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Identification of Inhibitors Against BAK Pore Formation using an Improved *in vitro* Assay System

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The pro-apoptotic BCL-2 family protein BID activates BAK and/or BAX, which form oligomeric pores in the mitochondrial outer membrane. This results in the release of cytochrome *c* into the cytoplasm, initiating the apoptotic cascade. Here, we utilized liposomes encapsulating sulfo-rhodamine at a controlled temperature to improve upon a previously reported assay system with enhanced sensitivity and specificity for measuring membrane permeabilization by BID-dependent BAK activation. BAK activation was inhibited by BCL-X_L protein but not by a mutant protein with impaired anti-apoptotic activity. With the assay system, we screened a chemical library and identified several compounds including trifluoperazine, a mitochondrial apoptosis-induced channel blocker. It inhibited BAK activation by direct binding to BAK and blocking the oligomerization of BAK.

Key Words : BAK, BAX, Trifluoperazine, Apoptosis, High throughput

Introduction

BAK (Bcl-2 homologous antagonist killer) and BAX (Bcl-2 associate X protein) serve as a critical control point of apoptosis in the mitochondrial cell death pathway.[1,2] They are members of the BCL-2 (B-Cell Lymphoma-2) family[2] and normally remain inactive in the mitochondrial outer membrane and in the cytosol, respectively. Upon arrival of cell death signals, which are mediated by other members of the BCL-2 family such as BID, BIM, or PUMA, BAK is set free from VDAC2 (an isoform of the voltage-dependent anion channel)[3,4] and forms oligomeric pores. Similarly, BAX forms oligomeric pores in the mitochondrial outer membrane upon liberation from Ku70 protein.[5] It is through these pores that cytochrome *c* and other apoptosis-promoting factors are released from the mitochondrial inter-membrane space into the cytoplasm where they initiate protease activation cascades, leading to cell death.[6] Ischemic brain damage after stroke as well as neuronal cell death in neurodegenerative diseases occur *via* this pathway.[7,8] Thus, BAX/BAK serves as a critical control point of apoptosis in the mitochondrial cell death pathway.[9] Information on the structure of BAK/BAX and the mechanism of pore formation has been accumulated. However, the molecular details of conformational change of BAK/BAX during pore formation and the atomic structure of the pore in membrane are still elusive. Inhibitors of BAX channel[10-13] along with BAX-inhibiting peptides derived from Ku70[14,15] have been shown to have cytoprotective effects in certain animal models, demonstrating BAX/BAK as a potential therapeutic target for minimizing apoptotic processes in diseases such as stroke.[7]

We previously reported a method for the recapitulation of mitochondrial apoptotic pore formation induced by BID and BAK in an artificial membrane system.[16] In this study, we developed a high throughput assay system capable of identifying compounds that inhibit the functions of BAK after improving the sensitivity of the assay by encapsulating highly concentrated sulforhodamine B (Sulfo-Rho) (Fig. 1). With this assay method, we screened a chemical library consisted of drug compounds and identified trifluoroperazine

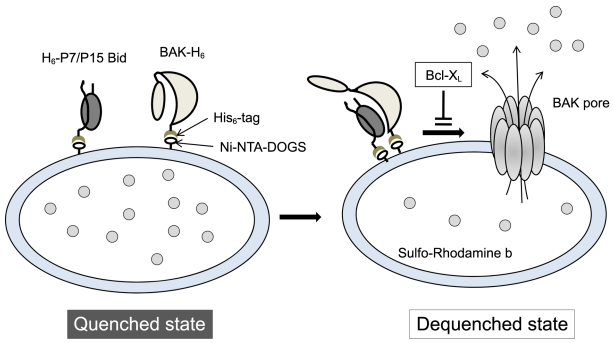

Figure 1. Schematic representation of BID-induced formation of BAK pore and the liposome dye release assay. N-terminally His_6-tagged p7/p15 BID and C-terminally His_6-tagged soluble form of BAK are targeted to the membrane *via* affinity of the His-tag for the Ni-NTA moieties present on the surface of liposomes that encapsulate the fluorescent dye Sulfo-Rho. p7/p15 BID activates BAK *via* BH3 domain engagement, triggering BAK oligomerization and release of Sulfo-Rho (grey dots) through the pores.

Abbreviations: BCL-2, B-Cell Lymphoma-2; LUV, large unilamellar vesicles; Sulfo-Rho, sulforhodamine B; TFP, trifluoperazine; POPC, 1-Palmitoyl-2-oleoyl-*sn*glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl*sn*-glycero-3-phosphoethanolamine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; DOGS-NTA-Ni, 1,2-dioleoyl-*sn*-glycero-3-{[*N*-5-amino-1-carboxylpentyl)-iminodiacetic acid]succinyl}(nickel salt); ITC, isothermal titration calorimetry

(TFP) and other compounds as inhibitors against the leakage of Sulfo-Rho from BAK pores. Furthermore, we showed that the oligomerization of BAK after the activation of BID protein was prevented by these compounds. These results advance our understanding of the mechanism of action of this compound and further provide a convenient method for the high throughput screening of chemical compounds capable of inhibiting BAK pore formation.

Materials and Methods

Materials. 1-Palmitoyl-2-oleoyl-*sn*glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl*sn*-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS), beef heart cardiolipin, cholesterol, and 1,2-dioleoyl-*sn*-glycero-3-{[*N*-5-amino-1-carboxylpentyl)-iminodiacetic acid]succinyl} (nickel salt) (DOGS-NTA-Ni) were purchased from Avanti Polar Lipids, Inc (AL, USA). Sulforhodamine B (Sulfo-Rho hereafter) was obtained from Invitrogen (NY, USA). Trifluoperazine were from Sigma-Aldrich (MO, USA). A commercial chemical library, NINDS Custom Collection II was purchased from MicroSource Discovery Systems Inc. (Gaylordsville, CT, USA). All other chemicals were reagent grade.

Expression and Purification of Recombinant Proteins. The hexahistidine-tagged soluble mouse BAK (sBAK-ΔC-His), glutathione sulfur-transferase fusion protein of BCL-X_L (residues 3–212) with C-terminal transmembrane domain substituted with a hexahistidine tag (GST-BCL-X_LΔC-His (wt)), and its G138E/R139L/I140N triple amino acid substitution mutant (GST-BCL-X_LΔC-His (m8)) which is incapable of heterodimerization with other BCL-2 proteins were prepared as described.[15] p7/p15 BID or tBID, activated forms of p22BID, was prepared from p22BID protein after cleavage with caspase-8, as described.[17,18] All protein preparations were stored in 18% glycerol, 20 mM HEPES buffer (pH 7.2), and 150 mM KCl at −80 °C. Protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce)[19] with bovine serum albumin as a standard.

Preparation of Lipid Vesicles. Lipid mixture (total 2 mg) was prepared as a thin film in glass test tubes under nitrogen gas and dried under vacuum for an additional 16 h. The weight ratio of POPC, POPE, POPS, cholesterol, cardiolipin, and DOGS-NTA-Ni in the lipid mixture was 36:22:9:8:20:5. The dried lipids were resuspended in 0.4 mL of 100 mM Sulfo-Rho, 20 mM HEPES, and 150 mM KCl (pH 7.0), freeze-thawed three times, and extruded 11 times through a polycarbonate membrane with a pore size of 100 nm using an extruder (Avanti Polar Lipids) for the preparation of large unilamellar vesicles (LUVs).[20] Liposomes encapsulating Sulfo-Rho were recovered by ultracentrifugation at 110,000 × g for 20 min using an airfuge (Beckman, USA). The free fluorescence dyes in the liposome preparations were removed by three additional ultracentrifugations.

Liposomal Release Assay. The fluorescence dequenching assays described by Terrones *et al.*[21] and Kuwana *et al.*[22,23] were adapted to assess the biological activities of sBAK-ΔC-His and tBID or p7/p15 BID. BAK pore formation was initiated by adding sBAK-ΔC-His protein to the mixture of liposomes and p7/p15 BID (or tBID). The release of Sulfo-Rho from the LUVs was monitored by fluorometry (Cary Eclipse, Varian) using a 1-cm path length quartz cuvette at 25 °C or at the indicated temperatures. Excitation and emission wavelengths were 565 and 585 nm for Sulfo-Rho (slits, 5 nm). The extent of marker release was quantified on a percentage basis according to the following equation: $((F_t - F_0)/(F_{100} - F_0) \times 100)$, where F_t is the measured fluorescence of reagent-treated LUVs at time t, F_0 is the average fluorescence value of the LUV suspension for the initial 1–2 min before the addition of BAK protein, and F_{100} is the average fluorescence value for the final 1–2 min after complete disruption of LUVs by addition of Triton X-100 (final concentration, 0.1%). Lipid concentration was 50 mg/mL unless otherwise stated.

Screening of BAK inhibitor. To screen the library for BAK inhibitor, 1 μL of dimethylsulfoxide solution containing 10 mM of the chemical compound was added to 150 μL reaction mixture (p7/p15 BID BID protein 20 nM, Sulfo-Rho-charged liposome 50 μg/mL, sBAK-ΔC-His 50 nM, 20 mM HEPES, 150 mM KCl, pH 7.2). After 10 min of incubation, the intensity of the fluorescence emission at 585 nm was measured at an excitation wavelength of 565 nm using a fluorimeter (Cary Eclipse, Varian).

Isothermal Titration Calorimetry (ITC). ITC analysis of the binding of TFP to sBAK-ΔC-His was carried out using an ITC-200 microcalorimeter (MicroCal, USA). Aliquots of TFP (2.1 mM) were added at an interval of 2 min to the reaction chamber filled with sBAK-ΔC-His protein (50 μM) with gentle stirring using a computer controlled micro-syringe system. The binding stoichiometry and binding constants were calculated by fitting the data to one site binding model using GraphPad Prizm (version 5.2, GraphPad Software, Inc.).

Cross-linking. LUVs (0.5 mg lipid/mL) that consist of POPC, POPE, POPS, cholesterol, cardiolipin, and DOGS-NTA-Ni were prepared as described above without Sulfo-Rho and incubated with sBAK-ΔC-His (1 μM) and p7/p15 BID (0.1 μM) for 3 h at 37 °C in the presence or absence of tested chemicals. The cross-linking reaction was performed for 30 min in the presence of 1% glutaraldehyde. After termination of cross-linking reaction by adding 100 mM of glycine, the proteins in the reaction mixture were separated by SDS-PAGE, and the BAK protein was analyzed by western blotting using HRP-conjugated His tag specific antibody.

Results

BID-dependent Activation of BAK Pore Assembly Can be Efficiently Measured using His-tagged Soluble BID and BAK Proteins with Ni-NTA-decorated Liposomes Encapsulating Sulfo-Rho. In the absence of BID (50 nM), membrane-targeted sBAK-ΔC-His (100 nM) marginally released the encapsulated Sulfo-Rho within 10 min at 37 °C (Fig. 2(a)), consistent with previous observations.[16] The rate of release significantly increased in the presence of p7/p15

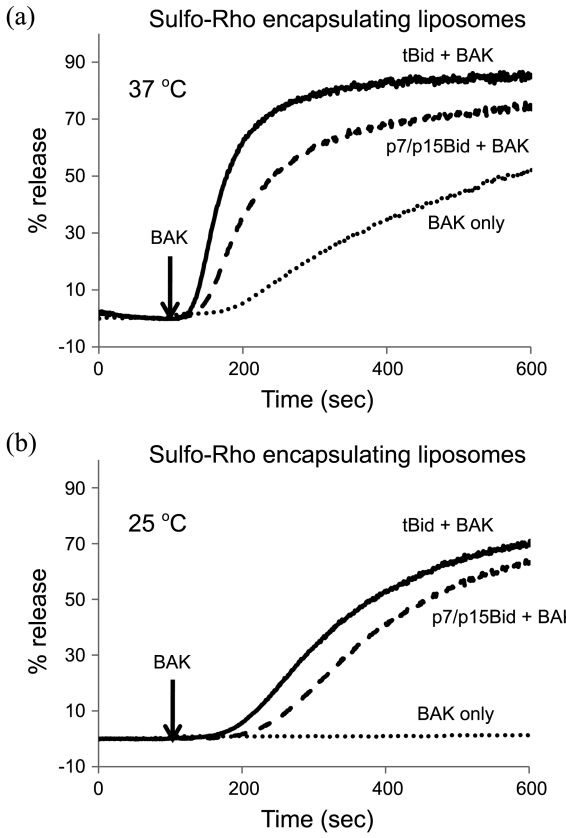

Figure 2. Temperature dependence of BID-dependent or -independent membrane permeabilization by BAK. Permeabilization of Sulfo-Rho-loaded liposomes was observed by mixing His_6-tagged sBAK (sBAK-ΔC-His, 100 nM) with liposomes (50 μg/mL) in the presence of 50 nM tBID (solid line), His_6-tagged p7/p15 BID (broken line), or without BID protein (dotted line) at 37 °C (a) and 25 °C (b).

BID or tBID (Fig. 2(a)) indicating that p7/p15 BID or tBID induced the formation of BAK pore. To differentiate between BID-dependent and -independent membrane permeabilization by sBAK-ΔC-His, the release of Sulfo-Rho was measured at lower temperatures in the presence or absence of tBID or p7/p15 BID. Any detectable release of Sulfo-Rho upon BAK pore formation without BID protein was not observed at 25 during the test period. However, BID-induced membrane permeabilization by sBAK-ΔC-His was readily observed with either tBID or p7/p15 BID (Fig. 2(b)). These results showed that BID-independent BAK pore formation could be effectively eliminated at 25 °C. With this assay condition, the spontaneous formation of BAK pore which occurs substantial rate at 37 °C without BID protein[16] could be suppressed. The signal-to-noise ratio, *i.e.*, the ratio of maximum difference in fluorescence intensity (F_{max}-F_{min}) to the background noise level in the Sulfo-Rho release measurement, was about 200. Since p7/p15 BID displayed activity comparable to that of truncated BID (tBID) in activating sBAK-ΔC-His (Fig. 2), p7/p15 BID was used for convenience in all the subsequent analyses. Using this assay system, inhibitory compounds against BID-dependent BAK pore formation could be screened.

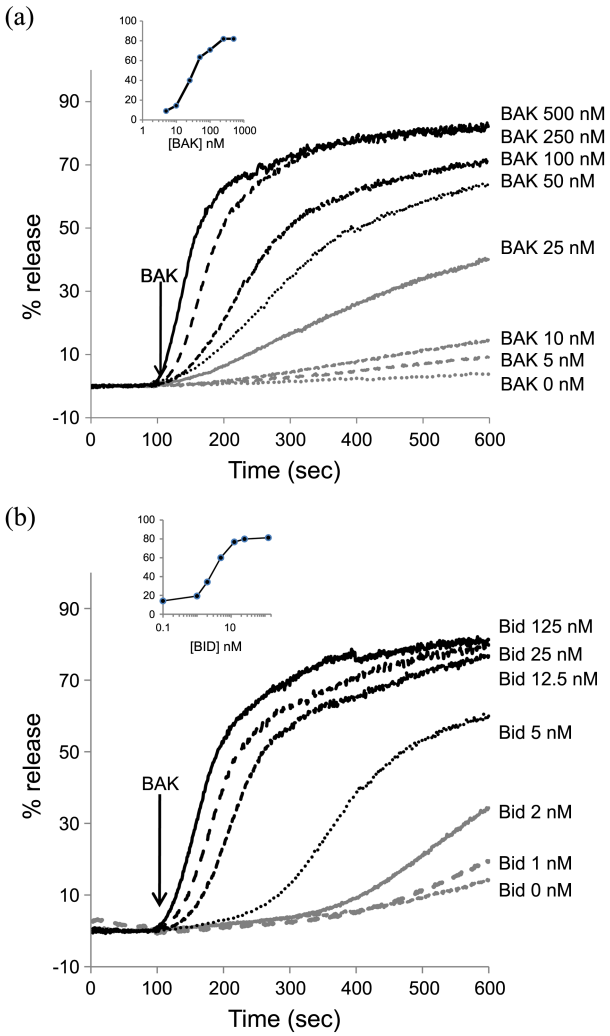

Figure 3. Dependence of membrane permeabilization on concentrations of His_6-tagged p7/p15 BID and His_6-tagged BAK. (a) Percent release of Sulfo-Rho from liposomes was monitored by mixing various concentrations of sBAK-ΔC-His (hereafter indicated as BAK) with liposomes (50 μg/mL) in the presence of 50 nM of His_6-tagged p7/p15 BID (hereafter indicated as BID) at 25 °C. The time point of BAK addition was indicated as arrow. (b) Percent release of Sulfo-Rho from liposomes was measured by mixing BAK (100 nM) with liposomes (50 μg/mL) in the presence of various concentrations of BID at 25 °C. Insets represent the maximum percent release at 10 min as a function of the concentration of BAK (a) or BID (b).

Rate of Sulfo-Rho Release from Liposomes Depends on Concentrations of BAK and BID. To determine the optimum conditions for BID-induced BAK pore formation, the release of Sulfo-Rho was examined at various concentrations of sBAK-ΔC-His and a fixed concentration of p7/p15 BID (50 nM) at 25 °C. As shown in Figure 3(a), the rate of Sulfo-Rho release was almost saturated at or above 100 nM sBAK-ΔC-His (inset of Fig. 3(a)) within 10 min. Similarly, the optimum concentration of p7/p15 BID was determined at various concentrations of p7/p15 BID at a concentration of 100 nM sBAK-ΔC-His at 25 °C (Fig. 3(b)). The release rate was saturated at or above 12.5 nM p7/p15 BID (inset, Fig. 3(b)). Based on this result, we concluded that BID-depen-

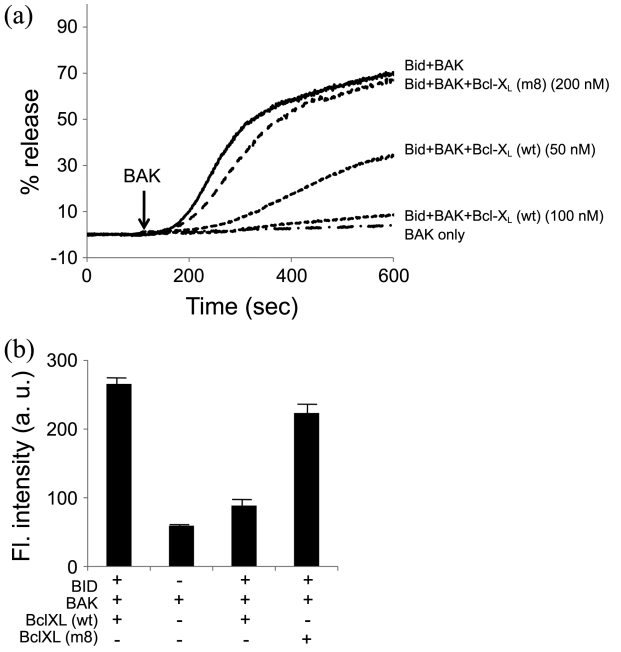

Figure 4. Inhibition of BAK-mediated membrane permeabilization by BCL-X_L. (a) Effect of an anti-apoptotic BCL-2 family protein, BCL-X_L, on BID-induced membrane permeabilization by BAK (sBAK-ΔC-His). GST-fusion protein of a soluble domain of wild-type BCL-X_L (GST-BCL-X_L-ΔChis (wt), 100 nM) or a protein with defective point mutations (GST-BCL-X_L-ΔChis (m8), 200 nM) was pre-incubated with 50 nM p7/p15 BID and Sulfo-Rho-containing liposomes (50 μg/mL). Permeabilization of liposomes was measured at 25 °C. BAK (100 nM) was added at the indicated time (arrow). (b) Fluorescence intensity of Sulfo-Rho-encapsulating liposomes after incubation for 10 min at 25 °C in the presence of various combinations of p7/p15 BID (50 nM), BAK (100 nM), BCL-XL (wt)(100 nM), or BCL-XL (m8)(200 nM).

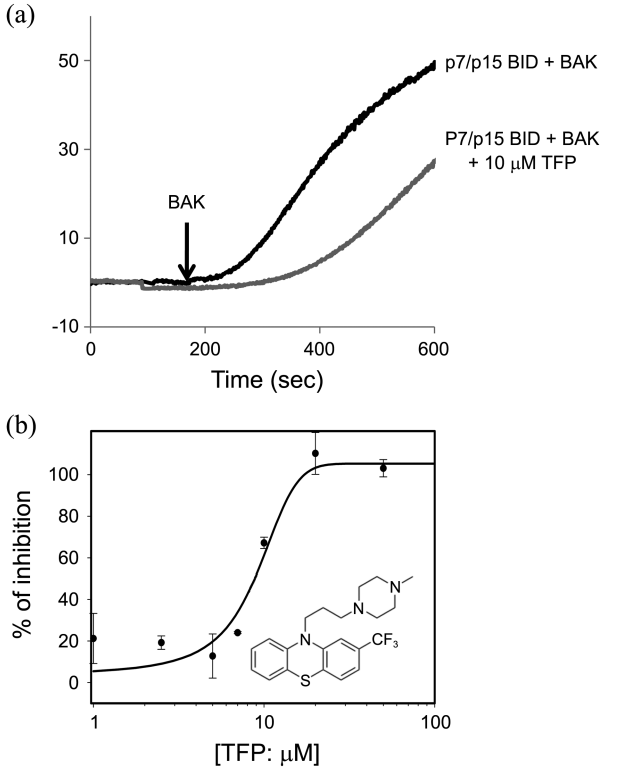

Figure 5. Inhibition of the BAK-mediated membrane permeabilization by trifluoperazine. (a) Effect of trifluoperazine on BID-dependent permeabilization by BAK. BAK (100 nM) was added at the indicated time (arrow) to Sulfo-Rho-encapsulating liposomes (50 μg/mL) in the presence of TFP (10 μM) at 25 °C. (b) Membrane permeabilization of Sulfo-Rho-loaded liposomes was measured after incubation of liposomes for 10 min at 25 °C in the presence of BID (50 nM), BAK (100 nM), and various concentrations of TFP. Inhibitory activity was quantified using the following equation: % inhibition = $100 \cdot (F_{100} - F)/(F_{100} - F_0)$, where F is the fluorescence intensity from the reagent-treated liposomes after 10 min of incubation, F_0 is the average fluorescence intensity from the liposomes treated only with BAK, and F_{100} is the average fluorescence intensity from the liposomes treated with both BAK and p7/p15 BID.

dent BAK pore formation can be optimally achieved with high sensitivity under the experimental conditions of ≥ 12.5 nM p7/p15 BID and ≥ 100 nM sBAK-ΔC-His in a short period of time.

BCL-X_L Inhibits BID-dependent Membrane Permeabilization by BAK. We next examined whether or not BCL-X_L could prevent the release of Sulfo-Rho from liposomes by inhibiting the BID-dependent membrane permeabilization by BAK. At 50 nM, a half equivalent of sBAK-ΔC-His, BCL-X_L significantly inhibited the release of Sulfo-Rho from liposomes treated with BID and sBAK-ΔC-His (Fig. 4(a)). In the presence of 100 nM BCL-X_L, Sulfo-Rho release was almost completely inhibited (Figs. 4(a) and 4(b)). In contrast, the triple amino acid substitution mutant of BCL-X_L, BCL-X_L (m8), which lacks heterodimerization capability[24], did not have any significant effect (Figs. 4(a) and 4(b)). These results confirm that the *in vitro* assay system consisting sBAK-ΔC-His and synthetic liposomes containing Sulfo-Rho is applicable for Bid-induced formation of BAK pore in membrane.

Inhibitors Against BID-dependent BAK Pore Formation were Identified. A chemical library consisted of bioactive chemicals including drug compounds was screened using the above assay system. Several compounds that reduced the release of Sulfo-Rho from liposomes from BAK pore were identified. Among them, TFP, perphenazine, and fluoxetine reduced the release of Sulfo-Rho from BAK pore by more than 90% at 67 μM. It is noticeable that TFP had been reported to block mitochondrial apoptosis-induced channel (MAC).[12] However, the inhibition mechanism of TFP against MAC has not been elucidated. Since TFP is the only compound that related to anti-apoptotic activity among the identified hits, we select TFP for detailed inhibitory mechanism against BAK pore formation. The time-dependent release of Sulfo-Rho from BAK pore was significantly retarded in the presence of TFP (Fig. 5(a)), and its IC_{50} value was further measured as 8.3 μM (Fig. 5(b)). These results indicated that TFP inhibits BAK pore either by preventing the formation of BAK pore or blocking the transport of Sufo-Rho through BAK pore.

TFP Directly Binds to BAK and Inhibits the Oligomerization of BAK. The formation of BAK pore requires the

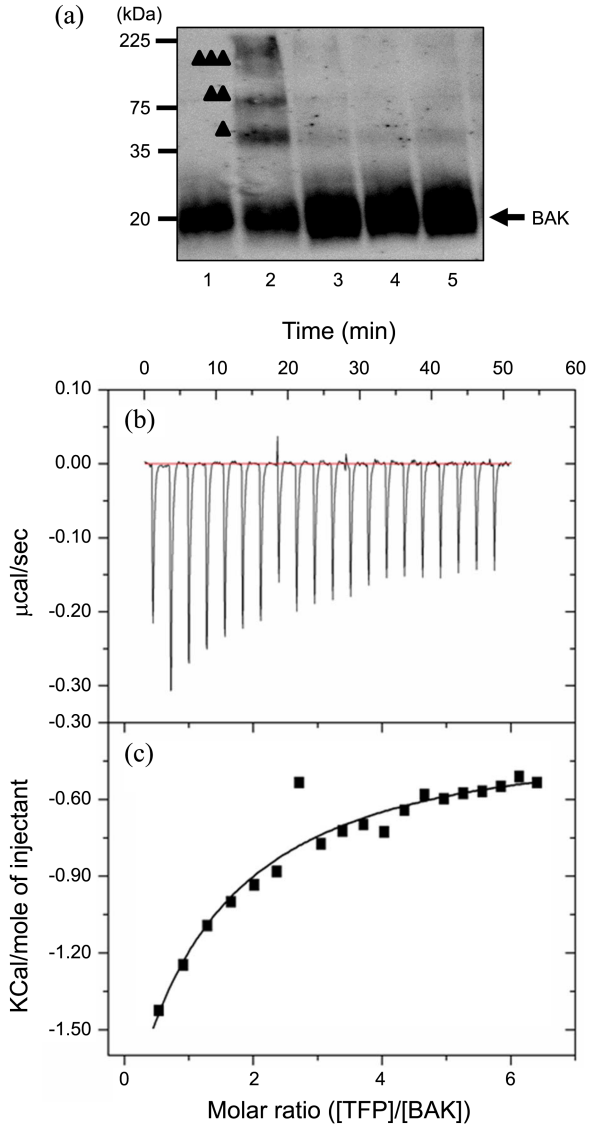

Figure 6. Effect of TFP on the oligomerization of BAK in membrane and ITC analysis of the specific binding of trifluoroperazine to sBAK-ΔC-His. (a) BAK was cross-linked with 1% glutaraldehyde in the absence of BID and liposomes (lane 1). Alternatively, BAK was mixed with LUVs containing Ni-NTA lipids at 37 ºC for 3 h in the absence (lane 2) or presence of 200 μM TFP (lane 3), perphenazine (lane 4), fluoxetine (lane 5). After cross-linking reaction with 1% glutaraldehyde, the His-tag containing BAK protein was analyzed by SDS-PAGE and western blotting using anti-His-tag antibody. (b) Calorimetric titration of BAK with TFP. Aliquots of TFP (5 μL of 2.1 mM) were injected into a sample cell containing BAK (50 μM) at 25 °C with a 120 s interval. The heat of dilution of TFP into buffer has been subtracted. (c) The plot of enthalpy versus molar ratio of TFP to BAK.

conformational change of BAK induced by BID and oligomerization of BAK to form multi-subunit pore structure in membrane.[25,26] To examine whether the identified inhibitors block the process of BAK pore formation, the BID-dependent oligomerization of BAK in the membrane was examined using chemical crosslinking. Monomeric form of BAK became oligomerized in the presence of BID and liposomes (Fig. 6(a), lane 1 and 2), and dimer (triangle), tetramer (double triangle) and highly oligomerized products (triple triangle) were observed after chemical cross-linking (Fig. 6(a), lane 2). In the presence of indentified inhibitors, however, the cross-linking products of BAK oligomer were significantly reduced (Fig. 6(a), lane 3-6), indicating that the inhibitors prevents the oligomerization process of activated BAK. We also examined whether TFP directly binds to BAK protein using isothermal titration calorimetry. As shown in Figure 6(b), a concentration dependent increase of heat was observed by mixing sBAK-ΔC-His with TFP with a dissociation constant (K_D) of 70 μM. These results suggest that TFP binds to sBAK-ΔC-His and interferes its oligomerization which requires for the formation of BAK pore.

Discussion

Anti-apoptotic BCL-2 proteins are considered to be potent targets of anticancer agents since they are overexpressed in many cancer cells, which makes them resistant to chemo- or radiotherapies.[27] Small molecule inhibitors of BCL-2, BCL-X_L, BCL-w, and MCL-1 have been developed for cancer therapy, with many currently in clinical trials.[28-30] In contrast, progress in targeting pro-apoptotic proteins such as BAX or BAK in other diseases such as stroke has been slow despite the discovery of relevant inhibitors.[10-15] For the identification of novel inhibitors against BID-dependent BAK pore formation, a quantitative assay method easily adaptable to a high-throughput format is required. In this study, we established experimental conditions in which BID-dependent membrane permeabilization by BAK can be selectively observed. Hence, a rapid multi-well screening system measuring the end-point level of release could be easily established for identifying compounds capable of inhibiting BID-dependent activation of BAK. By extension, this assay system could be used to identify inhibitors of anti-apoptotic proteins such as BCL-2 or BCL-X_L (Fig. 4). The assays suggested above could be used as an efficient 'pre-screening' method to identify compounds that can be further tested in biological screening systems utilizing cells or animal models. In this study, we observed evidence for the inhibitory mechanism of TFP on BAK, a compound previously shown to directly block the mitochondrial apoptosis-induced channel.[12] We showed that TFP prevents the oligomerization of BID-activated BAK in membrane by direct binding to BAK. The apparent K_D value of TFP to BAK is several-fold higher than the IC_{50} value of TFP on the BAK formation. This discrepancy could be due to the difference in stage of inhibition. As for the nature of TFP and other inhibitors identified in this study regarding to their inhibition site to BAK, validation of the inhibitory activity of the identified compounds other than TFP on calluar apoptosis and structural studies on the BAK-inhibitor complex are needed, which will help develop more potent inhibitors of BAK.

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