Application of the nuclear factor-κB inhibitor BAY 11-7085 for the treatment of endometriosis: an in vitro study

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Application of the nuclear factor-κB inhibitor BAY 11-7085 for the treatment of endometriosis: an in vitro study. Am J Physiol Endocrinol Metab 293: E16–E23, 2007. First published August 8, 2006; doi:10.1152/ajpendo.00135.2006.—Most of the current medical treatments for endometriosis aim to downregulate estrogen activity. However, a high recurrence rate after medical treatment has been the most significant problem. BAY 11-7085, a soluble inhibitor of NF-κB activation, has been shown to inhibit cell proliferation and induce apoptosis of a variety of cells. To examine the potential application of BAY 11-7085 in the treatment of endometriosis, we investigated the effects of this agent on the cell proliferation and apoptosis of cultured ovarian endometriotic cyst stromal cells (ECSCs) by a modified methylthiazole tetrazolium assay, a 5-bromo-2′-deoxyuridine incorporation assay, and internucleosomal DNA fragmentation assays. The effect of BAY 11-7085 on the cell cycle of ECSCs was also determined by flow cytometry. The expression of apoptosis-related molecules was examined in ECSCs with Western blot analysis. BAY 11-7085 significantly inhibited the cell proliferation and DNA synthesis of ECSCs and induced apoptosis and the G0/G1 phase cell cycle arrest of these cells. Additionally, downregulation of the B-cell lymphoma/leukemia-2 (Bcl-2) and Bcl-XL expression with simultaneous activation of caspase-3, -8, and -9 was observed in ECSCs after treatment with BAY 11-7085. These results suggest that BAY 11-7085 induces apoptosis of ECSCs by suppressing antiapoptotic proteins, and that caspase-3, -8, and -9-mediated cascades are involved in this mechanism. Therefore, BAY 11-7085 could be used as a therapeutic agent for the treatment of endometriosis.

Address for reprint requests and other correspondence: K. Nasu, Dept. of Obstetrics and Gynecology, Faculty of Medicine, Oita Univ., Idaigaoka 1-1, Hasama-machi, Yufu-shi, Oita 879-5593, Japan (e-mail: nasu@med.oita-u.ac.jp).

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the signal transduction in cell proliferation and differentiation. The JNK signaling pathway is often involved in the signal transduction in cell proliferation and differentiation. The JNK signaling pathway can be activated by a variety of stresses, including genotoxic stress, heat shock, osmotic shock, and metabolic stress, as well as proinflammatory cytokines (7). The persistent activation of the JNK pathway often mediates intracellular signaling that leads to cell death. The p38 MAPK signaling pathway is also stimulated by cellular stress and proinflammatory cytokines in a way that is similar to the JNK signaling pathway. ERK, JNK, and p38 MAPK are present in the endometriotic cells as well as in the endometrial cells (34). Significant activation of p38 MAPK has been demonstrated in endometriotic cells (34), and it is considered that MAPKs play pathophysiological roles in the development of endometriosis.

A synthetic compound, BAY 11-7085, has been shown to inhibit IkB phosphorylation and the expression of NF-κB-regulated gene products (26). In addition to its inhibition of IkB phosphorylation via IkB kinase (IKK) and the resultant decrease in the DNA-binding function of NF-κB (30), BAY 11-7085 has also been shown to activate JNK and p38 MAPK (26). It has been reported that BAY 11-7085 inhibits cell proliferation and induces apoptosis of colonic epithelial cells (30), keratinocytes (6), chondrocytes (28), and endothelial cells (26).

Molecular targeted treatment strategies are becoming an increasingly attractive option for the treatment of endometriosis, particularly because of the dismal clinical results of more traditional therapies. Our laboratory and others have focused on molecular strategies to alter the antiapoptotic response of endometriotic cells to both novel and traditional therapeutic agents (18, 20). In the present study, we evaluated the effects of BAY 11-7085, an inhibitor of IkB phosphorylation and activator of JNK and p38 MAPK, on the proliferation, cell cycle, and apoptosis of cultured endometriotic cells, using methods that were previously proposed for evaluating novel therapeutic agents for endometriosis (18, 20). We also discuss new therapeutic strategies for the treatment of endometriosis.

MATERIALS AND METHODS

Endometriotic cyst stromal cells and normal endometrial stromal cells: isolation procedure and cell culture conditions. Endometriotic cyst stromal cells (ECSCs) were obtained from premenopausal patients who had undergone salpingo-oophorectomy or ovariectomy for ovarian endometriotic cysts (n = 10). Normal endometrial stromal cells (NESCs) were obtained from premenopausal patients who had undergone hysterectomy for subserosal or intramural leiomyoma (n = 9). The indication of hysterectomy was the size of the tumor or hypermenorrhea. None of the patients had received any hormonal treatments at least 2 yr prior to operation. All specimens were considered to be unaffected by the presence of leiomyoma and were considered to have been free of endometriotic lesions (18). Each experiment was performed in triplicate and repeated at least six times.

Assessment of cell proliferation and cell viability of ECSCs and NESCs. The cell proliferation and cell viability of ECSCs and NESCs after BAY 11-7085 treatment were determined in 96-well plates by a modified methylthiazoletetrazolium (MTT) assay using WST-1 (Roche Diagnostics, Penzberg, Germany) following the manufacturer’s protocol. We distributed 5 × 10^4 cells in DMEM supplemented with 10% FBS into each well of a 96-well flat-bottomed microplate (Corning, New York, NY) and incubated them overnight. The medium was then removed, and the cells were incubated for 48 h with 200 µl of experimental medium containing various concentrations of BAY 11-7085 (0.01–10 µM, Nagara Science, Gifu, Japan). Thereafter, 20 µl of WST-1 dye were added to each well, and the cells were further incubated for 4 h. All experiments were performed in the presence of 10% FBS. Cell proliferation was evaluated by measuring absorbance at 540 nm. Data were calculated as the ratio of the values obtained for the BAY 11-7085-treated cells to those for the untreated controls.

Cell proliferation of ECSCs and NESCs after BAY 11-7085 treatment was determined by 5-bromo-2′-deoxyuridine (BrdU) incorporation using cell proliferation enzyme-linked immunosorbent assay (ELISA, Roche). We placed 1 × 10^4 cells in DMEM supplemented with 10% FBS into each well of a 96-well flat-bottomed microplate and incubated them overnight. The medium was then removed, and the cells were incubated for 48 h with 100 µl of experimental medium containing various concentrations of BAY 11-7085 (0.01–10 µM). We then added 10 µl of BrdU (10 mM) to each well and incubated the samples for 2 h. BrdU incorporation was then evaluated according to the manufacturer’s protocols. All experiments were performed in the presence of 10% FBS. Cell proliferation was evaluated by measuring absorbance at 450 nm. Data were calculated as the ratio of the values obtained for the BAY 11-7085-treated cells to those for the untreated controls.

Assessment of internucleosomal DNA fragmentation in ECSCs. Internucleosomal DNA fragmentation in ECSCs after BAY 11-7085 treatment was evaluated using a Quick Apoptotic DNA Ladder Detection Kit (BioVision Research Products, Mountain View, CA), as previously described (20). Cells (1 × 10^5) of ECSCs were plated onto 100-mm culture dishes (Corning) in 10 ml of DMEM supplemented with 10% heat-inactivated FBS and cultured overnight. The supernatant was then replaced with fresh culture medium (DMEM + 10% FBS) containing various concentrations of BAY 11-7085 (0.01–10 µM). Twenty-four hours after stimulation, the DNA was extracted from these cells according to the manufacturer’s protocol. DNA fragmentation was analyzed by electrophoresis on an agarose gel (1.2%). The DNA bands were visualized by staining with ethidium bromide and were photographed under ultraviolet light using a transilluminator.
Assessment of BAY 11-7085-induced apoptosis in ECSCs and NESCs.

The BAY 11-7085-induced apoptosis of ECSCs and NESCs was quantified by direct determination of nucleosomal DNA fragmentation by Cell Death Detection ELISA (Roche) as previously described (20). The assay used specific monoclonal antibodies directed against histones from fragmented DNA, allowing the determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. Briefly, 1 × 10^6 cells were plated onto 24-well culture plates (Corning) in 1 ml of culture medium with 10% heat-inactivated FBS and cultured overnight. The supernatant was replaced with fresh culture medium (DMEM + 10% FBS) containing various amounts of BAY 11-7085 (0.01–10 μM). Twenty-four hours after stimulation, the cells were lysed according to the manufacturer’s instructions, followed by centrifugation (200 g, 5 min). The mono- and oligonucleosomes contained in the supernatants were determined using an antihistone-biotin antibody. The concentration of nucleosomes-antibody was determined photometrically at a wavelength of 405 nm using 2,2′-azino-di-(3-ethylbenzthiazoline-sulfonate) as a substrate. Data were calculated as the ratio of the values obtained for the BAY 11-7085-treated cells to those for the untreated controls.

Analysis of cell cycle by flow cytometry. The cell cycle was analyzed by flow cytometry after 2 days of culture with or without BAY 11-7085, as previously described (18). Briefly, ECSCs were cultured in DMEM supplemented with 10% FBS at <60% confluence for 2 days with or without the presence of BAY 11-7085 (10 μM). They were then trypsinized, washed in phosphate-buffered saline (PBS), fixed in methanol, and incubated for 30 min at 4°C in the dark with a solution of 5 μg/ml propidium iodide, 1 mg/ml RNase (Sigma Chemical, St. Louis, MO), and 0.1% Nonidet P-40 (Sigma). Flow cytometric analysis of the cell cycle was performed immediately after treatment with BAY 11-7085 (10 μM) for 48 h. Data are presented as percentages relative to untreated controls. *P < 0.0005, **P < 0.0001 vs. untreated controls (Bonferroni-Dunn test).

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Fig. 1. A: effects of BAY 11-7085 on cell viability of Endometriotic cyst stromal cells (ECSCs; filled bars) and normal endometriotic stromal cells (NESCs; open bars) as assessed by WST-1 assay. ECSCs and NESCs were treated with BAY 11-7085 (0.01–10 μM) for 48 h. Data are presented as percentages relative to the values of untreated controls. *P < 0.0025; **P < 0.0001 vs. untreated controls (Bonferroni-Dunn test). B: comparison of cell viability between ECSCs (n = 6) and NESCs (n = 6) after treatment with BAY 11-7085 (10 μM) for 48 h. Data are presented as percentages relative to untreated controls. *P < 0.005 vs. NESC group (Mann-Whitney U-test).

Fig. 2. A: effects of BAY 11-7085 on 5-bromo-2′-deoxyuridine (BrdU) incorporation of ECSCs (filled bars) and NESCs (open bars). ECSCs and NESCs were treated with BAY 11-7085 (0.01–10 μM) for 48 h. Data are presented as percentages relative to the values of untreated controls. *P < 0.0005, **P < 0.0001 vs. untreated controls (Bonferroni-Dunn test). B: comparison of BrdU incorporation between ECSCs (n = 7) and NESCs (n = 6) after treatment with BAY 11-7085 (10 μM) for 48 h. Data are presented as percentages relative to untreated controls. *P < 0.005 vs. NESC group (Mann-Whitney U-test).
staining using the CellFIT program (Becton-Dickinson, Sunnyvale, CA), in which the S-phase was calculated using an RFIT model.

Assessment of the expression of apoptosis-related proteins in ECSCs. The expression of apoptosis-related proteins (Bcl-2, Bcl-XL, Bax, Fas, and Fas ligand) was determined by Western blotting analysis (20). Subconfluent ECSCs were cultured on 100-mm dishes for 24 h with or without the presence of BAY 11-7085 (10 µM). The cells were then washed with PBS, and whole cell extracts were prepared by lysing the cells in lysis buffer (50 mM Tris-HCl, 125 mM NaCl, 0.1% Nonidet P-40, 5 mM ethylenediamine tetraacetic acid, 50 mM NaF, and 0.1% phenylmethylsulfonyl fluoride). The suspension was centrifuged at 15,000 g for 15 min at 4°C, and the supernatant was collected. The total protein concentration was quantified using the Coomassie protein assay reagent (Pierce, Rockford, IL). The whole cell protein extract was resolved with sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 10% polyacrylamide gel under reduced conditions. After transfer to an Immobilon-P transfer membrane (Millipore, Bedford, MA), the protein was stained with Ponceau S (Sigma) to verify uniform loading and transfer. The membranes were blocked with 5% skim milk (Becton-Dickinson) in Tris-buffered saline with Tween 20 (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.4) (TBS-T) overnight and subsequently incubated with primary antibodies (Bcl-2, Bcl-XL, Bax, Fas, and Fas ligand obtained from BD Biosciences, San Jose, CA; cleaved caspase-3, cleaved caspase-8, and cleaved caspase-9 from Cell Signaling, Beverly, MA; and GAPDH from Ambion, Austin, TX) at appropriate dilutions for 1 h at room temperature. The membrane was washed three times with TBS-T and incubated with the appropriate secondary antibodies. The complexes were detected using an ECL chemiluminescence system (Amersham, Piscataway, NJ). The bands were quantified using a densitometer. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

Fig. 3. Effects of BAY 11-7085 on internucleosomal DNA fragmentation of ECSCs. DNA was extracted from ECSCs cultured for 24 h with or without BAY 11-7085 (10 µM) and analyzed by agarose gel electrophoresis. A DNA ladder was detected in the BAY 11-7085-treated ECSCs, suggesting the presence of apoptotic cells. Representative results of 6 repeated experiments are shown.

Fig. 4. A: effects of BAY 11-7085 on apoptosis of ECSCs (filled bars) and NESCs (open bars) as assessed by Cell Death Detection ELISA. The assay used specific monoclonal antibodies directed against histones from fragmented DNA, allowing determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. ECSCs and NESCs were treated with BAY 11-7085 (0.01–10 µM) for 24 h. Data are presented as percentages relative to values of untreated controls. *P < 0.0001 vs. untreated controls (Bonferroni-Dunn test). B: comparison of apoptosis between ECSCs (n = 6) and NESCs (n = 6) after treatment with BAY 11-7085 (10 µM) for 48 h. Data are presented as percentages relative to untreated controls. *P < 0.005 vs. NESC group (Mann-Whitney U-test).
appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Subsequently, the membrane was washed three times with TBS-T and analyzed by enhanced chemiluminescence (Amersham Pharmacia Biotech, Chicago, IL).

**Statistical analysis.** Data were calculated as percentages relative to the untreated controls, presented as means (SD), and appropriately analyzed by the Bonferroni-Dunn test or Mann-Whitney U-test with StatView 4.5 (Abacus Concepts, Berkeley, CA). Values of $P < 0.05$ were considered to be statistically significant.

**RESULTS**

**Effects of BAY 11-7085 on cell proliferation and cell viability of ECSCs and NESCs.** The effects of BAY 11-7085 on the cell proliferation and cell viability of ECSCs and NESCs were investigated by modified MTT assay. As shown in Fig. 1A, the number of viable ECSCs and NESCs was significantly decreased by the addition of increasing amounts of BAY 11-7085 (66.1 and 54.7% decreases, respectively, at a concentration of 10 μM). BAY 11-7085 showed a stronger inhibitory effect on the cell viability of ECSCs than that of NESCs (Fig. 1B).

To further assess the effects of BAY 11-7085 on cell proliferation, the DNA synthesis of ECSCs and NESCs after BAY 11-7085 treatment was evaluated by BrdU incorporation assay. As shown in Fig. 2, BAY 11-7085 treatment resulted in a significant inhibition of the BrdU incorporation of ECSCs in a dose-dependent manner (53.2% decrease at a concentration of 10 μM), whereas BAY 11-7085 was associated with only a weak inhibitory effect on the BrdU incorporation of NESCs (38.2% decrease at a concentration of 10 μM).

**Effects of BAY 11-7085 on apoptosis of ECSCs and NESCs.** The apoptotic effects of BAY 11-7085 on ECSCs and NESCs were assessed by evaluating the presence of internucleosomal DNA fragmentation. BAY 11-7085 induced the fragmentation of internucleosomal DNA in ECSCs (Figs. 3 and 4). A DNA ladder was detected by electrophoresis in BAY 11-7085-treated ECSCs, suggesting the presence of apoptotic cells (Fig. 3). The apoptosis of both ECSCs and NESCs was significantly induced by the addition of increasing amounts of BAY 11-7085 (725.1 and 368.2% increases, respectively, at a concentration of 10 μM; Fig. 4A). BAY 11-7085 showed a stronger stimulatory effect on the apoptosis of ECSCs than that of NESCs (Fig. 4B).

**Effects of BAY 11-7085 on the cell cycle of ECSCs.** The effect of BAY 11-7085 on the cell cycle of ECSCs was determined by flow cytometry. As shown in Fig. 5, the culturing of ECSCs cultured for 2 days in the presence of BAY 11-7085 (10 μM) resulted in an accumulation of these cells in the G0/G1 phase of the cell cycle, with a concomitant decrease in the proportion of those in the S-phase. Similar results were obtained in all repeated experiments.

**Effects of BAY 11-7085 on expression of apoptosis-related proteins in ECSCs.** To analyze the underlying mechanisms of the above-mentioned findings, we evaluated the expression of...
apoptosis-related proteins in ECSCs. As shown in Fig. 6, BAY 11-7085 downregulated the expression of Bcl-2 and Bcl-XL proteins and upregulated the expression of cleaved caspase-3, cleaved caspase-8, and cleaved caspase-9 proteins in ECSCs. The levels of Bax, Fas, and Fas ligand protein in ECSCs remained unchanged. Similar results were obtained in all repeated experiments.

**DISCUSSION**

It has been suggested that decreased apoptosis in endometriotic cells plays the essential role in the development and progression of endometriosis (15, 20). Recently, we (20) demonstrated that ECSCs are resistant to cytokine-induced apoptosis compared with NESCs. Izawa et al. (13) have also demonstrated that ECSCs are resistant to staurosporine-induced apoptosis; enhanced expression of antiapoptotic molecules was considered to be involved in this phenomenon (20). We have also demonstrated (18) that bufalin, an apoptosis-inducing agent, can be a promising drug for the medical treatment of endometriosis. In the present study, we demonstrated for the first time that BAY 11-7085 inhibits cell proliferation by inducing apoptosis and the G0/G1 arrest of the cell cycle of ECSCs in vitro. Western blot analysis clearly showed the downregulation of the expression of Bcl-2 and Bcl-XL in ECSCs after BAY 11-7085 treatments, suggesting that BAY 11-7085 induces the apoptosis of ECSCs by suppressing the antiapoptotic proteins. We also observed the activation of caspase-8 and caspase-9, initiator caspases closely coupled to proapoptotic signals, and caspase-3, a downstream effector caspase, after BAY 11-7085 treatments, suggesting that these caspase-mediated cascades are involved in the mechanism of BAY 11-7085-induced apoptosis (22). Interestingly, BAY 11-7085 showed only weak effects on the cell proliferation and apoptosis of NESCs compared with those of ECSCs, suggesting that BAY 11-7085 has cell-specific effects on ECSCs and that its effects could be weaker on the normal endometrium. These results indicate that BAY 11-7085 could be used as a novel therapeutic agent for the medical treatment of endometriosis.

It has been suggested that NF-κB, JNK, and p38 MAPK may play significant roles in the proliferation of endometriotic lesions (29, 34). Therefore, it would be reasonable to hypothesize that BAY 11-7085 might stimulate the expression of a specific proapoptotic gene that eventually triggers the downstream signaling pathway, leading ECSCs to apoptotic cell death, via NF-κB-, JNK-, and/or p38 MAPK-mediated pathways (26, 30). Interestingly, Bcl-2 and Bcl-XL proteins that were downregulated by BAY 11-7085 are under the transcriptional regulation of NF-κB (16), JNK (24), and p38 MAPK (24). Treatment with BAY 11-7085 results in the complementation and activation of the mitochondrial pathway and activation of the downstream regions of caspase-9 and -3, resulting in apoptosis. Therefore, BAY 11-7085 could enhance apoptosis and diminish antiapoptotic signaling in ECSCs. We speculated that higher constitutive expression of Bcl-2 and Bcl-XL proteins in ECSCs than in NESCs might be involved in this phenomenon (18, 20). Further investigations are necessary to fully elucidate the specific action mechanisms of BAY 11-7085 on ECSCs.

Endometriosis is considered to be a local inflammatory-like phenomenon (11, 19). Inflammatory responses are now thought to be mediated by cytokines and chemokines, which are activated by the transcription factor NF-κB (1). NF-κB can be activated by different stimuli, including proinflammatory cy-

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**Fig. 6.** Western blot analysis for expression of apoptosis-related proteins (Bcl-2, Bcl-XL, Bax, Fas, Fas ligand, cleaved caspase-3, cleaved caspase-8, and cleaved caspase-9) and GAPDH protein in ECSCs. ECSCs were cultured with or without BAY 11-7085 (10 μM) for 24 h, and total protein was extracted and subjected to Western blotting. BAY 11-7085 suppressed expression of Bcl-2 and Bcl-XL proteins and simultaneously induced expression of cleaved caspase-3, cleaved caspase-8, and cleaved caspase-9 proteins in ECSCs. Levels of Bax, Fas, and Fas ligand protein in ECSCs remained unchanged. Representative results of 6 repeated experiments are shown.
tokines, which are considered to be important in the progression of endometriosis (11, 12, 19, 29). The endometriotic cells themselves may be an important source of cytokines in the peritoneal cavity (19, 29). Therefore, in addition to the induction of cell growth arrest and apoptosis, the control of NF-κB activation seems to be a powerful therapeutic strategy for preventing pathological responses in endometriosis as a consequence of the release of excessive amounts of inflammatory mediators.

Current and standard medical treatments for endometriosis include GnRH agonists, contraceptive steroids, progestogens, and androgens (27), all of which aim to reduce circulating estradiol concentrations. Progesterone, which is commonly used in the clinical management of endometriosis, can also suppress NF-κB activity by stimulating the synthesis of IkB and/or by inducing the binding of progesterone receptor and NF-κB subunits (35). Sakamoto et al. (29) showed that GnRH agonist, currently the most effective drug for the treatment of endometriosis, also inhibits the NF-κB pathway in endometriotic cells. Such inhibition of NF-κB appears to suppress the expression of critical downstream effectors, including cytokines, growth factors, and apoptosis-regulatory proteins. It has been reported that N-tosyl-L-phenylalanine chloromethyl ketone, a synthetic NF-κB inhibitor, decreases the interleukin-8/H9260 from the Japan Society for the Promotion of Science (no. 16591672 to K. Nakshatri et al.).

In summary, we have demonstrated that BAY 11-7085 is able to induce apoptosis and the G0/G1-phase cell cycle arrest of ECSCs. Downregulation of Bcl-2 and Bcl-XL expression with simultaneous upregulation of cleaved caspase-3, cleaved caspase-8, and cleaved caspase-9 expression was induced by BAY 11-7085 treatment, suggesting that BAY 11-7085 may be applicable for the medical treatment of endometriosis as an adjuvant approach in combination with current medical treatment for this disease. Further studies with other inhibitors of NF-κB, JNK, or p38 MAPK on the cell proliferation and apoptosis of endometriotic cells may contribute to the establishment of more effective and sophisticated treatment strategies for endometriosis.

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