Synthetic Bichalcone TSWU-BR23 Induces Apoptosis of Human Colon Cancer HT-29 Cells by p53-Mediated Mitochondrial Oligomerization of BAX/BAK and Lipid Raft Localization of CD95/FADD

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Abstract. *A synthetic bichalcone analog, (E)-1-(3-((4-(4-acetylphenyl)piperazin-1-yl)methyl)-4-hydroxy-5-methoxyphenyl)-3-(pyridin-3-yl)prop-2-en-1-one (TSWU-BR23), has been shown to induce apoptosis in human colon cancer HT-29 cells involving the induction of CD95 and FAS-associated protein death domain (FADD), but its precise mechanism of action has not been fully elucidated. Using cell-surface biotinylation and sucrose density-gradient-based membrane flotation techniques, we showed that the disruption of TSWU-BR23-induced lipid raft localization of CD95/FADD by cholesterol-depleting agent (methyl-β-cyclodextrin) was reversed by cholesterol replenishment. Blockade of p53 expression by short-hairpin RNA (shRNA) suppressed oligomeric Bcl-2-associated x protein (BAX)/Bcl-2 antagonist killer 1 (BAK)-mediated mitochondrial apoptosis but did not inhibit lipid raft localization of CD95/FADD and pro-caspase-8 cleavage induced by TSWU-BR23. Co-expression of p53 shRNA and dominant-negative mutant of FADD completely inhibited TSWU-BR32-induced mitochondrial apoptotic cell death. Collectively, these data demonstrate that TSWU-BR23 leads to HT-29 cell apoptosis by inducing p53-mediated mitochondrial oligomerization of BAX/BAK and the localization of CD95/FADD with lipid rafts at the cell surface.*

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Apoptosis is a key regulatory mechanism of tissue and cellular homeostasis that can be induced *via* activation of either the extrinsic or the intrinsic signaling pathway (1). The extrinsic pathway is triggered by binding cluster of differentiation 95 (CD95) ligand to CD95 receptor on the target cells, which in turn leads to recruitment of cytosolic adaptor FAS-associated death domain-containing protein (FADD) and pro-caspase-8. Upon co-localization with FADD, pro-caspase-8 undergoes auto-proteolytic cleavage, releasing activated caspase-8 into the cytosol, where it causes cleavage of BH3-interacting domain death agonist (BID) into truncated BID (tBID) to engage the intrinsic pathway and initiates apoptosis by activating executioner caspase (1). The intrinsic pathway is closely linked to permeabilization of the mitochondrial outer membrane by the activation and oligomerization of pro-apoptotic Bcl-2-associated x protein and Bcl-2 antagonist killer 1, that lead to cytochrome *c* release from the mitochondria into the cytosol (2). Cytosolic cytochrome *c* triggers the processing of pro-caspase-9, thereby initiating the formation of an apoptosome composed of apoptotic protease-activating factor 1, dATP, caspase-9, and cytochrome *c*. Apoptosome formation leads to activation of the executioner caspase-3, which causes apoptotic cell death (1).

Membrane lipid rafts are specialized, detergent-insoluble, cholesterol/glycosphingolipid-rich microdomains that can serve as recruitment platforms for membrane-associated receptors and downstream specific adaptors to affect signaling transduction (3). CD95 and FADD concentration in lipid rafts can form apoptosis-promoting clusters (4). Evidence also exists that recruitment of CD95 and FADD to lipid rafts can be driven not only by the interaction between CD95 ligand and their receptors at the cell surface, but also through induction of chemotherapeutic agent in a CD95 ligand-

independent manner (4). Thus, it has garnered intense interest as a potential agent that can induce CD95 ligand-independent activation of apoptosis signal transduction pathways.

We previously used a Mannich base reaction to synthesize a new synthetic bichalcone analog (*E*)-1-(3-((4-(4-acetylphenyl)piperazin-1-yl)methyl)-4-hydroxy-5-methoxyphenyl)-3-(pyridin-3-yl)prop-2-en-1-one, or TSWU-BR23, with a single B-ring pyridyl moiety (5). Although we found that TSWU-BR23 potently activated the caspase cascade and induced apoptosis of human colon cancer HT-29 cell line through the induction of CD95 and FADD, the mechanism by which TSWU-BR23 causes HT-29 cells to undergo apoptotic cell death was not elucidated. In the present study, we investigated the molecular mechanism by which TSWU-BR23 induces apoptotic effects in HT-29 cells and how these effects are modulated by CD95/FADD and mitochondria.

Materials and Methods

Cell culture. The human colon cancer HT-29 cell line was obtained as previously described (5). The HT-29 cell line was cultured routinely in Roswell Park Memorial Institute 1640 (RPMI-1640) medium supplemented with 5% fetal bovine serum (FBS) and grown in 10-cm tissue culture dishes at 37˚C in a humidified incubator containing 5% CO_2.

Chemicals, reagents and plasmids. Bismaleimidohexane (BMH), Brij 98, Tris-HCl, cyclosporine A, methyl-β-cyclodextrin, and Triton X-100, were obtained from Sigma-Aldrich (St. Louis, MO, USA). The TSWU-BR23 was dissolved in and diluted with dimethyl sulfoxide (DMSO) and then stored at −20˚C as a 100 mM stock. DMSO and potassium phosphate were purchased from Merck (Darmstadt, Germany). Lipofectamine 2000 was obtained from Invitrogen (Carlsbad, CA, USA). RPMI-1640, FBS, trypsin-EDTA, and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). The caspase-3 activity assay kit was purchased from OncoImmunin (Gaithersburg, MD, USA). The plasmids encoding p53 shRNA, green-fluorescent protein (GFP) shRNA, or dominant-negative mutant form (dn) FADD were obtained from Addgene (Cambridge, MA, USA). BAX small-interfering RNA (siRNA), BAK siRNA, control siRNA, and western blotting luminol reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The BAX siRNA, BAK siRNA, and control siRNA were dissolved in RNase-free water.

Antibodies. The antibodies against BAK, BAX, and cytochrome c were obtained from BD Pharmingen (San Diego, CA, USA). Caspase-3, caspase-8, and caspase-9 antibodies were purchased from Calbiochem (San Diego, CA, USA). Anti-cycle-oxygenase subunit II (COX2) and calnexin antibodies were obtained from Abcam (Cambridge, MA, USA). The antibodies against p53 and -phospho (p)-p53 (Ser 15) were purchased from Santa Cruz Biotechnology. The antibody against β-actin was obtained from Sigma-Aldrich. Anti-CD55, CD71, CD95, and FADD antibodies were provided by Santa Cruz Biotechnology. Peroxidase-conjugated anti-mouse IgG, -goat IgG, and -rabbit IgG secondary antibodies were purchased from Jackson ImmunoResearch Laboratory (West Grove, PA, USA).

Assays for the detection of caspase-3, -8, and -9 activities. Caspase-3, -8, and -9 activities were measured using the PhiPhiLux G1D2 kit (OncoImmunin, College Park, MD, USA), Caspase-8 Colorimetric Assay Kit (Abcam), and Caspase-9 Colorimetric Assay Kit (Calbiochem, San Diego, CA, USA) according to the manufacturer's protocols. For the detection of caspase-3 activity, the TSWU-BR23- or vehicle-treated cells were incubated with the PhiPhiLux fluorogenic Caspase substrate at 37˚C for 1 h and were then analyzed using a FACSCount flow cytometer. For the detection of caspase-8 and -9 activities, lysates of treated cells were incubated with Ile-Glu-Thr-Asp (IETD)-*p*-nitroanilide (p-NA) or Leu-Glu-His-Asp (LEHD)-p-NA substrate at 37˚C for 1 h and were then analyzed using a microtiter plate reader at 405 nm.

Measurement of DNA fragmentation. Histone-associated DNA fragments were determined using the Cell Death Detection enzyme-linked immunosorbent assay (ELISA) kit (Roche Applied Science, Mannheim, Germany). Briefly, vehicle- or TSWU-BR23-treated cells were incubated in hypertonic buffer for 30 min at room temperature. After centrifugation, the cell lysates were transferred into an anti-histone–coated microplate to bind histone-associated DNA fragments. Plates were washed after 1.5 h of incubation, and nonspecific binding sites were saturated with blocking buffer. Plates were then incubated with peroxidase-conjugated anti-DNA for 1.5 h at room temperature. To determine the amount of retained peroxidase, 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) was added as a substrate, and a spectrophotometer (Thermo Labsystems Multiskan Spectrum, Frankin, MA, USA) was used to measure the absorbance at 405 nm.

Western blot analysis. Treated or transfected cells were lysed in lysis buffer (50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 1 μg/ml aprotinin, 100 mM Na_3VO_4, 50 mM NaF, 0.5% NP-40). Protein concentration was determined by the Bradford method (Bio-Rad, Hercules, CA, USA). Proteins were separated by electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA). Membranes were blocked overnight with phosphate buffered saline (PBS) containing 3% skim milk and then incubated with primary antibody against CD95, FADD, CD55, CD71, caspase-8, caspase-9, BAX, BAK, calnexin, BAX, BAK, BID, tBID, caspase-3, caspase-8, caspase-9, cytochrome *c*, or COX2. Proteins were detected with horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit, or donkey anti-goat antibodies and western Blotting Luminol Reagent. To confirm equal protein loading, β-actin was measured.

Cell surface biotinylation. This assay was performed as previously described (6). Briefly, treated cells were washed twice in ice-cold PBS and incubated with 0.5 mg/ml of EZ-Link Sulfo-NHS-SS-Biotin (Pierce, Rockford, IL, USA) for 30 min at 4˚C. Biotinylated cells were washed twice in ice-cold PBS and treated with 50 mM NH_4Cl for 10 min at 4˚C to stop the biotinylation reaction. The avidin-agarose beads (Pierce, Rockford, IL, USA) were then added to the biotinylated cells, and the mixture was incubated with gentle rocking at 4˚C for 16 h. The beads were pelleted and washed three times with 500 μl of ice-cold PBS. Bound proteins were mixed with 1× SDS sample buffer and incubated for 5 min at 100˚C. The proteins were then separated by 10% SDS-PAGE and immunoblotted with antibody against CD95 or FADD.

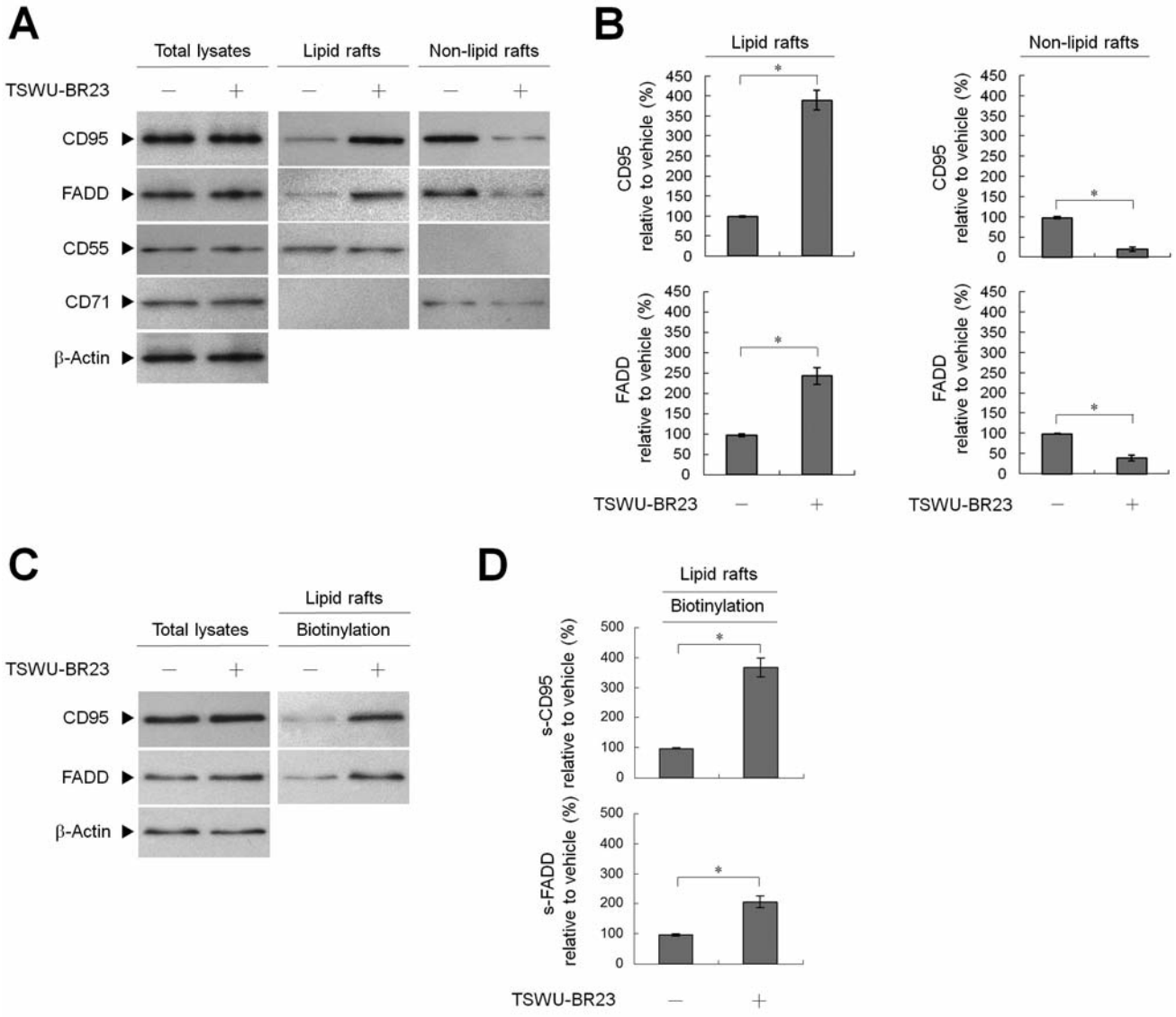

Figure 1. *TSWU-BR23 induces localization of cluster of differentiation 95 (CD95) and Fas-associated death domain (FADD) at the cell surface lipid rafts. A: The effect of TSWU-BR23 on the lipid raft localization of CD95 and FADD. Cells were treated with either vehicle or TSWU-BR23 (30 μM) for 36 h. Crude homogenates of the cells were treated with 1% Brij 98 and fractionated by discontinuous sucrose gradient centrifugation. The levels of the indicated proteins in the total cell lysates, the lipid rafts fractions or the non-lipid rafts fractions were analyzed using specific antibodies. B: Quantitation of the relative levels of lipid raft-associated and non-lipid raft-associated CD95 and FADD in the treated cells. The percentage protein stated above the figure represents the relative density of the bands normalized to that for β-actin. The values presented are the mean±the standard error from three independent experiments. *p<0.05: significantly different from the vehicle-treated cells. C: Cells were treated with either vehicle or TSWU-BR23 (30 μM) for 36 h. Treated cells were biotinylated as described in the Materials and Methods section. Crude homogenates of the cells were treated with 1% Brij 98 and fractionated by discontinuous sucrose gradient centrifugation. Biotinylated proteins were pulled-down using streptavidin agarose beads. The biotin-streptavidin complexes were immunoblotted with CD95 and FADD antibodies. D: Quantitation of the relative expression levels of cell-surface CD95 (s-CD95) and s-FADD in the lipid rafts of the treated cells. The percentage of s-CD95 or s-FADD represents the relative density of the bands normalized to that for β-actin. β-Actin was used as internal control for sample loading. The values presented are the mean±the standard error from three independent experiments. *p<0.05: Significantly different from the vehicle-treated cells.*

Detection of cytochrome c. Subcellular fraction was as previously described (7). The treated cells were washed twice with ice-cold PBS and scraped into a 200 mM sucrose solution containing 25 mM HEPES (pH 7.5), 10 mM KCl, 15 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 1 μg/ml aprotinin. The cells were disrupted by passage through a 26-gauge hypodermic needle 30 times and then centrifuged for 10 min in an Eppendorf microcentrifuge (5804R) at 750 × *g* at 4˚C to remove unlysed cells and nuclei. The supernatant was collected and then centrifuged for 20 min at 10,000 × *g* at 4˚C to form a new supernatant and pellet. The resulting pellet was saved

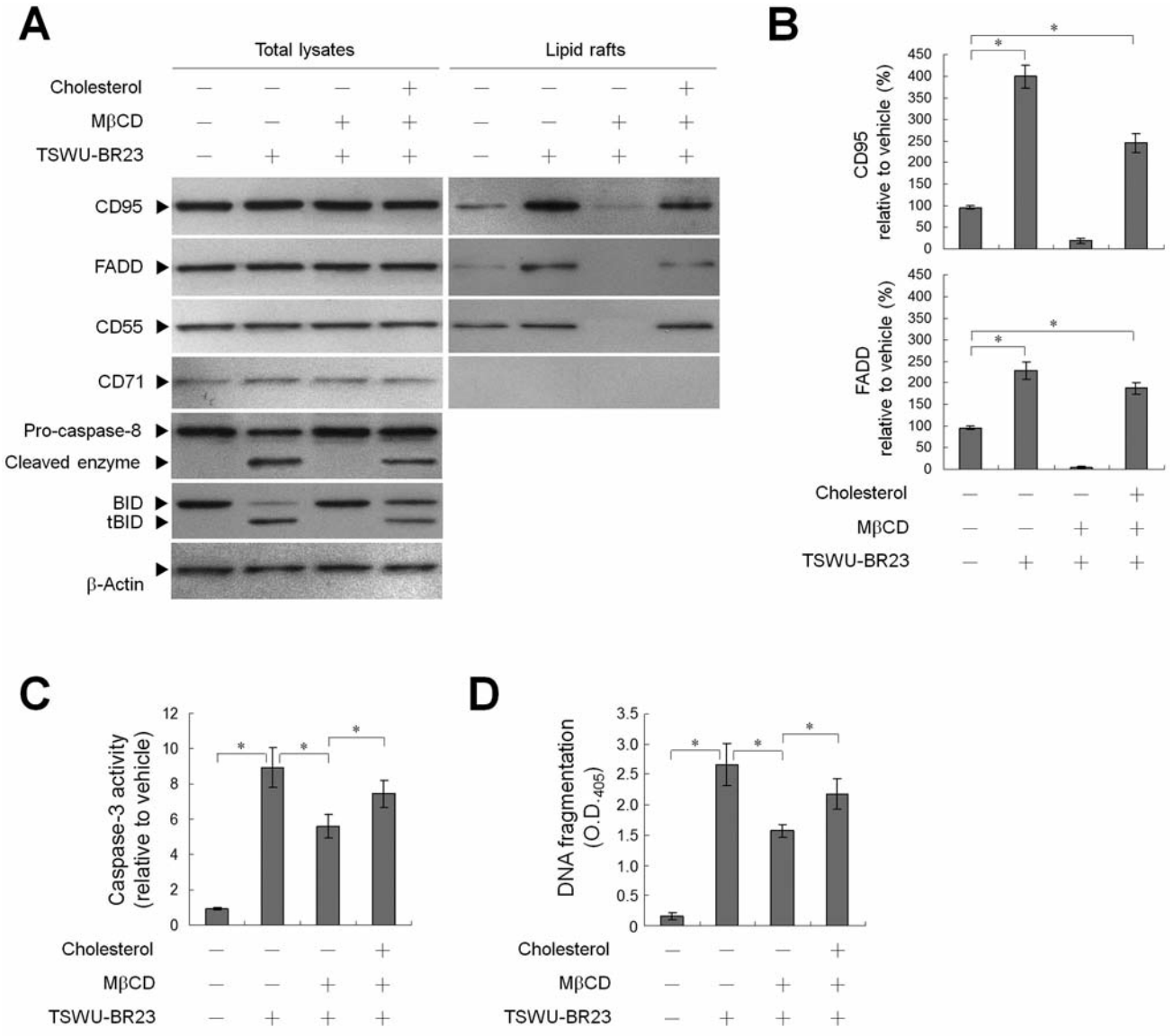

Figure 2. *The lipid raft-disrupting agent methyl-β-cyclodextrin (MβCD) inhibits the formation of the lipid raft-associated cluster of differentiation 95 (CD95)/Fas-associated death domain (FADD) and apoptosis. A-D: HT-29 cells were pre-treated with vehicle or MβCD (3 μM) for 1 h at 37˚C. Following pre-treatment, the cells were treated with vehicle, TSWU-BR23 alone or in combination with cholesterol (50 μg/ml) for 36 h. The crude homogenates of the treated cells were treated with 1% Brij 98 and fractionated by density-based sucrose gradient centrifugation. The levels of the indicated proteins in the total lysates and lipid rafts were determined using specific antibodies. β-Actin was used as internal control for sample loading. DNA fragmentation and caspase-3 activities were measured using a Cell Death Detection kit and flow cytometry, respectively. The values presented are the mean±standard errors from three independent experiments. *p<0.05: Significantly different from vehicle control-treated cells, TSWU-BR23 treated cells, or MβCD pre-treated and TSWU-BR23 treated cells.*

as the mitochondrial fraction, and the supernatant was further centrifuged at 100,000 × *g* for 1 h at 4˚C. The new supernatant was saved as the cytosolic fraction, and the pellet was reserved as the endoplasmic reticulum (ER)/microsomal fraction. The resulting mitochondrial and microsomal fractions were lysed in RIPA buffer (1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 10 mM Tris-HCl [pH 8.0], and 0.14 M NaCl) for western blot analysis. The purity of each subcellular fraction was confirmed by western blotting using specific antibodies against the mitochondrial marker COX2, and the cytosolic marker β-actin.

Measurement of mitochondrial membrane potential. Mitochondrial membrane potential (ψ_m) was determined by measuring the retention of the dye 3,3'-dihexyloxacarbocyanine ($DiOC_6$). Briefly, treated cells were incubated with 40 nM $DiOC_6$ for 30 min at 37˚C. Cells were then pelleted by centrifugation at 160 × *g*. Pellets were

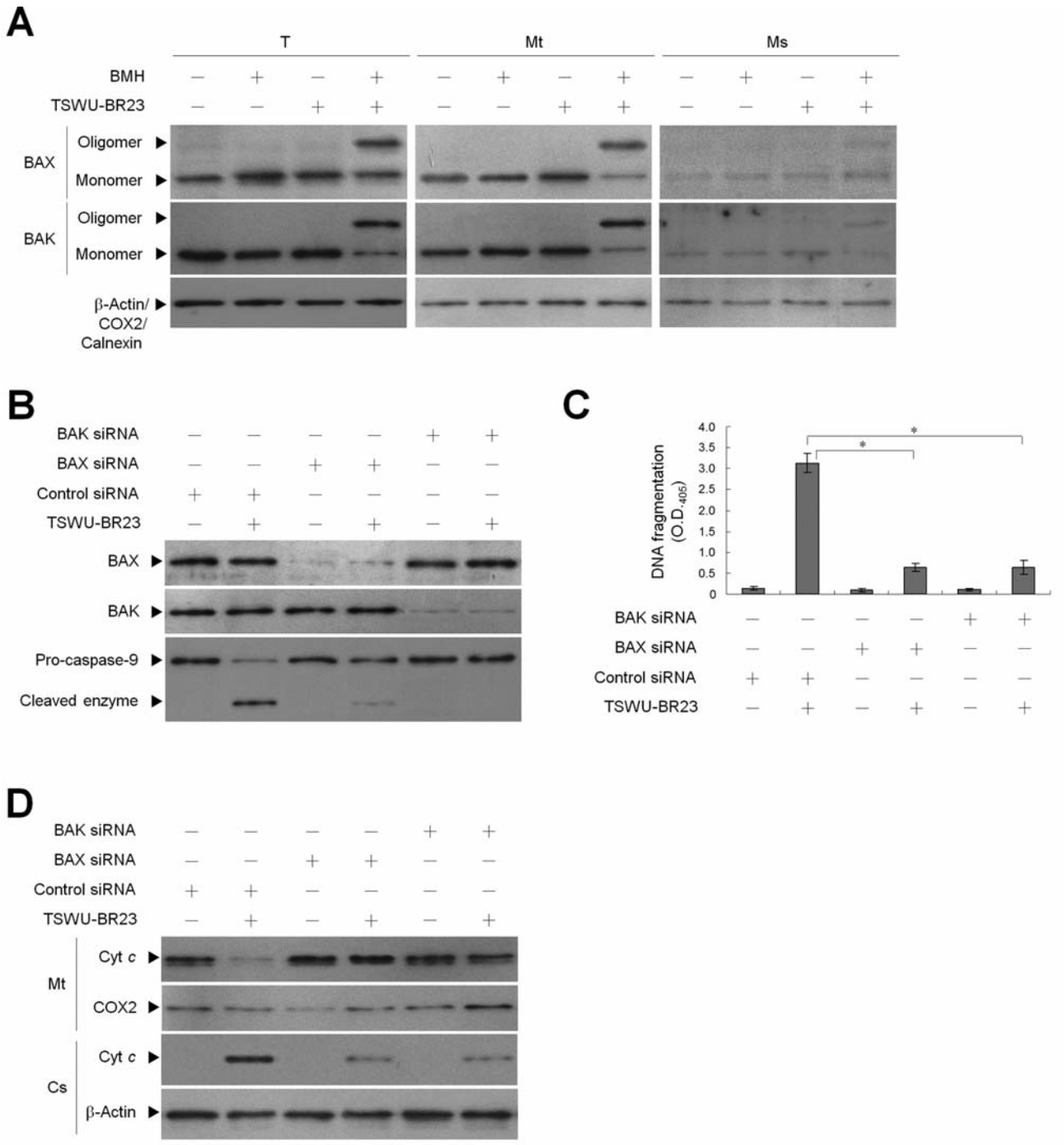

Figure 3. *TSWU-BR23 induces Bcl-2-associated x protein (BAX)/Bcl-2 antagonist killer 1 (BAK) oligomerization in mitochondria and mitochondria-dependent apoptosis. A: Cells were harvested 36 h after treatment with vehicle or TSWU-BR23, and cell pellets were re-suspended in hypotonic buffer. Crude homogenates were incubated with 5 mM BMH in phosphate buffered saline for 30 min at room temperature and then subjected to subcellular fractionation to obtain the mitochondrial (Mt) and ER/microsomal (Ms) fractions. In total, 20 μg of total protein from the recovered fractions was analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed with specific antibodies, as indicated. Cytochrome c oxidase subunit 2 (COX2), calnexin, and β-actin were used as internal controls for mitochondria, ER, and cytosol, respectively. B and C: At 12 h after transfection with control, BAX, or BAK small interfering RNA (siRNA), cells were treated with vehicle or TSWU-BR23 for 36 h. After western blotting was used to examine the BAX, BAK, pro-caspase-9, and cleaved caspase-9 levels, the DNA fragmentation was determined using a Cell Death Detection ELISA kit, respectively. *p<0.05: Significantly different from control siRNA-transfected TSWU-BR23-treated cells. D: The levels of cytochrome c (Cyt c) in the cytosol and mitochondria were determined by western blotting using specific antibodies. COX2 and β-actin were used as internal controls for the mitochondria and cytosol, respectively.*

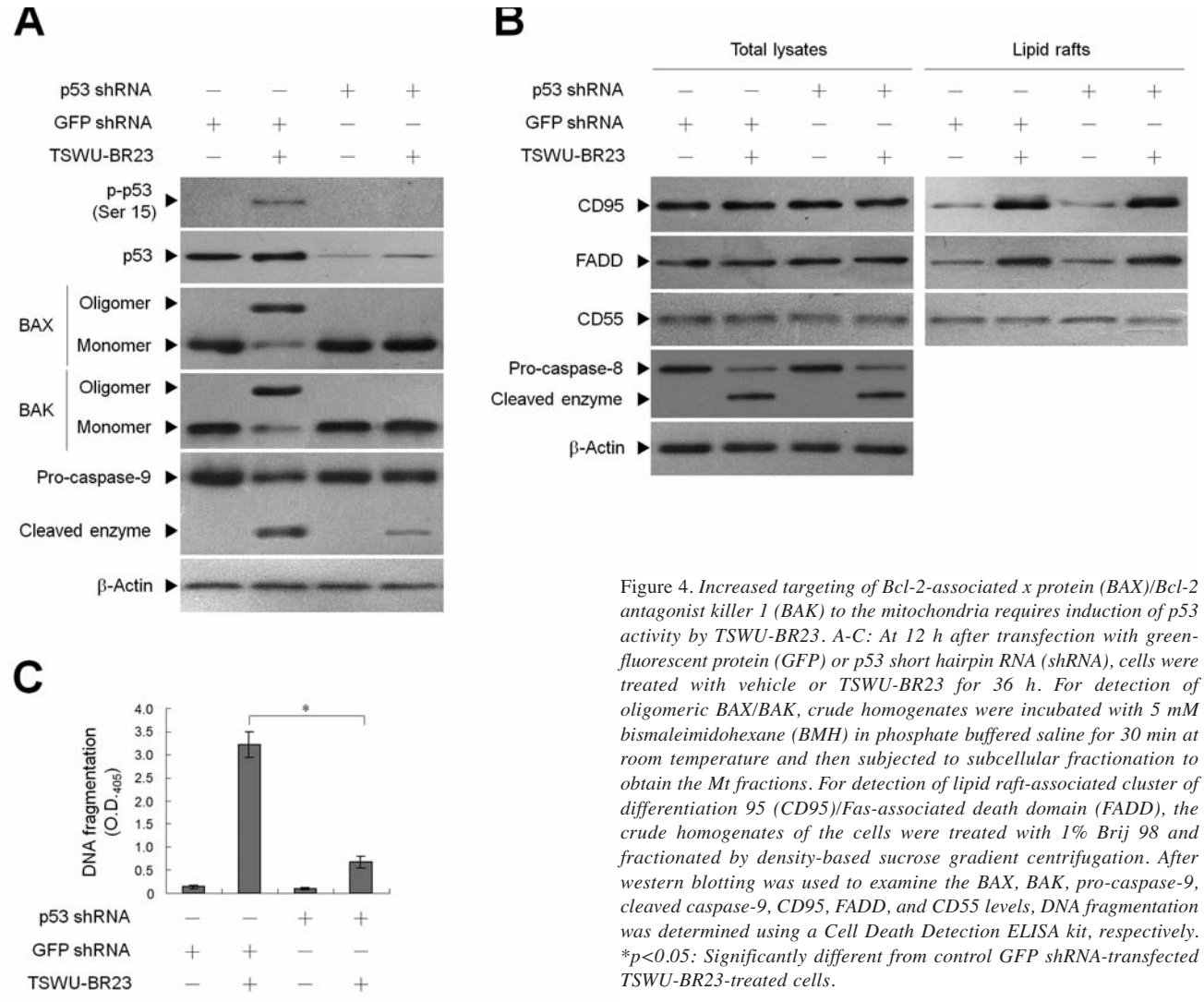

Figure 4. *Increased targeting of Bcl-2-associated x protein (BAX)/Bcl-2 antagonist killer 1 (BAK) to the mitochondria requires induction of p53 activity by TSWU-BR23. A-C: At 12 h after transfection with green-fluorescent protein (GFP) or p53 short hairpin RNA (shRNA), cells were treated with vehicle or TSWU-BR23 for 36 h. For detection of oligomeric BAX/BAK, crude homogenates were incubated with 5 mM bismaleimidohexane (BMH) in phosphate buffered saline for 30 min at room temperature and then subjected to subcellular fractionation to obtain the Mt fractions. For detection of lipid raft-associated cluster of differentiation 95 (CD95)/Fas-associated death domain (FADD), the crude homogenates of the cells were treated with 1% Brij 98 and fractionated by density-based sucrose gradient centrifugation. After western blotting was used to examine the BAX, BAK, pro-caspase-9, cleaved caspase-9, CD95, FADD, and CD55 levels, DNA fragmentation was determined using a Cell Death Detection ELISA kit, respectively. *p<0.05: Significantly different from control GFP shRNA-transfected TSWU-BR23-treated cells.*

resuspended and washed twice with PBS. The $\Delta\psi m$ was determined with a FACSCount flow cytometer (8).

Detection of reactive oxygen species (ROS). Briefly, treated cells were then resuspended in 500 μl of 2,7-dichlorodihydrofluorescein diacetate (10 μM) and incubated for 30 min at 37˚C. The level of ROS was determined using a FACSCount flow cytometer (8).

Plasmid and siRNA transfection. Cells (at 60-70% confluence in a 12-well plate) were transfected with the p53 sh RNA or dn FADD expression plasmid or with BAX, BAK, or control siRNA using Lipofectamine 2000. The expression of p53, dn FADD, BAX, and BAK in transfected cells was assessed by western blotting using antibodies specific to p53, FADD, BAX, and BAK.

Density-based membrane flotation technique. Detergent-resistant membranes were prepared as described previously (7). Briefly, treated cells were washed twice in ice-cold PBS before being removed from dishes by scraping. Cells were then harvested by centrifugation, resuspended in 1 ml of hypotonic lysis buffer [10 mM Tris (pH 7.5), 10 mM KCl, 5 mM $MgCl_2$] containing 1% Brij 98, incubated at 37˚C for 5 min, and ruptured by passage through a 25-gauge hypodermic needle 20 times. Unbroken cells and nuclei were removed by centrifugation at 1,000 × *g* for 5 min in a microcentrifuge at 4˚C. The crude homogenates were kept on ice for an additional 5 min, mixed with 3 ml of 72% sucrose, and overlaid with 4 ml of 55% sucrose and 1.5 ml of 10% sucrose; all sucrose solutions were dissolved in low-salt buffer (50 mM Tris-HCl [pH 7.5], 25 mM KCl, 5 mM $MgCl_2$). Samples were centrifuged for 14 h in a Beckman SW41 rotor at 280,000 × *g* and 4˚C. Fractions were collected from the top of the gradient in 1-ml increments and concentrated to approximately 100 μl by passage through a 50-kDa Centricon filter (Millipore, Bedford, MA, USA). The purity of detergent-resistant membranes fractions and detergent-soluble membrane fractions was confirmed by western blotting using specific antibodies against the lipid raft marker CD55, and the non-lipid raft marker CD71.

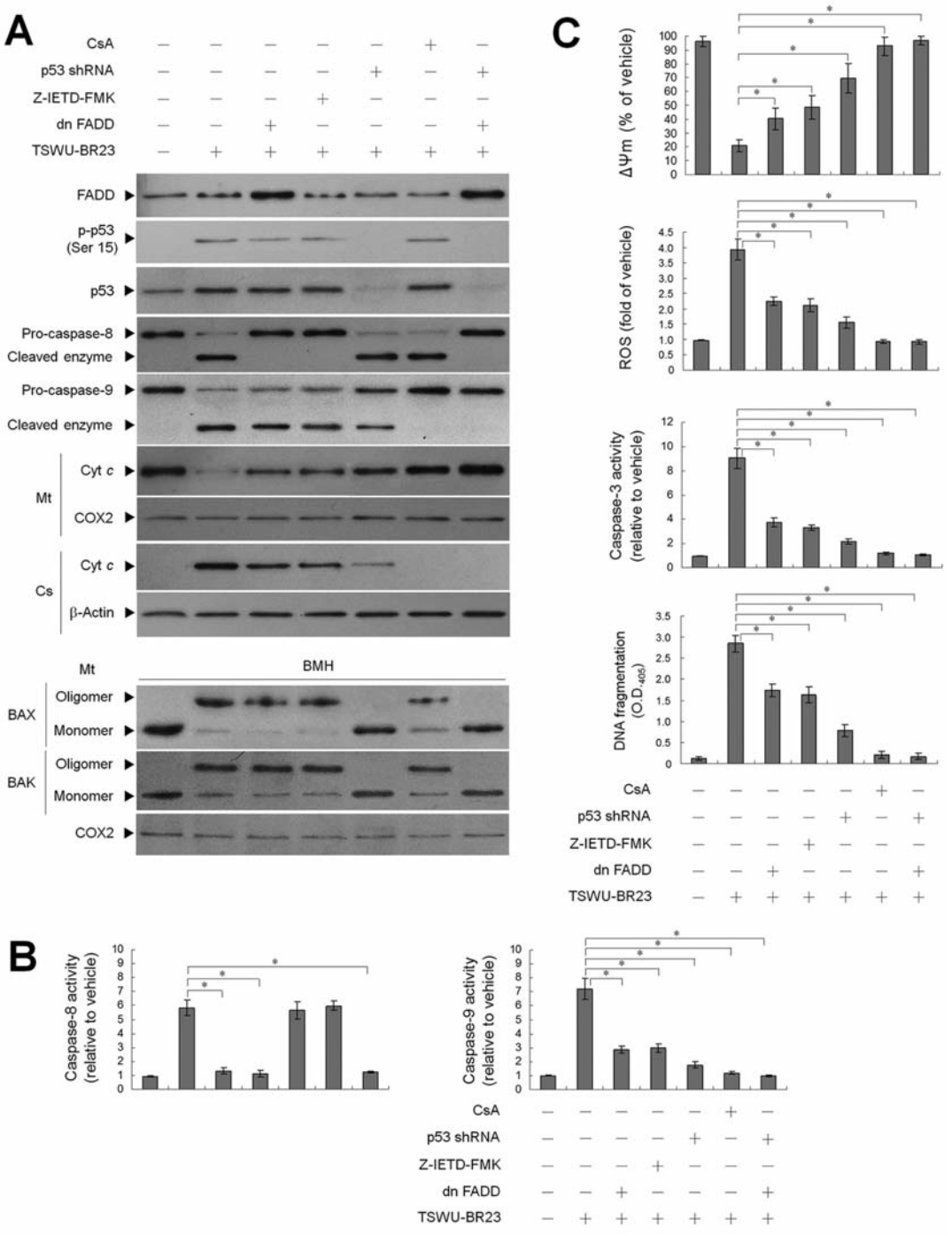

Figure 5. *Involvement of lipid raft-associated cluster of differentiation 95 (CD95)/Fas-associated death domain (FADD)-mediated caspase-8 activity and p53-mediated Bcl-2-associated x protein (BAX)/Bcl-2 antagonist killer 1 (BAK) apoptotic activity in TSWU-BR23-induced mitochondrial apoptosis. A-C: At 12 h after transfection with vector alone, or the dominant-negative mutant FADD (dn FADD) or p53 short hairpin RNA (shRNA) expression vector, cells were treated with either vehicle, TSWU-BR23, caspase-8 inhibitor Z-IETD-FMK, or cyclosporin A (CsA) for 36 h. The levels of the indicated proteins in the total lysates and mitochondrial (mt) were determined by western blot analysis using specific antibodies. The levels of cytochrome c (Cyt c) in the cytosol (Cs) and mitochondria were determined by western blotting using specific antibodies. Cytochrome c oxidase subunit 2 (COX2) and β-actin were used as internal controls for the mitochondria and cytosol, respectively. Caspase-8 and -9 activities were analyzed using spectrophotometry. The decrease in 3,3'-dihexyloxacarbocyanine Iodide fluorescence was measured by flow cytometry. The generation of reactive oxygen species (ROS) was monitored by measuring increased fluorescence of Indo-1 by flow cytometry. DNA fragmentation and caspase-3 activities were measured using a Cell Death Detection kit and flow cytometry, respectively. The values presented are the mean±standard errors from three independent experiments. *p<0.05, significantly different from vehicle control treated cells, TSWU-BR23 treated cells.*

Statistical analysis of data. Statistical calculations of the data were performed using the unpaired Student's *t*-test and ANOVA analysis. $p<0.05$ was considered statistically significant.

Results

TSWU-BR23 induces lipid raft localization of CD95/FADD at the cell surface of human colon cancer HT-29 cells. To investigate whether TSWU-BR23 treatment induces CD95/FADD-lipid raft co-cluster in HT-29 cells, we used 1% Brij 98 solubilization of TSWU-BR23-treated cells and density-based membrane flotation technique to isolate detergent-resistant membranes, which are thought to be lipid rafts based on their composition and properties (9). Western blot analysis showed that TSWU-BR23 treatment did not affect the levels of CD95 and FADD expressed by cells, whereas the treatment increased CD95 and FADD levels in lipid rafts by decreasing the amounts of CD95 and FADD in the cytosol (Figure 1A). Quantitation of immunoblot band intensities from the lipid raft and non-lipid raft fractions revealed that the levels of CD95 and FADD in lipid rafts were increased by an average of 4.1-fold and 2.5-fold after treatment with TSWU-BR23 relative to vehicle-treated cells (Figure 1B). To further examine the effect of TSWU-BR23 on the cell surface localization of CD95 and FADD, we used 1% Brij 98 solubilization of biotinylated cells to isolate lipid rafts and cell surface proteins. Figure 1C shows that a small amount of CD95 and FADD was detected in cells treated with vehicle. With TSWU-BR23 treatment, the levels of CD95 and FADD were increased 3.7-fold and 2.1-fold, respectively (Figure 1D). The results indicate that TSWU-BR23 can induce localization of CD95 and FADD at the cell surface lipid rafts.

To verify the lipid raft localization of CD95/FADD at the cell surface, cells were pre-treated with methyl-β-cyclodextrin (MβCD). MβCD is a water-soluble cyclic oligosaccharide containing a hydrophobic cavity, which enables a selective extraction of cholesterol from the cell membrane to disrupt lipid rafts (10). Pre-treatment of cells with MβCD completely disrupted CD55 association with membrane lipid rafts without interfering with the expression of CD55 (Figure 2A). As a consequence, MβCD blocked lipid raft localization of CD95/FADD, cleavage of caspase-8 and BID, and apoptosis induced by TSWU-BR23; however, MβCD did not completely inhibit TSWU-BR23-induced induction of caspase-3 activity and apoptosis (Figures 2A-D). Cholesterol supplementation significantly reversed TSWU-BR23-induced effects on the induction of the clustering of CD95/FADD in lipid rafts, caspase-3 activity, pro-caspase-8 and BID cleavage, and apoptosis (Figures 2A-D). These results suggest the involvement of the lipid raft localization of CD95/FADD at the cell surface in TSWU-BR23-induced apoptosis.

Oligomerization of p53-mediated BAX/BAK in mitochondria is important for TSWU-BR23-induced mitochondrial apoptosis of HT-29 cells. In response to apoptotic stimuli, BAX and BAK change their conformations to forms oligomers that associate with the mitochondrial membrane (2). We sought to determine whether apoptosis involved the oligomerization of BAX and BAK in mitochondria. To this end, the sulfhydryl-reactive agent BMH was applied to crosslink the oligomerized proteins in treated cells, followed by isolation of mitochondrial and ER subcellular fractions. As shown in Figure 3A, TSWU-BR23 treatment increased the mitochondrial localization and oligomerization of BAX/BAK. Similarly, a small amount of oligomerized BAX/BAK was detected in the ER. To address whether oligomerized BAX/BAK modulates TSWU-BR23-induced apoptotic activity, cells were transfected with siRNA specific to BAX or BAK transcript. The level of BAX or BAK protein was decreased after transfection with siRNA, which was accompanied by inhibition of TSWU-BR23-induced pro-caspase-9 cleavage, mitochondrial cytochrome c release, and apoptosis (Figures 3B-D), indicating that pro-apoptotic activity of BAX and BAK was required for TSWU-BR23-induced mitochondrial cell death.

It has been documented that p-p53 (Ser 15) can induce conformational change in BAX/BAK, which converts these proteins into oligomeric forms, leading to their localization to the mitochondria and the subsequent induction of apoptosis (11-13). To explore whether increased targeting of BAX/BAK to the mitochondria by TSWU-BR23 requires p53 activity, we examined the expression and phosphorylation levels of p53 protein. The protein level of p53 appeared up-regulated in control GFP shRNA-transfected, TSWU-BR23-treated cells. p53 phosphorylation on Ser 21 was also induced by TSWU-BR23 (Figure 4A). The treatment of cells with p53 shRNA resulted in suppression of TSWU-BR23-induced BAX/BAK oligomerization, pro-caspase-9 cleavage, and apoptosis. However, p53 shRNA transfection had no detectable effect on the inhibition of the TSWU-BR23-induced CD95/FADD lipid raft localization and pro-caspase-8 cleavage (Figures 4A-C). These finding indicate that increased p53 activity is required for TSWU-BR23-induced apoptotic potency of BAX/BAK in the mitochondria but not for the induction of the recruitment of CD95/FADD-caspase-8 apoptotic signaling in lipid rafts.

Lipid raft-associated CD95/FADD-mediated caspase-8 activity and p53-mediated BAX/BAK oligomerization in TSWU-BR23-induced apoptosis. We next investigated whether both lipid raft-associated CD95/FADD and p53-mediated BAX/BAK apoptotic activities were acting in connection with TSWU-BR23-induced apoptosis. Ectopic expression of dn FADD lacking the caspase-8-binding site, similar to that of inhibition by p53 shRNA or caspase-8 inhibitor Z-IETD-FMK treatment, does not completely suppress TSWU-BR23-induced loss of ψm and increase in cellular ROS production, procaspase-9 cleavage,

mitochondrial cytochrome c release, caspase-3 activation, and apoptosis, although p53 shRNA appeared to have a higher inhibitory effect on mitochondria-mediated apoptosis (Figure 5). The alteration of ψm and ROS production, and induction of the release of cytochrome *c* from mitochondria, procaspase-9 cleavage, and caspase-3 were dependent on FADD- and p53-mediated activities, since transfection of both p53 shRNA and dn FADD completely blocked TSWU-BR23-induced mitochondrial apoptosis (Figure 5). Co-treatment with cyclosporine A also completely inhibited the increasing level of ROS, mitochondrial cytochrome *c* release, pro-caspase-9 cleavage, caspase-3 activity, and apoptosis, as well as reducing the ψm by TSWU-BR23 (Figure 5). These findings indicate that both lipid raft-associated CD95/FADD-mediated caspase-8 activity and p53-mediated BAX/BAK apoptotic activity were required for TSWU-BR23-induced mitochondrial apoptosis in HT-29 cells.

Discussion

Although there are major differences in the activation mechanism and the subsequent enzymatic cascade of caspases, both extrinsic and intrinsic pathways can converge into the mitochondrial apoptotic death pathway. Based on accumulating evidence, intrinsic pathway is linked to the mitochondrial apoptotic death pathway through caspase-8-mediated cleavage of BID into tBID (14). In the present study, we showed that TSWU-BR23-induced loss of ψm and increase of ROS, caspase-3 and -9 activities, and apoptosis were partially inhibited by Z-IETD-FMK co-treatment, and dn FADD overexpression. p53 shRNA efficiently inhibited mitochondrial apoptosis at a low level and completely blocked the mitochondrial oligomerization of BAX/BAK, but had no influence on the TSWU-BR23-induced CD95/FADD-mediated caspase-8 activity. Although tBID has been shown to be capable of inducing mitochondrial translocation and oligomerization of BAX/BAK, thereby promoting release of mitochondrial cytochrome *c* (15-17), mitochondrial dysfunction and ROS generation can occur dependently of the induction of caspase-3 activity (18). We observed that the blockade of caspase-8 activation by Z-IETD-FMK or dn FADD did not appear to affect the TSWU-BR23-induced formation of BAX/BAK oligomer. These findings suggest that capapse-8 acts downstream of lipid raft-associated FADD and that activation of caspase-8 was responsible for BID cleavage and strengthening of TSWU-BR23-induced p53-mediated mitochondrial apoptosis but not for induction of BAX/BAK dimerization. How TSWU-BR23 leads to on our observation that the localization of CD95/FADD with lipid rafts at cell surface is required for the induction of caspase-8 activity in HT-29 cells remains to be determined.

Our findings show that Ser 15-phosphorylated p53 contributes to TSWU-BR23-induced mitochondrial oligomerization of BAX/BAK. Although caspase-8-mediated tBID activity is correlated with the induction of BAX/BAK oligomerization in mitochondrial outer membrane (15), silencing of p53 expression by shRNA suppressed TSWU-BR23-induced formation of BAX/BAK oligomer. TSWU-BR23-induced Ser 15 phosphorylation of p53 does not depend on the extrinsic pathway of caspase-8 activation, because the ectopic expression of dn FADD or the treatment of cells with Z-IETD-FMK did not attenuate the phosphorylation of p53 on Ser 15. Mitochondrial p-p53 (Ser 15) was shown to be required for its mitochondrial translocation and for its interactions with the BAK–BCL-X_L complex to subsequently activate BAK, which contributes to its capacity for mitochondria-dependent apoptosis (13). There exist reports that the phosphorylation status of cytoplasmic p53 is associated with its transcription-independent apoptotic activity (19, 20), which raises the question as to whether TSWU-BR23-induced apoptosis mediates p53 phosphorylation independently of its target gene induction. A pharmacological approach provided evidence that extracellular signal-regulated protein kinase (ERK) activity was required for p53-Ser 15 phosphorylation to induce the pro-apoptotic action of p53 in mitochondria (21, 22). A study using epithelial cells of mouse mammary gland found that Myc-induced AMP-activated protein kinase (AMPK) activity is able to induce p53 to form p-p53 (Ser 15) in the mitochondria, thereby triggering BAK or BAX oligomerization (13). Therefore, the possibility that ERK or AMPK activity may regulate p53 activity via Ser 15 phosphorylation must be considered.

The result provided by western blot analysis of subcellular fractions in the present study showed that the oligomerization of BAX/BAK in the ER was induced by TSWU-BR23. Although we do not exclude the possibility that ER-associated oligomeric BAX/BAK is involved in apoptosis induction, we did not observe any significant induction of unfold protein response (UPR) regulator GRP78, UPR-activated transcriptional factor CHOP, or ER-associated pro-caspase-12 cleavage in the cells treated with TSWU-BR23. ER stress can induce conformational changes in BAX and BAK, which converts these proteins into oligomeric forms, leading to their localization to the ER and subsequent induction of pro-apoptotic activity (23). The physiological relevance of the TSWU-BR23-induced ER-associated oligomeric BAX/BAK needs to be further investigated.

In summary, the present study showed that TSWU-BR23-induced apoptosis of HT-29 cells is dependent on the p-p53 (Ser 15)-mediated oligomerization of BAX/BAK in the mitochondria and lipid raft-associated CD95/FADD-regulated caspase-8 activity. The characterization of this mechanism in human colon cancer cells may provide a theoretical basis for utilizing the bichalcone analog TSWU-BR23 to treat cancer.

Conflicts of Interest

The Authors disclose that there exist no financial or personal relationships with other people or organizations that could inappropriately influence (bias) our work

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