

The Bcl-2 Family Antagonist ABT-737 Significantly Inhibits Multiple Animal Models of Autoimmunity

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The Bcl-2 family of proteins plays a critical role in controlling immune responses by regulating the expansion and contraction of activated lymphocyte clones by apoptosis. ABT-737, which was originally developed for oncology, is a potent inhibitor of Bcl-2, Bcl-x_L, and Bcl-w protein function. There is evidence that Bcl-2-associated dysregulation of lymphocyte apoptosis may contribute to the pathogenesis of autoimmunity and lead to the development of autoimmune diseases. In this study, we report that ABT-737 treatment resulted in potent inhibition of lymphocyte proliferation as measured by in vitro mitogenic or ex vivo Ag-specific stimulation. More importantly, ABT-737 significantly reduced disease severity in tissue-specific and systemic animal models of autoimmunity. Bcl-2 family antagonism by ABT-737 was efficacious in treating animal models of arthritis and lupus. Our results suggest that treatment with a Bcl-2 family antagonist represents a novel and potentially attractive therapeutic approach for the clinical treatment of autoimmunity. *The Journal of Immunology*, 2009, 182: 7482–7489.

Proper functioning of the immune system depends on the tight control of cell proliferation and cell death. Apoptosis represents one of the central mechanisms for eliminating potentially autoreactive cells during lymphocyte development (1) and for clearing various inflammatory cells in the resolution of inflammation (2). Impairment of apoptosis in immune effector cells may cause the breakdown of immune tolerance to self-Ags, leading to autoimmunity (3). Dysregulation of apoptosis has been implicated in the development of human autoimmune diseases such as rheumatoid arthritis (RA)³ and systemic lupus erythematosus (SLE). RA is characterized by the infiltration of lymphocytes, NK cells, macrophages, dendritic cells, mast cells, and platelets in the synovium; pronounced hyperplasia of synovial fibroblasts; expression of proinflammatory cytokines; enhanced angiogenesis; and, finally, cartilage degradation and bone erosion (4). RA is primarily a tissue-specific disease with an etiology caused in part by infiltrating lymphocytes in the target tissues. In contrast, SLE is a systemic autoimmune disease, with some tissue-specific

characteristics, distinguished by the production of T cell-dependent antinuclear autoantibodies. Compared with RA, the etiology of SLE pathogenesis seems to be primarily driven by lymphocytes through Ab-mediated inflammation, immune complex deposition, chemokine expression in target organs, and production of cytokines by activated T lymphocytes (5). In addition, it has recently been shown that a dysregulation of the Bcl-2 pathway alters the function of APCs, leading to SLE-like symptoms in mice (6). Restoration of lymphocyte apoptosis in the context of autoimmunity may therefore present a novel therapeutic approach.

Programmed cell death mediated by the intrinsic apoptotic pathway is controlled in the mitochondria through the interaction of opposing proapoptotic and antiapoptotic Bcl-2 family proteins. The multidomain, proapoptotic proteins Bax and Bak are essential mediators that, once activated, cause mitochondrial permeabilization, cytochrome *c* release, caspase activation, and cell death (7). The BH3-only proapoptotic proteins (e.g., Bim, Bad, Noxa, Puma) act upstream in response to a variety of cellular stimuli to propagate the apoptotic signal and induce the activation of Bax and Bak. Antiapoptotic proteins, including Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, and Bfl-1, promote cell survival by binding to and sequestering their proapoptotic counterparts, thus preventing Bax/Bak activation.

Because of the prevalent role of dysregulated apoptosis in tumorigenesis and resistance to chemotherapy, Bcl-2 family proteins represent promising targets for cancer therapy (8). ABT-737 is a small molecule inhibitor of antiapoptotic Bcl-2 proteins that binds with high-affinity ($K_i < 1$ nM) to Bcl-2, Bcl-x_L, and Bcl-w but not to the less homologous proteins Mcl-1 and Bfl-1 (9). In cancer cells, ABT-737 has been shown to disrupt the interactions of Bcl-2 family proteins and induce apoptosis (8). Administration of ABT-737 to tumor-bearing animals results in rapid tumor regression in multiple murine xenograft models. ABT-737 has also been reported to elicit lymphopenia in naive mice (10). Given the potential role of aberrant antiapoptotic Bcl-2 family member activity in the maintenance of activated and/or autoreactive lymphocytes and the observed effects of ABT-737 on circulating lymphocytes, we

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³ Abbreviations used in this paper: RA, rheumatoid arthritis; BAFF, B-cell activating factor; CBC, complete blood count; CIA, collagen-induced arthritis; CsA, cyclosporin A; DTH, delayed-type hypersensitivity; KLH, keyhole limpet hemocyanin; LN, lymph node; MAS, mean arthritic score; mBSA, methylated BSA; MMF, mycophenolate mofetil; PU, proteinuria; SLE, systemic lupus erythematosus; Dex, dexamethasone.

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sought to examine the efficacy of this agent in two disparate models of autoimmune disease, specifically animal models of RA and SLE.

Materials and Methods

Human and mouse lymphocyte *in vitro* assays

CD19⁺ human B cells (1×10^5 cells/well), isolated from blood by Ab-based negative selection (Biological Specialty), were cultured in the presence of anti-IgM (10 μ g/ml; R&D Systems) for 48 h. Negatively selected CD3⁺ human T cells (1.5×10^5 cells/well; Biological Specialty) were stimulated with anti-CD3 (5 μ g/ml; R&D Systems) and anti-CD28 (2 μ g/ml; R&D Systems) mAbs for 3 days. For both assays, cells were pulsed with [³H]thymidine (PerkinElmer) for 16 h. A MLR using unfractionated C57BL/6 and BALB/c (The Jackson Laboratory) splenocytes were plated at a density of 5×10^5 cells/well and cultured for 72 h. Untouched naive B cells were separated from C57BL/6J mouse spleens by magnetic cell sorting (Miltenyi Biotec) and were cultured with 0.25 μ g/ml LPS (Sigma-Aldrich) for 72 h. Mouse cells were labeled for 6 h with [³H]thymidine. [³H]thymidine incorporation was measured using a TopCount liquid scintillation beta counter (PerkinElmer). The Caspase-Glo 3/7 luminescent assay was used according to the manufacturer's protocol (Promega).

Mice, ABT-737 treatment, and complete blood counts (CBCs)

All mice were obtained from vendors and were handled under procedures approved by the Abbott Institutional Animal Care and Use Committee Association, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The vehicle for i.p. administration of ABT-737 consisted of 30% polyethylene glycol, 5% Tween 80, 65% of a 5% dextrose solution, and <1% DMSO. Serum concentrations of ABT-737 were measured by liquid chromatography-mass spectrometry in plasma taken from mice 24 h after the last dose. Cell counts were performed on a Cell-Dyn 3700 (Abbott Laboratories) automated hematology analyzer.

Keyhole limpet hemocyanin (KLH) restimulation of *in vivo*-primed T cells

C57BL/6J mice were immunized s.c. with 100 μ g of KLH (Sigma-Aldrich) in CFA (Difco Laboratories). Control mice were treated with cyclosporin A (CsA) (Alexis Biochemicals) at 50 mg/kg/day. Mice were sacrificed 4 days later and inguinal lymph nodes (LNs) were harvested. Lymphocytes from these LNs were restimulated with KLH (50 μ g/ml) or 2 μ g/ml Con A (Sigma-Aldrich) for 48 h before pulsing with [³H]thymidine for 16 h. [³H]Thymidine incorporation was then measured.

Delayed-type hypersensitivity (DTH) in C57BL/6 mice

C57BL/6 mice were immunized intradermally with 200 μ g of methylated BSA (mBSA; Sigma-Aldrich) in an emulsion with CFA. Mice were treated i.p. once daily with vehicle, dexamethasone (Sigma-Aldrich), or ABT-737 from day of immunization through day 7. Seven days after immunization, mice were challenged in the right hind footpad with 100 μ g of mBSA in 20 μ l of PBS. The left footpad received PBS alone. Footpad swelling (Δ right– Δ left) was measured 24 h after challenge using a dial thickness caliper (Dyer Gauge).

Collagen-induced arthritis (CIA) in DBA/J mice

Bovine type II collagen (lyophilized; obtained from M. Griffiths, University of Utah, Salt Lake City, UT) was dissolved in 0.1 N acetic acid and then emulsified in CFA. Six- to 8-wk-old male DBA/J mice (The Jackson Laboratory) were immunized intradermally at the base of the tail with 100 μ l of emulsion containing 100 μ g of bovine type II collagen. On day 21 after immunization, mice were boosted with 1 mg of zymosan (Sigma-Aldrich) i.p. Mice were enrolled for the study at the first clinical signs of disease (24–28 days). The mean arthritic score (MAS) was determined by scoring each paw for arthritis using a scale of 0–3, where 0 = normal, 1 = swelling in one site, foot or ankle, 2 = swelling in foot and ankle, and 3 = ankylosis. In addition to clinical scores, mice were also evaluated for paw edema using a caliper. Treatment of mice with ABT-737 (50 mg/kg), dexamethasone (1 mg/kg), or vehicle was performed once daily for 21 days.

Microcomputed tomography imaging

Microcomputed tomography imaging was performed on a Scanco μ CT-40 unit (Scanco Medical). The hind paws (stored in 80% ethanol) were secured in imaging tubes and the tarsal bone volume was measured for a 1.8-mm section of the mouse ankle from the base of the tibia to the tarsal/

metatarsal joint at a resolution of 18 μ m. The raw microcomputed tomography image data were then analyzed using Scanco software.

Adenoviral IFN- α -induced model of lupus nephritis

(NZB \times NZW)F₁ mice (The Jackson Laboratory), 13–15 wk old, were injected with a single i.v. dose of IFN- α adenovirus (Qbiogene) at a concentration of 5×10^9 viral particles/mouse. In addition to ABT-737, some mice were treated 7 days after adenovirus injection with 100 mg/kg/day mycophenolate mofetil (MMF; Henry Schein Veterinary) orally. Following adenovirus injection, mice were monitored weekly for proteinuria (PU) using Albustix test strips (VWR). Severe PU was defined by consecutive weekly measurements of PU \geq 300 mg/dl. When mice became moribund, they were sacrificed according to Institutional Animal Care and Use Committee protocols.

Histologic assessment of renal injury in lupus model

Kidneys were bisected and then fixed in 10% neutral-buffered formalin or cryopreserved (snap frozen). For H&E staining, 5- μ m sections from paraffin-embedded tissues were semiquantitatively scored (0–4) by an experienced pathologist for glomerulonephritis and tubular changes (dilation, basophilia, and casts). For IgG and C3 immunohistochemistry, 5- μ m cryosections were fixed with acetone, washed, and blocked with 10% normal goat serum. Sections were then incubated with FITC-conjugated goat anti-mouse IgG (Cappel/ICN Pharmaceuticals), HRP-goat anti-mouse C3 (MP Biomedical), or negative control HRP-goat IgG (Jackson ImmunoResearch Laboratories) and coverslipped using Vectashield with 4',6-diamidino-2-phenylindole (Vector Laboratories). Sections were evaluated for severity of IgG or C3 deposition using a semiquantitative scoring system (from 0–4).

Statistical analysis

IC₅₀ calculations were performed using Prism software (GraphPad). Prism was also used to calculate statistical significance for proliferation assays, CBC measurements, paw swelling, and flow cytometry using ANOVA. All graphs used the SEM. In collagen-induced arthritis (CIA) studies, MAS statistical significance ($p < 0.05$) was calculated using the nonparametric Mann-Whitney *U* test in Statistica software (Statsoft). In the lupus model, PU and survival data were presented as Kaplan-Meier survival curves using Prism and were considered significant at the level of $p < 0.05$. Histologic scores were analyzed using ordinal logistic regression in the SAS/STAT software package (SAS).

Results

ABT-737 inhibits lymphocyte proliferation *in vitro*

We previously established that certain human lymphomas are sensitive to Bcl-2 family inhibition (9). To determine whether normal human lymphocytes are likewise sensitive to Bcl-2 family inhibition, the ability of ABT-737 to prevent proliferation of activated T and B lymphocytes *in vitro* was examined. Proliferation of normal human B cells activated with anti-IgM was inhibited by ABT-737, with an observed IC₅₀ of 0.08 μ M (Fig. 1A). Similarly, human T cells stimulated with anti-CD3 were inhibited with an observed IC₅₀ of 0.40 μ M (Fig. 1A). The enantiomer, used as a less active control, was less potent in inhibiting human B cell (Fig. 1A) and T cell proliferation (Fig. 1A). Murine T cell proliferation, as measured by MLR, was also inhibited in a concentration-dependent manner with ABT-737 treatment (Fig. 1B). An observed IC₅₀ of 0.14 μ M for the MLR assay indicated that the potency of inhibition was likely sufficient to inhibit T cell proliferation *in vivo*. Similarly, ABT-737 inhibited LPS-induced murine B cell proliferation with an observed IC₅₀ of 0.11 μ M (Fig. 1C). These results show that proliferation of both human and murine lymphocytes can be inhibited by ABT-737 *in vitro*. Because proliferation is an indirect measure of cell death, induction of apoptosis was shown by measuring caspase 3/7 activation in lymphocytes using the murine MLR and LPS B cell stimulation assays. ABT-737 significantly induced caspase activation and apoptosis in a concentration-dependent manner shortly after treatment of murine lymphocytes (Fig. 1D). We have previously demonstrated that human B-cell lymphomas in mouse xenografts undergo caspase-3 activation and

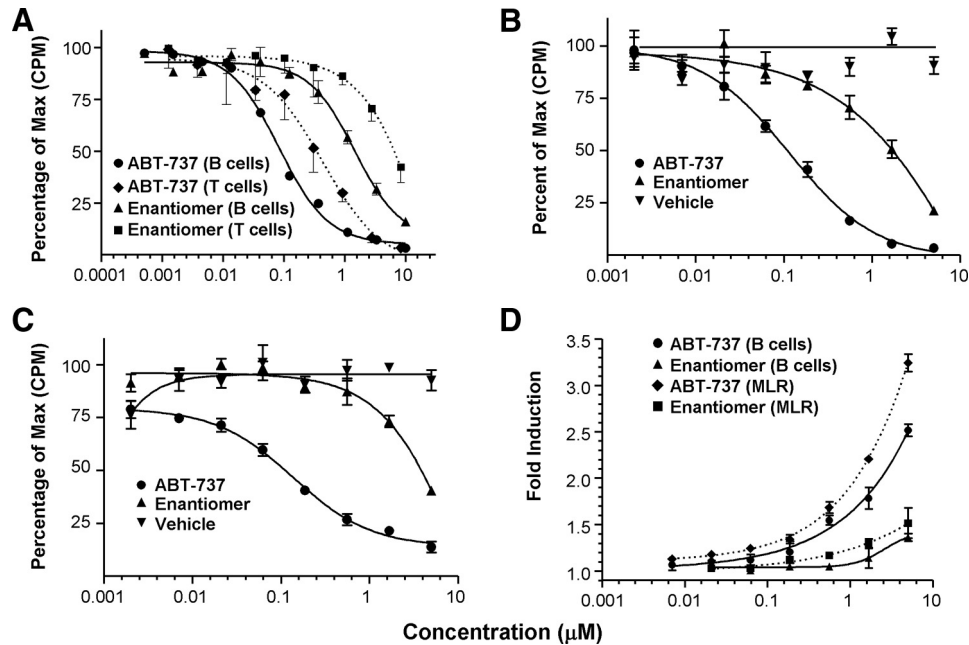


FIGURE 1. ABT-737 inhibits lymphocyte proliferation and triggers apoptosis. *A*, Human CD19⁺ B cells were stimulated with anti-IgM for 48 h in the presence of ABT-737 or the enantiomer before a 16-h pulse with [³H]thymidine. Human CD3⁺ T cells were stimulated with anti-CD3 and anti-CD28 Abs for 3 days and then treated with ABT-737 or the enantiomer for 72 h before a 16-h pulse with [³H]thymidine. *B*, Titration of ABT-737 and the enantiomer in a MLR assay. BALB/c and C57BL/6 splenocytes were cocultured for 2 days in the presence of ABT-737, the enantiomer, or vehicle control before a 6-h pulse with [³H]thymidine. *C*, Titration of ABT-737 and the enantiomer in a murine LPS B cell stimulation assay. B cells isolated by magnetic cell sorting were stimulated with LPS for 3 days in the presence of ABT-737, the enantiomer, or vehicle control before a 6-h pulse with [³H]thymidine. *D*, Caspase 3/7 activation was measured in the murine MLR and LPS B cell stimulation assays after a 1-h incubation with a titration of ABT-737 or the enantiomer. Luminescence was normalized to baseline and expressed as fold induction. One representative experiment is shown for all. Values are mean ± SEM.

apoptosis following treatment with ABT-263 (11), a compound highly related to ABT-737 that is being used in clinical trials.

KLH-primed T cells are sensitive to ABT-737

To understand the effectiveness of ABT-737 in preventing T cell proliferation *in vivo*, we immunized C57BL/6J mice with KLH and the draining LNs were harvested. Isolated lymphocytes were restimulated *in vitro* with KLH and T cell proliferation was measured. We recently reported the pharmacodynamic profile of ABT-737 in mice upon *i.p.* dosing (9). Based on this study, a pilot dose-response experiment was performed with once-daily dosing of ABT-737 at 50, 25, and 5 mg/kg for 4 days in the KLH model. Mice treated with 50 mg/kg ABT-737 showed complete inhibition of KLH-restimulated T cell proliferation (Fig. 2A). This inhibition was greater than that obtained with control animals treated with CsA (50 mg/kg/day) or 25 mg/kg/day ABT-737.

To assess the effect of a single 50-mg/kg dose of ABT-737 on resting vs activated T cells, we took LNs from naive and KLH-immunized animals that were treated with either vehicle or ABT-737. Harvested lymphocytes were subsequently stimulated with either KLH or Con A and proliferation was measured (Fig. 2B). As expected, because they were not primed, lymphocytes from naive animals treated with the vehicle or ABT-737 showed limited T cell proliferative capacity in response to KLH stimulation *in vitro*. In contrast, lymphocytes from KLH-immunized mice treated with ABT-737 showed a significantly reduced capacity to proliferate in response to KLH restimulation compared with lymphocytes from vehicle-treated animals ($p < 0.01$). Thus, *in vivo* ABT-737 treatment prevented KLH-induced T cell proliferation *ex vivo*. Somewhat surprisingly, Con A-stimulated T cell proliferation was similar among all groups, suggesting that the T lymphocytes remaining after ABT-737 treatment are functional.

Because ABT-737 is known to deplete lymphocytes and platelets (9), CBCs were taken for all experiments. Both naive and KLH-immunized mice treated with ABT-737 showed significant reductions in lymphocyte and platelet numbers (Fig. 2C). There was no significant difference between the lymphocyte and platelet counts in naive or immunized mice treated with ABT-737. Other cell types, such as neutrophils, monocytes, eosinophils, and basophils, did not show significant changes between the groups (data not shown).

ABT-737 inhibits DTH reaction in mice

The tuberculin-type DTH response is a T cell-dependent Ag response often used as a model to study mechanisms of chronic inflammation (12). To assess the efficacy of Bcl-2 antagonism in this model, we treated C57BL/6 mice daily with different dosages of ABT-737 from the time of immunization with mBSA (day 0) through Ag challenge on day 7. Treatment with ≥10 mg/kg/day ABT-737 during the priming phase resulted in significantly decreased paw swelling ($p < 0.01$), similar to dexamethasone treatment (1 mg/kg/day) (Fig. 3A). Doses that inhibited the DTH response also caused significant lymphocyte and platelet depletion (Fig. 3, B and C). It is well understood that a component of DTH is a T cell-mediated response, which can be used as an acute model of Th1 effector function (12). In fact, anti-T cell mAbs work well in preventing DTH (10) and CIA (13). Therefore, based on our results in DTH, we would predict an effect in the predominantly Th1-driven CIA animal model (14).

ABT-737 prevents disease progression in CIA

The CIA model is a well-established rodent model for RA, which is clinically characterized by paw swelling leading to ankylosis. At the first clinical signs of disease, mice were treated once daily with

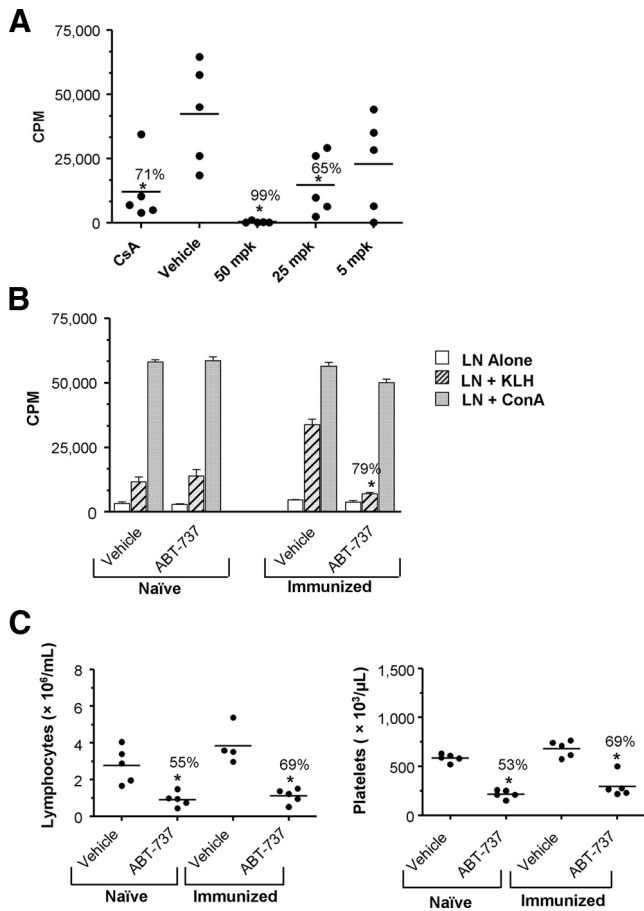


FIGURE 2. ABT-737 inhibits KLH restimulation of in vivo-primed T cells. *A*, A dose-dependent inhibition of KLH-induced proliferation is seen with ABT-737 treatment in C57BL/6J mice. C57BL/6J mice were immunized with KLH and were sacrificed 4 days later; their inguinal LNs were then harvested. Lymphocytes isolated from these LNs were restimulated with KLH for 48 h before pulsing with [³H]thymidine (16 h). Alternatively, mice were treated with 50 mg/kg/day CsA or the vehicle (30% polyethylene glycol solution). *B*, The T cell proliferative capacity was determined in LN lymphocytes isolated from naïve and KLH-immunized animals that were treated with either vehicle or ABT-737 (50 mg/kg). The isolated lymphocytes were stimulated with KLH or Con A or were not stimulated (LN alone). One representative experiment is shown. *, *p* < 0.01 compared with the respective vehicle control. *C*, CBCs were taken on the day of the LN harvest. Lymphocyte and platelet counts are shown. *, *p* < 0.01 compared with respective vehicle control.

ABT-737 (50 mg/kg) for 21 days. Treatment with ABT-737 and dexamethasone (1 mg/kg) significantly reduced the MAS and paw swelling compared with the vehicle-treated group (Fig. 4, *A* and *B*). With ABT-737 treatment, MAS and paw swelling were reduced by 45% (*p* < 0.05) and 89% (*p* < 0.05), respectively. Dexamethasone reversed the disease course, resulting in complete protection as assessed by both MAS and paw swelling. Moreover, ABT-737 and dexamethasone treatment also inhibited anti-collagen Ab production by 62% (*p* < 0.05) and 83% (*p* < 0.05), respectively (supplemental Fig. 1⁴). The impact of ABT-737 treatment on tarsal bone damage was assessed by microcomputed tomography. ABT-737 treatment protected mice from bone and cartilage destruction (Fig. 4*C*). The total bone volume in the group receiving ABT-737 treatment was similar to age-matched naïve controls and significantly different from the vehicle-treated group

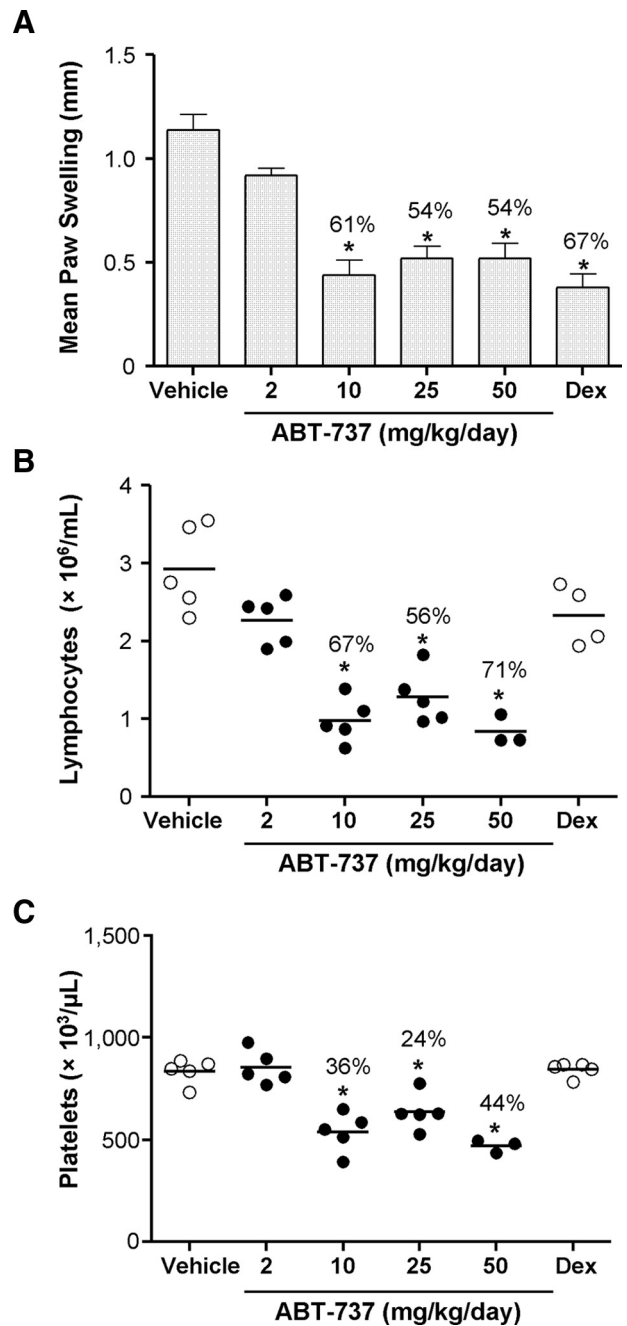


FIGURE 3. ABT-737 inhibits the tuberculin-type DTH response in C57BL/6 mice. *A*, The graph shows the effect of different doses of ABT-737 (mg/kg/day) and dexamethasone (Dex; 1 mg/kg/day) on the DTH response in C57BL/6 mice by measuring the percentage of paw swelling inhibition in comparison to vehicle, 1 day after Ag challenge on day 7 of treatment. CBCs were taken 1 day after the challenge and lymphocyte (*B*) and platelet counts (*C*) are shown. *, *p* < 0.01 compared with vehicle control.

(*p* < 0.05; supplemental Fig. 2). ABT-737 and dexamethasone treatment reduced the total lymphocyte count in whole blood by 80% (*p* < 0.01) and 21%, respectively (Fig. 4*D*). Additionally, ABT-737 and dexamethasone treatment reduced the platelet count by 43 and 55% (*p* < 0.01), respectively (Fig. 4*D*). Collectively, these data demonstrate that ABT-737 prevented disease progression in CIA mice, whereas dexamethasone, which has additional anti-inflammatory properties besides suppression of lymphocyte proliferation, was able to reverse the disease.

⁴ The online version of this article contains supplemental material.

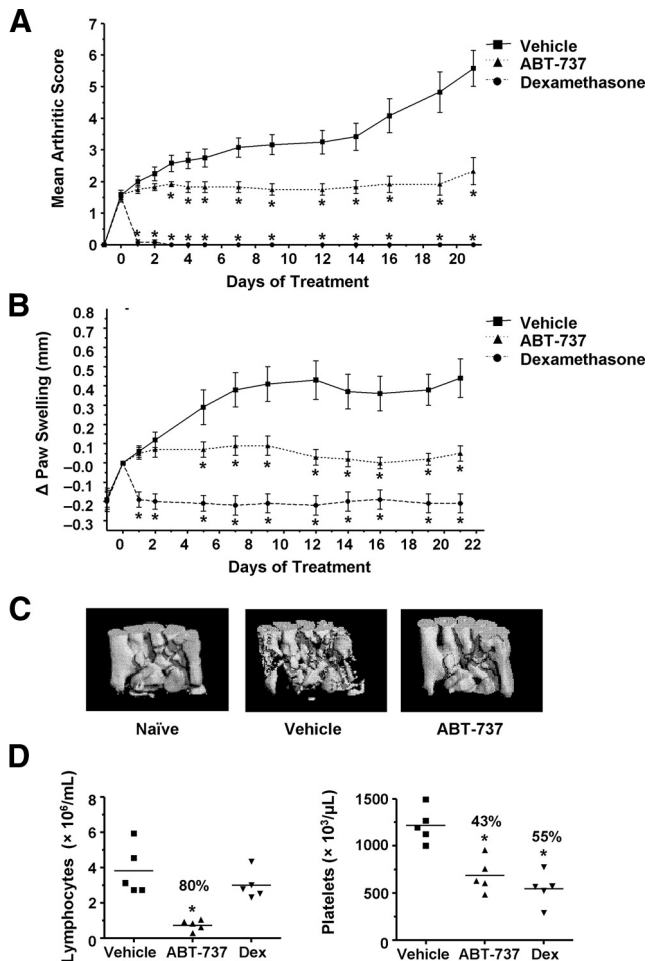


FIGURE 4. ABT-737 is efficacious when treatment is initiated at the first clinical signs of disease in a mouse model of CIA. Disease was induced in DBA/1J mice by immunizing mice with collagen in adjuvant on day 0. The mice were boosted with zymosan on day 21 and were monitored for clinical signs of arthritis. Mice were enrolled in the study and drug treatment was initiated at the first signs of disease, i.e., from days 24 to 28 (normalized to day 0 in the graphs). Once enrolled, arthritic mice were treated once per day with ABT-737 (50 mg/kg), dexamethasone (Dex; 1 mg/kg), or vehicle for 21 days. Serum exposure of ABT-737 was measured on day 21 at the time of sacrifice ($C_{\text{min}} = 2.5 \mu\text{M}$). **A**, The MAS was measured beginning on day 0 (normalized, days 24–28 after enrollment) by scoring clinical signs of disease as described in *Materials and Methods*. *, $p < 0.05$ compared with vehicle (Mann-Whitney U test). **B**, The mean change in paw swelling was calculated by normalizing to the paw size on day 0 (normalized, days 24–28 enrollment). *, $p < 0.05$ compared with vehicle control. **C**, Microcomputed tomography measurements were taken to monitor bone destruction 21 days after the onset of CIA disease. **D**, CBCs were taken from mice sacrificed on day 21. The respective lymphocyte and platelet counts are shown. *, $p < 0.01$ compared with vehicle control. One representative experiment is shown ($n = 3$).

ABT-737 inhibits IFN- α -induced lupus nephritis in (NZB \times NZW) F_1 mice

Lupus, unlike RA, predominantly results in chronic inflammation and tissue damage throughout the body. IFN- α has been implicated in the pathogenesis of SLE, but a direct role for IFN- α in triggering lupus pathogenesis has been lacking until recently (15). Adenovirus vector-mediated delivery of murine IFN- α in lupus-prone (NZB \times NZW) F_1 mice induces a rapid and severe disease with many characteristics of SLE, including death due to severe glomerulonephritis (15). To assess the role of Bcl-2-mediated apo-

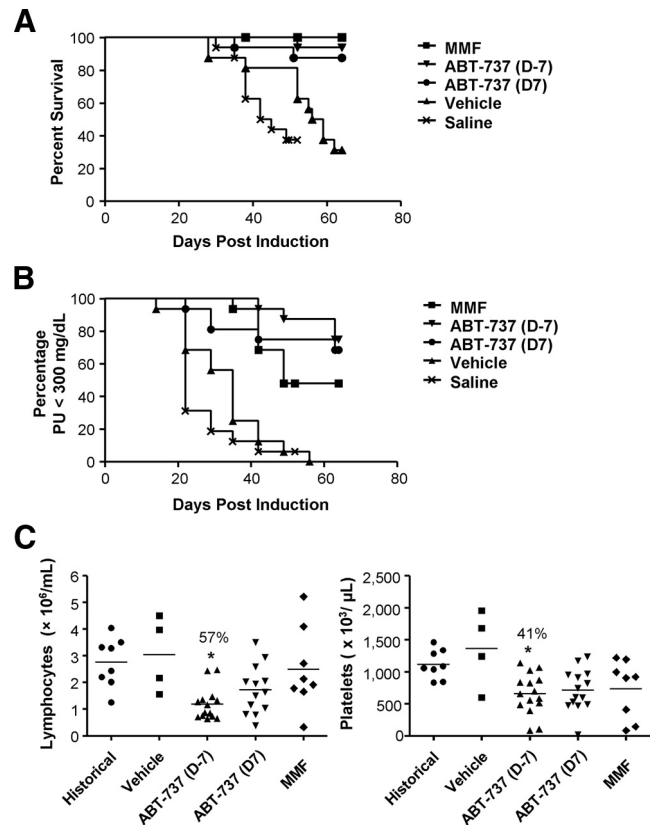


FIGURE 5. ABT-737 is efficacious in a prophylactic and late-prophylactic mouse model of SLE. Disease was induced in (NZB \times NZW) F_1 mice by treatment with IFN- α adenovirus on day 0. Mice were treated once daily with ABT-737 (50 mg/kg/day) either 7 days before (day -7) or 7 days after (day 7) the administration of IFN- α adenovirus, until the time of death or the end of the study. MMF (100 mg/kg/day) and the vehicle (both treatments began day -7) served as controls. Serum exposure of ABT-737 was measured on day 64 at the time of sacrifice ($C_{\text{min}} = 1.66 \mu\text{M}$). **A**, Kaplan-Meier cumulative survival was plotted for the mice under the four treatment scenarios. The vehicle group was statistically different ($p < 0.01$) from the other treatment groups. **B**, The incidence of PU (<300 mg/dl) was determined for each treatment group. The vehicle group was statistically different ($p < 0.01$) from the other treatment groups. **C**, CBCs were taken at the end of the study and the respective lymphocyte and platelet counts are shown. Historical controls are from saline-treated and similarly aged mice as the vehicle group. Significant differences were observed only in the day -7 treatment group ($p < 0.01$). One representative experiment is shown ($n = 2$).

ptosis in lupus pathogenesis, we treated (NZB \times NZW) F_1 mice with ABT-737 (50 mg/kg/day) either 7 days before (prophylactic) or 7 days after (late prophylactic) the administration of IFN- α -containing adenovirus vector. Similar to results obtained with the clinically validated benchmark MMF, survival was prolonged (Fig. 5A) and the onset of severe PU was delayed (Fig. 5B) with both ABT-737 treatment regimens, compared with vehicle control. At the conclusion of the study, lymphocyte and platelet counts were determined (Fig. 5C). Although there were significant differences in lymphocyte and platelet counts in the day -7 group of ABT-737-treated mice compared with controls ($p < 0.05$), the differences between the ABT-737- and MMF-treated groups were not appreciable (Fig. 5C).

Histologic analysis revealed that vehicle control-treated IFN- α -induced (NZB \times NZW) F_1 mice had severe glomerular injury, indicative of lupus nephritis (Fig. 6). In contrast, mice treated

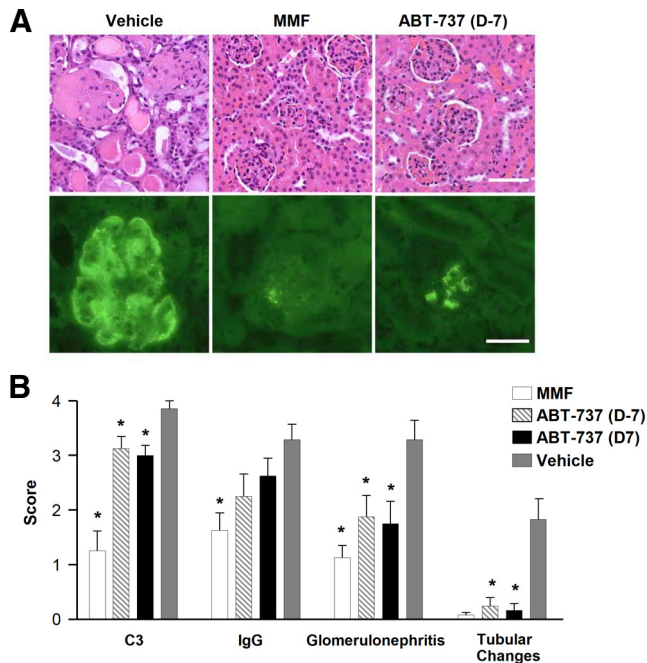


FIGURE 6. ABT-737 treatment in (NZB × NZW)_{F1} IFN- α -induced lupus nephritis model significantly reduced renal damage. *A*, The *top panel* shows representative images of H&E-stained kidney sections. Vehicle control-treated mice have severe glomerulonephritis and dilated renal tubules with casts (*top left*). MMF-treated and day -7 ABT-737-treated mice show minimal glomerular and tubular changes (*top middle and right*, respectively). The *bottom panel* shows representative immunofluorescent images of IgG deposition. Severe IgG deposition is seen in the vehicle control section (*bottom left*). Minimal segmental glomerular IgG deposits are seen in the MMF-treated and day -7 ABT-737-treated sections (*bottom middle and right*, respectively). There were no appreciable differences between the ABT-737 treatment groups; only the day -7 group is represented. Scale bar, 40 μ m. *B*, Summary graph of the average histologic scores indicative of lupus nephritis: C3 deposits, IgG immune complex, glomerulonephritis, and tubular changes (dilation, basophilia, and casts). Values are mean \pm SEM. *, $p < 0.05$ compared with vehicle control (ordinal logistic regression).

with ABT-737 or MMF were protected from significant glomerulonephritis (Fig. 6), compared with controls ($p < 0.05$). There was significant protection from renal tubular dilatation, casts, and basophilia in the ABT-737- and MMF-treated groups ($p < 0.05$). IgG and complement C3 deposits in the kidney are other hallmarks of lupus nephritis. As expected, vehicle control-treated mice showed significant increases in IgG (Fig. 6) and C3 (Fig. 6B) deposits. Interestingly, MMF treatment had a greater impact on C3 and IgG deposition than ABT-737 treatment (Fig. 6B). C3 deposits were significantly inhibited in both the ABT-737- and MMF-treated groups, while IgG deposits were significantly inhibited in only the MMF-treated group (Fig. 6B). However, qualitatively the IgG deposition in the ABT-737-treated groups was less severe than in the vehicle controls (Fig. 6A). In summary, treatment with ABT-737 prolonged survival and delayed the onset of severe PU in an IFN- α -induced (NZB × NZW)_{F1} mouse model of SLE, comparable to results observed with MMF.

Discussion

Autoimmune diseases, either tissue-specific or systemic, are in part caused by uncontrolled proliferation of autoreactive lymphocytes, eventually leading to tissue and organ damage. Inducing apoptosis of autoreactive lymphocytes constitutes an attractive therapeutic

strategy for RA (16), SLE (17), and possibly other rheumatic diseases. As a Bcl-2 family inhibitor, ABT-737 presented a unique opportunity to test a proapoptotic agent in animal models of RA and SLE. First, we demonstrated that ABT-737 was able to prevent proliferation of in vitro-activated T and B lymphocytes. Treating KLH-immunized mice with ABT-737 allowed us to demonstrate the immunosuppressive properties of ABT-737. We observed profound inhibition of KLH Ag-specific proliferation of murine LN T cells. Interestingly, T cells that were spared after treatment with ABT-737 were shown to be responsive to Con A, suggesting that these remaining T cells were still functional. We then proceeded to evaluate the efficacy of ABT-737 in two well-established animal models for human autoimmune diseases (i.e., CIA (arthritis) and IFN- α -induced lupus nephritis (lupus)).

The lymphopenia elicited by ABT-737 may contribute directly to the efficacy observed in both mouse CIA and lupus. It has been shown that CD4 T cell depletion acts synergistically with anti-TNF treatment to ameliorate disease in CIA (18). B cell depletion has also been implicated in CIA, but primarily as a prophylactic treatment (19). The CBC measurements from the CIA mice in the present study showed that lymphopenia and thrombocytopenia were the only systemic effects seen. Further study will be needed to examine the effect of ABT-737 on the microenvironment of the inflamed joints of CIA mice to understand whether pathogenic, nonlymphoid cells are affected. In lupus mouse models, the most striking example of T cell involvement has been seen with CTLA-4Ig treatment, which mechanistically inhibits both CD4 T cell activation and help provided by T cells to autoantibody-producing B cells (20). In addition, anti-CD20 treatment that depletes B cells has been shown to be effective for both CIA (19) and lupus (21). Therefore, targeting lymphocytes for depletion with a Bcl-2 inhibitor such as ABT-737 may be an effective clinical strategy.

Persistent or high-concentration expression of antiapoptotic Bcl-2 family proteins has been suggested to sustain the resistance of immune-effector cell death in both animal models and human diseases of autoimmunity. In mice with CIA, adenovirus-mediated Fas ligand-induced apoptosis has been shown to ameliorate disease (22). In related animal models, Bcl-2 overexpression was subsequently shown to correlate with disease in rat adjuvant-induced arthritis (23), and dysregulated apoptosis was shown to exacerbate arthritis in Bim-deficient recipients of K/BxN serum (24). These observations were supported by clinical studies in which elevated expression concentrations of antiapoptotic Bcl-2 proteins were detected in synovial fibroblasts of patients with RA compared with patients with osteoarthritis (25). It was further shown that synovial fibroblasts underwent apoptosis following knockdown of Bcl-2 expression. Furthermore, T cells isolated directly from the RA joints of patients exhibited increased Bcl-x_L expression and were resistant to spontaneous apoptosis (26). Thus, induction of apoptosis of immune and nonimmune effector cells may be an effective strategy for treating chronic inflammatory diseases such as RA (27).

The role of apoptosis in the pathogenesis of SLE is complex (28, 29). In animal models, the failure of lymphocyte apoptosis in promulgating disease pathogenesis is clearly illustrated in Bcl-2-transgenic mice (30) and Bim-deficient mice (31), both of which show evidence of SLE-like clinical symptoms caused by inappropriate survival of autoreactive lymphocytes. It has been postulated that disease in some patients with lupus may have a similar etiology, as elevated Bcl-2 expression in lymphocytes has been reported (32). In fact, persistent activation and inhibition of apoptosis has been implicated in sustaining chronic systemic inflammation (32–34). In contrast, the somewhat confusing etiology of excessive cell death and impaired clearance of apoptotic cell material has also been implicated in the pathogenesis of lupus (35). What is clear is that

defects in regulating the apoptotic pathway are often correlated with disease manifestation.

Clinically, there are a variety of drugs in use or under evaluation that target B-lymphocytes in patients with autoimmune disease. Examination of their mechanisms of action may provide insight into our results with ABT-737. Rituximab (anti-CD20), a B cell-depleting mAb, is effective in the treatment of CIA (19) and RA (21) and has shown promising clinical results for SLE (36). Also under clinical evaluation are two B cell-activating factor antagonists: belimumab (anti-B cell-activating factor) for RA and SLE (37) and atacept (transmembrane activator and calcium modulator and cyclophilin ligand interactor-Ig) for RA and SLE (38). ABT-737 treatment in one of the animal models reported here showed desirable effects on B cells, with anti-collagen Ab concentrations decreased in CIA.

In the clinic, T cell-directed therapies for autoimmunity have a long and complicated history (39). As an example, alemtuzumab (anti-CD52) causes profound depletion of peripheral T cells in humans but has not been efficacious in the treatment of autoimmune diseases such as RA and SLE. One potential explanation for this lack of success was found in a study of patients with RA. These patients had T cell infiltrates in the joints, even though peripheral depletion was complete (40). However, recent success in blocking T cell costimulation and activation has been seen with abatacept (CTLA4-Ig), which is effective in treating RA (41) and is currently in clinical trials for glomerulonephritis caused by SLE. We have demonstrated that ABT-737 treatment impaired T cell immunity in mice immunized with KLH. Taken together, depletion of potentially pathogenic B and T cells with ABT-737 represents an appealing approach for the treatment of autoimmune diseases.

The clinical development of B and T cell-targeted therapies in SLE is proving to be challenging (42). Phase I trial results with belimumab have shown a lack of efficacy in reducing SLE disease activity (43). In addition, rituximab and abatacept were recently reported to have failed to achieve positive clinical outcomes (42). As Lipsky (42) pointed out in a recent editorial, it is currently unclear why these therapies are not efficacious. It is possible that targeting B or T cells alone is not sufficient, that the mechanisms of these drugs are incomplete in suppressing lymphocyte activities, or that the pharmacodynamics of these therapies need further elucidation. Additional studies will be necessary to determine whether targeting B cells, T cells, or both, as seen with ABT-737, will yield novel SLE therapies.

In conclusion, the Bcl-2 family of proteins plays a central role in the control of lymphocyte immune responses by regulating the expansion and contraction of clones by apoptosis. Treatment with ABT-737, a potent inhibitor of Bcl-2, Bcl-x_L, and Bcl-w protein function, resulted in potent inhibition of lymphocyte proliferation in vitro. In vivo, ABT-737 treatment induced profound lymphopenia in normal and CIA mice and less pronounced lymphopenia in IFN- α -induced (NZB \times NZW)F₁ lupus mice. Nevertheless, mice treated with ABT-737 in animal models of arthritis (CIA) and lupus (IFN- α -induced (NZB \times NZW)F₁) showed a significant decrease in disease severity. These results suggest that treatment with a Bcl-2 family antagonist may provide a novel and potentially promising therapeutic approach for the clinical treatment of autoimmunity.

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