ear 24 h after challenge.

OVA-specific T cell response

OVA at 2 mg/mL in phosphate-buffered saline (PBS) was emulsified in an equal volume of complete Freund's adjuvant. The emulsion (100 μL containing 100 μg OVA) was injected subcutaneously into the backs of the C57BL/6 mice on day 1. OVA-immunized mice were administered orally with vehicle or compound**s** once daily for 14 days. On day 14, splenocytes (4×10^5 cells/well) from each group were stimulated with or without OVA (100 μg/mL) in 96-well flat-bottom plates. Cells were pulsed with 0.5 μCi/well [^{3}H]thymidine for 8 h before harvesting and assessed for [^{3}H]thymidine incorporation at 48 h.

We gratefully acknowledge the financial support for new century excellent talents in university (NCET). And we also thank Lab of Organic Functional Molecules, the Sino-French Institute of ECNU for support. This work was supported by National Science Fair Committee (NSFC): 1072652, National Science & Technology Major Project 2009ZX09103-070, 2009ZX09102-019 and 2009ZX09301-001.

The authors have declared no conflict of interest.

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