Bioorganic & Medicinal Chemistry 16 (2008) 9212–9216

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis and biological evaluation of immunosuppressive agent DZ2002 and its stereoisomers

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ARTICLE INFO

Article history:
Received 31 July 2008
Revised 1 September 2008
Accepted 5 September 2008
Available online 9 September 2008

Keywords:
DZ2002
Stereoisomers
Synthesis
Immunosuppressive potency
S-Adenosyl-L-homocysteine hydrolase

ABSTRACT

DZ2002 and its related stereoisomers were efficiently synthesized. The optical data of (*R*)- and (*S*)-DZ2002 were disclosed here for the first time. Their inhibitory potency was evaluated on SAHase and MLR assay in the mean time. In accordance with respective inhibitory potency of SAHase, the immunosuppressive potency order was demonstrated as (*S*)-DZ2002 > (*Rac*)-DZ2002 > (*R*)-DZ2002 > (*Keto*)-DZ2002. These results indicate (*S*)-configuration of 2-chiral center in DZ2002 is important for binding with SAHase.

1. Introduction

S-Adenosylmethionine (AdoMet)-dependent biological transmethylations are involved in a variety of important physiologic processes, including most methylations of proteins,[1] lipids,[2] nucleic acids,[3] and small molecules.[4] These reactions are primarily catalyzed by AdoMet-dependent methyltransferases, which convert AdoMet to *S*-adenosyl-L-homocysteine (AdoHcy). AdoHcy is then hydrolyzed by *S*-adenosyl-L-homocysteine hydrolase (SAHase) to prevent feedback inhibition of transmethylations.[4–9] Inhibiting SAHase leads to accumulation of intracellular AdoHcy and most intracellular transmethylations cease or halt. Since lymphocytes seem more dependent on transmethylation reactions than most other cell types for their functions,[10] SAHase is regarded as a suitable target for the design of immunosuppressive, anti-inflammatory, and antiviral agents.[4]

Generally, three types of SAHase inhibitors have been categorized based on binding mechanism: the irreversible type I, type II, and reversible type III inhibitors.[4] Type I inhibitors, like aristeromycin, neplanocin A, MDL28,842, C^3-Ado, etc., are irreversibly trapped in the active site of SAHase utilizing its 3′-oxidative activity. Type II inhibitors, such as 6′-bromo-5′,6′-didehydro-6′-deoxy-6′-fluorohomoadenosine, act by permanently altering the active site of the enzyme using its 5′-hydrolytic activity. Among three types of inhibitors, type 1 inhibitors have been explored most extensively. However, irreversible inhibitors manifest severe toxicity and side effects due to the nature of irreversible binding with the enzyme, which circumscribe their development to clinically useful drugs.[4] In yet another aspect, type III inhibitors can reversibly bind to the open form of the enzyme, maintaining a similar potency with much reduced toxicity.

Within recent years, 4-(6-aminopurine-9-yl)-2-hydroxybutyric acid methyl ester (DZ2002), has been recognized as a potent reversible inhibitor of SAHase, whose reversibility was proved by the rapid restoration of SAHase activity after treatment discontinuation.[5–9] Biological studies show that DZ2002 reduces DNFB-induced delayed-type hypersensitivity responses,[6] suppresses OVA-induced lymphocyte proliferation and Th1-type cytokine production,[8] ameliorates MOG35-55-induced experimental autoimmune encephalomyelitis (EAE).[9] DZ2002 is regarded as a promising therapeutic agent for immune-related diseases. The presence of 2-chiral center allows DZ2002 to exist in two optical forms, both of which have the same physical and chemical properties in an achiral environment, but probably have different

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doi:10.1016/j.bmc.2008.09.017

biological properties. However, the lack of disclosed optical information on DZ2002 from reported data makes it difficult to develop a practical manufacturing process.[11] All of mentioned above intrigued us to synthesize all its related stereoisomers with an aim to investigate the necessity of chiral center in DZ2002. Here, we described efficient approaches to prepare DZ2002 and its related stereoisomers. Their inhibitory activity against SAHase and immunosuppressive potency, were examined in the mean time. The ketone form of DZ2002, a possible existing form in vivo, was also prepared and examined (Fig. 1).

2. Chemistry

Different from patented synthesis of DZ2002, which either commenced with the reaction of adenine and 3-hydroxybutyrolactone,[11a] or utilized 9-(3,4-*O*-isopropylidene-3,4-dihydroxybutyl)adenine as starting material,[11b,11c] our synthesis started with cheap and commercially available optical malic acid (Scheme 1). In brief, (*S*)-malic acid was first transformed to acetonide **1**. Acetonide **1** was reduced to alcohol **2**,[12] which was immediately converted to tosylate **3**. Coupling of tosylate **3** with adenine gave compound **4** in a yield of 32% after silica-gel chromatograph. Compound **4** was readily transformed to (*S*)-DZ2002 upon spontaneous deprotection and esterification in a methanol solution of HCl. Because of the rigid conformation of acetonide during reaction sequences, 2-chiral center was highly reserved and potential racemization was avoided. Optical test results of newly prepared sample were, $[\alpha]_D^{25}$ +19.2 (*c* 0.3, MeOH/H_2O = 1:1, v/v), ee 92%. Following the same process, (*R*)-DZ2002 was synthesized smoothly from (*R*)-malic acid, $[\alpha]_D^{25}$ −17.9 (*c* 0.3, MeOH/H_2O = 1:1, v/v), ee 94%.

Attempts to synthesize (*Keto*)-DZ2002 by direct oxidation of (*S*)- or (*R*)-DZ2002 with Jones agent, PCC or IBX, failed and an alternative approach was employed (Scheme 2). Ester **5**, prepared via a Michael-type addition of adenine to ethyl acrylate, was hydrolyzed in refluxing 3 N HCl solution.[13] The obtained acid **6** was then coupled with easily accessible (triphenylphosphoranylidene)acetonitrile in the presence of EDCI to afford compound **7**.[14] After ozonolysis of compound **7** in methanol, the desired (*Keto*)-DZ2002 was obtained cleanly. Upon treatment with $NaBH_4$ in methanol, (*Rac*)-DZ2002 was obtained in a yield of 60% after silica-gel chromatograph.

3. Biological assay

The inhibitory potency against SAHase was measured by DTNB-coupled assay of SAHase hydrolytic activity, a mature and reliable method developed by Lozada-Ramirez,[15] later further proved by Wu et al[16]. Test results (Fig. 2) showed that (*S*)-DZ2002 was the strongest inhibitor among four compounds (IC_{50} = 21.2 μM, Table 1). The order of enzyme inhibition potency was (*S*)-Z2002 > (*Rac*)-DZ2002 > (*R*)-DZ2002 > (*Keto*)-Z2002.

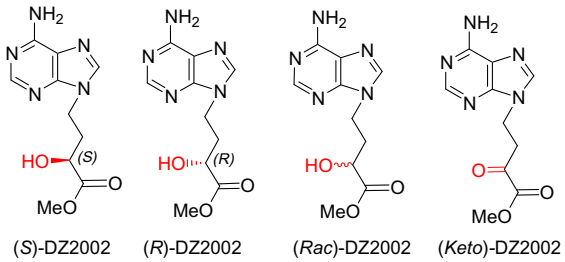
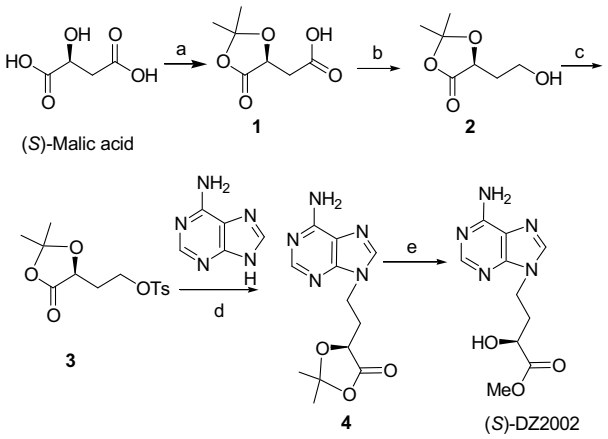
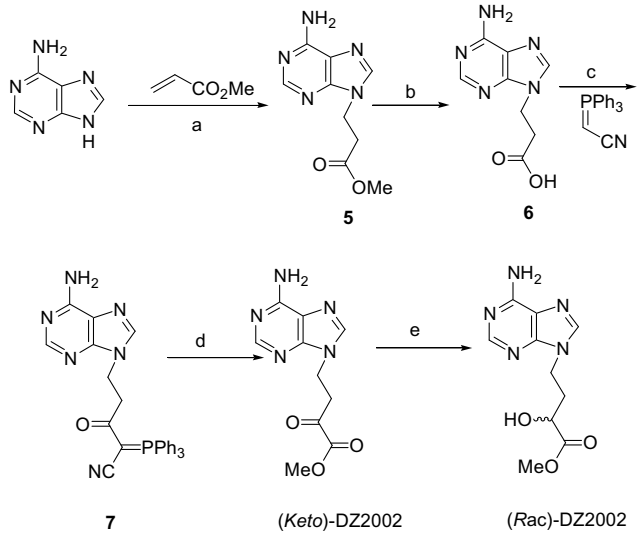


Figure 1. DZ2002 and related stereoisomers.



Scheme 1. Reagents and conditions: (a) 2,2-dimethoxypropane, cat. *p*-TSA, 6 h, 61%; (b) $(CH_3)_2S{\cdot}BH_3$, THF, 0 °C–room temperature, 12 h, quantitative yield; (c) TsCl, pyridine, 0 °C–room temperature, 5 h, 67%; (d) K_2CO_3, adenine, 18-crown-6, DMF, 50 °C, 12 h, 32%; (e) AcCl, MeOH, 0 °C–room temperature, 5 h, 50%.



Scheme 2. Reagents and conditions: (a) EtONa, benzene, reflux, 12 h, 83%; (b) 3 N HCl, reflux, 3 h, 95%; (c) EDCI, DMAP, DMF, room temperature, 12 h, 71%; (d) O_3, MeOH, −78 °C, 20 min, 88%; (e) $NaBH_4$, MeOH, room temperature, 30 min, 60%.

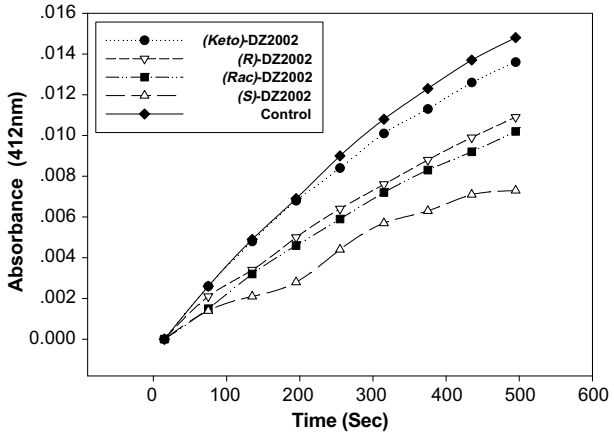


Figure 2. Inhibition of SAHase by (*S*)-, (*R*)-, (*Rac*)-, and (*Keto*)-DZ2002. Data are representative of three independent experiments.

Table 1
Inhibitory value on SAHase of each compound

Compound	IC_{50} (μM)	CC_{50} (μM)
(*S*)-DZ2002	21.2	>1000
(*Rac*)-DZ2002	24.4	>1000
(*R*)-DZ2002	61.3	>1000
(*Keto*)-DZ2002	193.9	>1000

IC_{50}, concentration, at which the lymphocyte proliferation was inhibited by 50%; CC_{50}, concentration, at which cell viability was reduced by 50%.

The immunosuppressive potency of each compound was examined by mixed lymphocyte reaction (MLR) (Fig. 3).[6] Respective suppressive potency appeared to match the inhibitory potency of the SAHase. The order of immunosuppressive activity in vitro was in accordance with that of inhibitory potency of enzyme. Of the four compounds, (*S*)-DZ2002 displayed the most potent immunosuppressive activity in vitro.

4. Discussion

Chirality is an important issue for current pharmaceuticals because the body always recognizes chirality. In most times, both chiral forms of a drug can be taken safely without serious side effects. In some cases, one chiral form is active and the other is not. In our DZ2002 case, (*S*)-, (*R*)-, and (*Rac*)-DZ2002 were evaluated for their biological efficacy. The results indicated that maintenance of 2-chiral center in the molecule as (*S*)-configuration is important for its binding affinity. Further evidence in support of this hypothesis comes from simplified docking results of (*S*)-, (*R*)-, and (*Keto*)-DZ2002 into the active site of SAHase (Fig. 4). The crystal structure of SAHase complexed with neplanocin (PDB code: 1Ii4)[16] was chosen to represent the enzyme structure and the computation was performed on Discovery Studio™. When the conformation of adenine rings were fixed for a simplicity reason, their aliphatic side chains orientated differently, resulting in different numbers of hydrogen bonds interacted with SAHase, a critical factor determining affinity with the enzyme, particularly for reversible inhibitors. The side chain of (*S*)-DZ2002 interacted with Asp55, Asp131, and Asp301 via four hydrogen bonds. The side chain of (*R*)-DZ2002 interacted via three hydrogen bonds with Asp131, Asp186, and Asp190 and the side chain of (*Keto*)-DZ2002 interacted via only two hydrogen bonds with Asp157 and Asp346. Combined these results together, we determined the critical role of (*S*)-configuration of 2-hydroxy group in DZ2002 for binding with SAHase.

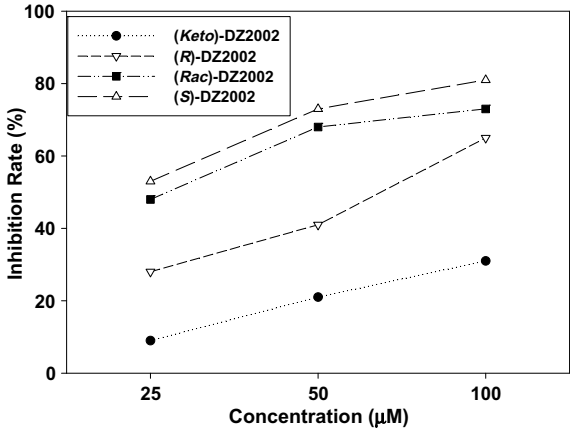


Figure 3. (*S*)-, (*R*)-, (*Rac*)-, and (*Keto*)-DZ2002 (three concentrations: 25, 50, and 100 μM) suppressed cell proliferation in mixed lymphocyte reaction. Data are representative of three independent experiments.

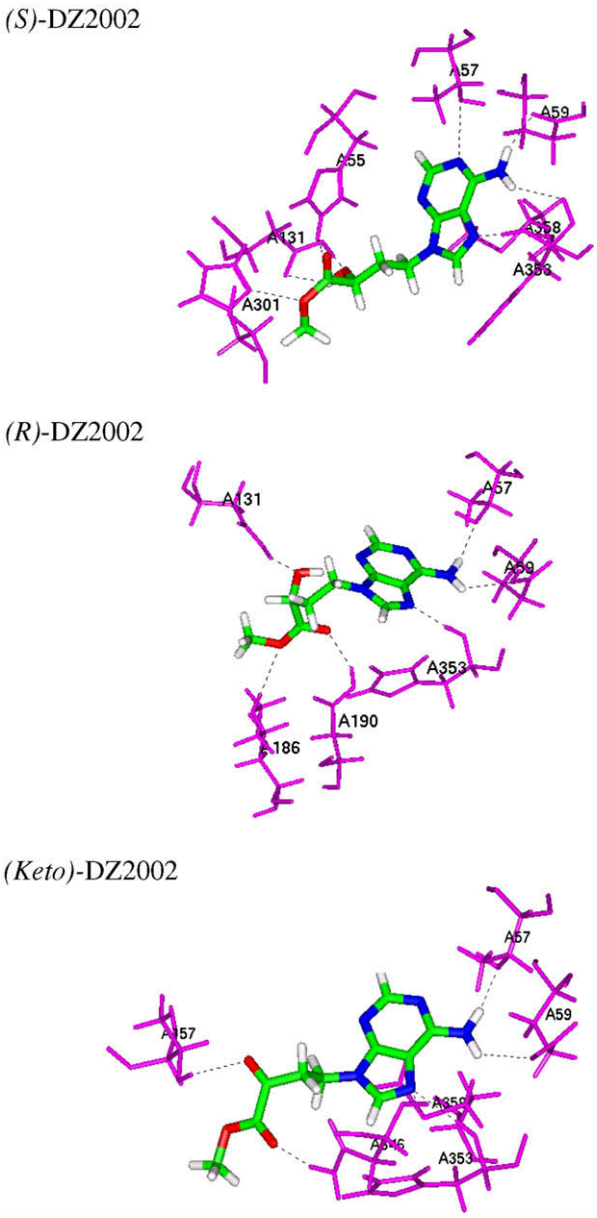


Figure 4. (*S*)-, (*R*)-, and (*Keto*)-DZ2002 docked into the active site of SAHase. For clarity, only the residues providing the main hydrogen bond interactions are shown. Hydrogen bonds are represented as dotted lines.

In summary, we described a practical approach to prepare optical DZ2002 and related isomers. The optical data of (*R*)- and (*S*)-DZ2002 were disclosed here for the first time. (*Keto*)-DZ2002 and (*Rac*)-DZ2002 were also synthesized conveniently. Biological studies demonstrated that their immunosuppressive potency was in accordance with respective inhibitory potency of SAHase, following the order of (*S*)-DZ2002 > (*Rac*)-DZ2002 > (*R*)-DZ2002 > (*Keto*)-DZ2002. (*S*)-DZ2002 displayed the strongest inhibitory potency against SAHase and immunosuppressive potency in vitro. These experimental results indicate that 2-chiral center in the molecule of DZ2002 is necessary and important for its activity, possibly by affecting the formation of hydrogen bonds with the enzyme. Therefore, our results provide useful information for quality control in development of practical manufacturing process.

5. Experimental

5.1. General procedure

^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectra were performed on a Varian Mercury-VX300 spectrometer, using residue $CHCl_3$, DMSO or H_2O as internal standard. HRMS (ESI) were recorded on a waters-micromass Q-TOF Ultima Global electrospray mass spectrometer. Melting point was measured on a ShengGuang WRR Melting Points apparatus and was not corrected. Optical rotation data was recorded on a Perkin-Elmer Model 341 Polarimeter. ee values were determined by HPLC: Chiralcel OJ-H column; *n*-hexane/2-propanol (90:10); 1.0 mL/min; 263 nm; t_R (*R*) 69.2 min; t_R (*S*) 98.1 min.

5.1.1. (4*S*)-Toluene-4-sulfonic acid 2-(2,2-dimethyl-5-oxo-[1,3]dioxolan-4-yl)-ethyl ester (3)

To a solution of compound **2** (60.0 g, 375 mmol), prepared from (*S*)-malic acid,[12] in pyridine (300 mL), was added *p*-toluenesulfonyl chloride (71.5 g, 375 mmol) at 0 °C. The mixture was stirred at that temperature for 1 h and then at room temperature for 4 h. The solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate (600 mL). The organic solution was washed with HCl solution (5%), satd $NaHCO_3$ solution, and brine, dried on $MgSO_4$ and concentrated. Crude product was purified by silica-gel chromatograph eluting with petroleum ether/ethyl acetate (5:1). Compound **3** was obtained (67%) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.80 (2H, d, J = 8.1 Hz), 7.35 (2H, d, J = 8.4 Hz), 4.43 (1H, dd, J = 4.5 and 7.8 Hz), 4.23 (2H, m), 2.45 (3H, s), 2.22 (1H, m), 2.02 (1H, m), 1.57 (3H, s), 1.51 (3H, s).

5.1.2. (5*S*)-5-[2-(6-Aminopurine-9-yl)-ethyl]-2,2-dimethyl-[1,3]dioxolan-4-one (4)

A mixture of compound **3** (72.0 g, 229 mmol), adenine (62.0 g, 459 mmol), K_2CO_3 (92.0 g, 665 mmol) and 18-crown-6 (2.0 g, 7.6 mmol) in dry DMF (360 mL) was heated to 50 °C for 12 h. The mixture was cooled to room temperature and filtered. The solid was washed with ethyl acetate. The combined organic solution was concentrated to remove most of DMF to afford a brown oil. The crude product was purified by silica-gel chromatograph eluting with chloroform/methanol (20:1, then 10:1). Compound **4** was obtained (20.0 g, 32%) as a pale yellow solid: ^{1}H NMR (DMSO-d_6) δ 8.14 (2H, s), 7.19 (2H, br s), 4.67 (1H, dd, J = 4.2 and 7.8 Hz), 4.27 (2H, t, J = 7.2 Hz), 2.40 (1H, m), 2.23 (1H, m), 1.56 (3H, s), 1.52 (3H, s).

5.1.3. (2*S*)-4-(6-Aminopurine-9-yl)-2-hydroxybutyric acid methyl ester [(*S*)-DZ2002]

Acetyl chloride (7.0 mL, 98 mmol) was added dropwise to a solution of compound **4** (18.0 g, 65 mmol) in methanol (540 mL) at 0 °C within 30 min. The mixture was then allowed to stir at room temperature for 3 h. $NaHCO_3$ (14 g, 167 mmol) was added carefully to neutralize the generated acid. The mixture was filtered. To the filtrate was added silica-gel (60 g) and concentrated under reduced pressure until a freely running solid was obtained. This solid was transferred to a silica-gel column and eluted with chloroform/methanol (15:1, then 10:1). The obtained crude product was further purified by recrystallization in methanol. (*S*)-DZ2002 was obtained (8.1 g, 50%) as white crystalline powders: ^{1}H NMR (DMSO-d_6): δ 8.13 (1H, s), 8.07 (1H, s), 7.18 (2H, br s), 5.69 (1H, br d, J = 5.7 Hz), 4.24 (2H, t, J = 6.9 Hz), 4.01 (1H, m), 3.56 (3H, s), 2.23 (1H, m), 2.06 (1H, m); ^{13}C NMR (DMSO-d_6): δ 174.53, 156.63, 153.06, 150.18, 141.67, 119.49, 67.97, 52.22, 40.34, 34.21; HR ESIMS m/z 274.0921 $[M+Na]^+$ (calcd. for $C_{10}H_{13}N_5NaO_3$ 274.0916); mp 162–164 °C; $[\alpha]_D^{25}$ +19.2 (c 0.3, MeOH/H_2O = 1:1); ee 92%.

5.1.4. (2*R*)-4-(6-Aminopurine-9-yl)-2-hydroxybutyric acid methyl ester [(*R*)-DZ2002]

In the same manner, (*R*)-DZ2002 was obtained as a white solid: ^{1}H NMR (DMSO-d_6): δ 8.14 (1H, s), 8.07 (1H, s), 7.18 (2H, br s), 5.71 (1H, br d, J = 5.7 Hz), 4.24 (2H, t, J = 6.9 Hz), 4.01 (1H, m), 3.56 (3H, s), 2.21 (1H, m), 2.06 (1H, m); HR ESIMS m/z 252.1100 $[M+H]^+$ (calcd. for $C_{10}H_{14}N_5O_3$ 252.1097); $[\alpha]_D^{25}$ −17.9 (c 0.3, MeOH/H_2O = 1:1, v/v); ee 94%.

5.1.5. 3-(6-Aminopurine-9-yl)-propionic acid methyl ester (5)

To a mixture of adenine (5.0 g, 37.0 mmol) in absolute ethanol (130 mL) and dry benzene (16 mL) was added sodium metal (60 mg, 1.6 mmol) carefully at room temperature. Upon the disappearance of sodium, ethyl acrylate was added dropwise. The resulting mixture was heated to reflux for 12 h, and then cooled to room temperature. The solvent was removed under reduced pressure. The obtained residue was titrated with cold ethanol. The precipitate was collected by filtration, washed with cold ethanol and dried in vacuum. Compound **5** was obtained (7.2 g, 83%) as an off-white solid: ^{1}H NMR ($CDCl_3$) δ 8.36 (1H, s), 7.90 (1H, s), 5.71 (2H, br s), 4.50 (2H, t, J = 6.3 Hz), 4.13 (2H, q, J = 7.2 Hz), 2.92 (2H, t, J = 6.3 Hz), 1.22 (2H, t, J = 7.2 Hz).

5.1.6. 3-(6-Aminopurine-9-yl)-propionic acid (6)

A mixture of compound **5** (4 g, 17 mmol) and aqueous 3 N HCl solution was heated to reflux for 3 h and was cooled to room temperature. The mixture pH was adjusted to 3 by careful addition of solid NaOH. The precipitate was collected by filtration, washed with cold water and dried in vacuum. Compound **6** was obtained (3.35 g, 95%) as a white solid: ^{1}H NMR (D_2O) δ 8.42 (1H, s), 8.37 (1H, s), 4.58 (2H, t, J = 6.3 Hz), 3.02 (2H, t, J = 6.3 Hz).

5.1.7. 5-(6-Aminopurine-9-yl)-3-oxo-2-(triphenyl-λ^5-phosphanylidene)-pentanenitrile (7)

A mixture of compound **6** (1.3 g, 6 mmol), (triphenylphosphoranylidene)acetonitrile (3.8 g, 13 mmol),[14] EDCI (1.3 g, 7 mmol) and DMAP (77 mg, 0.6 mmol) in dry DMF (35 mL) was allowed to stir at room temperature for 12 h. The mixture was diluted with water (35 mL) and extracted with ethyl acetate (3× 40 mL). The combined organic phase was washed with water and brine, dried on $MgSO_4$, and concentrated to afford the title product. Compound **7** was obtained (2.2 g, 71%) as a pale yellow powder: ^{1}H NMR ($CDCl_3$) δ 8.38 (1H, s), 7.75 (1H, s), 7.65 (4H, m), 7.49 (11H, m), 5.54 (2H, br s), 4.53 (2H, t, J = 6.0 Hz), 3.32 (2H, t, J = 6.0 Hz).

5.1.8. 3-(6-Aminopurine-9-yl)-2-oxo-propionic acid methyl ester [(*Keto*)-DZ2002]

Ozone was bubbled into a mixture of compound **7** (300 mg, 0.6 mmol) in dichloromethane (20 mL) and methanol (20 mL) at −78 °C until the color turned pale blue and then for additional 10 min. N_2 was then bubbled through to remove excess ozone. Silica-gel (1.2 g) was added to the mixture and was then concentrated until a free-running solid was obtained. The solid was transferred to a silica-gel column and eluted with chloroform/methanol (15:1). After removal of solvents, (*Keto*)-DZ2002 was obtained (134 mg, 88%) as a white solid: ^{1}H NMR ($CDCl_3$): δ 8.34 (1H, s), 7.90 (1H, s), 5.58 (2H, br s), 4.54 (2H, t, J = 6.0 Hz), 3.86 (3H, s), 3.53 (2H, t, J = 6.0 Hz); ^{13}C NMR (DMSO-d_6): δ 190.90, 160.07, 155.88, 152.24, 149.43, 140.95, 118.70, 52.54, 38.27, 37.64; HR ESIMS m/z 272.0758 $[M+Na]^+$ (calcd. for $C_{10}H_{11}N_5NaO_3$ 272.0760).

5.1.9. (2*Rac*)-4-(6-Aminopurine-9-yl)-2-hydroxybutyric acid methyl ester [(*Rac*)-DZ2002]

A mixture of (*Keto*)-DZ2002 (38 mg, 0.15 mmol) in methanol (20 mL) was added $NaBH_4$ (7 mg, 0.18 mmol). The mixture was stirred for 30 min at room temperature. Silica-gel (120 mg) was

added to the mixture and concentrated until a free-running solid was obtained. The solid was transferred to a silica-gel column and eluted with chloroform/methanol (20:1). Removal of solvents afforded (*Rac*)-DZ2002 (23 mg, 60%) as a white solid: ^{1}H NMR (DMSO-d_6): δ 8.13 (1H, s), 8.07 (1H, s), 7.19 (2H, br s), 5.72 (1H, br d, J = 5.7 Hz), 4.24 (2H, t, J = 6.6 Hz), 4.01 (1H, m), 3.56 (3H, s), 2.23 (1H, m), 2.05 (1H, m); HR ESIMS m/z 274.0936 $[M+Na]^+$ (calcd. for $C_{10}H_{13}N_5NaO_3$ 274.0916).

5.2. SAHase and MLR assay

5.2.1. Reagents and animals

S-Adenosyl-L-homocysteine hydrolase (SAHase) from rabbit erythrocytes, *S*-adenosyl-L-homocysteine (SAH), and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) were obtained from Sigma. RPMI 1640 medium was purchased from Gibco BRL/Life Technologies Inc. (Gaithersburg, MD), fetal calf serum (FCS) was obtained from Hyclone Laboratories (Logan, UT), [^{3}H]thymidine was provided by GE Healthcare.

BALB/c and C57BL/6 mice were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences. The animals were housed in specific pathogen-free conditions. All 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.

5.2.2. DTNB-coupled assay of SAHase hydrolytic activity

SAHase and compound were cultured in phosphate buffer (pH 8.0) for 10 min before initiated the reaction. The reaction mixture (200 μL) containing 50 μM SAH, 100 μM DTNB, 0.066 U SAHase, and 10 μM compound was measured using a spectraMAX-190 spectrophotometer, and O.D. at 412 nm (maximum absorbance of the product HCY-TNB) was continuously read every minute from 0 to 8 min. All operational works proceeded at 37 °C.

5.2.3. Mixed lymphocyte reaction (MLR)

BALB/c splenic lymphocytes (1×10^7cells/mL) were γ-irradiated with a ^{60}Co source for 7 min, and then cultured with fresh C57BL/6 splenic lymphocytes and compound in a 96-well plate. After a 72 h incubation at 37 °C in a 5% CO_2 water jacketed CO_2 incubator, cells were pulsed with 1 μCi/well of [^{3}H]thymidine and incubated for another 24 h. Then cells were harvested onto glass fiber filters and incorporated radioactivity was counted using a Beta Scintillation Counter (MicroBeta Trilux).

Acknowledgments

This work was supported by National Natural Science Foundation of China (Grants 30725049 to F.N.), the Shanghai Commission of Science and Technology (Grant 064319014 to J.Z.), National Natural Science Foundation of China (No. 30572195 to J.Z.), and Program for New Century Excellent Talents in University (NCET to W.L.). We thank for Dr. Zhan-Li Wang and Ms. Cui-Fang Lin of Neotrident Inc. for their kindly help on docking computation.

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