Supplemental Data

Table S1, related to Figure 1. IC_{50} values of BDA-366 analyzed by SRB assay in NSCLC cell lines

IC_{50} (μM)	SAEC	H157	Calu-1	H358	H460	A549	HCC827	H292	H1299	H1975
BDA-366	10.64± 1.37	0.72± 0.03	0.67± 0.02	0.78± 0.06	0.21± 0.01	1.68± 0.17	0.84± 0.07	0.63± 0.04	1.73± 0.12	0.72± 0.05

Table S2, related to Figure 1. IC_{50} values of BDA-366 analyzed by MTS assay in SCLC cell lines

IC_{50} (μM)	DMS53	DMS153	DMS114	H128	H146	H69
BDA-366	0.21± 0.08	0.2± 0.07	1.48± 0.15	1.25± 0.02	0.51± 0.09	0.41± 0.05

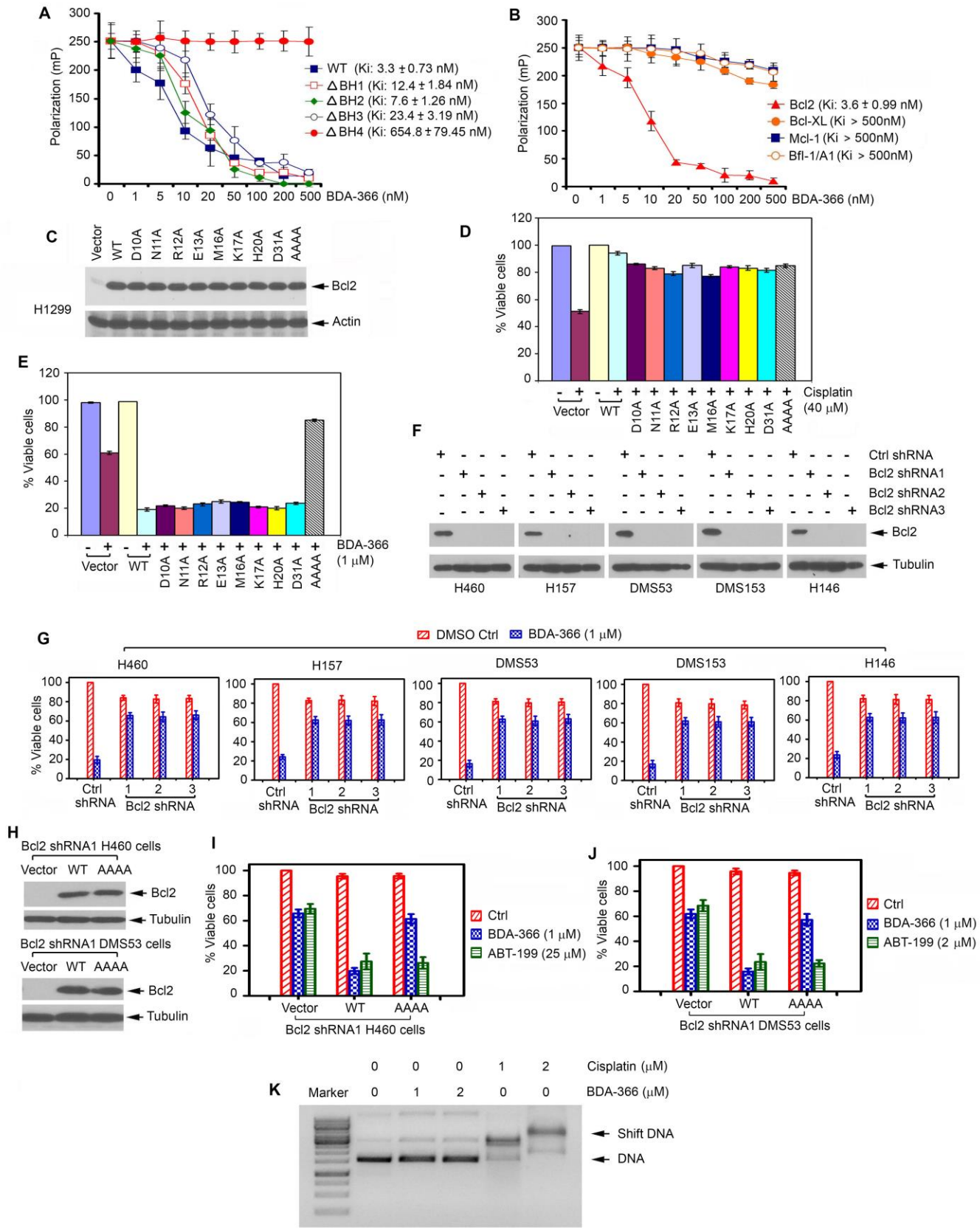

A
Polarization (mP)
WT (Ki: 3.3 ± 0.73 nM)
ΔBH1 (Ki: 12.4 ± 1.84 nM)
ΔBH2 (Ki: 7.6 ± 1.26 nM)
ΔBH3 (Ki: 23.4 ± 3.19 nM)
ΔBH4 (Ki: 654.8 ± 79.45 nM)
BDA-366 (nM)
B
Polarization (mP)
Bcl2 (Ki: 3.6 ± 0.99 nM)
Bcl-XL (Ki > 500nM)
Mcl-1 (Ki > 500nM)
Bfl-1/A1 (Ki > 500nM)
BDA-366 (nM)
C
Vector
WT
D10A
N11A
R12A
E13A
M16A
K17A
H20A
D31A
AAAA
H1299
Bcl2
Actin
D
% Viable cells
Vector
WT
Cisplatin (40 μM)
E
% Viable cells
BDA-366 (1 μM)
F
Ctrl shRNA
Bcl2 shRNA1
Bcl2 shRNA2
Bcl2 shRNA3
Bcl2
Tubulin
H460
H157
DMS53
DMS153
H146
G
DMSO Ctrl
BDA-366 (1 μM)
H460
H157
DMS53
DMS153
H146
% Viable cells
Ctrl shRNA
Bcl2 shRNA
H
Bcl2 shRNA1 H460 cells
Vector
WT
AAAA
Bcl2
Tubulin
Bcl2 shRNA1 DMS53 cells
I
% Viable cells
Ctrl
BDA-366 (1 μM)
ABT-199 (25 μM)
Bcl2 shRNA1 H460 cells
J
% Viable cells
Ctrl
BDA-366 (1 μM)
ABT-199 (2 μM)
Bcl2 shRNA1 DMS53 cells
K
Marker
Cisplatin (μM)
BDA-366 (μM)
Shift DNA
DNA

Figure S1, related to Figure 1. BDA-366 selectively binds to Bcl2 at BH4 domain, which is required for BDA-366 induction of apoptosis in human lung cancer cells. (A) Binding affinities of BDA-366 with WT or each of BH domain deletion Bcl2 mutants were analyzed using a competition fluorescence polarization assay. (B) Binding affinities of BDA-366 with Bcl2 and other Bcl2 family members were analyzed using a competition fluorescence polarization assay. Error bars represent ± S.D. (C) WT and various *Bcl2* mutants were stably overexpressed in H1299 cells. The levels of exogenous WT and various Bcl2 mutants were analyzed by Western blot. (D) and (E) H1299 cells overexpressing quantitatively similar levels of exogenous WT and various Bcl2 mutants were treated with cisplatin (40 μM) or BDA-366(1 μM) for 48 hr. Cell viability was determined by analyzing annexin-V binding on FACS. Data represent the mean ± S.D. of three determinations. (F) and (G) *Bcl2* shRNA1, 2, 3 or control shRNA was transfected into NSCLC cell lines **(**H460 and H157) and SCLC cell lines (DMS53, DMS153 and H146) that express endogenous Bcl2. Then, cells were treated with BDA-366 (1μM) for 72 hr. Cell viability was determined by analyzing Annexin-V/PI binding on FACS. Data represent the mean ± S.D. of three determinations. (H) WT, D10A/N11A/R12A/E13A (AAAA) *Bcl2* mutant or vector-only control was transfected into H460 and DMS53 cells expressing Bcl2 shRNA1. Because *Bcl2* shRNA1 targets the 5'UTR of endogenous *Bcl2*, the silencing effect of shRNA1 can be rescued by transfection of exogenous Bcl2 cDNA. After transfection, expression levels of exogenous Bcl2 were analyzed by Western blot. (I) and (J) The *Bcl2* silenced H460 and DMS53 cells expressing exogenous WT, AAAA Bcl2 mutant or vector-only control were treated with BDA-366 or ABT-199 as at the indicated concentrations for 72 hr. Cell viability was determined by analyzing Annexin-V/PI binding on FACS. Data represent the mean ± S.D. of three determinations. (K) Plasmid pUC19 DNA (200ng) was incubated with BDA-366 or cisplatin at 37 °C for 3 h. After electrophoresis, gels were stained with ethidium bromide and photographed.

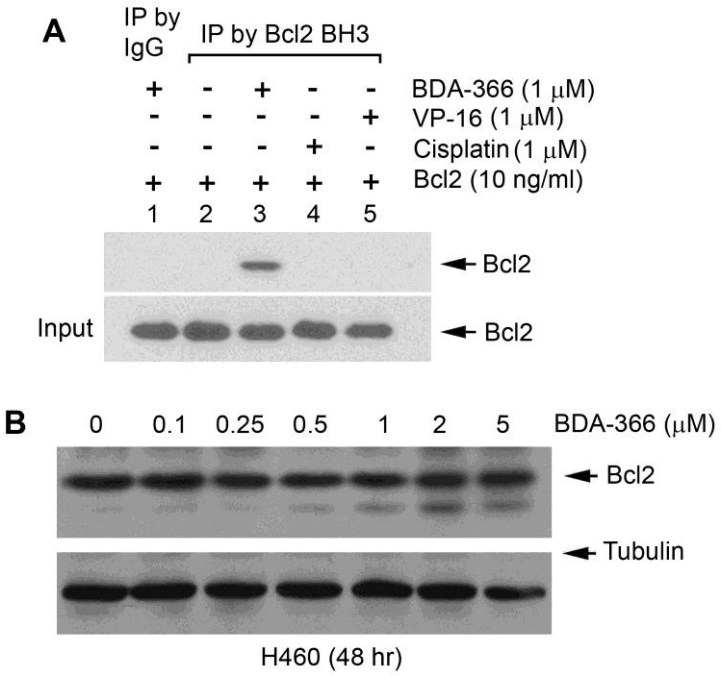


Figure S2, related to Figure 2. BDA-366 directly induces Bcl2 conformational change but does not significantly affect expression level of Bcl2 in lung cancer cells. (A) Purified recombinant Bcl2 was treated with BDA-366, VP-16 or cisplatin in 1% CHAPS lysis buffer, followed by IP using anti-Bcl2/BH3 domain antibody. BH3 domain-exposed Bcl2 was analyzed by Western blot using Bcl2 antibody. (B) H460 cells were treated with increasing concentrations of BDA-366 for 48 hr. Bcl2 expression was analyzed by Western blot.

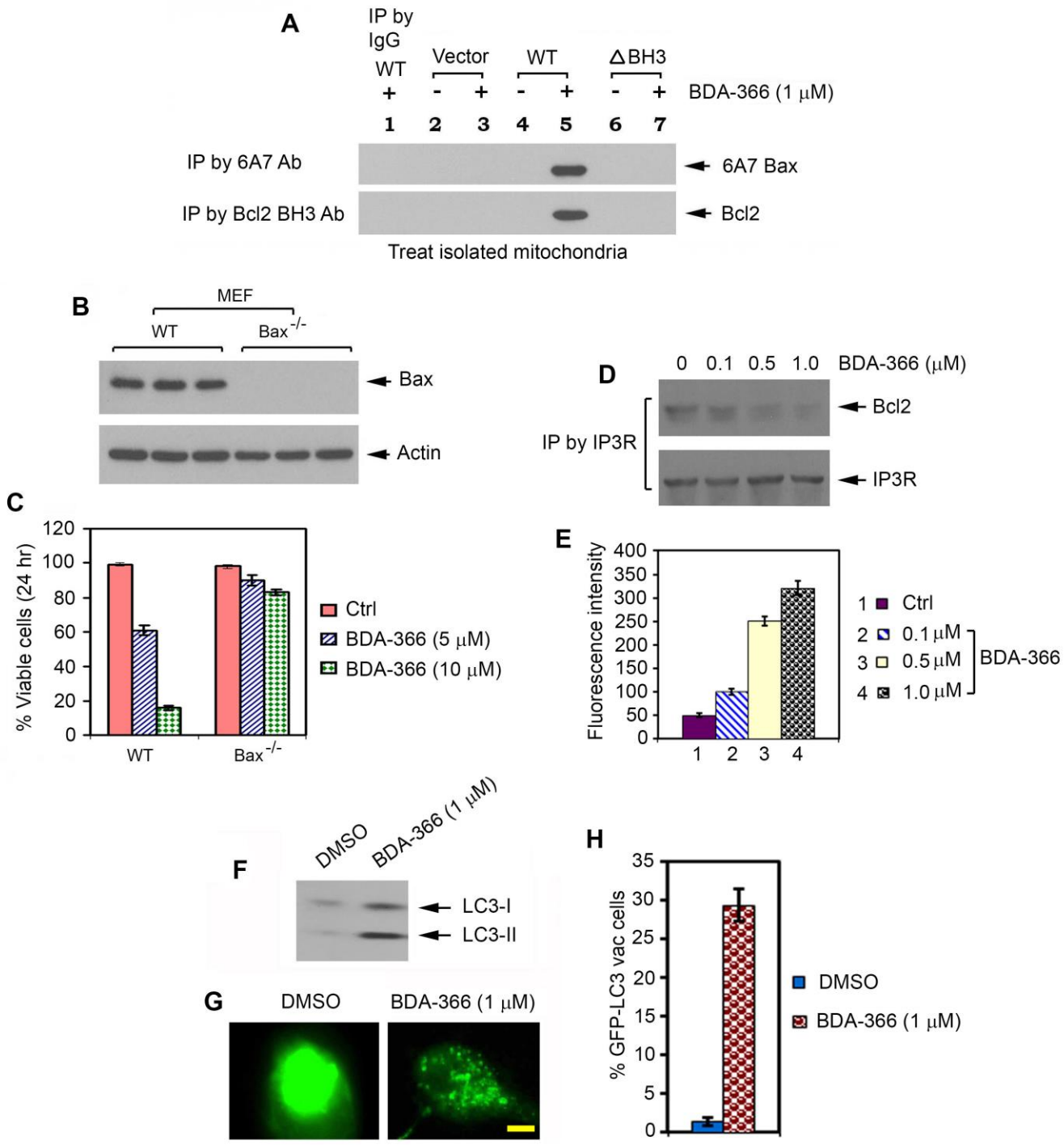
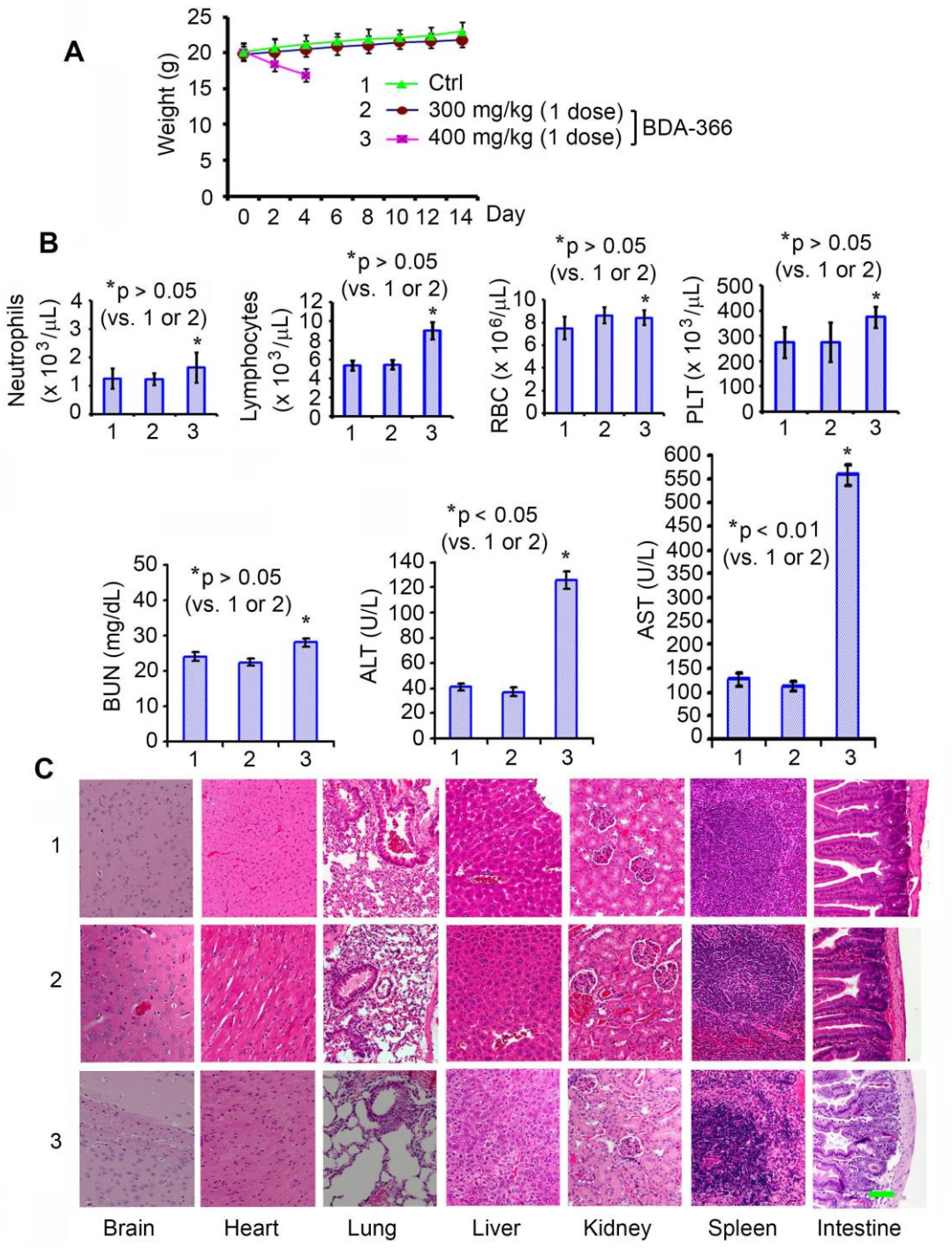

A
IP by IgG
WT
Vector
WT
ΔBH3
+ - + - + - +
BDA-366 (1 μM)
1 2 3 4 5 6 7
IP by 6A7 Ab
6A7 Bax
IP by Bcl2 BH3 Ab
Bcl2
Treat isolated mitochondria
B
MEF
WT
Bax-/-
Bax
Actin
C
% Viable cells (24 hr)
120
100
80
60
40
20
0
WT
Bax-/-
Ctrl
BDA-366 (5 μM)
BDA-366 (10 μM)
D
0 0.1 0.5 1.0 BDA-366 (μM)
IP by IP3R
Bcl2
IP3R
E
Fluorescence intensity
400
350
300
250
200
150
100
50
0
1 2 3 4
1 Ctrl
2 0.1 μM
3 0.5 μM
4 1.0 μM
BDA-366
F
DMSO
BDA-366 (1 μM)
LC3-I
LC3-II
G
DMSO
BDA-366 (1 μM)
H
% GFP-LC3 vac cells
35
30
25
20
15
10
5
0
DMSO
BDA-366 (1 μM)

Figure S3, related to Figure 3. BDA-366 directly induces Bcl2 conformational change leading to activation of Bax, calcium (Ca^{2+}) release and autophagy. (A) Isolated mitochondria from H1299 cells expressing WT, ΔBH3 or vector-only control were treated with BDA-366 in mitochondrial buffer for 30 min at 30°C, followed by IP in lysis buffer using Bcl2 BH3 specific antibody or 6A7 antibody, respectively. (B) Expression of Bax was analyzed in wild type (WT) and *Bax* knockout (*Bax*$^{-/-}$) MEF cells. (C) WT and *Bax*$^{-/-}$ MEF cells were treated with BDA-366 for 24 hr. Cell viability was determined by analyzing annexin-V binding on FACS. Data represent the mean ± S.D. of three determinations. (D) and (E) H460 cells were treated with increasing concentrations of BDA-366 for 24 hr. A co-IP was performed using an IP3R antibody. Bcl2 and IP3R were analyzed by Western blot. Ca^{2+} release was analyzed as described in "Supplementary Experimental Procedures". Error bars represent ± S.D. (F) H460 cells were treated with BDA-366 (1 μM) for 24 hr. The levels of LC3-I and LC3-II were analyzed by Western blot. (G) and (H) H460 cells were transfected with *GFP-LC3*. After 24 hr, cells were treated with BDA-366 (1 μM) for 24 hr. Autophagic vacuoles in the representative cells from various treatments were shown in (G). Scale bar represents 5 um. The percentage of *LC3-GFP*-transfected cells bearing LC3-GFP aggregates (LC3-FGPvac) were quantified as shown in (H). Error bars represent ± S.D.


A
Weight (g)
Day
1 Ctrl
2 300 mg/kg (1 dose)
3 400 mg/kg (1 dose)
BDA-366
B
Neutrophils (x 10^3/μL)
Lymphocytes (x 10^3/μL)
RBC (x 10^6/μL)
PLT (x 10^3/μL)
BUN (mg/dL)
ALT (U/L)
AST (U/L)
*p > 0.05 (vs. 1 or 2)
*p < 0.05 (vs. 1 or 2)
*p < 0.01 (vs. 1 or 2)
C
1
2
3
Brain
Heart
Lung
Liver
Kidney
Spleen
Intestine

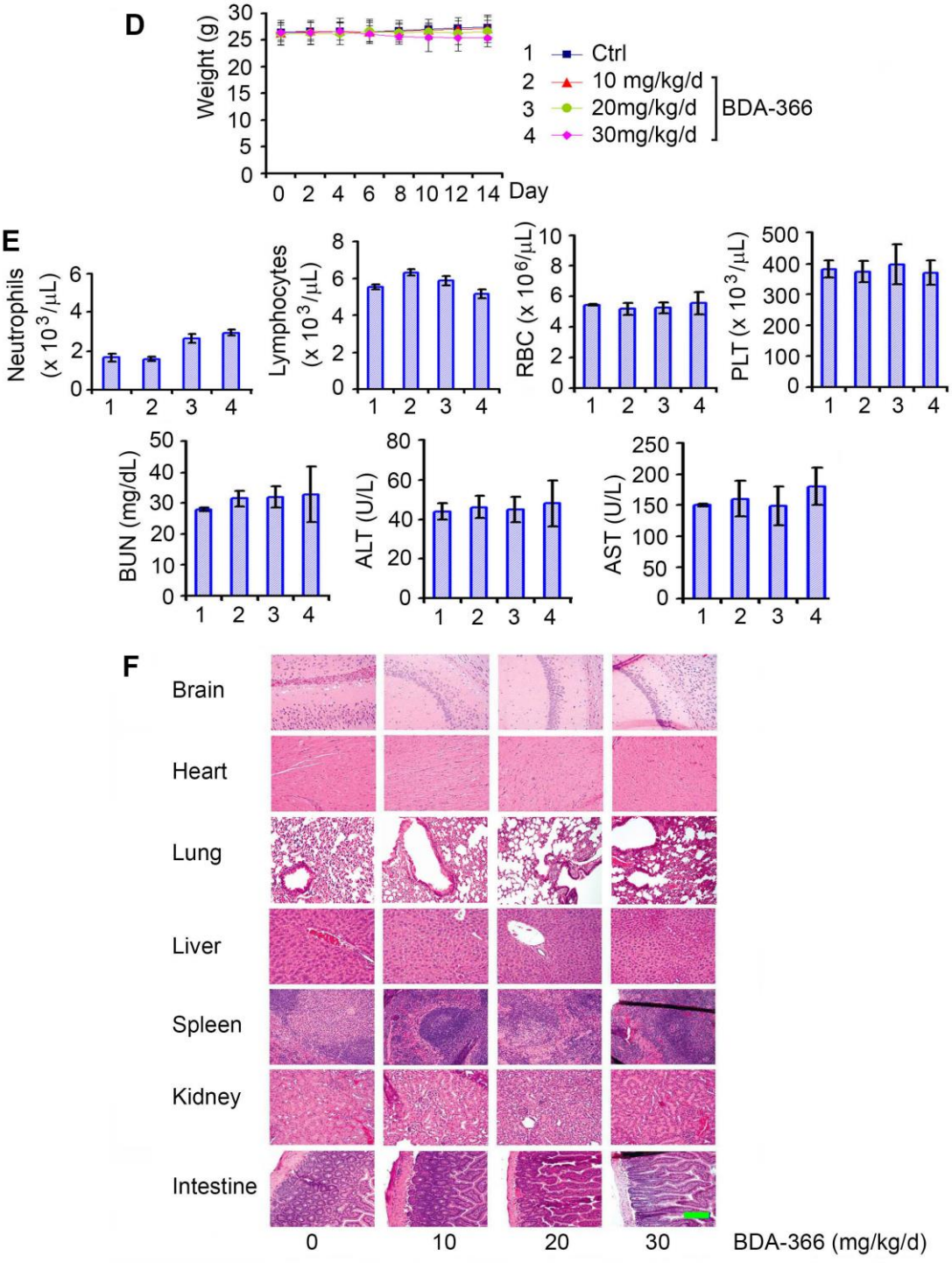


Figure S4, related to Figure 4. Determination of single dose maximum tolerated dose (MTD) and evaluation of in vivo toxicity of BDA-366. (A) Normal C57BL/6 mice were treated with single dose (*i.e*. 0, 300mg/kg or 400mg/kg) of BDA-366 via i.p. (n = 6 mice per group). After treatment, body weight of mice was measured once every other day for 2 weeks. (B) Blood analysis of mice after treatment with single dose (s) of BDA-366. Error bars represent ± S.D. (C) H&E histology of various organs from mice after treatment with single dose (s) of BDA-366. Scale bar represents 25 um. (D-F) Nu/Nu nude mice with H460 lung cancer xenografts were treated with increasing doses of BDA-366 (0, 10, 20 and 30mg/kg/d)

for 14 days (n = 6 mice per group). Body weight of mice was measured once every other day during treatment with various doses of BDA-366 (D). Blood analysis of mice after treatment with various doses of BDA-366 for 14 days (E). Error bars represent ± S.D. H&E histology of various organs from mice after treatment with various doses of BDA-366 for 14 days (F). Scale bar represents 50 um.

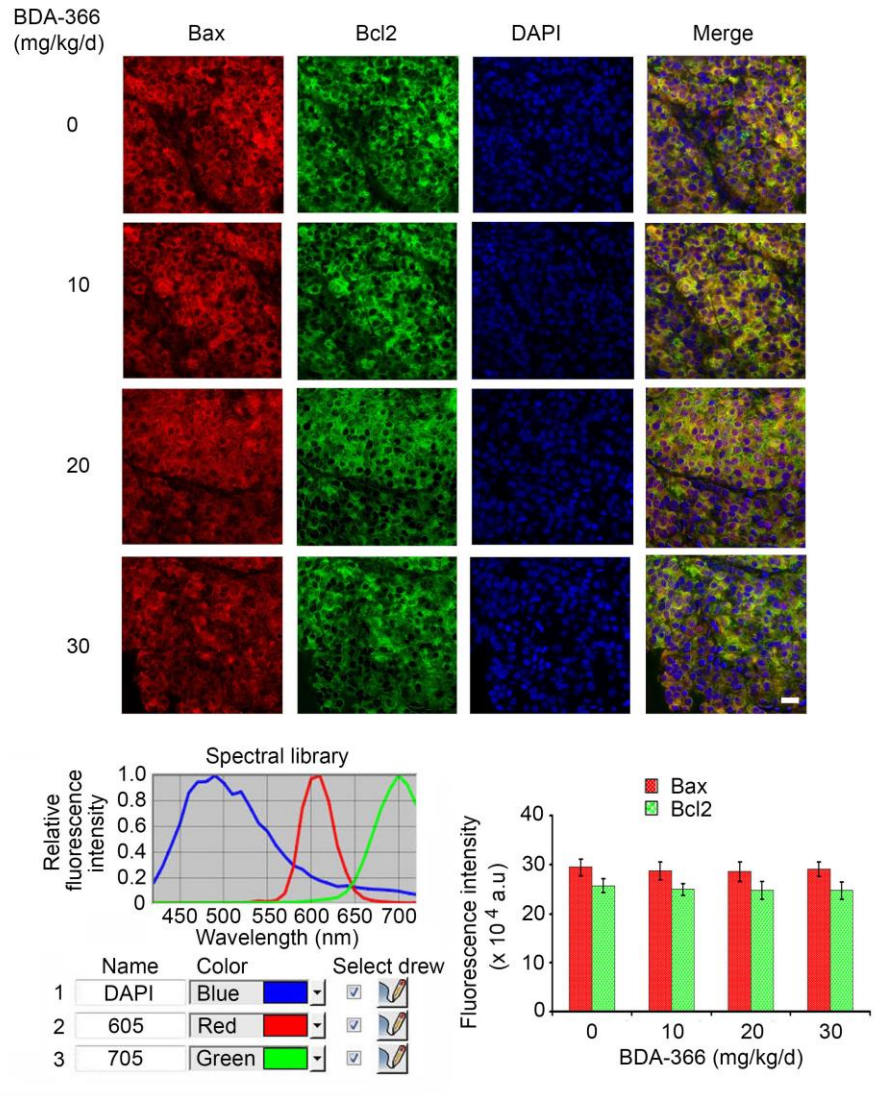


Figure S5, related to Figure 6. Quantum dot-based immunohistofluorescence (QD-IHF) analysis of total Bcl2 and Bax in tumor tissues after treatment mice with BDA-366. H460 lung cancer xenografts were treated with increasing doses of BDA-366 for 14 days (n = 6 mice per group). Total Bcl2 and Bax were analyzed in tumor tissues at the end of experiments by QD-IHF using Bcl2 and Bax, antibody and quantified as described in “Supplemental Experimental Procedures”. Scale bar represents 10 um. Error bars represent ± S.D.

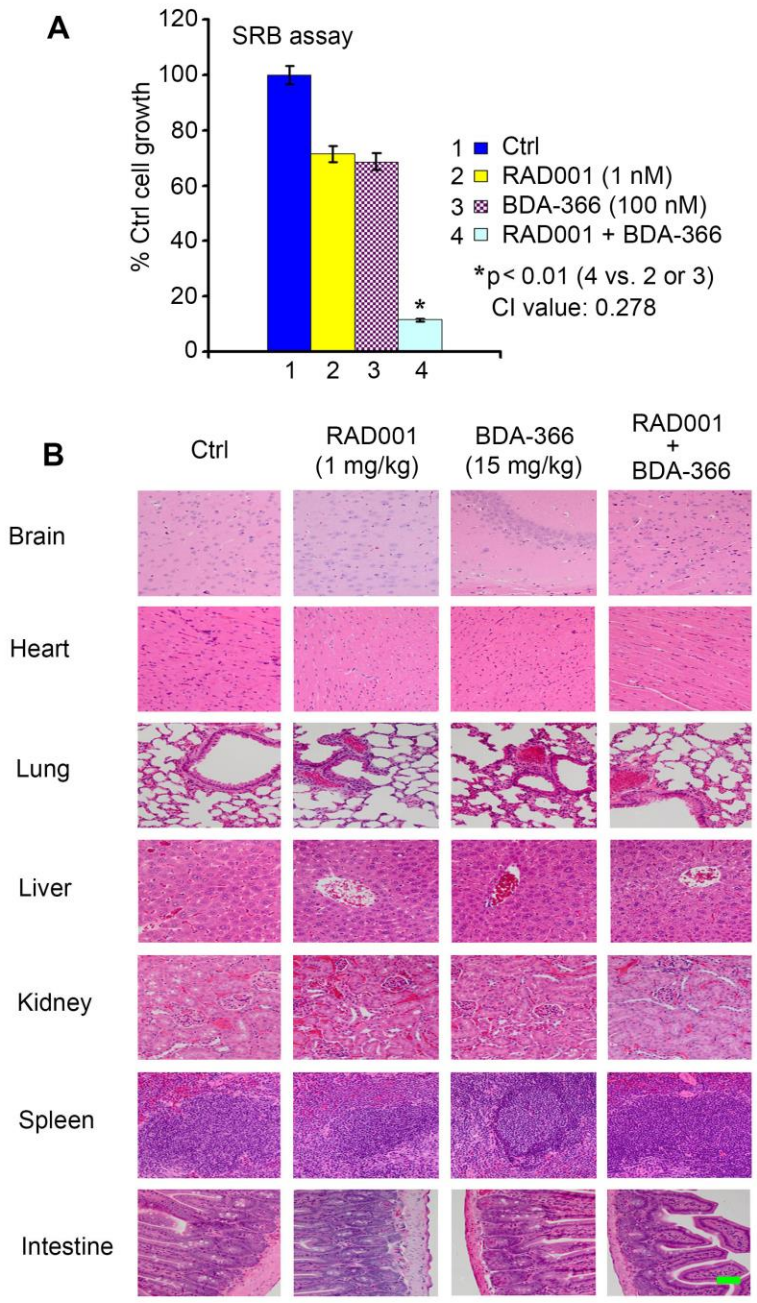


Figure S6, related to Figure 8. BDA-366 synergizes with RAD001 against lung cancer cells in vitro and combination of BDA-366 and RAD001 has no significant toxicity in vivo. (A) H460 cells were treated with RAD001 (1nM) or BDA-366 (100nM) alone or in combination for 72 hr. Cell growth was analyzed by sulforhodamine B (SRB) colorimetric assay. Combination index (CI) value for evaluating synergy of RAD001 and BDA-366 was calculated using the CompuSyn software. CI value between 0.1 and 0.3 represents strong synergism. Error bars represent ± S.D. (B) H&E histology of various organs after treatment of Nu/Nu mice bearing H460 lung cancer xenografts with RAD001, BDA-366 or in combination for 14 days (n = 6 mice per group). Scale bar represents 25 um.

Supplemental Experimental Procedures

Materials

Small molecule NSC639366 (BDA-366) was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutic Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (NCI, Bethesda, MD) (http://dtp.nci.nih.gov/RequestCompounds). ABT-737 and ABT-199 were purchased from Active Biochemicals (Wanchai, HongKong). RAD001 was purchased from Selleck Chemicals (Houston, TX). Cisplatin was obtained from Sigma (St. Louis, MO). pUC19 vector was purchased from New England Biolabs (Lpswich, MA). Bcl2 antibody was obtained from Calbiochem (Darmstadt, Germany). The Bcl2/BH3 domain-specific antibody was obtained from Abgent (San Diego, CA). Mouse 6A7 Bax antibody was purchased from BD PharMingen (San Diego, CA). Active caspase 3-specific antibody was purchased from Cell Signaling Technology. Fluorescent Bak peptide (FAM-GQVGRQLAIIGDDINR) and Bcl-XL protein were purchased from NeoBioSci™ (Cambridge, MA). Purified recombinant WT, ΔBH1, ΔBH2, ΔBH3, and ΔBH4 Bcl2 mutant proteins were obtained from Protein*X* Lab (San Diego, CA). Purified recombinant Mcl-1 protein was purchased from GenWay Biotech, Inc. (San Diego, CA). Purified recombinant Bfl-1/A1 proteins were obtained from R&D systems (Minneapolis, MN). Purified recombinant Bax protein was obtained from Novus Biologicals (Littleton, CO). BAM7 was purchased from Sigma-Aldrich (St. Louis, MO). MitoSOX™ kit was obtained from Molecular Probes, Inc (Eugene, OR). TUNEL Kit was purchased from Promega Corporation (Madison, WI). QD605 goat anti-rabbit IgG conjugate (red), QD705 goat anti-mouse IgG conjugate (green) and ProLong® Gold antifade reagent with 4', 6-diamidino-2-phenylindole (DAPI) were obtained from Invitrogen Life Technologies Inc (Carlsbad, CA). *GFP-LC3* plasmid DNA was kindly provided by Dr. William

A. Dunn (University of Florida). All other reagents used were obtained from commercial sources unless otherwise stated.

Measurement of cytotoxicity *in vitro*

For adhesive cells (*i.e.* NSCLC cell lines), cells were seeded at a density of 6 x 10^3 - 8 x 10^3 per well in 96-well plates and allowed to grow overnight. Cells were treated with BDA-366 or other agent(s) for 72h. The surviving cell fraction was determined using the sulforhodamine B (SRB) assay. For non-adhesive cells (*i.e.* SCLC cell lines), cells were seeded in 96-well plates at 1–2 $\times$ 10^4 cells per well. After 48 h, exponentially growing cells were treated BDA-355 for 72 h. The surviving cell population following drug exposure was detected using MTS [(3-(4,5-dimethylthiazol-2-yl)-5- (3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)/phenazine methosulfate (PMS)] colorimetric assay Kit from Promega (Madison, WI) according to the manufacturer's protocol.

Cell apoptosis assay

Apoptotic and viable cells were detected using an Annexin V /PI kit (BD Pharmingen, CA) according to the manufacturer's instructions. The percentage of viable cells or apoptotic cells was determined by fluorescence-activated cell sorter (FACS) analysis.

Preparation of cell lysate and Western blot

Cells were washed with cold PBS and resuspended in ice-cold EBC buffer (0.5% Nonidet P-40, 50mM Tris, pH 7.6, 120mM NaCl, 1mM EDTA, and 1mM-β-mercaptoethanol) containing protease inhibitor mixture set I. Following cell lysis by sonication and centrifugation at 14,000 x g for 15 min at 4 °C, the resulting supernatant was collected as the total cell lysate. As previously described, Western blot was performed by loading 50μg of protein per lane on an

8–12% SDS-PAGE, followed by protein transfer to nitrocellulose membrane for analysis of specific protein(s) (Ito et al., 1997).

Fluorescence polarization assay

To measure the binding affinity of BDA-366 to Bcl2 family proteins (*i.e*. Bcl2, Bcl-XL, Mcl-1 or Bfl-1/A1), a competition fluorescence polarization assay was employed. Fluorescent Bak peptide (FAM-GQVGRQLAIIGDDINR) was used for competition fluorescence polarization assay. Fluorescent Bak peptide was incubated with purified, human Bcl2, Bcl-XL, Mcl-1 or Bfl-1/A1 protein (6nM) in the absence or presence of increasing concentrations (*i.e*. 0~500nM) of BDA-366 in binding affinity assay buffer (50mM Tris (pH8.0), 150mM NaCl, 0.1% BSA, and 5mM DTT) in a 96-well assay plate. The plate was mixed on a shaker for 1 min and incubated at room temperature for an additional 15 min. Polarization, measured as millipolarization units (mP), was assessed at room temperature with a fluorescence microplate reader at 485/530nm (Gemini XPS^{TM}, Molecular Devices, CA). A negative control (DMSO, 3nM peptide and assay buffer) and a positive control (DMSO, 3nM peptide, 6nM Bcl2 and assay buffer) were used to determine the range of the assay. The percentage of inhibition was determined by the equation: 1-[(mP value of well - negative control)/range)] × 100%. Inhibitory constant (*K*i) value was determined by the formula: $Ki = [I]_{50}/([L]_{50}/Kd + [P]_0/Kd + 1)$ as described (Nikolovska-Coleska et al., 2004). Reported values are the mean ± S.D. for three separate experiments run in duplicate.

Subcellular fractionation

Cells (2×10^7) were washed with cold 1× PBS and resuspended in isotonic mitochondrial buffer (210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM Hepes, pH 7.5) containing 10% protease inhibitor mixture set I, homogenized with a polytron homogenizer, and then

centrifuged at 1000 × *g* for 5 min to remove the nuclei and unbroken cells. The supernatant was centrifuged at 13,000 × *g* for 10 min to pellet mitochondria. The resulting supernatant is the cytosolic fraction. Mitochondria were washed twice with mitochondrial buffer and resuspended in 1% Nonidet P-40 lysis buffer, rocked for 60 min, and then centrifuged at 17,530 × *g* for 10 min at 4 °C. The resulting supernatant containing mitochondrial proteins was collected. Protein (50 μg) from each fraction was subjected to SDS-PAGE. Cyt c was analyzed by Western blot.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed for measurement of small molecule/DNA binding. Cisplatin, a known DNA binding agent, was used as positive control. Plasmid pUC19 DNA (200ng) was incubated with BDA-366 or cisplatin in a total volume of 40 μl in 10mM Tris-EDTA buffer at 37 °C for 3 hr. After incubation, 4 μl loading dye was added to a 20 μl aliquot of the sample, and electrophoresis was performed for 90 min at 80 V in a 1% agarose gel using 0.5× Tris–borate-EDTA buffer. Gels were stained with 0.2 μg/mL ethidium bromide solution, illuminated with UV light, and photographed using the gel documentation system GelDoc-It (UVP, Upland, CA, USA).

Generation of various *Bcl2* mutants in BH4 domain

The BH1, BH2, BH3, and BH4 *Bcl2* deletion mutants were created by using a mutagenesis kit (Clontech) and confirmed by sequencing of the cDNA as described (Xie et al., 2015). The WT and various Bcl2 deletion mutants were then cloned into the pCIneo (Promega) mammalian expression vector. To mutate specific residues that interact with BDA-366 in the BH4 domain of Bcl2, the 5′-phosphorylated mutagenic primers for various precise deletion mutants were synthesized as follow: D10A, 5’-GAG AAC AGG GTA TGC TAA CCG GGA GAT C-3’; N11A,

5’-GAA CAG GGT ATG ATG CCC GGG AGA TCG TG-3’; R12A, 5’- CAG GGT ATG ATA ACG CGG AGA TCG TGA TG-3’; E13A, 5’- CAG GGT ATG ATA ACC GGG CGA TCG TGA TGA AGT AC-3’ ; M16A, 5’- CGG GAG ATC GTG GCG AAG TAC ATA C-3’; K17A, 5’- CCG GGA GAT CGT GAT GGC GTA CAT ACA TTA TAA GC-3’; H20A; 5’- GGG AGA TCG TGA TGA AGT ACA TAG CTT ATA AGC TGT CAC AGA GGG GC-3’; D31A, 5’- GGC TAC GAG TGG GCT GCT GGA GAT GCG-3’. The WT *Bcl2*/pUC19 construct was used as the target plasmid, which contains a unique NdeI restriction site for selection against the unmutated plasmid. The NdeI selection primer is 5′-GAG TGC ACC ATG GGC GGT GTG AAA-3′. These Bcl2 mutants were created using a site-directed mutagenesis kit (Clontech) according to the manufacturer's instructions. Each single mutant was confirmed by sequencing of the cDNA and was then cloned into the pCIneo (Promega) mammalian expression vector. The pCIneo plasmid containing each *Bcl2* mutant cDNA was transfected into H1299 cells using Lipofectamine 2000 (Invitrogen). Clones stably expressing WT or mutant Bcl2 were selected in medium containing G418 (0.6 mg/ml). The expression levels of exogenous Bcl2 were compared by Western blot analysis using a Bcl2 antibody. Three separate clones for each mutant expressing similar amounts of exogenous Bcl2 were selected for analysis.

Silencing of *Bcl2* and Bcl2 rescue in *Bcl2*-silenced lung cancer cells

Bcl2 shRNA1, *Bcl2* shRNA2, *Bcl2* shRNA1 and control shRNA in pRS shRNA vector were obtained from Origene (Rockville, MD). Hairpin sequence of human *Bcl2* shRNA1: ACG TGC CTC AAA TAA AGA TCC GAA AG; *Bcl2* shRNA2: GAG GAT TGT GGC CTT CTT TGA GTT CGG TG; *Bcl2* shRNA3: TGA TTT CTC CTG GCT GTC TCT GAA GAC TC. Control shRNA: GCA CTA CCA GAG CTA ACT CAG ATA GTA CT. For pseudovirus production, *Bcl2* shRNA1, 2, 3 or control shRNA was cotransfected into 293FT cells with a retrovirus packaging plasmid

mixture (Agilent technologies, CA) using the Nanojuice transfection kit (EMD Chemical, Inc.). After 48h, the virus-containing media were harvested by centrifugation at 20,000 × g. Various human lung cancer cells were infected with the virus-containing media in the presence of polybrene (8 μg/ml) for 24h. Stable positive clones were selected using 1 μg/ml puromycin. The silencing efficiency of the targeted Bcl2 gene was confirmed by Western blotting. It is known that if the shRNA is directed to the 3′ UTR or 5’ UTR of the gene, the effect of the shRNA can be rescued by ectopically expressing the protein using the wild-type or mutant cDNA. Because the Bcl2 shRNA1 used here targets the 5’UTR of endogenous *Bcl2*, the silencing effect of shRNA1 could be rescued by transfection of exogenous *Bcl2* cDNA. Vector, WT or BDA-366 binding deficient AAAA *Bcl2* mutant was transfected into H460 and DMS53 cells expressing *Bcl2* shRNA1 using the Nanojuice transfection kit (EMD Chemical, Inc.). Then, cells were treated with BDA-366 or ABT-199 as indicated. Cell viability was analyzed by annexin-V/PI binding by FACS.

TUNEL assay for apoptotic cells *in vivo*

Apoptosis was assessed on tumor xenograft tissues using the TUNEL method with DeadEnd™ Colorimetric TUNEL System from Promega Corporation (Madison, WI) according to the manufacturer's instructions.

Calcium fluorometry

H460 cells were treated with increasing concentrations (0.1 ~1.0 μM) BDA-366 for 15 min, then washed with PBS and incubated with 0.5 μM Fura-2 AM in PBS for 30 min at room temperature. Cells were washed twice with PBS and then incubated for another 30 min to allow complete de-esterification of intracellular AM esters. Fluorescence was recorded in a fluorometer (Photon Technology Inc.).

Immunohistochemical (IHC) staining

Tumors from patients or mice xenografts were harvested, fixed in formalin and embedded in paraffin. Representative sections from paraffin-embedded tumor tissues were analyzed by IHC staining using active caspase 3-specific antibody. Active caspase 3-positive cells in tumor tissues were scored at 400 × magnification. The average number of positive cells per 0.0625 mm^2 area was determined from three separate fields in each of three independent tumor samples. Bcl2 expression in tumor tissues from NSCLC patients were analyzed by IHC using a Bcl2 antibody and quantified by immunoscore.

Quantum dot-based immunohistofluorescence (QD-IHF)

QD-IHF analysis of the inactive form of Bcl2 and active form of Bax in tumor tissues after treatment with BDA-366 was performed as described previously (Xin et al., 2014). Briefly, harvested tumors were embedded in paraffin and cut into 4-μm sections. After deparaffinization and rehydration, antigen retrieval was performed by heating with citric acid (10 mmol/L, pH 6.0) in a microwave for 10 min. The tissue slides were blocked with 2.5% normal horse serum for 10 min before the primary antibody incubation. Primary Bcl2/BH3-domain specific antibody from rabbit and 6A7 Bax antibody from mouse were mixed at 1:50 dilution in 1×PBS containing 2.5% horse serum. Normal rabbit IgG was used as negative control. Tissue sections were incubated with a mixed solution of Bcl2/BH3-domain specific and 6A7 Bax antibodies overnight at 4°C. After washing with 1×PBS three times, QD605 goat anti-rabbit IgG conjugate (red) and QD705 goat anti-mouse IgG conjugate (green) secondary antibodies were added to the slides with further incubation for 1h at 37°C. The slides were washed three times with 1×PBS, counterstained with DAPI, mounted and stored at 4^oC under dark conditions. QD imaging and quantification procedures were performed as described

previously (Xin et al., 2014). The Nuance^{TM} fluorescence microscope system (CRi consolidated with Caliper, a PerkinElmer company, Hopkinton, MA) was used for quantification of the QD-IHF signals. All cubed image files were collected from tumor tissue slides at 10 nm wavelength intervals from 420-720 nm, with an auto exposure time per wavelength interval at 200~400x magnification. Taking the cube with a long wavelength band pass filter allowed transmission of all emission wavelengths above 420 nm. Both separated and combined QD images were obtained after establishing the QD spectral library and unmixing the image cube. For each tissue slide, 10 cubes were taken. The background signal was removed for accurate quantification of the QD signals. The average of each QD signal was obtained by selecting tumor areas on each cube for quantification by Nuance imaging software (Caliper/PerkinElmer). An average reading from the 10 cubes was obtained as a total average signal count of each tissue slide for both QD signals.

Mouse blood analysis

Whole blood (250μL) was collected in EDTA-coated tubes via cardiac puncture of anesthetized mice for hematology studies. Specimens were analyzed for white blood cells (WBC), red blood cells (RBC), platelets (PLT), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and blood urea nitrogen (BUN) in the Clinical Pathology Laboratory at the University of Georgia (Athens, GA) as described (Xin et al., 2014).

Analysis of combination index (CI) value

CI value for evaluating drug synergy was calculated using the CompuSyn software (Combo-Syn, Inc.; Paramus, NJ) as described (Chou, 2010). A CI value of >1 is defined as antagonism, equal to 1 as additive effect, and < 1 as synergy ($<0.1\rightarrow$ very strong synergism; 0.1~0.3$\rightarrow$ strong synergism).

Supplemental References

Chou, T. C. (2010). Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res *70*, 440-446.
Ito, T., Deng, X., Carr, B., and May, W. S. (1997). Bcl-2 phosphorylation required for anti-apoptosis function. J Biol Chem *272*, 11671-11673.
Nikolovska-Coleska, Z., Wang, R., Fang, X., Pan, H., Tomita, Y., Li, P., Roller, P. P., Krajewski, K., Saito, N. G., Stuckey, J. A., and Wang, S. (2004). Development and optimization of a binding assay for the XIAP BIR3 domain using fluorescence polarization. Anal Biochem *332*, 261-273.
Xie, M., Park, D., You, S., Li, R., Owonikoko, T. K., Wang, Y., Doetsch, P. W., and Deng, X. (2015). Bcl2 inhibits recruitment of Mre11 complex to DNA double-strand breaks in response to high-linear energy transfer radiation. Nucleic Acids Res *43*, 960-972.
Xin, M., Li, R., Xie, M., Park, D., Owonikoko, T. K., Sica, G. L., Corsino, P. E., Zhou, J., Ding, C., White, M. A.*, et al.* (2014). Small-molecule Bax agonists for cancer therapy. Nat Commun *5*, 4935.