OPEN

Experimental & Molecular Medicine (2013) 45, e47; doi:10.1038/emm.2013.91

www.nature.com/emm

ORIGINAL ARTICLE

Aspirin-induced Bcl-2 translocation and its phosphorylation in the nucleus trigger apoptosis in breast cancer cells

Bo-Hwa Choi[1,2], Goutam Chakraborty[1], Kwanghee Baek[3] and Ho Sup Yoon[1,3]

Here, we report that B-cell lymphoma 2 (Bcl-2) is a novel target molecule of aspirin in breast cancer cells. Aspirin influenced the formation of a complex by Bcl-2 and FKBP38 and induced the nuclear translocation of Bcl-2 and its phosphorylation. These events inhibited cancer cell proliferation and subsequently enhanced MCF-7 breast cancer cell apoptosis. Bcl-2 knockdown using small interfering RNA (siRNA) delayed apoptotic cell death, which correlated with increased proliferation following aspirin exposure. In contrast, Bcl-2 overexpression enhanced the onset of aspirin-induced apoptosis, which was also associated with a significant increase in Bcl-2 phosphorylation in the nucleus. Therefore, this study may provide novel insight into the molecular mechanism of aspirin, particularly its anticancer effects in Bcl-2- and estrogen receptor-positive breast cancer cells.
Experimental & Molecular Medicine (2013) **45,** e47; doi:10.1038/emm.2013.91; published online 11 October 2013

Keywords: apoptosis; aspirin; Bcl-2; breast cancer; FKBP38; MCF-7

INTRODUCTION

Aspirin, a nonsteroidal anti-inflammatory drug, is a well-known antipyretic and analgesic agent[1] and is used for the prevention of recurrent transient ischemic attacks or stroke.[2] In addition to its classical anti-inflammatory function, clinical and epidemiological studies have also demonstrated that prolonged aspirin use reduces the risk of colorectal, breast, prostate, lung and skin cancers.[3–7] The anticancer effect of aspirin is attributable to its ability to induce apoptosis or inhibit cell proliferation. The mechanism of aspirin's therapeutic effects has been established as the irreversible inhibition of cyclooxygenases (COXs) by cross-acetylation.[8] Overexpression of COX-2 leads to cellular proliferation and angiogenesis and inhibits apoptosis, ultimately contributing to carcinogenesis.[9,10] Recent studies have reported COX-2 overexpression in various types of cancers, including colon, lung, breast and head and neck cancers,[11–14] and it is typically associated with poor prognosis.[15,16] Interestingly, aspirin has been shown to exhibit an antiproliferative effect and to induce apoptosis in a COX-independent manner in colon cancer cells and COX-null mouse embryo fibroblasts.[17,18] Moreover, the difference in the clinical activities of aspirin at low and high doses cannot explain all the therapeutic effects of aspirin, suggesting that multiple targets might be involved in the biological effects of aspirin.

Several mechanisms have been proposed for aspirin-mediated apoptosis by COX-independent pathways, including downregulation of the activity of the transcription factor nuclear factor-κB (NF-κB),[19] inhibition of proteasome function[20] and modulation of B-cell lymphoma 2 (Bcl-2) family proteins.[21,22] Aspirin has been shown to inhibit the NF-κB pathway in Jurkat cells through the inhibition of IKK-β, which phosphorylates IκB, leading to its degradation and the translocation of NF-κB to the nucleus where it binds specific DNA sequences in genes that influence cell growth and proliferation,[19,23] such as Bcl-2 and Bcl-X_L.[24] In fact, aspirin is associated with the downregulation of Bcl-2 expression and the upregulation of Bax and Bad expression in gastric cancer cells.[25] In addition, aspirin decreases proteasome activity and increases the accumulation of ubiquitinated proteins in Neuro 2a cells, which correlates with its effect on cell death.[20] Taken together, these findings suggest that aspirin-induced apoptosis in cancer cells involves multiple pathways. However, the exact target molecules of aspirin are still largely unknown.

[1]School of Biological Science, Nanyang Technological University, Singapore, Singapore; [2]Pohang Center for Evaluation of Biomaterials, Pohang Technopark, Pohang, South Korea and [3]Department of Genetic Engineering, College of Life Sciences, Kyung Hee University, Yongin-si, Republic of Korea
Correspondence: Professor HS Yoon, School of Biological Science, Nanyang Technological University, 60 Nnayang Drive, Singapore 637551, Singapore.
E-mail: hsyoon@ntu.edu.sg
Received 12 September 2012; revised 14 May 2013; accepted 24 June 2013

The inhibitory effects of aspirin on cell proliferation are dose and time dependent. Aspirin is generally present at a concentration of 1 to 5 mM in the plasma of patients treated for chronic inflammatory diseases,[23,26,27] and 3 to 10 mM aspirin exerts antiproliferative and proapoptotic effects in several cancer cell lines.[24,28] Here, we examined the potential molecular targets of aspirin that are involved in cancer cell apoptosis at these concentrations. Based on our structure-guided *in silico* screening approach using FKBP38, which is a noncanonical member of the immunosuppressive drug FK506-binding protein (FKBP) family and interacts with Bcl-2 in the absence of FK506, we have previously generated several lead compounds including salicylates and aspirin-like scaffolds, prompting us to investigate the effects of aspirin on the molecular interaction between Bcl-2 and FKBP38. Our results demonstrated that aspirin blocked the complex interaction between Bcl-2 and FKBP38, leading to Bcl-2 translocation to the nucleus and its related apoptotic dysregulation in MCF-7 breast cancer cells. In addition, higher levels of Bcl-2 expression enhanced and facilitated aspirin-induced apoptosis in breast cancer cells, and the phosphorylation of Bcl-2 in the nucleus induced by aspirin treatment was association with nuclear distortion and chromatin condensation.

MATERIALS AND METHODS

Plasmids, antibodies and reagents

Human Bcl-2 (GenBank: NM000633) fused to Flag-tag was cloned into the *Hin*dIII and *Xho*I sites of pXJ-Flag-S. All of the constructs were confirmed by DNA sequencing. Antibodies against Bcl-2, pBcl-2 (S70), pBcl-2 (S87), Bcl-X_L, α-tubulin and GFP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against pBcl-2 (T56) were obtained from Cell Signaling Technology (Denver, MA, USA). A monoclonal antibody against FKBP12 was obtained from BD Pharmingen (San Diego, CA, USA). Antibodies against Flag, actin, GAPDH and lamin A were obtained from Sigma-Aldrich (St Louis, MO, USA). Aspirin, salicylate and phosphatase inhibitors were purchased from Sigma-Aldrich, and cOmplete, Mini, Protease Inhibitor Cocktail Tablets were obtained from Roche Applied Science (Indianapolis, IN, USA).

Cell culture and transfection

Human breast cancer cell lines, including MCF-7 and MDA-MB231, were maintained in Dulbecco's modified Eagle's medium, which contained 10% fetal calf serum and 1% penicillin/streptomycin in a humidified 5% CO_2 incubator at 37 °C. In a six-well tissue culture plate (Falcon, Franklin Lakes, NJ, USA), cells were seeded at a density of $\sim 2 \times 10^5$ cells per well. When the cells were 70–80% confluent, they were transfected with plasmids using a Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA). After incubation for 24 h, the transfected cells were treated as indicated for analysis.

RNA interference experiments

Small interfering RNA (siRNA) duplexes that targeted Bcl-2 (5′-AAG UACAUCCAUUAUAAGCUG-3′) and control siRNA (5′-AAGUCUC CAAGCGGAUCUCGU-3′) were purchased from Dharmacon (Lafayette, CO, USA). Transfection of the siRNA duplexes at a final concentration of 100 nM was performed using a Lipofectamine reagent. After incubation for 48 h, the cells were processed for western blotting and fluorescence-activated cell sorting (FACS) as indicated.

Protein expression and purification

Full-length FKBP38 and Bcl-2 lacking the transmembrane motif were bacterially expressed as glutathione *S*-transferases (GST)-fusion and hexahistidine-tagged proteins, respectively, and purified as previously described.[29]

In vitro competition assay

Aspirin was incubated with 1 μg of the purified recombinant GST-FKBP38 for 2 h at 4 °C in a binding buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol (DTT), 10% glycerol) containing the protease inhibitor cocktail (Roche), followed by the addition of 1 μg of the purified recombinant His-Bcl-2. After a 2-h incubation with glutathione-sepharose beads (Amersham Biosciences, Uppsala, Sweden), the beads were washed four times and subjected to immunoblot analysis.

Immunoprecipitation and immunoblotting

Immunoblot analysis was performed as previously described.[30] For immunoprecipitation, cell lysates were prepared in a lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 1 mM PMSF). Equal amounts of protein were immunoprecipitated using anti-Flag and collected with Protein A/G-Sepharose beads (Santa Cruz Biotechnology) at 4 °C for 16 h. The immunoprecipitate was then washed four times in cold lysis buffer. The bound proteins were resolved by SDS-polyacrylamide gel electrophoresis, which was followed by western blotting analysis.

Immunocompetition assay

HeLa cells were co-transfected with YFP-Bcl-2 and Flag-FKBP38 and subsequently immunoprecipitated with an antibody against Flag. The immunoprecipitates were incubated with aspirin or salicylate in a reaction buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA and 1 mM PMSF) at 4 °C. After a 2-h incubation with Protein A/G-Sepharose beads, the beads were subjected to immunoblot analysis.

Confocal microscopy and image analysis

For immunocytochemistry, cells fixed with 3.7% paraformaldehyde were incubated with a blocking solution (2.5% bovine serum albumin and 2.5% horse serum in phosphate-buffered saline) for 30 min at 4 °C. Slides were incubated overnight at 4 °C with anti-FKBP38 and anti-Bcl-2 antibodies as indicated. After washing, samples were incubated with Alexa Fluor 488- and Alexa Fluor 546-conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. Slides were mounted and visualized at $\times 60$ magnification on a Zeiss LSM META confocal laser scanning microscope (Zeiss, Oberkochen, Germany). Image processing was performed with Adobe Photoshop 7.0 software (San Jose, CA, USA).

Preparation of mitochondrial and cytoplasmic extracts

Subcellular fractionation was performed as we have previously described in detail.[31] Briefly, cells were lysed in an isotonic mitochondrial buffer (300 mM sucrose, 10 mM HEPES, pH 7.4, 1 mM EGTA) containing protease inhibitors, homogenized and centrifuged at $1000 \times g$ for 10 min to discard nuclei and unbroken cells, and the resulting supernatant was centrifuged at $10\,000 \times g$ for 30 min to obtain the mitochondrial and cytoplasmic fractions.

Preparation of nuclear and cytoplasmic extracts

Cells were resuspended in hypotonic buffer (10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.2 mM PMSF, 0.5% Nonidet P-40, protease inhibitors and phosphatase inhibitors) and incubated at 4 °C for 30 min. Samples were agitated every 10 min and then centrifuged at 1800 × *g* for 4 min to collect the cytoplasmic fractions. To isolate nuclei, pellets were washed three times with and resuspended in nuclear extraction buffer (20 mM HEPES, 450 mM NaCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.2 mM PMSF, protease inhibitors and phosphatase inhibitors) for 20 min. Freeze–thawing was then repeated 5 times. The nuclear suspension was centrifuged at 16 000 × *g* for 20 min, and the supernatants were recovered as the nuclear fractions.

Cell cycle analysis

Cells were collected by trypsinization, washed with phosphate-buffered saline two times and resuspended in propidium iodide staining buffer (10 mM Tris-HCl 8.0, 10 mM NaCl, 50 mg l^{-1} propidium iodide, 10 mg l^{-1} RNase A, 0.1% Nonidet P-40) for 30 min at 4 °C in the dark. The cell cycle was immediately detected on a flow cytometer using a FACSCalibur instrument with ModFit LT software (Becton Dickinson, Singapore, Singapore).

Measurement of apoptosis

Apoptosis was measured as the percentage of cells in Sub-G_1 using flow cytometry. For all experiments, at least 10 000 events were collected per sample.

Cell proliferation assay

Cell proliferation was determined using the CellTiter 96 Aqueous One Solution Reagent kit (Promega, Madison, WI, USA) according to the manufacturer's instructions after treatment with aspirin, and absorbance was measured at 490 nm.

Statistical analysis

All experiments were independently repeated a minimum of three times. All quantitative data are presented as the mean ± s.e.m.

RESULTS

Aspirin inhibits the interaction of FKBP38 and Bcl-2 *in vitro*

Molecular docking studies on the interaction of aspirin with FKBP38 (Harikishore A and Yoon HS, unpublished data) led us to examine the effect of aspirin on the association of FKBP38 with Bcl-2. We first performed an *in vitro* competition assay using Bcl-2 and FKBP38 in the presence of aspirin and demonstrated that the molecular interaction between FKBP38 and Bcl-2 decreased with increasing concentrations of aspirin (Figure 1a). This finding was further confirmed in mammalian cells by immunocompetitive assays and coimmunoprecipitation assays using YFP-tagged Bcl-2 and Flag-tagged FKBP38. Our results demonstrated that the interaction between Bcl-2 and FKBP38 was significantly decreased after treatment with 10 mM aspirin (Figures 1b and d, Supplementary Figure 3). Under the same conditions, we also tested the effect of salicylate as a control. Salicylate is a metabolite of aspirin in cells, but it had no significant effect on the interaction between FKBP38 and Bcl-2 (Figures 1c and e), suggesting that aspirin affected the molecular interaction between FKBP38 and Bcl-2.

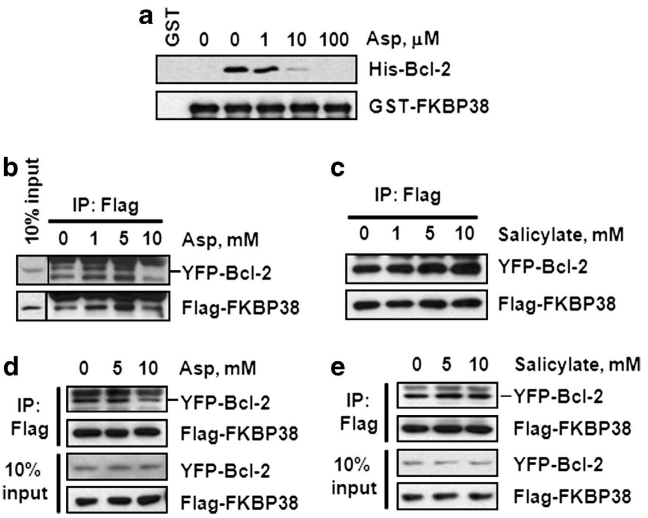

Figure 1 Aspirin hinders the interaction between FKBP38 and B-cell lymphoma 2 (Bcl-2). (**a**) *In vitro* competition assays. GST-FKBP38 and $(His)_6$-Bcl-2 were incubated with increasing concentrations of aspirin. Asp, aspirin. (**b**–**e**) Association of Flag-FKBP38 and YFP-Bcl-2. Transfected HeLa cell lysates were immunoprecipitated with a Flag antibody, the immunoprecipitated complex was incubated with aspirin or salicylate as shown for 2 h and associations between Flag-FKBP38 and YFP-Bcl-2 were analyzed by immunoblotting (**b**,**c**). Transfected HeLa cell were treated with aspirin or salicylate as indicated for 2 h, followed by immunoprecipitation using a Flag antibody (**d**,**e**). IP, immunoprecipitation.

Aspirin induces translocation of Bcl-2 to the nucleus in MCF-7 cells

In cells depleted of FKBP38 by specific siRNA, Bcl-2 was shown to aggregate in the perinuclear compartments.[32] Similarly, we tested the effect of aspirin on the localization of Bcl-2 in dose- and time-dependent manners in MCF-7 cells. Bcl-2 and FKBP38 were largely localized in the cytoplasm without aspirin treatment as expected (Figure 2a). However, a mild redistribution of Bcl-2 was observed in both the nucleus and the cytoplasm after 6 h in response to 5 mM aspirin treatment (Supplementary Figure 1), and Bcl-2 gradually accumulated in the nucleus (Figure 2a). Bcl-2 accumulation in the nucleus was also detected by subcellular fractionation analyses (Figure 2b). To further confirm the translocation of Bcl-2 induced by aspirin, we obtained mitochondrial fractions from untreated or aspirin-treated MCF-7 cells. As shown in Figure 2c, the level of endogenous Bcl-2 in the mitochondrial fraction was considerably reduced following treatment with 5 mM aspirin for 24 h, indicating that aspirin affects the localization of Bcl-2 in MCF-7 cells. Interestingly, a reduction in the level of endogenous FKBP38 protein was also observed at 24 h after treatment with 5 mM aspirin in MCF-7 cells (Figures 2a and c). However, neither transcriptional nor post-translational regulation was involved in the aspirin-mediated downregulation of FKBP38 (data not shown).

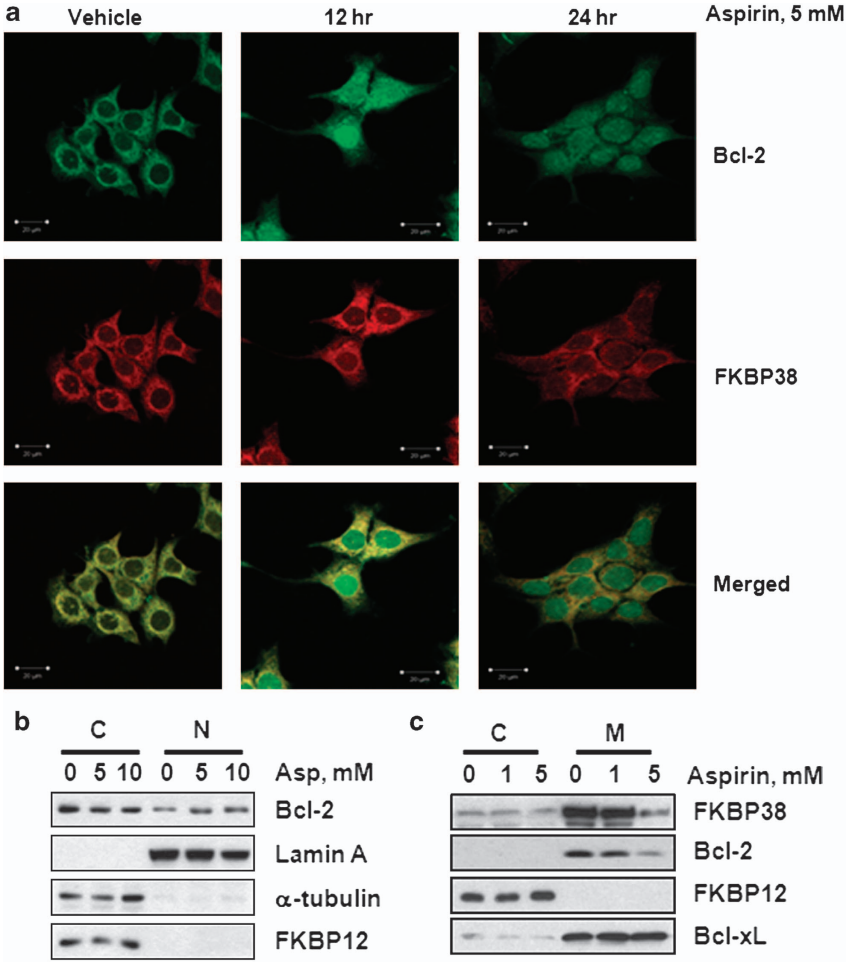

Figure 2 Aspirin-induced translocation of B-cell lymphoma 2 (Bcl-2) in MCF-7 cells. (**a**) The effect of aspirin on endogenous Bcl-2. MCF-7 cells were exposed to 5 mM aspirin for several time intervals as indicated. The distribution of Bcl-2 was visualized after immunostaining with anti-Bcl-2 and anti-FKBP38 antibodies. (**b**) MCF-7 cells were exposed to 5 mM aspirin for 24 h. Nuclear and cytoplasmic fractions were prepared as described in the MATERIALS AND METHODS and subjected to immunoblotting with the indicated antibodies. Lamin A, α-tubulin and FKBP12 were used as nucleus- and cytoplasm-specific controls. (**c**) FKBP38 and Bcl-2 are downregulated in mitochondrial fractions obtained from cells exposed to 5 mM aspirin for 24 h. C, cytosolic fraction, N, nuclear fraction, M, mitochondrial fraction.

Aspirin-induced apoptotic cell death is delayed by Bcl-2 knockdown in MCF-7 cells

Several studies have demonstrated that the antiapoptotic protein Bcl-2 contributes to the induction of apoptotic cell death through its nuclear localization.[33,34] In this study, we also observed the accumulation of Bcl-2 in the nucleus over a period of 24 h following treatment with aspirin (Figures 2a and b). A dose-dependent reduction in cell proliferation was observed in MCF-7 cells treated with aspirin for 24 h (Supplementary Figure 2), which was accompanied by an increased percentage of cells in the G_0/G_1 phase (upper panels in Figure 3b). To further delineate the role of Bcl-2 in MCF-7 cells, we knocked down endogenous Bcl-2 using siRNA (Figure 3a) and then performed cell cycle analysis in the presence or absence of aspirin in a time-dependent manner. Interestingly, Bcl-2 knockdown sustained the G_1-phase arrest until 48 h following aspirin exposure, whereas ~25% of cells transfected with scrambled siRNA progressed to apoptotic cell death (Figures 3b and c). To further confirm the effect of Bcl-2 on aspirin-induced apoptotic cell death, we performed a cell proliferation assay following the treatment of Bcl-2-knockdown cells and Bcl-2-expressing cells with aspirin for 48 h. As shown in Figure 3d, MCF-7 cells transfected with siRNA against Bcl-2 showed increased proliferation compared with cells transfected with scrambled siRNA, suggesting that Bcl-2 plays a role in facilitating aspirin-mediated apoptosis in MCF-7 breast cancer cells.

Overexpression of Bcl-2 facilitates the onset of aspirin-induced apoptosis in MCF-7 cells

To further understand the role of Bcl-2 in apoptotic cell death in MCF-7 cells, cells were transfected with a Flag-tagged Bcl-2 plasmid (Figure 4a), followed by treatment with aspirin for 24 h. The levels of Flag-Bcl-2 were increased slightly in the nuclear fraction of aspirin-treated cells compared with control cells (Figure 4b). In Bcl-2-overexpressed cells, the apoptotic

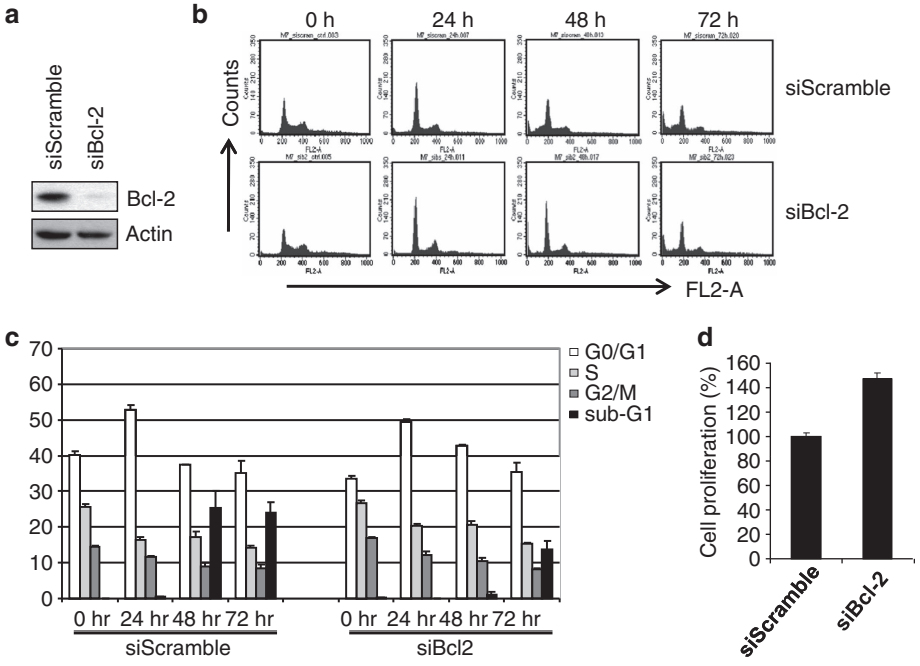

Figure 3 B-cell lymphoma 2 (Bcl-2) silencing by small interfering RNA (siRNA) delays aspirin-induced apoptotic cell death in MCF-7 cells. (**a**) Bcl-2 protein levels were detected by immunoblotting 72 h after siRNA transfection. (**b**, **c**) Cell cycle analysis. MCF-7 cells were transfected with Bcl-2-targeting or scrambled control siRNA and treated with 5 mM aspirin for the indicated incubation periods. The cell cycles were determined by flow cytometry (**b**) and quantitated by ModFit LT software (**c**). (**d**) Cell proliferation was measured 48 h after aspirin treatment using MCF-7 cells transfected with Bcl-2-targeting or scrambled control siRNA. Error bars represent the s.e. calculated from three independent experiments.

index was measured in the presence or absence of aspirin. Bcl-2 overexpression increased apoptotic cell death from 4 to 10% at 6 h after treatment with aspirin and to >17% at 24 h, whereas empty vector-transfected cells showed only a moderate increase in apoptotic cell death, from 2 to 10% at 24 h after aspirin treatment (Figure 4c). These data suggest that the overexpression of Bcl-2 is related to aspirin-induced apoptosis in MCF-7 cells. To examine whether Bcl-2 acts as a negative regulator of breast cancer cell proliferation, we used another breast cancer cell line, MDA-MB231, which expresses significantly less Bcl-2 compared with MCF-7 cells (Figure 5a). Almost 80% of MDA-MB231 cells continued to proliferate 24 h after treatment with 3 mM aspirin, whereas <40% of MCF-7 cells proliferated under the same conditions (Figure 5b). Taken together, these results suggest that the overexpression of Bcl-2 facilitates the shift from aspirin-induced G_1-phase arrest to apoptosis in MCF-7 breast cancer cells, which are estrogen receptor (ER) positive.

Bcl-2 phosphorylation by aspirin disrupts nuclear assembly linked to apoptosis

Bcl-2 is a central player in apoptosis, and its activity is regulated through phosphorylation in response to a variety of external stimuli.[35–37] This prompted us to examine Bcl-2 phosphorylation upon the addition of aspirin to cancer cells. We found that Bcl-2 phosphorylation in its flexible loop was dramatically increased in the nuclear fraction obtained from MCF-7 cells and Bcl-2-overexpressing cells after treatment with aspirin (Figures 6a and 4b). Because the phosphorylation of Bcl-2 is largely associated with its inactivation and because mutation of the phosphorylation sites enhances the anti-apoptotic activity of Bcl-2,[36] we monitored the progression of Bcl-2 phosphorylation in the nuclear-enriched fraction upon aspirin treatment. As shown in Figure 6b, Bcl-2 phosphorylation reached its maximum at 24 h and declined thereafter. The cleaved form of lamin A, which is an important player in chromatin condensation and nuclear disassembly in apoptosis,[38] was examined at 48 h, and its level increased up to 72 h (Figure 6b). To determine the correlation between Bcl-2 phosphorylation and nuclear disassembly, Bcl-2 phosphorylation was examined in the presence of 4′,6-diamidino-2-phenylindole staining. Most nuclear shapes became irregular and distorted at 48 h after aspirin treatment, and its phosphorylation was not evenly distributed (Figure 6c). However, Bcl-2 was highly phosphorylated in the damaged nucleus (Figure 6c), suggesting that the aspirin-mediated phosphorylation of Bcl-2 might trigger nuclear membrane disruption and chromatin condensation in MCF-7 cells.

DISCUSSION

The molecular mechanism of aspirin's effects on breast cancer remains elusive. Earlier studies showed a marginal reduction in breast cancer for those who had taken adult-strength aspirin for at least 5 years or low-dose aspirin (100 mg every other day) over an average of 10 years.[39,40] In contrast, assessment of 4 case–control and 7 cohort studies showed that aspirin is

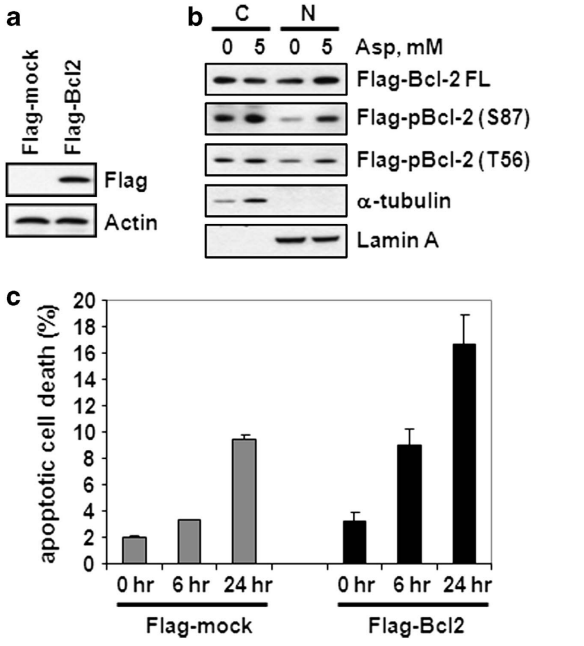

Figure 4 Overexpression of B-cell lymphoma 2 (Bcl-2) enhances aspirin-induced apoptotic cell death in MCF-7 cells. (**a**) Overexpressed Bcl-2 levels in MCF-7 cells transfected with the plasmids Flag-mock or Flag-Bcl-2 were detected by immunoblotting. (**b**) Transfected cells were treated with 5 mM aspirin for 24 h. Nuclear and cytoplasmic fractions were prepared and subjected to immunoblotting with the indicated antibodies. C, cytosolic fraction, N, nuclear fraction. (**c**) Transfected cells were exposed to 5 mM aspirin, and the sub-G_1 cell population was then measured by flow cytometry at the indicated time points. Error bars represent the s.e. calculated from three independent experiments.

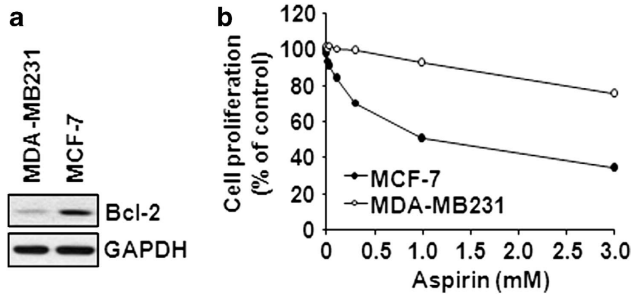

Figure 5 High levels of B-cell lymphoma 2 (Bcl-2) are associated with the antiproliferative effects of aspirin in breast cancer cells. (**a**) Cell lysates from MDA-MB-231 and MCF-7 cells were subjected to immunoblotting to detect Bcl-2 protein levels. (**b**) MDA-MB-231 and MCF-7 cells were treated with several concentrations of aspirin as indicated for 24 h, and cell proliferation was measured by MTT assay. Error bars represent the s.e. calculated from three independent experiments.

associated with a reduced breast cancer incidence of ~20% (relative risk, 0.77; 95% confidence interval, 0.69–0.86).[41] In addition, in a meta-analysis of 16 case–control studies, 18 cohort studies, 3 case–control studies nested in a well-defined cohort and 1 clinical trial, the relative risk of breast cancer was 0.87 (95% confidence interval, 0.82–0.92),[42] showing protection with a 15% reduction in incidence. The studies suggest that aspirin usage is associated with a significant decrease in the development of breast cancer and, furthermore, that it might be of benefit to women with breast cancer.

Using our structure-guided *in silico* screening approach, we found that aspirin-like small molecules may bind to FKBP38 through its FK506-binding domain (Harikishore A and Yoon HS, unpublished data). In fact, the domain is critical to the interaction between FKBP38 and Bcl-2.[32] In the present study, we showed that aspirin hindered the interaction between FKBP38 and Bcl-2 (Figure 1) and thus induced translocation of Bcl-2 to the nucleus in MCF-7 cells (Figure 2). However, further studies are required to determine the exact mechanism by which aspirin blocks the interaction between two proteins. FKBP12, which is a canonical member of the FKBP family, binds to calcineurin only in the presence of FK506 and the resulting FKBP12/FK506/calcineurin complex prevents T-cell proliferation by inhibiting the protein phosphatase activity of calcineurin.[43,44] However, FKBP38, a noncanonical FKBP family member, exhibits a distinct catalytic and ligand binding profile, with unique active site topology that would restrict FK506 binding and allow the binding of smaller molecule ligands.[45] It is therefore possible that small molecule ligands such as aspirin might bind to FKBP38 and consequently elicit the dissociation of Bcl-2 from FKBP38.

Bcl-2 was primarily targeted to mitochondria following complex formation with FKBP38, thus inhibiting apoptosis.[32] Perturbation of complex formation using siRNA against FKBP38 not only alters the subcellular localization of Bcl-2 but also influences its antiapoptotic activity.[32] Previously, nuclear Bcl-2 was detected in the aged hippocampus and cerebellum and failed to protect cells from apoptosis induced by oxidative stress.[46] Interestingly, the nuclear Bcl-2 functioned as a proapoptotic protein, and FKBP38 was unable to facilitate the delivery Bcl-2 to the mitochondria.[34] Nuclear Bcl-2 also reduces the levels of several transcription factors, particularly NF-κB, the proper levels of which are necessary to prevent cell death *in vitro*. In addition, it was also found that the BH4 domain of Bcl-2 is involved in the regulation of Bcl-2 movement and its interaction with FKBP38. Studies in the PC12 cell line have shown increased levels of apoptosis following the transient transfection of cells with Bcl-2 or Bcl-2ΔBH4[47] (both of which result in the nuclear localization of the protein compared with stable expression, which localizes primarily in the mitochondria and endoplasmic reticulum (ER)). Here, we found that the levels of nuclear Bcl-2 were slightly increased during the first 6 h of aspirin exposure, followed by a much higher level until 24 h (Supplementary Figure 1); this increase was linked to a reduction in cell proliferation (Supplementary Figure 2) and the subsequent induction of apoptosis in MCF-7 cells (Figure 3b). In addition, Bcl-2 knockdown by siRNA delayed the onset of aspirin-induced apoptosis (Figures 3b and c). These results suggest

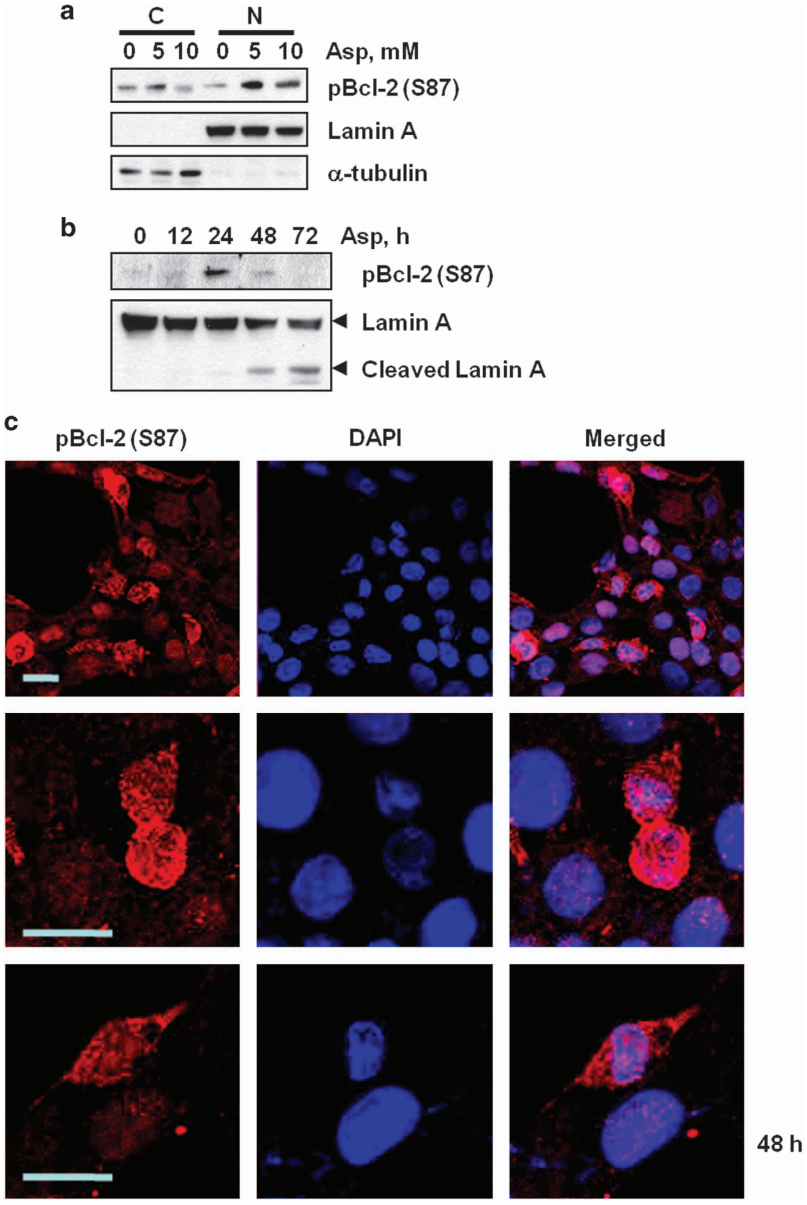

Figure 6 Phosphorylation of B-cell lymphoma 2 (Bcl-2) is associated with nuclear condensation. (**a**) MCF-7 cells were exposed to 5 mM aspirin for 24 h. Nuclear and cytoplasmic fractions were prepared as described in the MATERIALS AND METHODS and subjected to immunoblotting with the indicated antibodies. Lamin A and α-tubulin were used as nucleus- and cytoplasm-specific controls. C, cytosolic fraction, N, nuclear fraction. (**b**) MCF-7 cells were treated with 5 mM aspirin and incubated for the indicated time periods, and phosphorylation of Bcl-2 in the nuclear fraction was determined by immunoblotting. (**c**) MCF-7 cells were exposed to 5 mM aspirin for 48 h. Cells were stained with anti-phospho-Bcl-2 and 4′,6-diamidino-2-phenylindole (DAPI) and then analyzed by fluorescence microscopy.

that the nuclear translocation of Bcl-2 following aspirin exposure is one of the key events controlling cell death and survival.

Bcl-2 is expressed in a majority of breast tumor tissues. In addition, Bcl-2-positive tumors have been shown to correlate with menopause and ER-positive tumors.[48,49] The overexpression of Bcl-2 in primary breast carcinomas was associated with improved patient survival, suggesting that Bcl-2 inhibits cancer progression.[50,51] In agreement with these reports, we found that ER-positive MCF-7 cells expressed a higher level of Bcl-2 and responded well to aspirin (Figures 5a and b). In addition, the overexpression of Bcl-2 enhanced the onset of aspirin-induced apoptosis (Figure 4c), whereas the knockdown of Bcl-2 using siRNA delayed the effects of aspirin in MCF-7 cells (Figure 3). Therefore, we believe that aspirin may exhibit chemotherapeutic efficacy in ER-positive breast cancer cells overexpressing Bcl-2.

Aspirin has previously been shown to inhibit proteasome function indirectly, leading to the accumulation of ubiquitinated proteins, including Bax, p53, IκB-α and p27kip1 in

Neuro 2a cells.[20] In this study, we also demonstrated that aspirin induces the downregulation of FKBP38 through a transcription-independent mechanism (data not shown). However, the effects of aspirin were not associated with the proteasomal and lysosomal degradation pathways (data not shown). In fact, several studies have demonstrated that aspirin acts as a potent inhibitor of protein synthesis by inhibiting mRNA translation initiation.[52,53] Therefore, we believe that FKBP38 is downregulated through the inhibition of protein synthesis following exposure to aspirin. The detailed mechanism of FKBP38 downregulation by aspirin awaits further studies.

We conclude that Bcl-2 is a novel target molecule of aspirin in breast cancer cells. Aspirin affected the formation of a complex between Bcl-2 and FKBP38 and induced the nuclear translocation of Bcl-2 and its phosphorylation, leading to the inhibition of cancer cell proliferation and enhanced apoptotic cell death in MCF-7 cells. This study therefore provides novel insights into the molecular mechanism of aspirin, particularly its anticancer effects in Bcl-2- and ER-positive breast cancer cells.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank KT Kim for participating in discussions and providing suggestions. We also thank Joel Chia for his help with protein purification. This work was supported in part by a Ministry of Health Singapore National Medical Research Council Grant, NMRC/1177/2008, and a Ministry of Education Singapore AcRF Tier 1 Grant, RG 161/06.

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