CDK inhibitors upregulate BH3-only proteins to sensitize human myeloma cells to BH3 mimetic therapies

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SUPPLEMENTAL MATERIALS AND METHODS

Co-culture of MM cells with stromal cells

The human HS-5 stromal cell line, developed from human bone marrow, was obtained from ATCC. HS-5 cells were maintained in RPMI1640 medium containing 10% FBS, and sub-cultured twice weekly by trypsinization at a subcultivation ratio of 1:5. For co-culture experiments, HS-5 cells were cultured for 48 h prior to seeding MM cells (U266/GFP). After 24h of co-culture, equal volumes of fresh medium containing the indicated drugs were added. Alternatively, conditioned medium from HS-5 cells was prepared. Briefly, after HS-5 cells were grown to 80-90% confluence, medium was harvested and centrifuged to remove cells, and the supernatant was collected as HS-5 conditioned medium (CM). For drug treatment in the presence of CM, MM cells were cultured in suspension for 48 h, and then equal volumes of CM containing drugs was added.

Flow cytometry

The extent of apoptosis was evaluated by flow cytometric analysis utilizing annexin V-FITC/PI or DiOC6/7-AAD staining. Briefly, 1x10^6 cells were stained with annexin V-FITC (BD
PharMingen, San Diego, CA) and 5μg/ml propidium iodide (PI; Sigma) in 1x binding buffer for 15min at room temperature in the dark. Samples were then analyzed by BD Biosciences FACSCalibur flow cytometry (Becton-Dickinson, San Jose, CA) within 1h to determine the percentage of cells displaying annexin V positivity. In some cases, mitochondrial injury and cell death was assessed by double staining with 40nM 3,3-dihexyloxacarbocyanine (DiOC₆; Molecular Probes Inc., Eugene, OR) and 0.5μg/ml 7-AAD (Sigma, St. Louis, MO) in PBS at 37°C for 20min, and then analyzed using a flow cytometer.

**TUNEL**

In some cases, cytospin slides were stained for TUNEL using In Situ Cell Death Detective Kit (fluorescein; Roche, Penzberg, Germany) as per the manufacturer's instructions. Images were captured using an Olympus BX40 fluorescence microscope at 20x/0.50 (Olympus America Inc, Center Valley, PA) and a CE digital camera (Alpha Innotech Cor., San Leandro, CA) with a RS Image software Version 1.7.3. (Roper Scientific Photometrics, Tucson, AZ).

**Quantitative RT-PCR (qPCR)**

qPCR analysis using TaqMan Gene Expression Assays and 7900HT Real-Time PCR System (Applied Biosystems, Foster City, CA) were performed to quantify mRNA levels of human Mcl-1 or Bim expression in MM cells. Briefly, total RNA was isolated using TRIZOL Reagent (Invitrogen, Carlsbad, CA) as per the manufacturer’s instructions. cDNA was synthesized from 1μg of total RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). 2μl of cDNA was employed for qPCR assays (TaqMan Gene Expression Assays) using a 7900HT Real-Time PCR System (Applied Biosystems). Assay IDs for Mcl-1
and Bim were Hs03043899_m1 and Hs00708019 respectively. Reference for quantitation was human β-actin or GAPDH (Pre-Developed TaqMan Assay Reagents Control Kit, Applied Biosystems). Data analyzed by using SDS 2.3 software.

**Immunoblot**

Samples were prepared from whole-cell pellets. Total protein was quantified using Coomassie Protein Assay Reagent (Pierce, Rockford, IL). Equal amount of protein (30µg) were separated by SDS-PAGE and electro-transferred onto nitrocellulose membrane. Where indicated, the blots were reprobed with antibodies against β-actin (Sigma) or α-tubulin (Oncogene, La Jolla, CA) to ensure equal loading and transfer of proteins. The following antibodies were used as primary antibodies: BH3-only Protein Detection Set, anti-Bim, anti-Noxa, anti-Bik (ProSci Inc., Poway, CA); anti-Mcl-1, anti-caspase 9, and anti-caspase 3 (BD PharMingen); anti-cleaved caspase 3 (Asp175), anti-cleaved caspase 9 (Asp315), anti-cleaved PARP (Asp214), and anti-Bcl-xL (Cell Signaling, Beverly, MA); anti-human Bcl-2 oncoprotein (DAKO, Carpinteria, CA); anti-PARP (Biomol, Plymouth Meeting, PA); anti-phospho CTD (Ser2, Covance, Princeton, NJ) and anti-RNA Pol II (Santa Cruz Biotech, Santa Cruz, CA).

**Co-immunoprecipitation**

Interactions between Bim and Bcl-2, Bcl-xL and Mcl-1 were evaluated by co-immunoprecipitation analysis. For these studies, CHAPS buffer (150mmol/L NaCl, 10mmol/L HEPES pH7.4, protease inhibitors, and 1% CHAPS) was employed to avoid artifactual associations reported with buffers containing other detergents (e.g., NP-40 or Triton X-100) (30). Briefly, cells were lysed in CHAPS buffer and 200µg of protein per condition were incubated
with 1μg anti-Bcl-2 (Dako), anti-Bcl-xL (Cell Signaling), or anit-Mcl-1 (BD PharMingen) overnight at 4°C. 20μl/condition of Dynabeads (Dynal, Oslo, Norway) were then added and incubated for an additional 4h. After washing, the bead-bound protein was eluted by vortexing and boiling in 20μl 1x sample buffer. The samples were separated by SDS-PAGE and subjected to immunoblot analysis as described above. Anti-Bim (rat, Calbiochem) was used as a primary antibody.

Subcellular fractionation

2x10^6 cells were lysed in digitonin lysis buffer. Lysates were centrifuged, and the supernatant (S-100 cytosolic fraction) was collected and added to an equal volume of 2x sample buffer. The pellets (organelle/membrane fractions) were washed once in cold PBS and lysed in 1x sample buffer. The S-100 and pellet samples were quantified, separated by SDS-PAGE, and subjected to immunoblot. For analysis of release of mitochondrial pro-apoptotic factors, anti-cytochrome c (BD PharMingen) and anti-AIF (Santa Cruz Biotechnology) were used as primary antibodies. Anti-Bax antibody (Santa Cruz Biotechnology) was employed to evaluate translocation of Bax.

Bak and Bax conformational change

Cells were lysed in 1% CHAPS buffer, and 200μg of protein were immunoprecipitated using anti-Bax (6A7, Sigma) or anti-Bak (Ab-1, Calbiochem), which only recognizes Bax or Bak that have undergone conformation change, and Dynal Beads as described above. Immunoprecipitated protein was then subjected to immunoblot analysis using anti-Bax and anti-Bak (Santa Cruz) as primary antibodies.
SureSilencing shRNA plasmids (neomycin resistance) were purchased from SABioscience (Frederick, MD), which includes shBim (human BCL2L11; clone ID #1, GAGACGAGTTTAACGCTTACT; clone ID #2, CACCCATGAGTTGTGACAAAT), shNoxa (human PMAIP1, NM021127; CTCAGCACATTGTATATGATT), and negative control shRNA (shNC, GGAATCTCATTCGATGCATAC). U266 cells were stably transfected with these constructs by using the Amaxa Nucleofector device with Cell Line Specific Nucleofector Kit C (Amaxa GmbH, Cologne, Germany) as per the manufacturer's instructions, and clones with down-regulated expression of Bim or Noxa were selected with 400μg/ml G418.