Inhibition of S-Adenosyl-L-homocysteine Hydrolase Induces Immunosuppression

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ABSTRACT

Lymphocytes depend on transmethylation reactions for efficient activation and function. These reactions are primarily catalyzed by S-adenosylmethionine-dependent methyltransferases, which convert S-adenosylmethionine to S-adenosyl-L-homocysteine. S-adenosyl-L-homocysteine is then hydrolyzed by S-adenosyl-L-homocysteine hydrolase to prevent feedback inhibition of transmethylation reactions. By impeding S-adenosyl-L-homocysteine hydrolase, a build-up of S-adenosyl-L-homocysteine occurs, and most intracellular transmethylation reactions cease. Thus, a nontoxic inhibitor of this enzyme might be a useful immunosuppressive therapeutic agent. We identified a potent reversible type III inhibitor of S-adenosyl-L-homocysteine hydrolase, DZ2002 [methyl 4-(adenin-9-yl)-2-hydroxybutanoate], and determined its cytotoxic and immunologic effects. We demonstrated that DZ2002 blocked S-adenosyl-L-homocysteine hydrolase more effectively than a type I inhibitor, but cytotoxicity from DZ2002 was greatly reduced. Although DZ2002 did not prevent concanavalin A-induced T cell proliferation or interleukin (IL)-2 production, it significantly reduced both a mixed lymphocyte reaction and IL-12 production from in vitro-stimulated splenocytes. In addition, levels of CD80 and CD86 on human monocyteic THP-1 cells were decreased in a dose-dependent manner in the presence of 0.1 to 10 μM DZ2002, and decreases were also seen in IL-12 and tumor necrosis factor-α production from both mouse thioglycollate-stimulated peritoneal macrophages and THP-1 cells. In vivo, DZ2002 significantly suppressed a delayed-type hypersensitivity reaction as well as antibody secretion. We conclude that DZ2002’s immunosuppressive effects are likely not solely attributed to T cell inhibition but also to the obstruction of macrophage activation and function through reductions in cytokine output and/or T cell costimulation. These data suggest an important dual role for the S-adenosyl-L-homocysteine hydrolase in both macrophage and T cell function.

More than most cell types, lymphocytes require S-adenosylmethionine (AdoMet)-dependent transmethylation reactions for proliferation (German et al., 1983). Not surprisingly, an important role for a key enzyme involved in all transmethylation reactions, S-adenosyl-L-homocysteine (AdoHcy) hydrolase, has been identified in immune cell function. Biologic transmethylation that use AdoMet as a methyl donor are involved in a number of very important physiologic processes, including most methylation of proteins (Banerjee, 1980), lipids (Ueland et al., 1984), nucleic acids (Chiang et al., 1996), and small molecules (Yuan et al., 1999). Following demethylation, AdoMet is converted to AdoHcy, which functions as a potent feedback inhibitor of transmethylation. The enzyme AdoHcy hydrolase is a highly conserved homotetrameric 432-amino acid protein that catalyzes the reversible hydrolysis of AdoHcy to adenosine and Hcy. Blockade of AdoHcy hydrolase activity, therefore, causes a substantial accumulation of AdoHcy. AdoMet-dependent

ABBREVIATIONS: AdoMet, S-adenosylmethionine; AdoHcy, S-adenosyl-L-homocysteine; DTH, delayed-type hypersensitivity; MDL-28,842, (Z)-5’-fluoro-4’,5’-didehydro-5’-deoxyadenosine; DHCAa, 9’-[1’R,2’S,3’R]-2’,3’-dihydroxy-5-cyclopenten-4’-yl-1’-adenine; IL, interleukin; LPS, lipopolysaccharide; TNF, tumor necrosis factor; DZ2002, methyl 4-(adenin-9-yl)-2-hydroxybutanoate; Con A, concanavalin A; SAC, Staphylococcus aureus Cowan strain I; MT, 5’-4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; DNF, 2,4-dinitrofluorobenzene; IFN, interferon; FBS, fetal bovine serum; TG, thioglycollate; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; QHS, quantitative hemolysis of sheep red blood cells; SRBC, sheep red blood cell; MLR, mixed lymphocyte reaction; APC, antigen-presenting cell.
transmethylation reactions are particularly sensitive to changes in the ratio between AdoHcy and AdoMet, and because this ratio tips toward AdoHcy, intracellular AdoMet-mediated transmethylations slow down and eventually halt. Consequently, targeting of AdoHcy hydrolase has become an attractive strategy to prevent cellular transmethylation. In addition, AdoHcy is a competitive inhibitor of phosphatidyl inositol kinase, which is directly involved in T cell receptor and second message signaling, including the influx of intracellular calcium (Berridge, 1987; Pike and DeMeester, 1988; Berridge and Irvine, 1989).

AdoHcy hydrolase inhibitors are divided into three types (Yuan et al., 1999). Type I inhibitors use AdoHcy hydrolase's 3'-oxidative activity to reversibly reduce enzyme-bound NAD+ to NADH. These inhibitory molecules then become trapped in the active site of the closed form of the enzyme. Type II inhibitors exploit the 5'-hydrolytic activity of AdoHcy hydrolase to generate inhibitory molecules that covalently modify the enzyme's active site. And finally, type III inhibitors reversibly bind to the open form of AdoHcy hydrolase, employing neither the 3'-oxidative nor the 5'-hydrolytic activity of the enzyme.

AdoHcy hydrolase inhibitors demonstrate both prophylactic and therapeutic effects in several immune-mediated processes, including collagen- and peptidoglycan-induced arthritis (Wolos et al., 1999c; Saso et al., 2001), skin graft rejection (Wolos et al., 1999a), and delayed-type hypersensitivity (DTH) responses (Saso et al., 2001). In vitro, type I inhibitors, such as MDL-28,842 and 9-[(1R,2S,3R)-2',3'-dihydroxyyclo- pent-4'-en-1-yl]adenine (DHCAA), block T cell proliferation and IL-2 production (Wolos et al., 1993b; Saso et al., 2001). It is still unclear how blockade of AdoHcy hydrolase inhibits T cell activity. The prevention of cellular transmethylation and/or reducing intracellular Hcy levels are possibilities.

Further development of the type I irreversible inhibitors has been limited due to significant cytotoxic effects, which are likely attributable to the slow turnover rate of AdoHcy hydrolase (t1/2 ~ 24 h), leading to prolonged in vivo blockade of the enzyme (Yuan et al., 1999). In contrast, type III inhibitors reversibly inhibit AdoHcy hydrolase and, although generally less potent than type I inhibitors, exhibit considerably less cytotoxicity. In fact, no immunosuppressive effects have been previously reported for type III inhibitors apart from weak inhibition of lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)-α production from macrophages (Yuan et al., 1999).

In this paper, we report that a potent type III inhibitor, DZ2002 [methyl 4-(adenin-9-yl)-2-hydroxybutanoate], effectively blocks the activity of AdoHcy hydrolase and, importantly, that its blockade is reversible. We assessed DZ2002's binding affinity, cytotoxicity, and, in vitro and in vivo immunosuppressive effects. We demonstrated that DZ2002 had immunosuppressive activity similar to that of type I inhibitors, but with greatly reduced cytotoxicity. Moreover, we provide data that indicate that the immunosuppressive effects of DZ2002 might be attributed to suppression of mac- rophage activation and function, rather than direct inhibition of B or T cells, and that perhaps macrophage function is at least partially AdoHcy hydrolase-dependent. Together these data strongly suggest that DZ2002 may serve as an effective therapeutic agent for a number of human autoim- mune diseases.

Materials and Methods

Reagents. DZ2002 and DHCAA were synthesized. Concanavalin A (Con A), LPS (Escherichia coli 055:B5), and Staphylococcus aureus Cowan strain 1 (SAC) were obtained from Pansorbin cells (Strat- agene, La Jolla, CA). 3,4,5-Dimethoxytryptamine-2-yl-2,5-diphenyl- tetrazolium bromide (MTT) and 3,3',5,5'-tetramethylbenzidine (TMB) were Sigma-Aldrich (St. Louis, MO) products. Mitomycin-C was purchased from Kyowa Hakko (Tokyo, Japan). DNFB was pur- chased from Merck (Whitehouse Station, NJ). Recombinant human interferon (IFN)-γ was purchased from Shanghai Clonbiotech (Shanghai, China). Thioglycollate (TG) was obtained from Wako Pure Chemicals (Tokyo, Japan). RPMI 1640 medium was purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was pur- chased from Hyclone Laboratories (Logan, UT). THP-1 cells were obtained from American Type Culture Collection (Manassas, VA). Recombinant mouse IFN-γ, purified anti-mouse IL-2, IL-6, IL-10, IL-12 (p40, p70), TNF-α, purified anti-human IL-12 (p40, p70) and biotinylated anti-mouse IL-2, IL-6, IL-10, IL-12 (p40, p70), TNF-α, biotinylated anti-human IL-12 (p40, p70), fluorescein isothio- cyanate-anti-mouse-CD11b (Mac-1), PE-anti-human-CD14, -CD80, and -CD86 were BD Biosciences PharMingen (San Diego, CA) products.

Animals. Male and female BALB/c and C57BL/6 mice (6–8 weeks old) were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences. The animals were housed in specific pathogen-free conditions. All mice were allowed to acclima- tize in our facility for 1 week before any experiments were started. 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.

Cell Preparations. Erythrocytes were lysed with Tris-buffered ammonium chloride (0.155 M NH4CL and 16.5 mM Tris, pH 7.2) as previously described (Feng et al., 2002). Mononuclear cells were washed and resuspended in RPMI 1640 media (containing 10% FBS) supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml).

Peritoneal exudate cells were induced in female BALB/c mice by an i.p. injection of 0.5 ml of 3% TG. Four days later, peritoneal exudate cells were harvested by sterile lavage.

Human mononcytic THP-1 cells were maintained in RPMI 1640 medium supplemented with 10% FBS (Feng et al., 2004). Cell viability and concentration were determined by trypan blue exclusion for all cell types.

K of AdoHcy Hydrolase after the Addition of DZ2002. The kinetic constant of AdoHcy hydrolase inactivation was determined as previously described (Yuan et al., 1993). Briefly, DZ2002 was added to a reaction mixture containing 100 µM DNB and 180 µM AdoHcy in 50 mM phosphate-buffered saline, pH 7.4. AdoHcy hydrolase (0.5 µg) was then added, and the O.D. was continuously read from 0 to 7 min at 405 nm. K was calculated as follows: (V o/V c) - 1 = (1/K)[V o] - V c = A 1 - A i (control) and V c = A 1 - A i (inhibition). Therefore, if K o = [II]/(V o/V c) - 1, then K = K o/[1 + (SI/K)]

MTT Assay. Cytotoxicity was assessed by the MTT assay as previously described (Mosmann, 1983; Feng et al., 2004). Briefly, splenic lymphocytes were cultured in triplicate for 48 h with DZ2002 or DHCAA, MTT (5 mg/ml) reagent was added, and then cells were lysed with 10% sodium dodecyl sulfate and 50% N,N-dimethyl for- mide, pH 7.2. O.D. values were read at 570 nm, and the percentage of cell death was calculated. Five mice were analyzed for each data point.

Proliferation Assay. Splenic lymphocytes were cultured in triplicate for 48 h with 5 µg/ml Con A plus either DZ2002 or DHCAA. Cells were pulsed with 0.5 µCi/well of [3H]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). Five mice were analyzed for each data point.

**Mixed Lymphocyte Reaction.** BALB/c splenic lymphocytes (1 × 10^6 cells/ml) were pretreated with 50 μg/ml mitomycin C for 2 h, washed, and then cultured in triplicate with fresh C57BL/6 splenic lymphocytes and DZ2002. After 72 h, cells were pulsed with 0.5 μCi/well [3H]thymidine and incubated for another 24 h. Cells were harvested onto glass fiber filters, and incorporated radioactivity was counted using a Beta Scintillation Counter (MicroBeta Trilux). Five mice were analyzed for each data point.

**ELISA.** BALB/c splenic lymphocytes were cultured in triplicate for 24 h with SAC (1:10,000) or Con A (5 μg/ml), plus either DZ2002 or DHCaA. Mouse peritoneal cells were incubated for 2 h, nonadherent cells were washed away, and remaining adherent cells (peritoneal macrophages) were cultured for another 24 h with IFN-γ (25 U/ml), LPS (1 μg/ml), and DZ2002. Noncytic THP-1 cells were induced to differentiate with 1.2% DMSO for 24 h in the presence of DZ2002, then pretreated with IFN-γ (500 U/ml) for 16 h and stimulated with LPS (1 μg/ml) for another 24 h. All culture supernatants were stored at −20°C until assayed. Mouse IL-6, IL-10, IL-12p40 and p70, IFN-γ, TNF-α, and human IL-6, IL-12 p40 and p70, and TNF-α levels were determined by ELISA as indicated by the manufacturer.

**Flow Cytometry.** Cells were stained with antibodies to CD11b, CD14, CD80, and CD86. Data were acquired on a FACScalibur (BD Biosciences, San Jose, CA), then analyzed using CELLQuest software (BD Biosciences).

**DNFB-induced DTH Response.** Ten BALB/c mice were prepared for each group. Mice were initially sensitized with 0.5% DNFB dissolved in acetic-olive oil (4:1) on each hind foot on days 0 and 1. On day 9, mice were challenged with 0.4% DNFB on both sides of their left ear (Feng et al., 2002). Vehicle (acetic-olive oil solution), DHCaA (5 mg/kg), and DZ2002 (2, 10, and 50 mg/kg) were administered to each group by i.p. injection 1 h before and 24 h after challenge. Ear swelling was expressed as the difference between the weight of the left and right ear patches obtained from 8-mm punches 40 h after challenge. The patches were obtained in a blinded manner.

**Quantitative Hemolysis of Sheep Red Blood Cells (QHS).** Ten mice were prepared for each group. Vehicle, DZ2002 (0.08 and 2 mg/kg) was injected i.p. into BALB/c mice on 7 consecutive days. Mice were immunized by i.p. injection with 0.2 ml of a 1:6 dilution of sheep red blood cells (SRBCs) on day 4. On day 8, splenocytes were incubated with SRBC and guinea pig serum for 1 h at 37°C; hemolysis was determined by reading O.D. at 520 nm in a blinded manner.

**Statistical Analysis.** Results are expressed as mean ± S.E. One-way ANOVA followed by Dunnett's post test was used to determine variances between groups where appropriate. P < 0.05 was considered significant.

**Results**

**DZ2002 Inactivates AdoHcy Hydrolase.** In vivo, we proposed to use the ester form of DZ2002 to increase its permeability across the cell membrane. The ester form will then be hydrolyzed by intercellular esterases to an acidic active structure. In vitro, however, we used the acid form of DZ2002 for enzyme inhibition studies. The inactivation of AdoHcy hydrolase (NADH form) by DZ2002 exhibited pseudo-first order kinetics (Fig. 1). The pseudo-first-order rate constants were directly proportional to the concentration of the inhibitor, and a K_i value of 17.9 nM was calculated from the double-reciprocal plots of the rate constants versus the inhibitor concentration. These data indicate a clear dose-dependent inhibition of AdoHcy hydrolase by DZ2002.

**DZ2002 Is Less Cytotoxic Than DHCaA.** To test whether the type III inhibitor DZ2002 exhibits lower cytotoxicity compared with the type I inhibitor DHCaA, male BALB/c splenocytes were cultured with the indicated concentrations of DZ2002 or DHCaA. The cytotoxicity of DZ2002 is significantly less than DHCaA with an IC_{50} of 100 to 600 μM compared with 6 to 14 μM (Fig. 2) and shows very little cytotoxicity up to 100 μM.

**DZ2002 Does Not Affect Lymphocyte Proliferation or IL-2 Production.** It has been reported that type I AdoHcy hydrolase inhibitors block T cell proliferation and activation (Wolos et al., 1993b). We compared the effects of DHCaA (type I inhibitor) and DZ2002 (type III inhibitor) on proliferation and IL-2 production from Con A-stimulated mouse splenocytes. After 48 h of culture, DZ2002 had little effects on lymphocyte proliferation (0.1 μM = 150,604 ± 13,862, 1 μM = 159,894 ± 11,152, and 10 μM = 136,157 ± 21,943 cpm).

**Fig. 1.** Inhibition of AdoHcy hydrolase by DZ2002. DZ2002 was mixed with AdoHcy and DNTB in phosphate-buffered saline, after which 0.5 μg of AdoHcy hydrolase was added, and the O.D. at 405 nm was continuously read from 0 to 7 min.

**Fig. 2.** DZ2002 (broken line) is significantly less cytotoxic than DHCaA (solid line). BALB/c mouse splenocytes were cultured for 48 h with either DZ2002 or DHCaA. Cells were then pulsed with MTT (5 mg/ml) 4 h prior to the end of the culture, lysis solution was added, and plates were incubated for an additional 7 h, at which time the O.D. was read at 570 nm. Cell viability at increasing concentrations of DZ2002 and DHCaA was determined and expressed as the percentage of the control (untreated) O.D. value. Three independent experiments were performed with similar results.
versus untreated Con A-stimulated cells (168,725 ± 8025 cpm). Similarly, little effect was seen in regards to IL-2 production from DZ2002-treated cells (0.1 μM = 1,838 ± 88, 1 μM = 1,793 ± 58, and 10 μM = 1,731 ± 36 pg/ml) versus untreated Con A-stimulated cells (1,806 ± 43 pg/ml). In contrast, as previously reported, DHCaA inhibited both Con A-induced T cell proliferation (0.1 μM = 82,934 ± 3,071, 1 μM = 62,708 ± 4,220, and 10 μM = 39,059 ± 4,046 cpm) and IL-2 production (0.1 μM = 1941 ± 27, 1 μM = 1688 ± 2, and 10 μM = 950 ± 29 pg/ml) in a dose-dependent manner.

**DZ2002 Inhibits the Mixed Lymphocyte Reaction (MLR) Response.** The MLR was induced by allogeneic stimulation. To examine whether DZ2002 inhibits the MLR, mitomycin C-pretreated BALB/c (H-2^d^) stimulator cells were cultured with C57BL/6 (H-2^b^) responder cells, and proliferation of the responder population was measured by [3H]thymidine incorporation. Interestingly, although DZ2002 had little effect when T cells were stimulated with Con A, DZ2002 suppressed the MLR by 24.5, 42.3, and 46.0% at dosages of 0.1, 1, and 10 μM, respectively (Fig. 3).

**DZ2002 Inhibits IL-12 but Has No Effect on IFN-γ or IL-10 from SAC-Stimulated Cells.** SAC is a potent inducer of IL-10, IL-12, and IFN-γ production in mouse splenocytes (Cousens et al., 1997). We analyzed the effects of DZ2002 on SAC-stimulated cytokine production. As depicted in Fig. 4, DZ2002 reduced IL-12 p40 and p70 production in half from ~1200 pg/ml in untreated cells to ~600 pg/ml at 10 μM and ~350 pg/ml in untreated cells to ~150 pg/ml at 10 μM (p70), respectively, but DZ2002 had no effect on either IFN-γ or IL-10. Cytokine levels from unstimulated control splenocytes were 17.9 ± 3.71, 16.1 ± 2.78, 15.4 ± 3.23, and 12.3 ± 2.15 pg/ml for IFN-γ, IL-10, IL-12 p40, and IL-12 p70, respectively.

**DZ2002 Inhibits IL-12 and TNF-α Production from Both Mouse Peritoneal Exudate Cells and Human THP-1 Cells.** Although DZ2002 did not show the same strong suppressive effects as DHCaA on T cell proliferation and IL-2 production, it significantly inhibited both the MLR and IL-12 production from mouse splenocytes. These data indicate that DZ2002 may have strong immunosuppressive effects on antigen-presenting cells (APCs). We, therefore, analyzed the effects of DZ2002 on cytokine production from both mouse peritoneal TG-stimulated macrophages and a human monocytic cell line (THP-1). In TG-stimulated mouse peritoneal macrophages, DZ2002 again significantly blocked IL-12 p40 production from ~1800 pg/ml in untreated cells to ~850 pg/ml at 10 μM and, most importantly, drastically reduced the active p70 form from ~1200 pg/ml in untreated cells to ~50 pg/ml at 10 μM (Fig. 5). TNF-α production was reduced from ~850 pg/ml in untreated cells to ~70 pg/ml at 10 μM (Fig. 5).

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**Fig. 3.** DZ2002 suppressed cell proliferation in a mixed lymphocyte reaction. Mitomycin C-pretreated BALB/c splenocytes were cocultured with C57BL/6 splenocytes for 96 h in the presence of DZ2002. Cells were then pulsed with [3H]thymidine and assessed for [3H]thymidine incorporation. Results are expressed as mean ± S.E.M. ***, P < 0.01. Two experiments were performed with similar results.**

**Fig. 4.** DZ2002 reduces IL-12 p40 and p70 production from SAC-stimulated mouse splenocytes but has little effect on either IFN-γ or IL-10. Splenocytes were cultured with SAC for 24 h in the presence of DZ2002. Culture supernatants were collected, and IL-10, IL-12p40 and p70, and IFN-γ levels were determined by ELISA. Results are expressed as mean ± S.E.M. Two independent experiments were performed with similar results. ***, P < 0.01; ***, P < 0.001.

**Fig. 5.** DZ2002 inhibits IL-12 p40 and p70 and TNF-α production from macrophages; however, IL-10 and IL-6 were mostly unaffected. Mouse TG-induced peritoneal macrophages were cultured with LPS (1 μg/ml) and IFN-γ (25 U/ml) for 24 h in the presence of DZ2002. Culture supernatants were collected, and IL-6, IL-10, IL-12p40 and p70, and TNF-α levels were determined by ELISA. Results are expressed as mean ± S.E.M. Two independent experiments were performed with similar results. ***, P < 0.01; ***, P < 0.001.
also diminished from $\sim3600$ pg/ml in untreated cells to $\sim2000$ pg/ml at 10 $\mu$M, a 45% reduction. IL-6 levels showed a marginal decrease to $\sim7000$ pg/ml at 10 $\mu$M as compared with untreated cells at $\sim8000$ pg/ml. IL-10 levels were unaffected. Cytokine levels from unstimulated splenocytes were $17.5 \pm 4.2$, $15.1 \pm 2.45$, $13.5 \pm 2.78$, $11.3 \pm 2.51$, and $20.5 \pm 3.98$ pg/ml for IL-6, IL-10, IL-12 p40, IL-12 p70, and TNF-$\alpha$, respectively.

THP-1 cells are a human myeloid cell line that is used for determining cytokine production by human monocytes (Kubin et al., 1994; Feng et al., 2004). DMSO and IFN-$\gamma$ are potent inducers of myeloid cell differentiation, a process associated with a marked increase in cytokine output (Sakurada et al., 2000). We tested the effect of DZ2002 on cytokine production from DMSO-differentiated and IFN-$\gamma$- and LPS-activated THP-1 cells. Similar to TG-stimulated mouse peritoneal exudate cells, DZ2002, in the range of 0.1 to 10 $\mu$M, significantly inhibited IL-12 p40 and p70 production and slightly inhibited TNF-$\alpha$ levels from human THP-1 cells (Fig. 6). It did not, in this case, affect IL-6 levels, and under these culture conditions, IL-10 levels were too low to detect. Cytokine levels from unstimulated splenocytes were $12.5 \pm 1.25$, $14.7 \pm 3.25$, $10.7 \pm 4.78$, and $15.4 \pm 2.58$ pg/ml for IL-6, IL-12 p40, IL-12 p70, and TNF-$\alpha$, respectively.

**DZ2002 Down-Regulates Expression of the Costimulatory Molecules CD80 and CD86 on Differentiated THP-1 Cells.** DMSO inhibits THP-1 cell proliferation, and concurrently, several markers of differentiation, including cell surface molecules, are expressed (Agarwal and Marshall, 2000). IFN-$\gamma$-primed THP-1 cells incubated with 0.1 to 10 $\mu$M DZ2002 dramatically down-regulated CD80 and, in particular, CD86 expression in a dose-dependent manner (Fig. 7).

**DZ2002 Blocks the DNFB-Induced DTH Response.** DNFB-induced DTH is a Th1 cell-mediated immune response, in which IL-12 is highly expressed and macrophages have been shown to play an important role (Tsicopoulos et al., 1992). Table 1 illustrates the inhibitory effect of DZ2002 on DNFB-induced DTH. When administered 1 h before and 24 h after DNFB challenge at dosages of 2, 10, and 50 mg/kg, DZ2002, in a dose-dependent manner, appreciably suppressed ear swelling by 19.1, 28.7, and 33.1%, respectively. The type I inhibitor, DHCaA, also inhibited the DTH response by 36.3% when given at 5 mg/kg.

**DZ2002 Inhibits the QHS Assay.** Hemolysis of SRBCs is an established model of antibody production in response to antigenic stimulation. As compared with controls, consecutive 7-day i.p. injections of DZ2002 inhibited hemolysis by 24.5 and 18.4% at doses of 0.08 and 2 mg/kg, respectively, thus decreasing anti-SRBC antibody production in vivo.

**Discussion**

Here, we report the immunosuppressive effects of DZ2002, a type III AdoHcy hydrolase inhibitor that shows greatly reduced cytotoxic effects, as compared with type I inhibitors. Moreover, DZ2002 exhibits similar blockade of the AdoHcy hydrolase, suppression of the MLR, inhibition of IL-12 and TNF-$\alpha$ production, down-regulation of the costimulatory molecules CD80 and CD86, inhibition of the DTH response, and
reductions in cellular and humoral immune responses. This immunosuppression seems to be mediated by both lymphocytes and macrophages/microglia, indicating a dual and important role in immune regulation for AdoHcy hydrolase.

AdoHcy hydrolase has been identified as a potential therapeutic target for nearly 2 decades. The in vitro and in vivo efficacy of type I AdoHcy hydrolase inhibitors, such as MDL-28,842 and DHCaA, as effective immunosuppressants has been previously demonstrated. This approach, however, has yielded little success in generating clinically useful pharmacological agents. A primary impediment to developing useful AdoHcy hydrolase inhibitors is their associated cytotoxicity. The use of reversible type III inhibitors may be an effective approach to minimizing cytotoxicity; nevertheless, previous type III inhibitors were less potent as AdoHcy hydrolase inhibitors and less effective as immunosuppressors.

We demonstrated that DZ2002, a very potent reversible type III inhibitor of AdoHcy hydrolase, possessed strong immunosuppressive activity with low associated cytotoxicity. The acid form of DZ2002, selected out of hundreds of analogs, was the only type III inhibitor with an optimal $K_i$ (17.9 nM). The $K_i$ of DZ2002 is lower, in fact, than the type I inhibitors DHCaA (90 nM) and DHCeA ($K_i = 600$ nM), indicating that DZ2002 is an even more potent blocker of AdoHcy hydrolase (Saso et al., 2001). Importantly, the cytotoxicity of DZ2002 was 16- to 40-fold lower than DHCaA while still maintaining its immunosuppressive effects.

Although DZ2002 did not inhibit Con A induced T cell proliferation, it strongly suppressed proliferation in the MLR. Along with T cells, accessory cells, such as macrophages, are essential for the MLR, as removal of macrophages lessens the response (Naito et al., 1989). The expected function of these macrophages is to activate CD4$^+$ T cells. The acid form of DZ2002 also decreased the expression of IL-12 in SAC-stimulated mouse splenocytes, mouse peritoneal macrophages, and human monocyte TPH-1 cells. DZ2002 had little effect, however, on cytokines primarily expressed by T cells, such as IL-10 and IL-6. Thus, DZ2002 might influence a primarily Th1-type response to shift to a Th2-mediated immune response.

The type I AdoHcy hydrolase inhibitor, MDL-28,842, inhibited the synthesis of TNF-$\alpha$ and was effective in the treatment of collagen-induced arthritis (Wolos et al., 1993c). TNF-$\alpha$ is a major proinflammatory cytokine largely produced by activated macrophages and is implicated in the pathogenesis of many diseases, including rheumatoid arthritis, multiple sclerosis, and Alzheimer's (Owens and Sriram, 1995; Feldmann et al., 1996; Cacquevel et al., 2004). Similar to MDL-28,842 and DHCaA, DZ2002 also inhibited TNF-$\alpha$ production, which suggested a possible therapeutic role for DZ2002 in the treatment of diseases associated with the synthesis/release of TNF-$\alpha$.

Further evidence demonstrating the ability of DZ2002 to inhibit macrophage function was down-regulation of both CD80 and CD86 on THP-1 cells. Participation of macrophages as effective APCs requires cell surface expression of a number of glycoproteins, including CD80 and CD86. These molecules then interact with CD28 on the surface of T cells, providing the necessary second costimulatory signal for optimal T cell activation.

A third possible route by which DZ2002 blocks macrophage function is diminishing intracellular Hcy levels. Macrophages are deficient in endogenous nucleotide synthesis; therefore, they require uptake of exogenous nucleotides to meet their metabolic demands (Soler et al., 2001). Hcy is the methyl acceptor in the conversion of 5-methyltetrahydrofolate to tetrahydrofolate, which is essential for both purine and thymidylate synthesis (Saso et al., 2001). Thus, a molecule that exhausts intercellular Hcy could inhibit cell division. Moreover, Hcy has also been implicated in intercellular redox maintenance (Koch et al., 1998).

Hence, there could be three mechanistically distinct sites at which DZ2002 blocks macrophage function: reducing key cytokine levels such as IL-12 and TNF-$\alpha$, thus interfering with T cell activation; reducing expression of T cell costimulatory molecules such as CD86 and/or CD80 on APC; or indirectly, by reducing Hcy levels, thus lowering purine and thymidylate synthesis and/or interfering with redox equilibrium.

To assess the immunosuppressive effects of DZ2002 on cellular and humoral immunity, we used a DTH model and QHS assay, respectively. DNFB-induced DTH, a CD4$^+$ T cell-mediated response, was reduced by DZ2002 in a dose-dependent manner, signifying an inhibitory effect on cellular immune responses, in particular, CD4$^+$ T cells. In the early stages of a DTH response, IL-12 derived from activated APCs is a critical factor in directing a Th1-type CD4$^+$ T cell response, which enhances IFN-$\gamma$ production and cell-mediated immune responses (Trinchieri, 2003). Therefore, it is not too surprising that the DTH response was lessened due to DZ2002's ability to effectively block IL-12 production. The QHS assay quantitates the antibody-producing (especially IgM-producing) capacity of plasma cells responding to SRBC (Simpson and Gozzo, 1978). Suppression of QHS by DZ2002 administration indicates that it also has an effect upon antibody-secreting B cells. It has been reported that Hcy causes a significant enhancement of resting and LPS-induced B lymphocyte proliferation (Zhang et al., 2001). Moreover, the proliferation of B cells in response to LPS obtained from ApoE-knockout mice with hyperhomocysteinemia was greatly increased as compared with control cells (Zhang et al., 2001). These data imply that, similar to T cells, reductions in Hcy levels may also affect B cell proliferation. Although DZ2002 only modestly inhibited LPS-induced mouse splenocyte proliferation in vitro (unpublished data), it significantly inhibited antibody-secreting cells, even at relatively low doses.

In conclusion, the results from this study establish that DZ2002, a reversible type III AdoHcy hydrolase inhibitor, exhibits low cytotoxicity and compelling immunosuppressive effects, which might be attributed to its inhibition of macrophage function rather than to the direct restriction of lymphocytes. We believe that the data warrant further evaluation of DZ2002 as a possible therapeutic agent in the treatment of inflammatory and/or autoimmune diseases.
A Type III AdoHcy Hydrolase Inhibitor Leads to Immunosuppression

References


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