Minocycline Up-regulates Bcl-2 and Protects against Cell Death in Mitochondria*

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Robust neuroprotective effects have been shown for minocycline. Whether it also protects nonneuronal cells or tissues is unknown. More importantly, the mechanisms of minocycline protection appear multifaceted and remain to be clarified. Here we show that minocycline can protect kidney epithelial cells in vitro and protect the kidneys from ischemic injury in vivo. We further show that Bcl-2 is a key molecular determinant of minocycline protection. Minocycline protected kidney epithelial cells against apoptosis induced by hypoxia, azide, cisplatin, and staurosporine. The protection occurred at mitochondria, involving the suppression of Bax accumulation, outer membrane damage, and cytochrome c release. Minocycline induced Bcl-2, which accumulated in mitochondria and interacted with death-promoting molecules including Bax, Bak, and Bid. Down-regulation of Bcl-2 by specific antisense oligonucleotides abolished the cytoprotective effects of minocycline. Thus, minocycline can protect neuronal as well as nonneuronal cells and tissues. One mechanism for minocycline protection involves the induction of Bcl-2, an antiapoptotic protein.

Minocycline is a semisynthetic derivative of tetracycline, which has been used as an effective antibiotic for decades (1). Recent studies, however, have suggested bioactivities for minocycline that are unrelated to its antimicrobial property. For example, minocycline and related tetracyclines are anti-inflammatory (2). Strikingly, studies within the past a few years have demonstrated a robust neuroprotective effect of minocycline. Minocycline protects neuronal cells and tissues in experimental models of ischemic stroke (3–5), Huntington's disease (6), amyotrophic lateral sclerosis (7), traumatic brain injury (8), multiple sclerosis (9), and Parkinson's disease (10, 11). Neuroprotective effects of minocycline can be indirect, through the inhibition of microglial activation and proliferation (3, 11–14). On the other hand, direct neuronal protection by minocycline has been documented (4, 7, 15–17), probably involving the preservation of mitochondrial integrity and cytochrome c, followed by the suppression of caspase-dependent as well as -independent cell death (7, 15). Despite these important findings, two critical questions remain. First, is minocycline protection specific for nervous systems? does minocycline protect nonneuronal cells or tissues? Second and more importantly, what is the molecular basis for minocycline protection? Here we show that minocycline protects kidney epithelial cells against injury and death induced by different types of insults. We further show that minocycline protects kidney from ischemic injury in vivo. A major cellular site for minocycline protection is the mitochondrion. Finally, we have identified Bcl-2 as a key molecular determinant of the cytoprotective effects of minocycline. Bcl-2 is induced by minocycline, accumulates in mitochondria, and may protect the organelles by antagonizing proapoptotic Bax, Bak, and Bid. Down-regulation of Bcl-2 by specific anti-sense oligonucleotides abolishes the cytoprotective effects of minocycline. Thus, minocycline can protect neuronal as well as nonneuronal cells and tissues. One mechanism for minocycline protection involves the induction of Bcl-2.

EXPERIMENTAL PROCEDURES

Materials—Rat kidney proximal tubular cells were originally obtained from U. Hopfer (Case Western Reserve University, Cleveland, OH). The cells were cultured and plated for experiments as described (18–20). Other cell lines were purchased from ATCC. Antibodies were from the following sources: monoclonal anti-cytochrome c (TH8.2C12 and 6H2.B4) and anti-Bcl-xL (clone 4) from BD Pharmingen (San Diego, CA); monoclonal anti-Bax (1D1) and anti-Bcl-xL (2H12) from NeoMarkers (Fremont, CA); Polyclonal anti-lamin B, monoclonal (C-2) and polyclonal (D21) anti-Bcl-2 from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); polyclonal anti-Bak (NT) from Upstate Biotechnology, Inc.; monoclonal anti-COX IV (20E8) from Molecular Probes, Inc. (Eugene, OR); polyclonal anti-Bid from Dr. X. M. Yin at the University of Pittsburgh (Pittsburgh, PA); and polyclonal antibody specific for active caspase-3 from Dr. A. Srinivasan at Idun Pharmaceuticals Inc. (La Jolla, CA). All secondary antibodies were from Jackson Immuno-Research (West Grove, PA). Other reagents including minocycline were purchased from Sigma.

In Vitro Experimental Treatment—For minocycline pretreatment, cells were cultured in the presence of 2 μM minocycline for 18 h. Apoptosis was induced by hypoxia, staurosporine, or azide. Hypoxia and staurosporine incubation were conducted as previously (18, 21, 22). For azide experiments, cells were rinsed twice with phosphate-buffered saline to remove culture medium and then exposed to 10 mM azide in a glucose-free buffer. After azide incubation, cells were returned to culture medium to monitor the development of apoptotic morphology.

In Vivo Renal Ischemia—The in vivo effects of minocycline were tested in a bilateral clamping model of renal ischemia, as described in our recent work (23). Briefly, male Sprague-Dawley rats of 250–275 g were anesthetized with sodium pentobarbital for flank incisions to expose renal pedicles. Both renal pedicles were clamped for 35 min to induce ischemia. The clamps were then released for 48 h of reperfusion. Finally, kidneys were collected for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)1 assay of apoptosis (see below for details). Blood samples were also collected to measure

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1 The abbreviations used are: TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; AFC, N-acetyl-S-farnesyl-L-cysteine; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
serum creatinine levels to indicate renal function, using a kit purchased from Stanbio Laboratory (Boerne, TX) (23).

TUNEL Assay—Renal tissues were fixed in 4% paraformaldehyde and embedded in Paraffin. Paraffin sections were cut into 4 μm for the TUNEL assay using the in situ Cell Death Detection kit purchased from Roche Applied Science, following the manufacturer’s instructions. Briefly, tissue sections were deparaffinized and permeabilized by incubation at 65 °C in 0.1 M sodium citrate, pH 6.0. The sections were then exposed to a TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and nucleotides including fluorescein isothiocyanate-labeled dUTP. Positive staining was identified in cell nucleus with DNA fragmentation by fluorescence microscopy.

Antisense Treatment—The sequences of Bcl-2 antisense and mismatch control were 5′-TCCCGGCTTGCGCCAT-3′ and 5′-CTGTCGCGGCCTGACTC-3′, respectively (24). The oligodeoxynucleotides were synthesized to contain a phosphorothioate backbone and purified by HPLC (Integrated DNA Technologies, Coralville, IA). Cells were transfected with 0.5 μM oligonucleotides using Oligofectamine (Invitrogen) and examined 48 or 72 h later. Transfection of oligonucleotides was conducted as described by us recently (25).

Real-time PCR Analysis of Bcl-2 mRNA—RNA was extracted from cells using the Qiagen protocol including DNase treatment. RNA concentration was measured on the Bioanalyzer (Agilent Technologies). Reverse transcription was carried out according to the Superscript protocol (Invitrogen). Real time PCR amplifications were carried out using an iCycler qTMT Real Time PCR Detection System (Bio-Rad). SYBR Green dye was used to bind to the PCR products, which allows the CCD detector within the iCycler to provide immediate feedback on reaction success, thus reporting the data on the PCR in progress in a real time fashion. β-Actin was used as an internal control to normalize the results. Bcl-2-specific primers for PCR amplification were as follows: forward, 5′-GGAGGATTGTGGCCTTCTTTG-3′; reverse, 5′-CATCCCAGCCTCCGTTATCC-3′.

Morphological Analysis of Apoptosis (18, 21, 22)—Typical apoptotic morphology including cellular shrinkage and formation of apoptotic bodies was monitored by light microscopy. Cells were also stained with 5 g/ml Hoechst 33342 to reveal nuclear condensation and fragmentation by fluorescence microscopy. To quantify apoptosis, five fields with 200 cells per field in each dish were examined. The experiments were repeated for at least four times with duplicate dishes for each condition.

Measurement of Caspase Activity (18, 19, 22)—The enzymatic activity of caspases was measured using the fluorogenic peptide substrate DEVD.AFC (Enzyme Systems Products, Dublin, CA). Briefly, cells were extracted with 1% Triton X-100. The lysates of 25 μg of protein were added to the enzymatic reactions containing DEVD.AFC as substrates. After 60 min of reaction at 37 °C, fluorescence at 360-nm excitation/530-nm emission was monitored. For each measurement, a standard curve was constructed using free AFC. Based on the standard curve, the fluorescence reading from each enzymatic reaction was translated into the molar amount of liberated AFC to indicate caspase activity.

In Vitro Reconstitution of Caspase Activation (19, 22, 25)—Cytosolic capacity for caspase activation was determined by in vitro reconstitu-
tion assays. Cytosol was extracted with 0.05% digitonin and concentrated to 4–5 mg of protein/ml with 3-kDa cut-off microconcentrators. For reconstitution, 1 µl of 0.5 mg/ml rat heart cytochrome c and 1 µl of 10 mM dATP were added to 7.5 µl of cytosolic extracts containing 25 µg of protein and incubated for 1 h at 30 °C. The reconstitution mixture was then transferred to 200 µl of enzymatic reaction buffer containing 50 µM DEVD.AFC. The cleavage of DEVD.AFC was monitored as described above to determine reconstituted caspase activity.

**Cellular Fractionation**—To analyze cytochrome c release and Bax translocation, cells were fractionated into cytosolic and membrane-bound fractions using low concentrations of digitonin, which selectively permeabilizes the plasma membrane to release cytosol (21, 22, 25). Briefly, cells were permeabilized with 0.05% digitonin in isotonic sucrose buffer (250 mM sucrose, 10 mM Hepes, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, and 1 mM EGTA, pH 7.1) to collect cytosol. The digitonin-insoluble part was further extracted with 2% SDS to collect the membrane-bound organelar fraction.

**Immunoblot Analysis**—Proteins were analyzed by immunoblotting using NuPAGE Gel Systems (18, 20, 22). To analyze the subcellular distribution of cytochrome c and Bax, cytosolic and membrane fractions extracted from the same amounts (~1 × 10⁶) of cells were subjected to electrophoresis. For other blots, 50 µg of proteins were loaded for each lane. After electrophoresis, the proteins were electroblotted onto polyvinylidene difluoride membranes. Subsequently, the membranes were blocked with 5% milk and exposed to primary and horseradish peroxidase-conjugated secondary antibodies. Antigens on the blots were revealed by exposure to chemiluminescent substrates (Pierce).

**Co-immunoprecipitation** (21, 25, 26)—Cells were extracted with 2% CHAPS to collect whole cell lysate. The switzenon detergent CHAPS was used for extraction to avoid nonionic detergent-induced association between Bcl-2 family proteins (27). Proteins extracted from ~1 × 10⁶ cells were precleared by incubation with 1 µg of normal serum and 30 µl of agarose protein A/G (Santa Cruz Biotechnology). The precleared lysates were subsequently incubated with 2 µg of anti-Bcl-2 antibody (or nonimmune serum for mock immunoprecipitation) and agarose-protein A/G. Immunoprecipitates were collected by centrifugation and dissolved in 2% SDS sample buffer for immunoblot analysis of Bax, Bak, and BId.

**Immunofluorescence** (20–22)—Cells were grown on collagen-coated glass coverslips. After experimental incubation, the cells were fixed in a modified Zamboni’s fixative and permeabilized with 0.1% SDS. The cells were then blocked with 5% normal goat serum and exposed to primary antibodies, followed by secondary antibodies (fluorescein isothiocyanate-labeled goat anti-rabbit IgG or Cy-3-labeled goat anti-mouse IgG). Images were captured by fluorescence microscopy.

**Statistics**—Data were expressed as means ± S.E. (n ≥ 4). Statistical differences between means were determined using analysis of variance by two-tailed tests. p < 0.05 was considered to reflect significant differences.

**RESULTS AND DISCUSSION**

**Minocycline Protects Kidney Epithelial Cells against Injury Induced by Different Types of Insults**—We first determined whether minocycline could protect nonneuronal cells or tissues, using rat kidney proximal tubular cells, a cell line of epithelial origin. The basal level of apoptosis in control rat kidney proximal tubular cells was below 5% (Fig. 1a). Three hours of incubation with azide, a respiration inhibitor, induced apoptosis in 46% of cells (Fig. 1a). When the cells were pretreated with minocycline for 18 h, they became rather resistant to azide injury, and as a result, only 12% apoptosis was detected (Fig. 1a, +/P). In sharp contrast, minocycline added during azide incubation was not protective (Fig. 1a, +/D); brief (e.g., 2-h) pretreatment was not effective either (not shown). The cytoprotective effects of minocycline were further shown by measurements of caspase activity (Fig. 1b). Again, minocycline pretreatment for 18 h but not minocycline added during azide incubation drastically decreased caspase activation (Fig. 1b). These results were confirmed by subsequent analysis of the cleavage of an endogenous caspase substrate, lamin B (Fig. 1c). Azide incubation led to lamin B cleavage into an apoptotic fragment (lane 2), which was blocked by minocycline pretreatment (lane 3). We further examined apoptotic morphology (nuclear condensation and fragmentation) and caspase activation in the same cells. The results are shown in Fig. 1, d–i. Compared with control (Fig. 1, d and g), many nuclei in azide-treated cells appeared condensed and fragmented (Fig. 1e). The same cells showed caspase-3 activation (Fig. 1h). Minocycline treatment blocked azide-induced nuclear changes as well as caspase activation (Fig. 1, f and i). Similar protective effects of minocycline were demonstrated in the epithelial cells, following hypoxia, cisplatin, and staurosporine incubation (not shown). Thus, minocycline protected kidney epithelial cells against injury and death induced by various types of insults.

**Minocycline Protection of Kidneys in Vivo during Renal Ischemia-Reperfusion**—To further establish the in vitro findings obtained from a kidney cell line, we tested the in vivo effects of minocycline in a relevant model of renal ischemia-reperfusion. Rats with or without minocycline pretreatment were subjected to bilateral clamping to induce renal ischemia. After ischemia, the clamps were released for reperfusion (23). Ischemia-reperfusion induced renal failure, as shown by increases in serum creatinine level. For example, 35 min of ischemia followed by 48 h of reperfusion increased serum creatinine from 0.3 to 5.9 mg/dl. Minocycline treatment significantly ameliorated ischemic renal failure, showing a serum creatinine level of 1.7 mg/dl. Consistently, the TUNEL assay detected significantly amounts of apoptotic cells in renal tubules of the ischemia-reperfused kidneys (Fig. 2b), which were again suppressed by minocycline (Fig. 2c). Thus, minocycline not only protected cultured kidney epithelial cells; it also protected renal tubular cells in vivo in the kidneys.

**Minocycline Protection Occurs at the Mitochondrial Level**—To identify the mechanism of minocycline protection, we came back to the in vitro model. The observation that minocycline-treated cells were cross-resistant to several types of insults suggest that molecular alterations in the common death pathways might be responsible. Two major pathways of apoptosis have been delineated (i.e., the death receptor-mediated intrinsic pathway and the mitochondrial-mediated extrinsic pathway) (28–30). Our earlier work indicated that hypoxia, staurosporine, and azide induce apoptosis in kidney epithelial cells through the extrinsic pathway (19–22, 23, 25). It has been shown that these stimuli activate proapoptotic Bcl-2 family proteins to permeabilize the outer membrane of mitochondria, leading to the release of apopptogenic proteins such as cytochrome c. Cytochrome c, after being released, binds Apaf-1 in the cytosol and activates caspase-9, which proteolytically activates downstream executioner caspases (19–22, 25). Thus, we first determined whether minocycline-pretreated cells were resistant to cytochrome c release, a hallmark of outer membrane damage in
mitochondria (31–33). The results are shown in Fig. 3a. In control cells, cytochrome c was detected exclusively in the membrane-bound organelar fraction, with no signal in cytosol (lane 1). After 2 h of azide incubation, noticeable amounts of cytochrome c were released from the organelar fraction into the cytosol (lane 3). By the end of 3 h of azide, over 50% of cytochrome c was released. In minocycline-treated cells, azide-induced cytochrome c release was markedly inhibited (lanes 5–8). As a result, less than 20% of cytochrome c was released after 3 h of azide incubation (lane 8). The inhibitory effects of minocycline on cytochrome c release were confirmed by immunofluorescence. As shown in Fig. 3b, control cells had perinuclear organelar staining of cytochrome c, consistent with its mitochondrial location (Control). After azide incubation, over 50% of cells lost mitochondrial cytochrome c (Azide). Minocycline treatment preserved mitochondrial cytochrome c in a majority of the cells (Azide + minocycline).

Targeting of mitochondria by proapoptotic Bcl-2 family proteins (e.g. Bax, Bak, and Bid) can be a primary cause of outer membrane leakage (32–35). Our previous work has suggested a critical role for Bax in mitochondrial damage during hypoxia-, azide-, and STS-induced apoptosis (20–22, 23, 25). Thus, to determine why minocycline-treated cells were resistant to cytochrome c release, we examined the targeting of mitochondria by Bax. The results are shown in Fig. 3c. In control cells, the majority of Bax was detected in the cytosolic fraction (lane 1). During azide incubation, Bax was lost from the cytosol and appeared in the membrane-bound organelar fraction, suggesting the translocation of Bax (lanes 2–4). By immunofluorescence, we showed that a major translocation site for Bax is mitochondria (not shown) (22). Of importance, targeting of Bax to mitochondria was ameliorated in minocycline-treated cells (Fig. 3c, lanes 5–8). For example, by the end of 3 h of azide incubation, less than 10% of Bax translocated to mitochondria in minocycline-treated cells (lane 8), whereas over 60% of Bax translocated in nontreated cells (lane 4). We further determined whether minocycline treatment also led to death resistance at levels downstream of cytochrome c release (i.e., in the cytosol). To this end, we isolated cytosols from control and minocycline-treated cells and compared their capacity of caspase activation after adding exogenous cytochrome c. The rationale for this experiment is that, if minocycline has inhibitory effects in the cytosol, then this cytosol isolated from minocycline-pretreated cells would reconstitute lower caspase activity after adding exogenous cytochrome c. The results of a typical reconstitution experiment are shown in Fig. 3d. Clearly, in the presence of the same amounts of cytochrome c, the cytosol of minocycline-treated cells reconstituted slightly higher (not lower) caspase activity. Thus, the primary targets for minocycline are not in the cytosol or downstream of cytochrome c release. Together, these results support previous neuronal studies (7, 15), indicating that minocycline protects cells at the level of mitochondria.

Minocycline Up-regulates Bcl-2 in Mitochondria—To identify the molecular changes in mitochondria that are responsible for minocycline protection, we focused on Bcl-2 family proteins. Bcl-2 family proteins are important regulators of apoptosis (34–36). They can be proapoptotic or anti-apoptotic. The interaction between the pro- and antiapoptotic Bcl-2 proteins may determine the fate of a cell, to live or to die. A major targeting site for these proteins has been identified to be the mitochon-
and untreated samples. The lysates were analyzed for various Bcl-2 family proteins by immunoblotting. The results demonstrate a specific up-regulation of Bcl-2 by minocycline. Immunoblot analysis of Bcl-2 in minocycline-treated cells was indeed the mitochondrion. Bcl-2 was down-regulated using a specific antisense oligonucleotide (24). The antisense specifically inhibited Bcl-2 expression without affecting the expression of Bcl-XL (Fig. 5a). We then examined the effect of Bcl-2 antisense on apoptosis induced by azide. The results are summarized in Fig. 5b. Without azide treatment, apoptosis was always below 5%. Azide incubation for 3 h led to 44% apoptosis. Consistent with previous results (Fig. 1), azide-induced apoptosis was drastically reduced by minocycline, to 8%. Notably, down-regulation of Bcl-2 by the antisense promoted apoptosis in minocycline-treated cells; as a result, 51% apoptosis was

**FIG. 4. Induction of Bcl-2 by minocycline.** a, immunoblot analysis of Bcl-2 family proteins. Whole cell lysates were extracted from cells with or without minocycline treatment. The lysates were analyzed for various Bcl-2 family proteins by immunoblotting. The results demonstrate a specific up-regulation of Bcl-2 by minocycline. b, densitometry of Bcl-2 and Bcl-xL. Immunoblots of Bcl-2 and Bcl-xL from four separate experiments were subjected to densitometry. The signals of untreated samples were arbitrarily set as 1; signals of minocycline-treated samples were normalized accordingly. Data are expressed as means ± S.E. (n = 4). *, significant difference between minocycline-treated and untreated samples. c, localization of Bcl-2 in minocycline-treated cells by immunofluorescence. Minocycline-treated cells were processed for double immunofluorescence of Bcl-2 and COX IV, an integral mitochondrial protein. The results suggest that minocycline induces Bcl-2 in mitochondria. d, co-immunoprecipitation of Bcl-2 with Bax, Bak, and Bid. Minocycline-treated cells were extracted with 2% CHAPS. The extracts were immunoprecipitated with anti-Bcl-2 antibodies. The immunoprecipitates were analyzed for various Bcl-2 family proteins by immunoblotting. The results demonstrate co-immunoprecipitation of Bcl-2 with the proapoptotic molecules to antagonize their toxicity in minocycline-treated cells.
FIG. 5. Down-regulation of Bcl-2 in minocycline-treated cells restores their sensitivity to apoptosis. 
a, down-regulation of Bcl-2 by specific antisense oligonucleotides. Control (lane 1) and minocycline-treated cells were mock-transfected (lane 2) or transfected with Bcl-2 antisense oligonucleotides (lane 3). Whole cell lysates were collected for immunoblot analysis of Bcl-2 and Bcl-xL. B, Bcl-2 antisense restores the sensitivity of minocycline-treated cells to azide-induced apoptosis. Cells transfected with Bcl-2 antisense or mismatch sequence were treated with minocycline and then subjected to azide incubation. Apoptosis was assessed by morphological methods. c, Bcl-2 antisense promotes caspase activation in minocycline-treated cells during azide incubation. Cells transfected with Bcl-2 antisense or mismatch sequence were treated with minocycline and then subjected to azide incubation. Caspase activity was measured enzymatically, using DEVD.AFC as substrates. d, comparison of minocycline protection in cells with normal Bcl-2 and cells with Bcl-2 down-regulated. Bcl-2 was down-regulated in one group of cells with Bcl-2 antisense. These cells and cells with normal Bcl-2 were subjected to azide incubation, with or without prior minocycline treatment. Apoptosis was assessed morphologically. The results show that, when Bcl-2 was down-regulated, minocycline protection was lost. e, representative images of cellular and nuclear morphology. Together, the results indicate that down-regulation of Bcl-2 restores cellular sensitivity to apoptosis in minocycline-treated cells, suggesting a critical role for Bcl-2 in cytoprotection by minocycline.
induced by azide in this group. A control oligonucleotide with scrambled sequence was without effect (Fig. 5b). These morphological observations were confirmed by measurements of caspase activity (Fig. 5c). Again, Bel-2 antisense and not the scrambled sequence promoted caspase activation in minocycline-treated cells during azide incubation (Fig. 5c). We further compared minocycline protection in cells containing normal Bel-2 and cells with Bel-2 down-regulated by antisense (Fig. 5d). Clearly, when Bel-2 was down-regulated, minocycline protection was lost, suggesting that minocycline protection was mediated by Bel-2 in this experimental model. Representative images of the cells are shown in Fig. 5e. Apoptotic cells assumed a shrunk and fragmented configuration, accompanied by nuclear condensation and fragmentation. Clearly, down-regulation of Bel-2 by antisense oligonucleotides restored the sensitivity of minocycline-treated cells to injury and death. Together, these results suggest an important role for Bel-2 in the cytoprotective effects of minocycline.

Conclusions—Since the first report in 1998 (3), beneficial effects of minocycline have been documented in various experimental models of neurological diseases including ischemic stroke (3–5), Huntington’s disease (6), amyotrophic lateral sclerosis (7), traumatic brain injury (8), multiple sclerosis (9), and Parkinson’s disease (10, 11). Using in vitro and in vivo models of kidney epithelial cell injury, we have now extended these observations to nonneuronal systems. Mechanistically, minocycline can protect cells and tissues indirectly, by suppressing inflammation and mitochondrial activation (3, 11–13). On the other hand, well controlled studies have also suggested direct cytoprotective actions of minocycline (4, 7, 15). In model systems of cells and isolated mitochondria, minocycline preserves mitochondrial integrity and cell viability are maintained. Considering the pivotal role played by Bel-2 family proteins in the regulation of mitochondrial homeostasis and cell death in general (34–36), it would certainly be interesting to investigate their involvement in minocycline protection in vitro including neurological diseases.

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