Bisindolylmaleimide VIII facilitates Fas-mediated apoptosis and inhibits T cell-mediated autoimmune diseases

TONG ZHOU[1], LING SONG[2], PINGAR YANG[1], ZHENG WANG[1], DI LUI[1] & RICHARD S. JOPE[2]

[1]*Division of Clinical Immunology and Rheumatology, Department of Medicine, and* [2]*Department of Psychiatry and Behavioral Neurobiology, The University of Alabama at Birmingham, Birmingham, Alabama 35294, USA*
Correspondence should be addressed to: T.Z; e-mail: tong.zhou@ccc.uab.edu; and R.S.J.; e-mail: jope@uab.edu

Fas-mediated apoptosis is essential for the elimination of cells, and impaired apoptosis can have severe detrimental consequences. Bisindolylmaleimide VIII potentiated Fas-mediated apoptosis in human astrocytoma 1321N1 cells and in Molt-4 T cells, both of which were devoid of apoptosis induced by anti-Fas antibody in the absence of bisindolylmaleimide VIII, and in Jurkat and CEM-6 T cells, which showed slight and moderate apoptotic responses, respectively, to low levels of Fas stimulation. Potentiation of Fas-mediated apoptosis by bisindolylmaleimide VIII was selective for activated, rather than non-activated, T cells, and was Fas-dependent, as it was not observed in T cells from Fas-deficient lpr/lpr mice. Administration of bisindolylmaleimide VIII to rats during autoantigen stimulation prevented the development of symptoms of T cell-mediated autoimmune diseases in two models, the Lewis rat model of experimental allergic encephalitis and the Lewis adjuvant arthritis model. Thus, the use of agents such as bisindolylmaleimide VIII may be therapeutically useful for supporting more effective elimination of detrimental cells through enhancement of Fas-dependent apoptosis signaling.

Apoptosis, or programmed cell death, is an essential mechanism used throughout life to selectively eliminate cells, and deficient apoptotic cell death is associated with a wide variety of disorders encompassing most cell systems[1]. Fas (Apo-1/CD95) is a member of the tumor necrosis factor (TNF) receptor family, one of the main signaling systems with the specialized function of inducing apoptosis[2]. Fas is a cell surface receptor that on activation (cross-linkage) by its natural ligand or by an agonistic antibody initiates a signaling cascade that leads to apoptosis[3,4]. Impairment of Fas-linked signaling, and thus of apoptosis, seems to contribute to a variety of severe disorders associated, for example, with cell proliferation, inflammation and autoimmunity[5]. Astrocytomas, which are among the most common lethal brain tumors, can express high levels of Fas and Fas ligand, but although often infiltrated by T cells, such infiltration does not improve patient prognosis, indicating that Fas apoptosis signaling may be dysfunctional[6,7]. Impaired Fas-mediated apoptosis in *lpr*/*lpr* mice and *gld*/*gld* mice caused by mutations of the Fas or Fas ligand genes, respectively, results in lymphoproliferation and autoimmune disease, indicating that Fas-mediated signaling plays important parts in the induction of lymphocyte apoptosis and the prevention of autoimmune disease[8,9]. Activation-induced cell death of T cells mediated by Fas-linked signaling is essential for down-modulating the T-cell response and the elimination of self-reactive T cells[10–12]. Thus, inadequate Fas-mediated apoptosis may contribute to proliferative disorders, and in T cells could produce loss of T-cell tolerance resulting in the development of autoimmune disease.

The expression of Fas or of Fas ligand can regulate Fas-mediated apoptosis, but it is evident that differences in cell susceptibilities to Fas-mediated apoptosis also can be controlled by the regulation of signaling cascades, because not all Fas-positive cell types undergo apoptosis similarly after stimulation of Fas[13,14]. For example, activated T cells are more susceptible to Fas-mediated apoptosis than are naive T cells[15]. Thus, interventions that facilitate Fas-induced apoptosis signaling processes may provide an ideal strategy for enhancing apoptosis for a variety of purposes, such as the elimination of self-reactive T cells in the treatment of autoimmune diseases. The advantage of this approach is that the intervention provides specificity for autoantigen-activated T cells that have failed to undergo activation-induced cell death, whereas the normal immune response to foreign antigens should remain less affected.

Here, several bisindolylmaleimide derivatives, which were originally described as inhibitors of protein kinase C (PKC)[16–18], were found to substantially facilitate Fas-mediated apoptosis in a human astrocytoma cell line and in several human T-cell lines. This facilitation of Fas-mediated apoptosis seemed to be independent of PKC inhibition. Facilitation of Fas-mediated apoptosis resulted in increased activation-induced cell death of activated T cells and this effect was specific for Fas-mediated apoptosis. Furthermore, *in vivo* administration of bisindolylmaleimide VIII to rats during autoantigen stimulation almost completely blocked the development of autoimmune diseases in two models: the Lewis rat model of experimental allergic encephalitis (EAE), and the Lewis adjuvant arthritis model.

Bisindolylmaleimide potentiates Fas-mediated apoptosis

Examination of cells that were stimulated with anti-Fas antibody alone or with bisindolylmaleimide VIII showed that bisindolylmaleimide VIII converted human astrocytoma 1321N1 cells from being essentially entirely resistant to apoptosis mediated by Fas to being highly sensitive to it. Although 1321N1 cells express functional Fas receptors on the cell surface (data not shown), they are relatively resistant to apoptosis induced by anti-Fas antibody. Thus, an overnight incubation of 1321N1 cells with a low

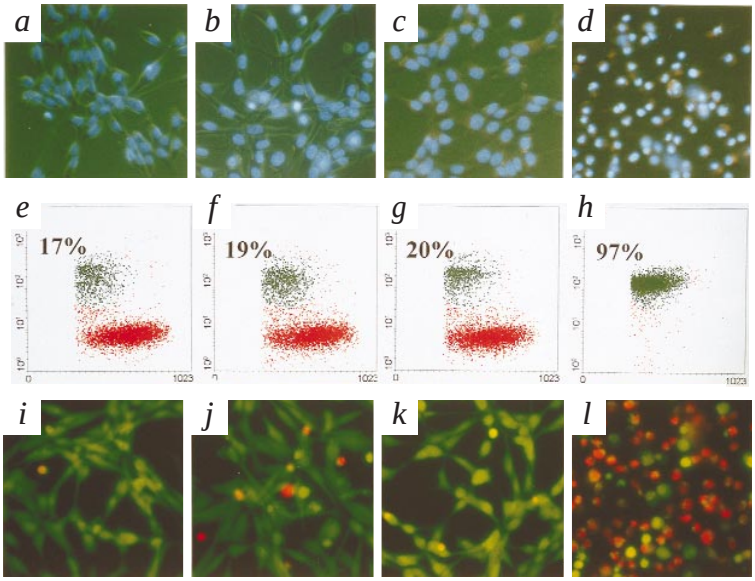

Fig. 1 Bisindolylmaleimide VIII enhances apoptosis induced by anti-Fas antibody in 1321N1 cells. 1321N1 cells were cultured overnight with control medium (*a, e* and *i*), 100 ng/ml anti-Fas antibody (*b, f* and *j*), 10 μM bisindolylmaleimide VIII (*c, g* and *k*) or both anti-Fas antibody and bisindolylmaleimide VIII (*d, h* and *l*). ***a–d***, Cells were stained with Hoechst 33342 and examined under ultraviolet fluorescence; *a–c* show staining of healthy cells, and *d* shows staining of condensed nuclei indicative of cell death. Original magnification, ×400. ***e–h***, For each treatment group 10,000 cells were analyzed by FACSvantage. Green dots indicate apoptotic cells; red dots indicate healthy cells. Percentages indicate the proportion of cell populations that were apoptotic ***i–l***, Cells were stained with a live/dead cell staining kit and examined by fluorescence microscopy. Healthy cells are stained green; dead or dying cells are stained red. Original magnification, ×400.

concentration of agonistic anti-Fas antibody (100 ng/ml) had no detectable effect on cell morphology or survival compared with that of cells maintained in growth medium (Fig. 1*a* and *b*). Incubation with 10 μM bisindolylmaleimide VIII alone also had no detectable effect (Fig. 1*c*). In contrast, after incubation with both anti-Fas antibody and bisindolylmaleimide VIII essentially all cells were round, membrane blebbing was apparent, and the cells had condensed nuclei, all changes that are typical of apoptosis (Fig. 1*d*). The condensed apoptotic nuclei were quantitatively analyzed by ultraviolet flow cytometry. This analysis confirmed that compared with control cells (Fig 1*e*) neither anti-Fas antibody (Fig. 1*f*) nor bisindolylmaleimide VIII (Fig. 1*g*) alone affected the cells. However, incubation of 1321N1 cells with 100 ng/ml of anti-Fas antibody in the presence of 10 μM bisindolylmaleimide VIII resulted in the induction of apoptosis in nearly 100% of the cells (Fig. 1*h*). The use of Calcein AM and ethidium homodimer-1 to distinguish live cells from dead cells in culture also confirmed that the proportion of healthy cells detected in control medium (Fig. 1*i*) was unaffected by anti-Fas antibody (Fig. 1*j*) or bisindolylmaleimide VIII (Fig.1*k*), but that combined treatment with anti-Fas antibody and bisindolylmaleimide VIII resulted in the death of most cells (Fig. 1*l*).

Apoptotic cell death of 1321N1 cells treated with both anti-Fas antibody and bisindolylmaleimide VIII was further verified by Annexin V staining, DNA laddering and TUNEL staining (Fig. 2). These results demonstrate that although bisindolylmaleimide VIII alone was not cytotoxic, it greatly potentiated Fas-mediated apoptosis, converting the Fas apoptosis resistant state of 1321N1 cells to a very sensitive state, indicating that bisindolylmaleimide VIII enhances the apoptosis signal generated by stimulation of Fas.

Concentration dependence of Fas-mediated apoptosis

The dose dependencies of anti-Fas antibody and bisindolylmaleimide VIII on apoptosis of 1321N1 cells were determined by measuring apoptosis in cells exposed to varying concentrations of each agent using Hoechst 33342 staining to quantify apoptotic cells. In the absence of anti-Fas antibody, 1, 3 or 10 μM bisindolylmaleimide VIII did not have a substantial effect on apoptosis (Fig. 3). Similarly, in the absence of bisindolylmaleimide VIII, anti-Fas antibody at concentrations less than 100 ng/ml had little effect on apoptosis of 1321N1 cells (Fig. 3). However, in the presence of bisindolylmaleimide VIII, there was a large increase in apoptosis induced by anti-Fas antibody. This potentiating effect of bisindolylmaleimide VIII on apoptosis induced by anti-Fas antibody was dose-dependent, with higher concentrations of bisindolylmaleimide VIII resulting in greater apoptosis induced by anti-Fas antibody. Thus, with 100 ng/ml anti-Fas antibody, the potentiating effect was clearly evident with a concentration of bisindolylmaleimide VIII as low as 1 μM, and nearly complete apoptosis was obtained with 3 μM bisin-

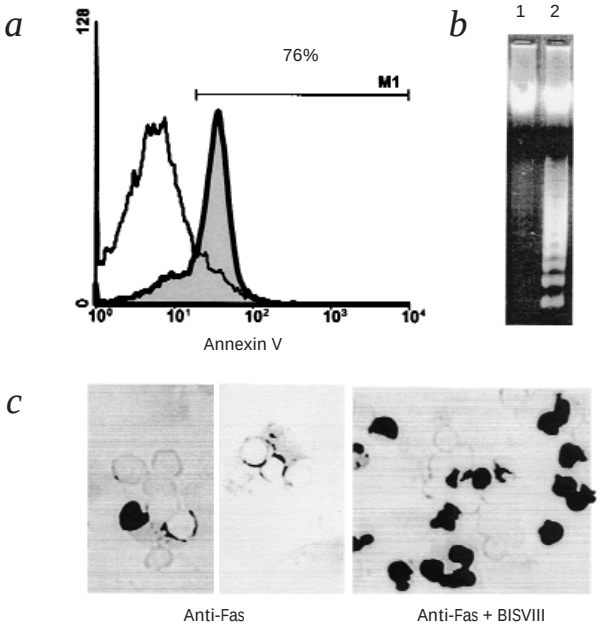

Fig. 2 Apoptosis analysis of 1321N1 cells. 1321N1 cells were cultured overnight with 100 ng/ml anti-Fas in the absence or presence of 10 μM bisindolylmaleimide VIII (BISVIII). ***a***, 1321N1 cells were stained with annexin V and analyzed by flow cytometry. Open histogram, cells treated with anti-Fas alone; filled histogram, cells treated with anti-Fas and bisindolylmaleimide VIII. Vertical axis, cell number; horizontal axis, fluorescence intensity. ***b***, DNA was extracted from cells treated with anti-Fas (lane 1) or with anti-Fas and bisindolylmaleimide VIII (lane 2) and resolved in 1% agarose gels. DNA laddering was evident after treatment with anti-Fas and bisindolylmaleimide VIII (lane 2) but not after treatment with only anti-Fas (lane 1). ***c***, TUNEL staining of cytospin preparations, showing that treatment with anti-Fas plus bisindolymaleimide VIII (right panel), but not with anti-Fas alone (left and center panels) resulted in positive staining in most cells.

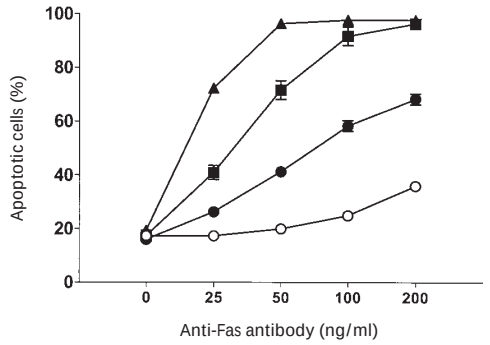

Fig. 3 Dose dependencies of bisindolylmaleimide VIII and anti-Fas antibody on potentiation of apoptosis. 1321N1 cells were incubated overnight with the indicated concentrations of anti-Fas antibody (horizontal axis) and 0 μM (○), 1 μM (●), 3 μM (■), or 10 μM (▲) bisindolylmaleimide VIII. Apoptosis was determined by flow cytometry analysis using Hoechst 33342. Values shown are mean ± s.e.m. of three independent experiments.

dolylmaleimide VIII. The potentiation by bisindolylmaleimide VIII also was dependent on the dose of anti-Fas antibody. For example, with 3 μM bisindolylmaleimide VIII, apoptosis was induced in more than 70% of 1321N1 cells by 50 ng/ml anti-Fas antibody. Thus, bisindolylmaleimide VIII decreased the threshold for apoptosis triggered by anti-Fas antibody in a concentration-dependent manner.

Bisindolylmaleimide potentiates TNF-mediated apoptosis

To determine if the potentiation of apoptosis induced by bisindolylmaleimide VIII is specific for Fas, we examined the effects of bisindolylmaleimide VIII on apoptosis of 1321N1 cells triggered by other apoptosis inducers, including TNF-α (to activate the TNF receptor, which belongs to the same receptor family as Fas), dexamethasone and irradiation. Bisindolylmaleimide VIII also enhanced apoptosis induced by TNF-α (Fig. 4). In the absence of bisindolylmaleimide VIII, 1321N1 cells were resistant to apoptosis mediated by TNF-α, as treatment with 5–20 ng/ml TNF-α caused no substantial increase in apoptosis (less than a 10% increase in apoptotic cells). Incubation with bisindolylmaleimide VIII facilitated apoptosis induced by TNF-α in a concentration-dependent manner, with 20 ng/ml TNF-α inducing apoptosis in over 60% of the cells in the presence of 10 μM bisindolylmaleimide VIII (Fig. 4*a*). In contrast, bisindolylmaleimide VIII had little effect on apoptosis of 1321N1 cells treated with dexamethasone (Fig. 4*b*) or irradiation (Fig. 4*c*). These results indicate that bisindolylmaleimide VIII selectively facilitates apoptosis signal transduction mechanisms induced by activation of the TNF receptor family.

Apoptosis with bisindolylmaleimides is PKC independent

Given the large potentiation of Fas-induced apoptosis by bisindolylmaleimide VIII, we next determined whether other bisindolylmaleimide derivatives had a similar effect, and if this interaction was related to inhibition of PKC. We assessed the effects of several bisindolylmaleimide derivatives and of other PKC inhibitors on Fas-mediated apoptosis in 1321N1 cells. Nine bisindolylmaleimide derivatives were tested, all of which (except for bisindolylmaleimide V) are relatively equally potent inhibitors of PKC, at a concentration of 10 μM (Table 1). Bisindolylmaleimides VIII and IX produced the greatest potentiation, increasing apoptosis induced by 100 ng/ml anti-Fas antibody from the basal level of near 19% to almost complete apoptosis (94%), an approximately fivefold increase. Bisindolylmaleimides III, X and XI produced intermediate potentiation, as demonstrated by the 2.2- to 3.6-fold increases in Fas-mediated apoptosis (from basal levels of 18–21% to treated levels of 45–66% apoptotic cells). Bisindolylmaleimides I, II and IV did not potentiate Fas-mediated apoptosis, although they are also potent inhibitors of PKC. Three other PKC inhibitors structurally unrelated to the bisindolylmaleimides (H7, calphostin C, and chelerythrine chloride) failed to potentiate Fas-mediated apoptosis. These results indicate that inhibition of PKC cannot account for the potentiation by bisindolylmaleimide VIII and IX of Fas-mediated apoptosis.

Potentiation of Fas-mediated apoptosis in T cells

The investigation of the effects of bisindolylmaleimide VIII was extended to T cells, because Fas-mediated apoptosis is well recognized as being essential for the maintenance of T-cell tolerance and in preventing the development of autoimmune diseases[8,9]. Therefore, we determined if bisindolylmaleimide VIII also facilitates Fas-mediated apoptosis in T cells using three human T-cell lines known to have different sensitivities to apoptosis induced by anti-Fas antibody. CEM-6 cells showed a moderate apoptotic response to anti-Fas antibody (100 ng/ml); bisindolylmaleimide VIII (10 μM) alone had no effect at 2 and 4 hours, but apoptosis was slightly increased after 8 hours (Fig. 5*a*). Exposure to both agents together more than doubled Fas-mediated apoptosis at 2, 4 and 8 hours. Jurkat cells showed a slight apoptotic response to anti-Fas antibody (100 ng/ml); bisindolylmaleimide VIII (which had no effect alone) greatly increased apoptosis induced by anti-Fas antibody, which reached 78% by 8 hours, compared with only 24% in the absence of bisindolylmaleimide VIII (Fig. 5*b*). Molt-4 cells were resistant to anti-Fas antibody in the absence of bisindolylmaleimide VIII, but incubation with both agents increased apoptotic cells to 54% at 8 hours, compared with 10% after exposure to either anti-Fas antibody or bisindolylmaleimide VIII alone (Fig. 5*c*). These results demonstrate that bisindolylmaleimide VIII greatly facilitates Fas-mediated apoptosis in human T cells, and that this effect occurs irrespective of the cell's basal sensitivity to apoptosis induced by anti-Fas antibody.

Table 1 Effect of bisindolylmaleimide derivatives and other PKC inhibitors on anti-Fas antibody induced apoptosis

Agents	Dose (μM)	Apoptosis (%) Without anti-Fas	With anti-Fas	Potentiation Index
Medium	-	18.5 ± 3.5	22.1 ± 2.9	1.19
Bisindolylmaleimide I	10	25.2 ± 4.2	27.8 ± 3.1	1.10
Bisindolylmaleimide II	10	19.2 ± 3.6	24.2 ± 3.2	1.26
Bisindolylmaleimide III	10	20.5 ± 2.6	44.5 ± 4.1	2.17
Bisindolylmaleimide IV	10	21.5 ± 2.8	24.5 ± 3.8	1.14
Bisindolylmaleimide V	10	18.9 ± 3.1	26.5 ± 3.2	1.40
Bisindolylmaleimide VIII	10	19.8 ± 2.4	92.5 ± 12.1	4.7
Bisindolylmaleimide IX	10	18.2 ± 2.3	95.2 ± 14.6	5.2
Bisindolylmaleimide X	10	18.2 ± 2.6	65.9 ± 8.9	3.6
Bisindolylmaleimide XI	10	21.3 ± 2.5	59.5 ± 11.2	2.8
H7	10	28.6 ± 3.4	32.1 ± 4.7	1.12
calphostin C	10	22.5 ± 3.2	25.6 ± 3.6	1.14
Chelerythrine chloride	10	72.5 ± 15.6	73.5 ± 16.3	1.01

1321N1 cells were cultured overnight with each agent at the indicated concentration in the absence or presence of anti-Fas antibody. Apoptosis was determined by flow cytometry using Hoechst 33342 staining. The potentiation index was calculated as the following ratio of apoptotic cells: (test agent with anti-Fas)/(test agent without anti-Fas). Each value is the mean ±s.e.m. of three independent experiments.

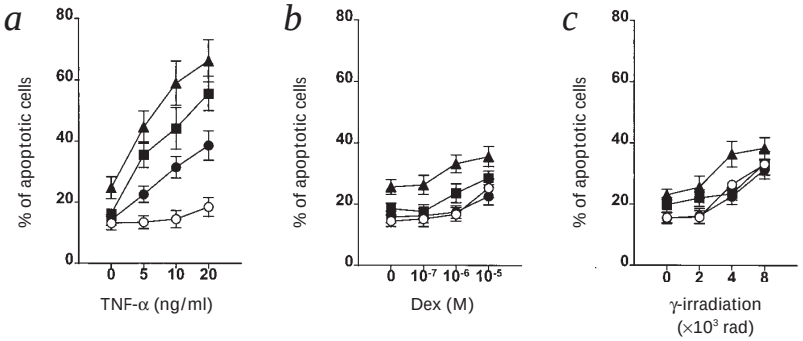

Fig. 4 Effect of bisindolylmaleimide VIII on apoptosis of 1321N1 cells induced by TNF-α, dexamathsone or gamma-irradiation. 1321N1 cells were incubated overnight with the indicated concentrations (horizontal axes) of TNF-α (***a***) or dexamathsone (***b***) in the presence of bisindolylmaleimide VIII; for gamma-irradiation (***c***), cells were irradiated at the indicated dose and incubated overnight with bisindolylmaleimide VIII. Apoptosis was determined by flow cytometry analysis using Hoechst 33342. ○, 0 μM bisindolylmaleimide VIII; ●, 1 μM; ■, 3 μM; ▲, 10 μM. Values shown are mean ± s.e.m. of three independent experiments.

Facilitation of activation-induced cell death of T cells

After activation, T cells express increased levels of both Fas and Fas ligand, and apoptosis mediated by Fas and Fas ligand is the main mechanism underlying activation-induced cell death of T cells[10–12]. Thus, if bisindolylmaleimide VIII facilitates signaling linked with Fas-induced apoptosis, bisindolylmaleimide VIII should promote activation-induced cell death of activated T cells. To test this, we isolated splenic T cells from wild-type mice and activated them using anti-CD3 antibody. Bisindolylmaleimide VIII greatly increased apoptosis of the activated T cells from wild-type mice in a dose-dependent manner, with almost 100% of the T cells stimulated by anti-CD3 antibody undergoing apoptosis in the presence of 10 μM bisindolylmaleimide VIII, compared with only 34% in the absence of bisindolylmaleimide VIII (Fig. 6*a*). Increased apoptosis of activated T cells in the presence of bisindolylmaleimide VIII was mediated mainly by Fas; soluble Fas–Ig fusion protein essentially completely blocked the increased apoptosis (Fig. 6*a*). Moreover, apoptosis of non-activated T cells was only slightly affected (Fig. 6*a*), indicating that enhanced apoptosis in activated T cells by bisindolylmaleimide VIII is Fas-dependent. Bisindolylmaleimide VIII only slightly increased apoptosis in either activated or non-activated T cells from *lpr*/*lpr* mice (Fig. 6*b*). This result also supports the conclusion that the increase in activation-induced cell death of T cells elicited by bisindolylmaleimide VIII is dependent on Fas-mediated apoptosis. To determine if the potentiation by bisindolylmaleimide VIII of Fas-mediated apoptosis in activated T cells could result from increased production of Fas ligand, we used a ^{51}Cr release assay to measure Fas ligand activity of the T cells after anti-CD3 re-stimulation. In the presence of bisindolylmaleimide VIII, Fas ligand activity was decreased in activated T cells from wild-type mice, which correlated with the increased cell death caused by bisindolylmaleimide VIII. In contrast, there was little effect of bisindolylmaleimide VIII on Fas ligand activity in activated T cells from *lpr*/*lpr* mice (Fig. 6*c*). Although these results do not rule out the possibility that Fas ligand expression was increased by bisindolylmaleimide VIII, which also would be able to enhance apoptosis of activated T cells, these results support the conclusion that bisindolylmaleimide VIII enhances apoptosis of activated T cells through a Fas-mediated signaling system, thus resulting in the rapid elimination, by an autocrine mechanism, of T cells expressing Fas ligand.

Table 2 Effect of bisindolylmaleimide VIII on the development of EAE

Treatment	Incidence	Onset (days)	Duration (days)	Clinical Score
Control	12 of 12	12.7 ± 0.9	7.0 ± 0.8	3.5 ± 0.5
Bis VIII	3 of 9	15.0 ± 0.9	3.5 ± 0.4	1.7 ± 0.3

Rats were immunized with MPB and injected with bisindolylmaleimide VIII (Bis VIII) or vehicle only (Control) and assessed for paralysis (Clinical Score: the average maximum achieved by each rat that attained a score of 1 or more). Onset, the day each rat achieved a clinical score of 1 or more.

Bisindolylmaleimide VIII prevents autoimmune diseases

Defective or insufficient activation-induced cell death in autoreactive T cells is thought to be an essential factor contributing to the development of some autoimmune diseases after autoantigen challenge. Thus, facilitation of activation-induced cell death during the activation of autoreactive T cells may be an ideal strategy for increasing the elimination of autoreactive T cells, and thus be useful in the treatment of certain autoimmune diseases. To determine whether the administration of bisindolylmaleimide VIII during the activation of autoreactive T cells can prevent the development of autoimmune diseases, we tested two T cell-mediated autoimmune disease models: the Lewis rat model of experimental allergic encephalitis (EAE), and the Lewis adjuvant arthritis model. To induce EAE, Lewis rats were immunized with myelin basic protein (MBP) to activate MBP-reactive T cells. The immunized rats were treated subsequently with 250 μg of bisindolylmaleimide VIII every other day for five doses. All of the control rats (12 of 12) treated with vehicle developed severe clinical symptoms of EAE, but only 33% of the bisindolylmaleimide VIII-treated rats (3 of 9) developed any symptoms of EAE. In the few bisindolylmaleimide VIII-treated rats that developed symptoms of EAE, the onset was slightly delayed and the recovery was twice as rapid as in control rats (Table 2). The EAE in the control rats (12 of 12) was progressive and severe. Most rats developed the manifestations of paralysis (clinical score, 3) . In contrast, the 33% of rats that showed any symptoms of EAE with bisindolylmaleimide VIII treatment did not progress to a severe form (clinical score, < 2). Thus, treatment with bisindolylmaleimide VIII reduced the incidence of symptoms, delayed the onset of disease, reduced the severity of disease, and enhanced recovery, indicating that bisindolylmaleimide VIII may facilitate apoptosis of MBP-reactive T cells *in vivo*.

Lewis rat adjuvant arthritis also is a T cell-mediated autoimmune disease. Five doses of bisindolylmaleimide VIII (250 μg per dose) or vehicle were administrated to rats as in the EAE model. Histological analysis showed that 100% of rats (12 of 12) treated with vehicle developed substantial symptoms of arthritis 30 days after immunization (Table 3). Severe inflammation, including cartilage erosion and bone destruction, was observed in most vehicle-treated rats. In contrast, less than 10% of the bisindolylmaleimide VIII-treated rats (1 of 12) had arthritic lesions, and the single positive rat attained a severity score of only 1, com-

Table 3 Effect of bisindolylmaleimide VIII on the development of adjuvant-induced arthritis

Treatment	Incidence	Severity Score
Control	12 of 12	2.7 ± 0.6
Bis VIII	1 of 12	1

Rats were immunized with Freund's complete adjuvant and injected with bisindolylmaleimide VIII (Bis VII) or vehicle (Control). Severity of arthritis was evaluated histologically 30 days after immunization; the severity score is given as the average maximum achieved by each rat that attained a score of 1 or more.

pared with an average score of 3 for the vehicle-treated rats. Thus, amelioration of autoimmune symptoms in two different models confirmed that bisindolylmaleimide VIII inhibits the development of T cell-mediated autoimmune disease.

To determine if increased activation-induced cell death in autoreactive T cells caused by bisindolylmaleimide VIII treatment is a chief mechanism contributing to its inhibition of the development of autoimmune disease, we examined the effect of *in vivo* treatment with bisindolylmaleimide VIII on the autoreactive T-cell response in both wild-type and *lpr*/*lpr* mice. C57BL/6 mice were immunized with myelin oligodendrocyte glycoprotein (MOG) peptide 35-55, which induces EAE by generating MOG-specific T cells in this strain of mice[19]. Treatment of wild-type B6 mice with bisindolylmaleimide VIII after autoantigen challenge substantially decreased the T-cell response to the subsequent MOG peptide stimulation compared with that of untreated control mice (Fig. 7*a*). This inhibited T-cell response is not due to a general immunodeficiency caused by bisindolylmaleimide VIII, as the T-cell response to anti-CD3 stimulation in treated animals was unimpaired (Fig. 7*a*). Furthermore, the T-cell response to the MOG peptide or anti-CD3 stimulation in bisindolylmaleimide-treated *lpr*/*lpr* mice was not substantially affected by bisindolylmaleimide VIII treatment (Fig. 7*b*). These results indicate that enhancement of activation-induced cell death in autoantigen-primed T cells by bisindolylmaleimide VIII is a chief mechanism by which autoantigen-reactive T cells are specifically eliminated.

Discussion

Deficient apoptosis can have severe, even lethal, consequences. Thus, with blocked apoptosis, transformed cells can evade elimination during tumor growth, and surviving autoreactive T cells can attack host tissues. Many therapeutic strategies are aimed at initiating apoptosis with exogenous agents, but the dose dependency and cell selectivity of such treatments can limit the degree of target cell elimination. An alternative strategy is to potentiate endogenous apoptotic signaling mechanisms that may be completely blocked or partially impaired in target cells, as may occur in transformed cells or autoreactive T cells. Here, cells resistant to Fas-mediated apoptosis were treated with bisindolylmaleimide VIII, which not only overcame the almost complete block of Fas-mediated apoptosis shown by both human 1321N1 astrocytoma cells and human Molt-4 T cells, but also potentiated weak and moderate apoptotic signals generated by a low dose of anti-Fas antibody in Jurkat and CEM-6 T cells, respectively. The facilitation of Fas-mediated apoptosis by bisindolylmaleimide VIII in autoreactive T cells was remarkably effective in blocking the development of two well-known autoimmune diseases, indicating that this may be a prototypical model for the development of improved therapeutic interventions in certain diseases associated with insufficient apoptotic signaling activity.

Fas-mediated apoptosis can be regulated by the levels of expression of Fas or Fas ligand, but these do not always correlate with cellular vulnerabilities to apoptosis, indicating that intracellular signaling processes associated with Fas-mediated apoptosis can be essential in determining if cells undergo apoptosis after activation of Fas[13,14]. Bisindolylmaleimide VIII seems to facilitate intracellular signaling initiated by activation of Fas, and this facilitation occurred in cells with a wide range of sensitivities to Fas-mediated apoptosis. Like most apoptotic processes, the Fas apoptosis signaling pathway is complex and has not been fully defined, so the sites affected by bisindolylmaleimide VIII remain to be identified. Although the bisindolylmaleimides have traditionally been considered selective inhibitors of PKC[16–18], this does not seem to be the action accounting for facilitation of Fas-mediated apoptosis, as not all bisindolylmaleimide derivatives that inhibit PKC potentiated Fas-mediated apoptosis, and because other PKC inhibitors had no effect. One possibility is that facilitation of Fas-mediated apoptosis by the bisindolylmaleimides occurs through inhibition of kinases other than PKC, which is consistent with previous reports that protein dephosphorylation, such as that mediated by hematopoietic cell protein tyrosine phosphatase, plays a part in the Fas apoptosis signaling pathway[13]. The main derivative studied here, bisindolylmaleimide VIII, modulates the activities of a variety of enzymes, such as mitogen-activated protein kinase phosphatase-1, Jun N-terminal kinase and tyrosine kinase signaling[20–22]. Thus, several candidate sites for the potentiating effect of bisindolylmaleimide VIII require investigation.

The interaction of bisindolylmaleimide VIII with Fas-mediated apoptosis was investigated in T cells as well as astrocytoma cells to address the cell selectivity of this interaction and because animal models of autoimmune diseases associated with impaired elimination of autoreactive T cells are well-described and are amenable to testing potential *in vivo* therapeutic interventions. A step towards *in vivo* use was the finding that bisindolylmaleimide VIII did not have a substantial effect on activated T

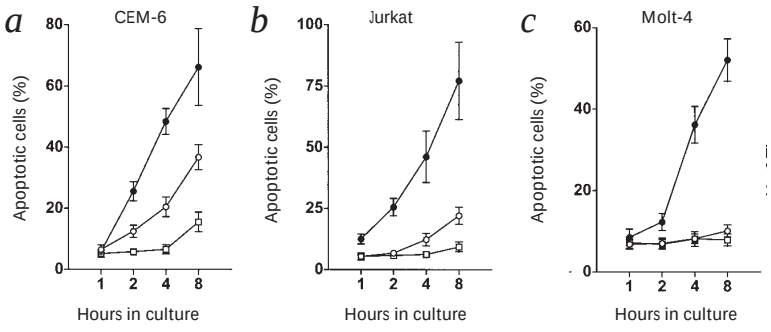

Fig. 5 Potentiation of Fas-mediated apoptosis by bisindolylmaleimide VIII in human T-cell lines. Human CEM-6 (***a***), Jurkat (***b***) and Molt-4 (***c***) T cells were cultured (1×10^6 cells/ml) in the presence of 10 μM bisindolylmaleimide VIII and/or 100 ng/ml anti-Fas antibody for the indicated times (horizontal axes). Apoptosis was determined by flow cytometry analysis using Hoechst 33342. ○, anti-Fas; □, bisindolylmaleimide VIII; ●, both reagents. Values shown are mean ± s.e.m. of three independent experiments.

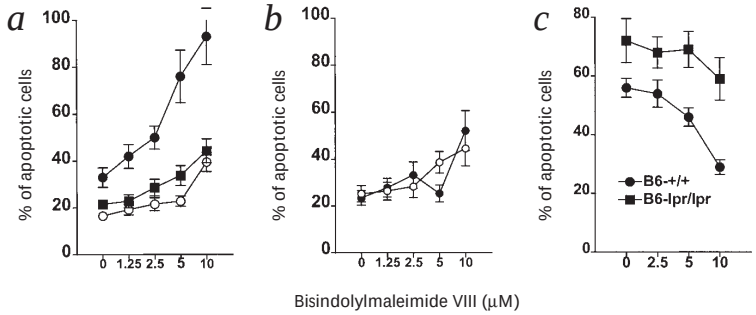

Fig. 6 Bisindolylmaleimide VIII enhances activation-induced cell death in T cells in a Fas-dependent fashion. T cells were isolated from spleens of C57BL/6(B6)+/+ (***a***) and *lpr*/*lpr* (***b***) mice. The T cells were stimulated with plate-bound anti-CD3 antibody for 48 h. Activated T cells were collected and maintained in the presence of interleukin-2 for three days, then re-stimulated overnight with anti-CD3 antibody in the presence of the indicated concentrations (horizontal axes) of bisindolylmaleimide VIII. To determine if the enhanced apoptosis is specifically mediated by Fas, 100 μg/ml soluble Fas–Ig fusion protein was added to the cultures of T cells from B6-+/+ mice. Freshly isolated spleen T cells without previous anti-CD3 stimulation were used as non-activated T cells. Apoptosis was determined by flow cytometry using Hoechst 33342 staining. ●, activated; ■, activated + Fas–Ig; ○, non-activated. Values shown are mean ± s.e.m. of T cells from three to five mice. ***c***, Effect of bisindolylmaleimide VIII on Fas ligand expression. T cells restimulated by anti-CD3 antibody (as described above), from B6-+/+ and *-lpr*/*lpr* mice, were analyzed for Fas ligand expression using the ^{51}Cr-release assay. Viable T cells were incubated with ^{51}Cr-labeled A20 cells (at a ratio of 1:10) for 8 h and Fas ligand activity was determined as the specific release of ^{51}Cr from A20 cells. Filled circles, B6-+/+; filled squares, B6-*lpr*/*lpr*. Values shown are means of triplicate cultures.

cells from Fas-deficient *lpr*/*lpr* mice, supporting the conclusion that the interaction is dependent on signaling through Fas. Moreover, bisindolylmaleimide VIII selectively promoted apoptosis in activated T cells, while having little effect on non-activated T cells. Because bisindolylmaleimides only promote Fas-mediated apoptosis in those T cells activated by autoantigens, it overcomes the disadvantage of non-specific immunosuppression. This raised the possibility that bisindolylmaleimide VIII may be especially useful for eliminating activated T cells that contribute to autoimmune diseases.

The finding that defective expression of Fas and Fas ligand causes the development of autoimmune diseases in *lpr*/*lpr* mice and *gld*/*gld* mice, respectively[8,9], indicates that insufficient Fas-mediated apoptosis may lead to loss of T-cell tolerance and hence to the development of autoimmune disease. Therefore, elimination of autoreactive T cells has been a therapeutic strategy for the treatment of autoimmune diseases. Enhancement of activation-induced cell death by the administration of high doses of autoantigens has been shown to effectively deplete autoreactive T cells and to abrogate the clinical and pathological symptoms of autoimmune encephalomyelitis[23,24]. As an alternative strategy, amelioration of autoimmune diseases may be attained by facilitation of Fas-mediated apoptosis in activated T cells, as was attained here by the administration of bisindolylmaleimide VIII in two animal models. In both the Lewis rat model of EAE and the Lewis adjuvant arthritis model, 100% of the vehicle-treated rats showed severe symptoms. In contrast, most of the rats treated with bisindolylmaleimide VIII showed no symptoms, and those rats with symptoms had greatly decreased severity of symptoms compared with those of vehicle-treated rats. These results demonstrate the feasibility of treatment of T cell-mediated autoimmune diseases by agents that facilitate Fas-mediated apoptosis of activated autoreactive T cells. Thus, these experiments indicate that agents that overcome blocked apoptotic signaling processes may be useful therapeutically, and, specifically, that bisindolylmaleimides might be potential leading compounds useful in treating T cell-mediated autoimmune diseases in humans.

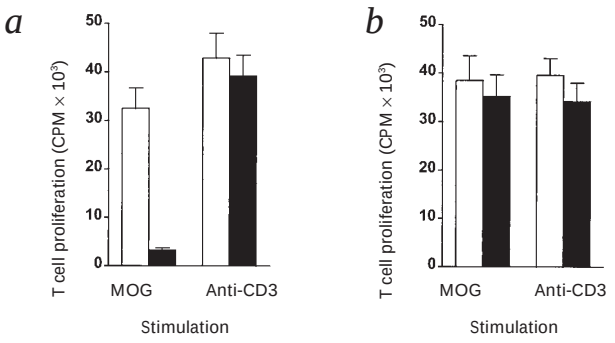

Fig. 7 Bisindolylmaleimide VIII inhibits antigen-specific T-cell response. C57BL/6-+/+ (***a***)and *-lpr*/*lpr* (***b***) mice were immunized with the MOG peptide and injected with bisindolylmaleimide VIII on day 3 after immunization; this treatment was repeated every other day for a total of three treatments. At day 10 after immunization, spleen T cells were purified and stimulated with the MOG peptide or anti-CD3 antibody. T-cell proliferative response was determined by the ^{3}H-thymidine incorporation assay. Basal proliferation was similar in the *lpr*/*lpr* and wild-type T cells in the absence of MOG or anti-CD3. Open bars, control; filled bars, bisindolylmaleimide VIII. Values shown are mean ±s.e.m. of five mice.

Methods

Cell lines and reagents. Human 1321N1 astrocytoma cells were a gift from J.H. Brown (University of California at San Diego), and the human T-cell leukemia cell lines, Jurkat, CEM-6 and Molt-4, were purchased from American Type Culture Collection (Rockville, Maryland). Bisindolylmaleimides I, II, III, IV, V, VIII, IX, X and XI, H7, calphostin C, and chelerythrine chloride were purchased from Alexis Biochemicals (San Diego, Californis). Anti-human Fas antibody (clone: CH11) was purchased from Upstate Biotechnology (Lake Placid, New York).

Induction of apoptosis. 1321N1 cells were grown in 48-well tissue culture plates in 5% FCS-DMEM to 70% confluence. The human T-cell lines (1×10^6 of log phase cells) were plated in 24-well plates in 1 ml of 12% FCS, RPMI-1640. Cells were incubated overnight, or for various times, with medium, anti-Fas antibody alone, bisindolylmaleimides or other test agents alone, or anti-Fas and bisindolylmaleimide or other agents together at various concentrations.

Detection of apoptosis. Cell death or apoptosis was determined by several methods. For cells in culture, the Live/Dead cell staining kit (Molecular Probes, Eugene, Oregon) was used according to the manufacturer's instructions. Stained cells were examined using an inverted fluorescence microscope. For Hoechst apoptosis staining and flow cytometric analysis, 5×10^5–10×10^5 cells were stained with 20 μl of 100 ng/ml Hoechst 33342 dye (Sigma) at room temperature for 20 min. After washing, the cells were fixed in 1% paraformaldehyde solution, and 10,000 ungated cells were analyzed by FACSvantage with a UV filter. For annexin V staining of apoptotic cells and flow cytometric analysis, FITC-conjugated annexin V (PharMingen, San Diego, California) was used according to the manufacturer's instructions, and 10,000 cells were analyzed by FACSvantage. For DNA laddering, high-molecular-weight DNA was extracted from cells using phenol-chloroform and was then resolved in 1% agarose gels and stained with ethidium bro-

mide. Finally, TdT-mediated dUTP nick-end labeling (TUNEL) was done according to the manufacturer's instructions (Amersham).

T-cell activation and induction of apoptosis. Splenic T cells were isolated from C57BL/6(B6)+/+ and *lpr*/*lpr* mice 4 to 6 weeks old using T-cell enrichment columns (R&D System, Minneapolis, Minnesota). Purified T cells (5×10^6 cells/ml) were cultured in plates precoated with 5 μg/ml anti-CD3 antibody (clone: F500; PharMingen, San Diego, California) for 48 h. The proliferative T cells were collected on Ficoll-Hypaque ($d = 1.077$) and cultured in 100 u/ml of interleukin-2 for 3 days. The T cells were then re-stimulated overnight with anti-CD3 in the presence or absence of bisindolylmaleimide VIII. Apoptosis was determined by Hoechst 33342 staining and flow cytometric analysis. Freshly isolated T cells that were not stimulated with anti-CD3 were used as non-activated controls.

^{51}Cr release assay for Fas ligand activity. Activated T cells from wild-type and *lpr*/*lpr* mice were re-stimulated with anti-CD3 antibody overnight in the presence or absence of bisindolylmaleimide VIII (as described above). Viable T cells were collected on Ficoll and used as effector cells for measuring Fas ligand activity. Fas ligand-sensitive A20 cells were used as the target cells and were labeled with ^{51}Cr. T cells (5×10^5) were co-cultured with A20 cells (5×10^4) in 96-well round-bottom plates for 8 h. A20 cells were cultured in medium to measure spontaneous release, or in medium containing 0.1 % SDS to measure maximum release. The supernatants were collected and radioactivity was assessed using a γ-counter. Fas ligand activity was calculated as the specific release of ^{51}Cr from A20 cells using the following equation: specific release (%) = (cpm of sample–cpm of spontaneous release)/(cpm of maximum release–cpm of spontaneous release).

Induction of autoimmune disease and treatment with bisindolylmaleimide VIII. For induction of EAE, female Lewis rats 6 weeks old (Jackson Laboratory, Bar Harbor, Maine) were immunized with 50 μg MBP (Sigma) in Freund's complete adjuvant (Difco, Detroit, Michigan) on day 0. Rats were injected intramuscularly with 250 μg bisindolylmaleimide VIII (dissolved in 10% DMSO–PBS) or vehicle. The treatment was started on day 1 and was repeated every other day for five doses. The development of EAE was evaluated every day for 10 days after onset; a clinical score was assigned to each individual rat as described[25]: 0, no disease; 1, loss of tail tone; 2, complete loss of tail tone; 3: lower leg paralysis; 4 paralysis. Onset was determined as the day each rat achieved a clinical score of 1 or more, and the clinical score was reported as the average maximum achieved by each rat that attained a score of 1 or more.

For induction of adjuvant arthritis, male Lewis rats 8 weeks old were immunized with 0.2 ml complete Freund's adjuvant at the base of the tail. The treatments with bisindolylmaleimide and control vehicle were exactly as used in the EAE model. Rats were killed 30 days after immunization and all joints were fixed in formalin and processed for histological evaluation to assess severity of arthritis. Histological lesions were evaluated for synovial proliferation, mononuclear cell infiltration, cartilage erosion and bone destruction as follows: 1, minor synovial proliferation; 2, severe synovial proliferation and inflammatory cell infiltration; 3, cartilage erosion; 4, bone destruction. Any joint showing any lesion was counted as positive; the severity score was reported as the average maximum achieved by each rat that attained a score of 1 or more.

The MOG peptide immunization and T-cell proliferative response. Female C57BL/6-+/+ and *lpr*/*lpr* mice 6 weeks old were immunized with 75 μg MOG peptide 25–35 in Freud's complete adjuvant[25], and were given 250 μg bisindolylmaleimide VIII (intraperitoneally) on day 3 after immunization and every other day for a total of three treatments. Ten days after immunization, splenic T cells were isolated and stimulated with 10 μg/ml MOG peptide or 1 μg/ml anti-CD3 antibody in 96-well round-bottomed plates. The T-cell proliferative response was determined by measuring ^{3}H-thymidine incorporation at 72 h after stimulation.

Acknowledgments

We thank W.J. Koopman and R.P. Kimberly for their critical comments and F. Hunter for editing the manuscript. T.Z. is supported by the Arthritis Foundation, the Juvenile Diabetes Foundation, a grant from the NIH (AR44982), and a grant from Sankyo (Japan). R.S.J. is supported by grants from the NIH (MH38752 and AG06569).

RECEIVED 6 OCTOBER; ACCEPTED 13 NOVEMBER 1998

1. Jacobson, M.D., Weil, M. & Raff, M.C. Programmed cell death in animal development. *Cell* **88**, 347–354 (1997).
2. Itoh, N. *et al*. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* **66**, 233–243 (1991).
3. Yonehara, S., Ishii, A. & Yonehara, M. A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. *J. Exp. Med.* **169**, 1747–1756 (1989).
4. Suda, T., Takahashi, T., Gostein, P. & Nagata, S. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell* **75**, 1169–1178 (1993).
5. Nagata, S. Apoptosis by death factor. *Cell* **88**, 355–365 (1997).
6. Saas, P. *et al*. Fas ligand expression by astrocytoma in vivo: Maintaining immune privilege in the brain? *J. Clin. Invest.* **99**, 1173–1178 (1997).
7. Weller, M. *et al*. Anti-Fas/APO-1 antibody-mediated apoptosis of cultured human glioma cells. *J. Clin. Invest.* **94**, 954–964 (1994).
8. Watanabe-Fukunaga, R. *et al*. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* **356**, 314–317 (1992).
9. Takahashi, T. *et al.* Generalized lymphoproliferative disease in mice caused by a point mutation in the Fas ligand. *Cell* **76**, 969–976 (1994).
10. Ju, S.T. *et al*. Fas(CD95)/FasL interactions required for programmed cell death after T-cell activation. *Nature* **373**, 444–448 (1995).
11. Brunner, T. *et al.* Cell-autonomous Fas(CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. *Nature* **373**, 441–444 (1995).
12. Dhein, J., Walczak, H., Baumler, C., Debatin, K.M. & Krammer, P.H. Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). *Nature* **373**, 438–441 (1995).
13. Su, X. *et al.* Defective expression of hematopoietic protein tyrosine phosphatase (HCP) in lymphoid cells blocks Fas-mediated apoptosis. *Immunity* **2**, 353–362 (1995).
14. Miyawaki, T. *et al.* Differential expression of apoptosis-related Fas antigen on lymphocyte subpopulations in human peripheral blood. *J. Immunol.* **149**, 3753–3758 (1992).
15. Ehl, S. *et al.* Different susceptibility of cytotoxic T cells to CD95 (Fas/Apo-1) ligand-mediated cell death after activation in vitro versus in vivo. *J. Immunol.* **156**, 2357–2360 (1996).
16. Toullec, D. *et al.* The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J. Biol. Chem.* **266**, 1571–1581 (1991).
17. Bit, R.A. *et al*. Inhibitors of protein kinase C. 3. Potent and highly selective bisindolylmaleimides by conformational restriction. *J. Med. Chem.* **36**, 21–29 (1993).
18. Jacobson, P.B. *et al*. Anti-inflammatory properties of Go 6850: a selective inhibitor of protein kinase C. *J. Pharmacol. Exp. Therap*. **275**, 995–1002 (1995).
19. Suen, W.E., Bergman, C.M., Hjelmstrom, P. & Ruddle, N.H. A critical role for lymphotoxin in experimental allergic encephalomyelitis. *J. Exp. Med.* **186**, 1233–1240 (1997).
20. Beltman, J., McCormick, F. & Cook, S.J. The selective protein kinase C inhibitor, Ro-31-8220, inhibits mitogen-activated protein kinase phosphatase-1 (MKP-1) expression, induces c-Jun expression, and activates Jun N-terminal kinase. *J. Biol. Chem.* **271**, 27018–27024 (1996).
21. Alessi, D.R. The protein kinase C inhibitors Ro 318220 and GF 109203X are equally potent inhibitors of MAPKAP kinase-1β (Rsk-2) and p70 S6 kinase. *FEBS Lett.* **402**, 121–123 (1997).
22. Yeo, E-J., Provost, J.J. & Exton, J.H. Dissociation of tyrosine phosphorylation and activation of phosphoinositide phospholipase C induced by the protein kinase C inhibitor Ro-31-8220 in Swiss 3T3 cells treated with platelet-derived growth factor. *Biochim. Biophys. Acta* **1356**, 308–320 (1997).
23. McFarland, H.I. *et al.* Amelioration of autoimmune reactions by antigen-induced apoptosis of T cells. *Adv. Exp. Med. Biol*. **383**, 157–166 (1995).
24. Tabi, Z., McCombe, P.A. & Pender, M.P. Antigen-specific down-regulation of myelin basic protein-reactive T cells during spontaneous recovery from experimental autoimmune encephalomyelitis: further evidence of apoptotic deletion of autoreactive T cells in the central nervous system. *Int. Immunol.* **7**, 967–973 (1995).
25. Sun, D., Qin, Y., Chluba, J., Epplen, J.T. & Wekerle, H.. Suppression of experimentally induced autoimmune encephalomyelitis by cytolytic T cell interactions. *Nature* **332**, 843–845 (1988).