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Functional Interaction of the Immunosuppressant Mizoribine with the 14-3-3 Protein

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Mizoribine (MIZ) is a novel imidazole nucleoside with immunosuppressive activity. MIZ has been approved in Japan and combination therapy with MIZ and glucocorticoids has been used after renal transplantation and for lupus nephritis and rheumatoid arthritis. In this study, we identify 14-3-3 proteins as MIZ-binding proteins. 14-3-3 proteins interact with many proteins involved in cellular signaling, including the glucocorticoid receptor (GR). The 14-3-3/GR interaction enhances the transcriptional activity of the receptor. We show that MIZ affects the conformation of 14-3-3 proteins and enhances the interaction of GR and 14-3-3η dose dependently *in vitro*. MIZ also has a stimulatory effect on transcriptional activation by the GR. Our results point to the possibility that one mechanism for the therapeutic effect of MIZ could be to regulate the GR function via 14-3-3 proteins. © 2000 Academic Press

Key Words: **mizoribine; immunosuppression; 14-3-3; glucocorticoid receptor; nuclear receptor; transactivation.**

Mizoribine (MIZ) (4-carbamoyl-1-β-D-ribofuranosylimidazolium-5-olate) was isolated from the culture media of *Eupenicillum brefaldianum* in 1974 in Japan (1). This agent is an imidazole nucleoside and possible immunosuppressive agent (2). The immunosuppressive effect of MIZ has been suggested to be due to inhibition of DNA synthesis in the S phase of the cell cycle (3). MIZ is phosphorylated by adenosine kinase (4), and then affects the synthesis of nucleic acids by inhibition of IMP dehydrogenase (4) or both IMP dehydrogenase and GMP synthetase (5). MIZ has been approved in Japan and used for immunosuppressive therapy after renal transplantation, and for lupus nephritis and rheumatoid arthritis. Combination therapy with MIZ and glucocorticoids has also been used. Sugiyama reported that by using MIZ treatment it was possible to decrease glucocorticoid administration without any recurrence of lupus nephritis (6).

The 14-3-3 proteins form a highly conserved family of acidic, dimeric proteins distributed widely among eukaryotic cells. 14-3-3 proteins interact with many intracellular signaling proteins and are known to regulate intracellular signal transduction processes (7). We previously showed using the yeast two-hybrid system that the COOH-terminal portion of the 14-3-3η protein interacts with the glucocorticoid receptor (GR) ligand-binding domain (LBD) and that the glucocorticoid agonist dexamethasone (Dex) induced the GR LBD/14-3-3η protein fragment interaction (8). The glucocorticoid receptor (GR) is a member of the nuclear receptor superfamily and mediates the effects of glucocorticoids by regulating the transcription of target genes (9). Furthermore, we reported that full-length 14-3-3η protein interacts with the ligand-activated GR and has stimulatory effect on transcriptional activation by the GR (8).

In a previous report, we have identified HSP60 as a MIZ-binding protein by using MIZ affinity column chromatography. The binding of MIZ to HSP60 resulted in the interference of chaperone activity of HSP60 *in vitro* (10). In this report, we identified 14-3-3 proteins as MIZ-binding proteins and demonstrated that MIZ has a functional effect on the 14-3-3 protein by modulating the conformation of the protein and enhancing the interaction between 14-3-3η and GR.

MATERIALS AND METHODS

MIZ. MIZ was kindly supplied by Asahi Chemical Industry Co., Ltd. (Tokyo, Japan).

Abbreviations used: MIZ, mizoribine; GR, glucocorticoid receptor; LBD, ligand-binding domain; Dex, dexamethasone; HPLC, high-pressure liquid chromatography; GST, glutathione *S*-transferase; PDB, pull-down buffer; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide.

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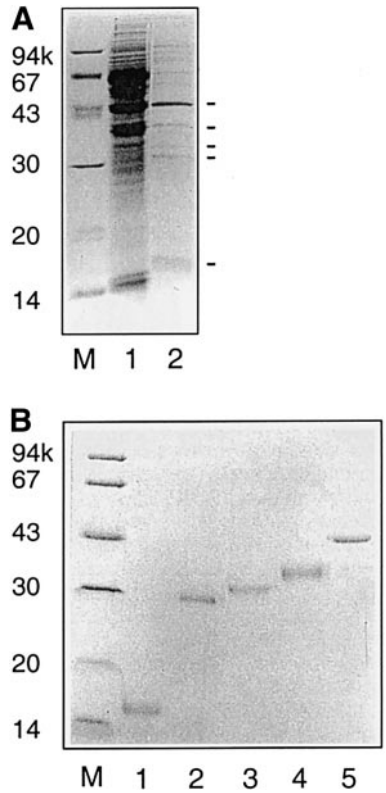

FIG. 1. Isolation and purification of MIZ-binding proteins from porcine kidney. SDS–polyacrylamide gel stained with Coomassie brilliant blue R-250. M denotes molecular standard proteins (Amersham Pharmacia Biotech) in kDa. (A) Lane 1: Porcine kidney extract applied to a MIZ-Sepharose column. Lane 2: Eluted proteins from the column with the buffer containing MIZ. Detailed conditions are given under Materials and Methods. Bars on the right indicate 17-, 30-, 32-, 36-, and 43-kDa proteins. (B) Lanes 1–5: 17-, 30-, 32-, 36-, and 43-kDa MIZ-binding proteins, respectively. Purification procedure is given under Materials and Methods.

Isolation and identification of MIZ-binding proteins from porcine kidney. One hundred milligrams of MIZ was coupled with 10 ml of Epoxy-Sepharose 6B (Amersham Pharmacia Biotech, Uppsala, Sweden), according to the manufacturer's instruction. The following steps were performed at 4 °C. Minced porcine kidney (150 g) was homogenized with 500 ml of buffer A (10 mM Tris–HCl, pH 7.4). After centrifugation at 20,000*g* for 15 min, the supernatant was applied to a MIZ-Sepharose column (1.5 × 5.2 cm) preequilibrated with buffer A. The column was washed with 200 ml of buffer A. Thereafter, MIZ-binding proteins were eluted with 30 ml of buffer A containing 20 mM MIZ (pH was adjusted to 7.4 by adding 1 N NaOH). The major peak of protein eluted (2 ml) was collected. The collected eluent containing MIZ-binding proteins was applied to long and thick SDS–polyacrylamide gels. After electrophoresis, gel was lightly stained with Coomassie brilliant blue R-250, and each separated protein band was carefully cut. Proteins with molecular mass of 17, 30, 32, 36, and 45 kDa were electrophoretically eluted from cut gels using Sample Concentrator (Model 1750; ISCO Inc., Lincoln, NE). Each concentrated protein sample was applied to SDS–polyacrylamide gels. After electrophoresis, production of peptides of the 17-, 30-, 32-, and 45-kDa proteins were carried out according to the method of Kawasaki *et al.* (11). Lysylendopeptidase-digested peptides were separated by reverse-phase HPLC on a C_{18} column (Wakosil $5C_{18}$; Wako Pure Chemical Industries, Osaka, Japan) as described previously (10). Amino acid sequences of the separated peptide were determined using a 491 Procise Protein Sequencer System (Perkin Elmer, Foster City, CA).

Purification of 14-3-3 proteins from porcine kidney. 14-3-3 proteins were extracted from porcine kidney in hypotonic buffer. After ammonium sulfate fractionation, 14-3-3 proteins were purified by subsequent DEAE (Whatman, Cambridge, UK), hydroxylapatite (Bio-Rad Laboratories, Hercules, CA), and Q-Sepharose (Amersham Pharmacia Biotech) column chromatographies. To select 14-3-3 protein-rich fractions, Western blot analysis with antibody against 14-3-3 proteins (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was performed at each purification step.

CD spectroscopy. CD measurements of purified 14-3-3 proteins from porcine kidney were performed on a Jasco J-720 spectropolarimeter (JASCO, Tokyo, Japan). Spectra were recorded in 50 mM Hepes buffer, pH 7.4, at 24°C in a cylindrical quartz cuvette with a 0.01-cm path length. The protein concentration was kept at 0.13 mg/ml in these experiments. Spectra were the average of four scans at 50 nm/min, each with the band width set at 2 nm, and were baseline-corrected by subtracting the corresponding blank. Spectra were recorded in the range 200–250 nm. The observed ellipticity was converted to mean residue ellipticity {$[\theta]$m.r.w. (degrees · cm^2 · $dmol^{-1}$)}. Smoothing was applied to the curves, by a mild function that increased the signal-to-noise ratio without altering the shape of spectra, with the Jasco J-720 program.

TABLE 1

Identification of MIZ-Specific-Binding Proteins from Porcine Kidney

Molec mass (kDa)	No. of peptides identified	Sequence determined	Homology (residues)
30	9	AVTEQGHELSNEERNLLSVA	14-3-3β (30–49)
		YLIPNATQP	14-3-3β (106–114)
		GDYFRYLSEVASGDN	14-3-3β (125–139)
		TAFDEAIAELDTLNEESYK	14-3-3β (196–214)
		GDYYRYLAEVAAGDDK	14-3-3 ζ (123–138)
		GIVDQSQQAYQEAFE	14-3-3 ζ (140–154)
		TAFDEAIAELDTLSEESYK	14-3-3 ζ (194–212)
		AVTELNEPLXNEDRNLLSVA	14-3-3η (29–48)
		GDYYRYLAEVASGEK	14-3-3η (128–142)
32	2	GDYHRYLAEFATGND	14-3-3 ϵ (126–140)
		AAFDDAIAELDTLSEESYK	14-3-3 ϵ (197–215)

Note. The species of 14-3-3 isoforms are human.

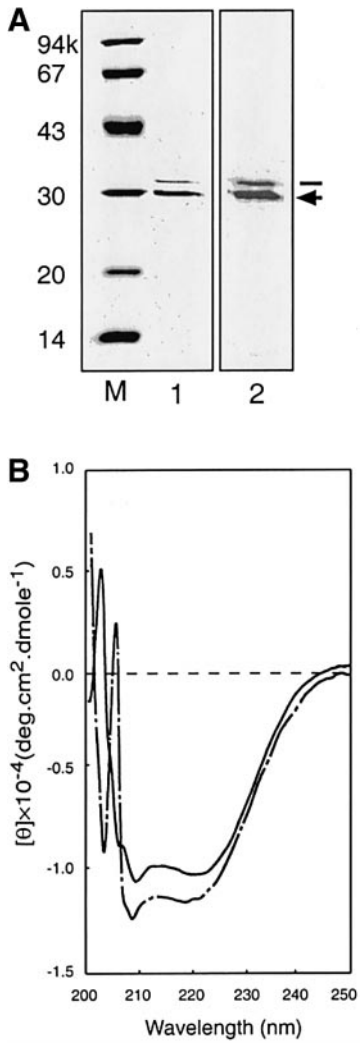

FIG. 2. Purification of 14-3-3 proteins from porcine kidney and the effect of MIZ on the CD spectrum of 14-3-3 proteins. (A) purified 14-3-3 proteins were analyzed by SDS–PAGE, stained with Coomassie brilliant blue R-250 (lane 1) or by Western blot analysis with antibody against 14-3-3 proteins (lane 2). The arrow and the bar indicate 30- and 32-kDa 14-3-3 proteins, respectively. M denotes molecular standard proteins as in Fig. 1. (B) the CD spectrum of the purified 14-3-3 proteins was measured in the absence (solid line) or presence of 5 mM MIZ (dot-dashed line) at 24°C as described under Materials and Methods.

Protein expression. [^{35}S]Methionine-labeled full-length hGR was translated *in vitro* using an *in vitro* translation kit (Promega) at 30°C for 90 min. 14-3-3η was expressed as a fusion protein with glutathione *S*-transferase (GST) from the vector pGEX-4T-1 (Amersham Pharmacia Biotech) (9). The original and 14-3-3η expressing pGEX vectors were transformed into *Escherichia coli,* BL21(DE3)pLysS cells. The induced GST and GST/14-3-3η proteins were purified from the bacterial lysate using glutathione-agarose beads (Sigma Chemical Co., St. Louis, MO) by standard procedure.

GST pull-down experiment. After the *in vitro* translation, 20 μl of the [^{35}S]-labeled GR translation mix was diluted into 625 μl of ice-cold GST pull-down buffer (PDB) [30 mM Hepes–KOH, pH 7.9, 10% glycerol, 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 mg/ml bovine serum albumin (BSA), 0.03% (v/v) IGEPAL CA-630 (Sigma Chemical Co.)] containing 1 μM Dex and incubated at 4 °C for 2 h. MIZ dissolved in PDB was added to the diluted GR in PDB at the concentrations 0–20 mM. 200 μl of each sample was then mixed either with 30 μl of GST- or GST/14-3-3η-glutathione beads at 4 °C overnight. Approximately 0.3 μg of GST proteins/μl of beads had been bound to the glutathione beads. Beads were collected by centrifugation and washed six times with PDB without BSA. Washed beads were resuspended in 30 μl 1 × SDS sample buffer, boiled for 5 min, and pelleted by centrifugation, and 10 μl of the supernatant were subjected to SDS–PAGE. Before autoradiography, gels were stained with Coomassie brilliant blue to control the stability of GST-fusion proteins and equal loading. For quantification, gels were analyzed using PhosphoImager (BAS-2000; Fujifilm, Tokyo, Japan). The nonspecific binding to GST was subtracted from the specific GST/14-3-3 binding, at each concentration of MIZ and the mean value and standard deviation from three experiments was calculated.

Transient transfection of mammalian cells. Monkey kidney COS-7 cells were maintained in DMEM supplemented by 10% fetal bovine serum (GIBCO BRL, Gaithersburg, MD), 100 units/ml penicillin, and 100 μg/ml streptomycin (GIBCO BRL). Cells seeded in 24 well plates were transiently transfected with 100 ng of p19LUC-TK reporter construct with two glucocorticoid responsible elements upstream of the reporter gene, 5 ng of pCMV-β-galactosidase reporter construct, 5 ng of pCMV4-hGR expression vector, and 5 ng of pBKCMV or pBKCMV-14-3-3η expression vector by using FuGENE 6 Transfection Reagent (Boehringer Mannheim, Mannheim, Germany). Following transfection, cells were exposed to various concentrations of MIZ in the absence or presence of Dex (1 μM) for 48 h and then cells were harvested for β-galactosidase assay using Galacto-Light Plus (TROPIX, Bedford, MA) and luciferase assay using GenGlow-1000 Kit (Bio-Orbit, Turku, Finland). To correct for variations in transfection efficiency, the luciferase activity was related to the β-galactosidase activity.

MTT assay. COS-7 cells were transiently transfected as described above. Three hours before the end of MIZ exposure, 125 μl tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide, MTT) (Sigma Chemical Co.) solution (2 mg/ml in phosphate-buffered saline) was added to each well. At the end of MIZ exposure the enzyme reaction was stopped by adding 1 N HCl:isopropanol (1:24) (500 μl/well) followed by thorough mixing with a pipette. Then, 200 μl of the mixture were transferred to a 96-well microtiter plate, and the plate was read on a SPECTRA MAX 250 (Molecular Devices, Sunnyvale, CA) at 540 nm.

RESULTS AND DISCUSSION

Using MIZ-affinity column chromatography, several low-molecular-weight proteins were isolated from a porcine kidney extract (Fig. 1A, lane 2). Among them 17-, 30-, 32-, 36-, and 43-kDa proteins were purified by the elution from SDS–polyacrylamide gels (Fig. 1B).

Amino acid sequences of lysylendopeptidase-digested fragments of the purified 17-, 30-, 32-, 36-, and 43-kDa proteins were determined. As shown in Table 1, the 30-kDa protein was identified as a mixture of β (12), ζ (13), and η (14) isoforms of 14-3-3 proteins, and the 32-kDa protein was 14-3-3ξ (15). The 17-, 36-, and 43-kDa proteins were identified as 17-kDa protein kinase C inhibitor (16), glyceraldehyde-3-phosphate dehydrogenase (17), and cytoplasmic actin (18), respectively (data not shown). Interestingly, all these MIZ-binding proteins can bind nucleotides. Athwal *et al.*

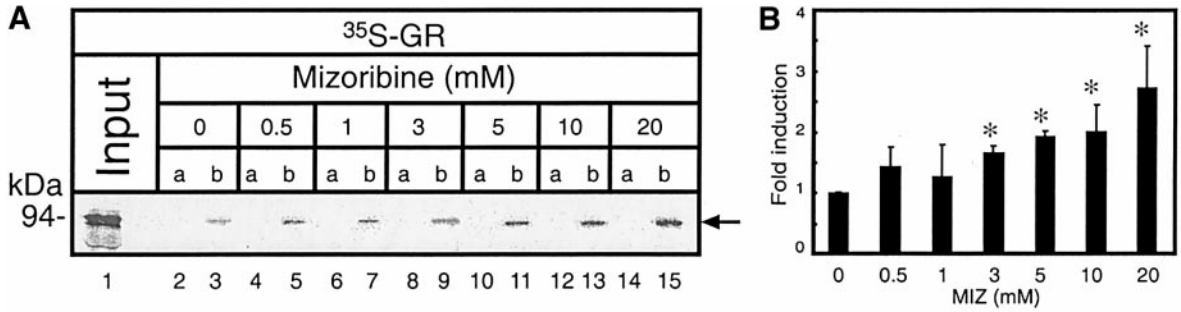

FIG. 3. Effect of MIZ on the interaction between hGR and 14-3-3η protein *in vitro.* (A) GST pull-down experiment. The translation mixture was incubated at 4°C for 2 h in the presence of 1 μM Dex and mixed with (a) GST- or (b) GST/14-3-3η-glutathione beads in the presence of various concentrations of MIZ. The input and eluted samples from washed beads were analyzed on SDS–PAGE and visualized by autoradiography. Molecular mass marker is shown in kDa. The arrowhead indicates the GR band. (B) GST pull-down experiments were performed in triplicate. Gels were analyzed using PhosphoImager (BAS-2000; Fujifilm, Tokyo, Japan). Data shown are means value ± standard deviation. * indicate $P < 0.05$ vs MIZ untreated group using Student's *t* test.

reported on an AMP-binding site on 14-3-3 proteins (19). Protein kinase C inhibitor is member of the histidine triad protein family which has been shown to bind purine monophosphate nucleotides (20). Glyceraldehyde 3-phosphate dehydrogenase (21) and cytoplasmic actin (22) has been reported as nucleoside-binding proteins.

The interaction of MIZ with the 14-3-3 proteins was studied further. To analyze any effect of MIZ on the conformation of the 14-3-3 proteins, CD spectroscopy was performed on purified 14-3-3 proteins from porcine kidney. As shown in Fig. 2A, we purified 30- and 32-kDa 14-3-3 proteins from porcine kidney (Fig. 2A, lane 1). Western blot analysis with antibody against 14-3-3 proteins was used to verify the identity of the purified proteins (Fig. 2A, lane 2). The purified 14-3-3 proteins were used for CD spectroscopy.

The CD spectrum of 14-3-3 protein showed minima at 208 and 220 nM and the addition of 5 mM MIZ gave reduction in the minima (Fig. 2B). The minima at 208 and 220 nm are characteristic of an α-helical conformation (23). The 14-3-3 protein exists as dimer and each subunit of the dimeric protein consists of a bundle of nine antiparallel α-helices that form a palisade around an amphipathic groove (24, 25). These results suggest that a conformational change of the 14-3-3 proteins was induced and the α-helical conformation of the proteins was increased after the addition of MIZ. 14-3-3 proteins act by binding to other signaling proteins in the cell. The MIZ induced conformational change of 14-3-3 might modulate the protein interaction characteristics of 14-3-3.

We have previously shown that 14-3-3η interacts with the GR, and we were interested in testing whether MIZ affected the 14-3-3/GR interaction. To investigate the MIZ effect on the binding of GR and 14-3-3η, we prepared fusion protein of human 14-3-3η with GST and tested its ability to bind *in vitro* translated [^{35}S] methionine-labeled GR in the presence of various concentrations of MIZ in GST pull-down assays. As shown in Fig. 3A, MIZ increased the specific interaction between GR and 14-3-3η dose-dependently *in vitro.* The specific binding at 20 mM MIZ showed a threefold increase above control (Fig. 3B).

To determine whether MIZ had any effect on transcriptional activation by the GR, we performed transient transfection of COS-7 cells (Fig. 4A). The results show that MIZ dose-dependently enhanced the induction of GR activity by Dex. This effect was seen already in the absence of cotransfected 14-3-3η. As previously reported, 14-3-3 enhanced the Dex induced GR transactivation (8) and MIZ further enhanced this effect. The effect of MIZ on the Dex-induced GR transactivation in the absence of cotransfected 14-3-3η is possibly due to enhanced interaction between GR and endogenous 14-3-3 proteins in the COS-7 cells. MIZ might also directly affect GR transactivation. To determine whether MIZ had any effect on the proliferation or viability of the transfected COS-7 cells, a MTT assay was performed (Fig. 4B). At MIZ concentrations 100 and 1000 μM, some cytotoxicity was observed. The presence or absence of transfected 14-3-3 and GR or Dex had no effect on the observed cytotoxicity (Fig. 4B and data not shown). This inhibition is not as remarkable as previously reported (26). Nakagawa *et al.* reported that the inhibitory effect of MIZ on the proliferation of cells was cell line dependent (26). 14-3-3 shows strong enhancement of Dex-induced GR transactivation at the dosage level where MIZ has some cytotoxic effect. However, at MIZ concentrations 1 and 10 μM ($\cong$0.26 and 2.6 μg/ml, respectively), MIZ enhanced the induction of GR activity by Dex without any cytotoxicity. Since Sonoda *et al.* reported that the effective range of the trough plasma concentration of MIZ in renal transplantation patients is $\geq$0.1 μg/ml, and the safe range of the trough plasma concentration is $\leq$3 μg/ml (27), the conditions of this transient transfection experiment can be considered physiologically

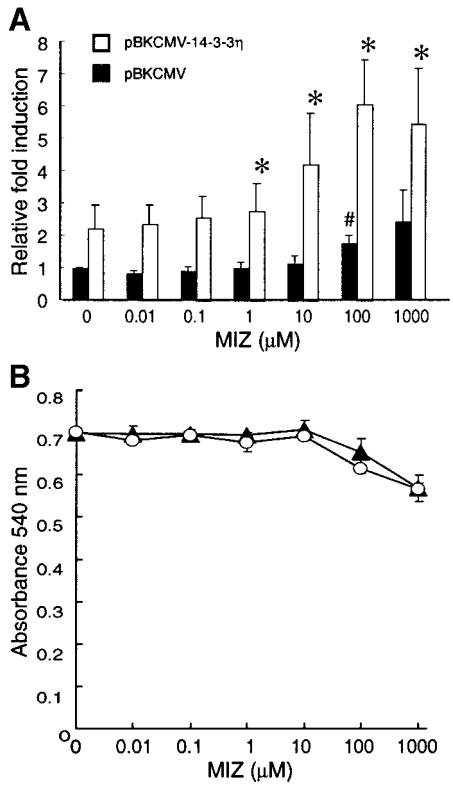

FIG. 4. The effect of MIZ on transcriptional activation by the hGR and cytotoxicity. (A) COS-7 cells were transiently transfected with 100 ng of p19LUC-TK reporter, 5 ng of pCMV-β -galactosidase, 5 ng of pCMV4-hGR expression vector, and 5 ng of pBKCMV or pBKCMV-14-3-3η expression vector. The cells were treated with various concentration of MIZ as indicated and with or without 1 μM Dex. The cells were harvested for luciferase assay after 48 h. Transfections were performed in triplicate. The data is expressed as Dex induced activity i.e., the luciferase activity in the presence of Dex related to the activity in the absence of Dex. The Dex induced activity in the absence of cotransfected 14-3-3 and added MIZ is set to 1. * indicate $P < 0.05$ vs. pBKCMV-14-3-3η transfected and MIZ untreated group, # indicate $P < 0.05$ vs. pBKCMV transfected and MIZ untreated group using Student's *t*-test. (B) COS-7 cells were transiently transfected with 100 ng of p19LUC-TK reporter, 5 ng of pCMV-β-galactosidase, 5 ng of pCMV4-hGR expression vector, and 5 ng of pBKCMV (▲) or pBKCMV-14-3-3η (○) expression vector. The cells were treated with various concentration of MIZ as indicated and with 1 μM Dex. The cells were incubated 48 h at 37°C after the transient transfection and MTT assay was performed as described under Materials and Methods. Data shown are mean value ± standard deviation.

relevant. However, in *in vitro* GST pull-down experiments using bacterially expressed protein, higher amounts of MIZ were needed.

In this study we show that 14-3-3 proteins are MIZ-binding proteins and that MIZ affects the conformation of 14-3-3 proteins. Since 14-3-3 proteins have been reported to interact with many proteins involved in cellular signaling, cell cycle regulation and apoptosis, it is possible that MIZ could affect the function of these proteins via the 14-3-3 interaction. One 14-3-3 binding protein is the GR. Ligand activation of GR results in immunosuppressive effects and this function has been widely used in therapy. We have here demonstrated that MIZ, an immunosuppressive therapeutic agent, can increase the binding between GR and 14-3-3. We also show that MIZ possibly has a strong effect on the activation of GR by Dex. Since 14-3-3 can regulate GR function, it is possible that one mechanism for the therapeutic effects of MIZ is to regulate GR function via 14-3-3.

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