Supporting Online Material

Materials and Methods

Protein expression and purification. Full-length human procaspase clones (residues 1-277 (procaspase-3), 1-293 (procaspase-6) and 1-303 (procaspase-7)) were generated with standard PCR-based cloning and verified with double-stranded plasmid sequencing. Procaspases-3, -6 and -7 were expressed with a C-terminal His_6-affinity tag from a pET-23b vector (Novagen) in *E. coli* BL21(DE3)pLysS cells (Stratagene). Cells were grown in 2xYT media supplemented with 200 μg/ml ampicillin and 50 μg/ml chloramphenicol at 37 °C to an OD_{600nm} of 0.8-1.0. Overexpression of procaspase was induced with 0.2 mM IPTG at 30 °C for 20 minutes to limit autolysis. Cells were immediately harvested and resuspended in ice cold 100 mM Tris, pH 8.0, 100 mM NaCl (buffer A) and subjected to 3 cycles of lysis by microfluidization (Microfluidics). The cell lysate was clarified by centrifugation at 45,000x*g* for 30 minutes at 4 °C and soluble fractions were loaded onto a 1 ml HisTrap HP Ni-NTA affinity column (GE Amersham) pre-equilibrated with buffer A and eluted with buffer A containing 200 mM Imidazole. The eluted protein was immediately diluted two-fold with buffer B (20 mM Tris, pH 8.0) and purified by anion-exchange chromatography (HiTrap Q HP, GE Amersham) with a 30-column volume gradient to 50% of buffer B containing 1 M NaCl. The protein was injected over a Superdex 200 16/60 gel filtration column (GE Amersham) in 20 mM Tris, pH 8.0, 50 mM NaCl to buffer exchange and to remove any remaining contaminants. Fractions corresponding to purified homodimeric procaspase-3, -6 or -7 were pulled and concentrated to approximately 1 mg/ml using Millipore Ultrafree-15 devices with a MWCO of 10,000 Da. All fast protein liquid chromatography (FPLC) procaspase purification steps were performed within 5 hours on an AKTA FPLC (GE Amersham) at 4 °C and immediately frozen and stored at -70 °C to eliminate potential spontaneous self-activation. Purified procaspase concentrations were measured using both Bio-Rad colorimetric assay and A_{280} absorbance in denaturing conditions (*1*). Protein purity was assayed by electrospray ionization mass spectrometry on an LCT Premier Mass Spectrometer (Waters) and SDS-PAGE under reducing conditions. All procaspases are >98% pure and have <0.1% the activity of fully-processed caspase. Active caspases-3 and -7 are produced using the same plasmids and purification protocols as the procaspases, except that overexpression is extended to 4 and 24 hours, respectively, in order to promote autocatalysis. Active caspase-6 clones were made by incorporation of the large subunit (residues 24-179) into pET-24b (Novagen) and the small subunit (residues 194-293 with a C-terminal His_6-affinity tag) into pET-23b. Both plasmids were co-transformed into *E. coli* BL21(DE3) cells and colonies were selected for resistance against 200 μg/ml ampicillin and 50 μg/ml kanamycin. Soluble active caspase-6 was overexpressed by induction with 0.2 mM IPTG in 2xYT media supplemented with 200 μg/ml ampicillin

and 50 μg/ml kanamycin overnight at 16 °C. Active caspase-6 was purified as described for procaspase.

High throughput screen for procaspase-3 activators. A total of 62,000 compounds were screened for their ability to activate procaspase-3 in a 384-well plate assay. Procaspase-3 was incubated at a physiologically-relevant concentration of 100 nM (*2, 3*) with 30 μM HTS compounds in a total volume of 50 μl consisting of a reaction buffer of 50 mM HEPES, pH 7.4, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT and 0.1% CHAPS (to reduce false positive hits due to compound aggregation). The procaspase-3/small molecule incubations were then agitated for 3 hours at 37 °C. A fluorogenic peptide substrate rhodamine 110, bis-(*N*-CBZ-DEVD) (Z-DEVD-R110) was subsequently added to a final concentration of 10 μM by a MultiMex bulk liquid dispenser (Beckman) and incubated for an additional 30 minutes at room temperature. The reaction was quenched with a final concentration of 40 mM HCl and the endpoint fluorescence was measured on an Analyst HT Assay Detection System (LJL Biosystem). The final concentration of DMSO in each well was 3% and had no effect on enzyme stability or activity. In order to obtain a large endpoint disparateness between the proenzyme and activated caspase species for Z', procaspase-3 was proteolytically cleaved with granzyme B to attain maximal activity. Granzyme B was added at a concentration 1:1000 of procaspase-3 and, thus, did not contribute to observable activity of substrate cleavage. The granzyme B-activated caspase-3 fluorescence averaged 100-fold greater than the background fluorescence by procaspase-3. A Z' ranging from 0.85 to 0.95 was conserved over the course of the HTS assays. Any compounds from the high throughput screens with an increased activity of 20% or more over the inherent activity of procaspase-3 were considered potential "hits". All components of the assay including protein, substrate and inhibitor were stored as frozen aliquots and thawed immediately prior to the assay.

Elimination of promiscuous and aggregating HTS hits. All potential procaspase-3 small molecule HTS activators were re-synthesized and analyzed to identify false-positives with potential for aggregation or promiscuity. All compounds were subjected to dynamic light scattering at concentrations ranging from 0.1 to 100 μM in a buffer consisting of 50 mM HEPES, pH 7.4, 50 mM KCl, 0.1 mM EDTA and 1 mM DTT to determine aggregation potential. Any compounds that exhibited a propensity towards aggregation or insolubility in the absence of detergent were eliminated from further analysis. Similarly, the functional effects of all HTS hits were analyzed for inhibition of β-lactamase, which is extremely sensitive to promiscuous inhibition, as previously described (*4, 5*). Briefly, 1 nM β-lactamase was incubated in the presence of various concentrations of HTS procaspase activators (25 to 100 μM) in a buffer consisting of 50 mM sodium cacodylate, pH 6.5 in the presence or absence of 0.01% Triton-X100 for 1 hour at 37 °C. A kinetic assay was initiated by addition of 40 μM CENTA™ (CalBiochem)

and monitored for 10 minutes on a SpectraMax M5 microplate reader (Molecular Devices) at an absorbance of 405 nm. Any compounds that inhibited β-lactamase in the presence or absence of 0.01% Triton-X100 were determined to be promiscuous and/or aggregating compounds and eliminated from further studies.

***In vitro* procaspase activator characterization.**

Time course assays of procaspase activation: Compounds were assayed for activation potential against 100 nM of each executioner procaspases-3, -6 and -7 with either 25 μM 1541, 1 nM granzyme B, DMSO or 100 μM PAC1 (TimTec) in an optimized reaction buffer (50 mM HEPES, pH 7.4, 0.01% Triton-X100, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT for procaspase-3; 100 mM HEPES, pH 7.4, 0.01% Triton-X100, 10 mM DTT for procaspase-6; 100 mM HEPES, 5 mM $CaCl_2$, 0.01% Triton-X100, 1 mM DTT for procaspase-7) (*6-12*). Kinetic assays were initiated after incubation at 37 °C by addition of the fluorogenic peptide Ac-DEVD-AFC (-3 and -7) or Ac-VEID-AFC (-6) to 25 μM. All sample incubations were harvested after 4 hours and subjected to SDS-PAGE to determine the extent of proteolysis of the procaspases.

Initial activity rates of procaspase-3 in presence of 1541B: Procaspase-3 was incubated at 100 nM in the presence of various amounts of compound 1541B (0.78 to 50 μM) in a buffer consisting of 50 mM HEPES, pH 7.4, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT and 0.1% CHAPS in a total volume of 100 μl. The kinetic activity of the mixtures were immediately assayed by addition of Ac-IETD-7-amino-4-trifluoromethylcoumarin (Ac-IETD-AFC) (1.2 to 300 μM) for approximately 15 minutes at 37 °C by monitoring the emission at 495 nm on excitation at 365 nm every 45 seconds. Ac-IETD-AFC was chosen as the cleavage reporter as it is the self-cleavage intradomain sequence in procaspase-3.

Granzyme B cleavage of procaspase-3 C163A: C163A procaspase-3 was incubated at 250 nM with 500 pM granzyme B in the presence of DMSO or 25 μM 1541. The reaction buffer consisted of 50 mM HEPES, pH7.4, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT and 0.1% CHAPS and mixtures were agitated at 37 °C with aliquots removed after 30 and 60 minutes. Aliquots were immediately quenched with addition of 1% SDS reducing gel loading buffer and boiled. Samples were subjected to silver stain gel electrophoresis to determine extent of granzyme B cleavage.

1541 EC_{50} (activation) and IC_{50} (inhibition) for procaspase-3 and active caspase-3: Procaspase-3, or active caspase-3, was incubated at 100 nM and 25 nM, respectively, in the presence of increasing amounts of compound 1541 (0.1 to 100 μM) in a reaction buffer consisted of 50 mM HEPES, pH7.4, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT and 0.1% CHAPS. The mixtures were assayed for kinetic activity

by incubation with 20 μM Ac-DEVD-AFC for 5 minutes after 4 hours of incubation at 37 °C (procaspase-3) or 10 minutes (active caspase-3).

Time course activation of procaspase-3 with 1541: Procaspase-3 was incubated at 100 nM in the presence of 1541 (0.1 to 100 μM) to assess speed of self-activation. The mixtures were agitated at 37 °C and assayed for kinetic activity every hour for 18 hours by incubation with 20 μM Ac-DEVD-AFC for 5 minutes.

Competitive vs. non-competitive inhibition of active caspases-3, -6 and -7 with 1541: Active caspases-3, -6 or -7 were incubated at 10 nM (caspase-3 or -7) or 50 nM (caspase-6) in the presence of compound 1541 (6.25 to 200 μM) in their optimal buffers in a total volume of 50 μl. The kinetic inhibition of the mixtures was immediately assayed by addition of Ac-DEVD-AFC (-3 and -7) or Ac-VEID-AFC (-6) (1.2 to 300 μM) for 10 minutes at room temperature.

Procaspase-1 incubation with 1541: Procaspase-1 (purified as described for procaspase-3) was incubated at 1 μM with 25 μM 1541 in an optimal reaction buffer consisting of 50 mM HEPES, pH 7.4, 0.01% Triton-X100, 200 mM NaCl, 50 mM KCl, 0.1 mM EDTA, 10 mM DTT overnight at 37 °C. The mixtures were agitated for 24 hours and aliquots were quenched with addition of 1% SDS reducing gel loading buffer and boiled. Samples were subjected to silver stain gel electrophoresis to determine extent of potential of procaspase-1 self-cleavage in the presence of 1541. All *in vitro* kinetic experiments were performed in 96-well plates with fluorescence read on a SpectraMax M5 microplate reader (Molecular Devices). Michaelis-Menten values (K_M and k_{cat}), Hill slopes, EC_{50}, IC_{50}, values were determined using GraphPad Prism software (GraphPad, Inc.).

Chemical Synthesis.

General procedure: 6-bromo-3-carboxy-coumarin was purchased from Alfa Aesar; O-(7-azabenzotriazole-1-yl)-N,N,N,N'-tetramethyluronium hexafluorophosphate was purchased from Applied Biosystems; 3-imidazo[1,2-a]pyridin-2-yl-phenylamine was purchased from Matrix Scientific; 8-methoxy-2-oxo-2H-chromene-3-carboxylic acid was purchased from Maybridge; 2,2-dimethyl-1,3-dioxane-4,6-dione (Meldrum's acid), 2,3-dihydroxybenzaldehyde, aniline, dimethylformamide and all other solvents and reagents were purchased from Sigma-Aldrich. The 3-carboxycoumarin ring was constructed from commercially available salicylaldehydes. The salicylaldehyde was reacted with Meldrum's acid in an aqueous solution (50-100 °C) to afford the 3-carboxycoumarin, as previously described (13). The resulting carboxylic acid was then converted to various amide derivatives using well-known coupling reactions, including direct coupling of the acid with amines using carbodiimide reagents. The products that precipitated out of solution were collected by filtration and dried under

vacuum. ^{1}H and ^{13}C NMR data were collected on a Varian 400 MHz spectrometer in DMSO-d_6. LCMS data was acquired on a Waters Micromass ZQ in ESI+ mode, equipped with a Waters 2996 photodiode array detector and Waters Alliance 2795 separations module. The LCMS protocol consisted of sample elution through an analytical Xterra C-18 column (2.1 mm x 50 mm x 3.5 μm) at a gradient of 5-95% methanol (0.2% formic acid)/water (0.2% formic acid) over 6 minutes at a flow rate of 1.0 mL/min.

8-Methoxy-2-oxo-2H-chromene-3-carboxylic acid (3-imidazo[1,2-a]pyridin-2-yl-phenyl)-amide (1541): Diisopropylethylamine (DIEA, 0.044 ml, 0.25 mmol) was added to 8-methoxy-2-oxo-2H-chromene-3-carboxylic acid (0.05 g, 0.23 mmol) and O-(7-Azabenzotriazole-1-yl)-N,N,N,N'-tetramethyluronium hexafluorophosphate (HATU, 0.096 g, 0.25 mmol) in 2 ml of dimethylformamide (DMF) with constant stirring at room temperature until a clear solution resulted. 3-Imidazo[1,2-a]pyridin-2-yl-phenylamine (0.048 g, 0.023 mmol) was then added and allowed to react for approximately 30 minutes. A yellow solid precipitated out of solution, was filtered and dried under suction and *in vacuo* to give 1541: ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.76 (s, 1H), 8.91 (s, 1H), 8.54 (ddd, J = 6.8, 1.1, 1.1 Hz, 1H), 8.44 (d, J = 0.6 Hz, 1H), 8.31 (dd, J = 1.8, 1.9 Hz, 1H), 7.73 (m, 2H), 7.60 (dd, J = 8.9, 0.7 Hz, 1H), 7.56 (dd, J = 7.7, 1.3 Hz, 1H), 7.44 (m, 3H), 7.26 (ddd, J = 8.9, 6.8, 1.2 Hz, 1H), 6.90 (ddd, J = 6.8, 6.8, 1.1 Hz, 1H), 3.96 (s, 3H); ^{13}C NMR (400 MHz, DMSO-d_6) δ 160.20, 159.87, 147.61, 146.33, 144.79, 143.87, 143.19, 142.36, 138.35, 134.72, 129.42, 126.92, 125.23, 125.04, 121.54, 121.19, 120.09, 119.05, 116.95, 116.66, 116.25, 112.33, 109.39, 56.24; LCMS (ESI) *m/z* 412 (MH^+).

8-Hydroxy-2-oxo-2H-chromene-3-carboxylic acid (3-imidazo[1,2-a]pyridin-2-yl-phenyl)-amide (1541B): 2,3-dihydroxybenzaldehyde (0.096 g, 0.69 mmol) and Meldrum's acid (0.100 g, 0.69 mmol) were combined in H_2O (1 ml). The solution was stirred at 75 °C for 2hr. After cooling to room temperature, the precipitate was filtered and dried at suction to give 0.123 g of 8-hydroxy-3-carboxy-coumarin in an 85% yield: LCMS (ESI) *m/z* 207 (MH^+). DIEA (0.046 ml, 0.27 mmol) was added to 8-hydroxy-3-carboxy-coumarin (0.050 g, 0.24 mmol) and HATU (0.101 g, 0.27 mmol) in 1 ml of DMF with constant stirring at room temperature until a clear solution resulted. 3-imidazo[1,2-a]pyridin-2-yl-phenylamine (0.051 g, 0.24 mmol) was then added and allowed to react overnight with a resulting yellow solid precipitation. The precipitate was filtered and dried under suction and *in vacuo* to give 0.021 g of 1541B in a 22% yield: ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.80 (s, 1H), 10.50 (s, 1H), 8.88 (s, 1H), 8.54 (ddd, J = 6.8, 1.1, 1.1 Hz, 1H), 8.44 (s, 1H), 8.31 (dd, J = 1.8, 1.7 Hz, 1H), 7.73 (m, 2H), 7.61 (d, J = 9.9 Hz, 1H), 7.45 (dd, J = 7.9, 7.8 Hz, 1H), 7.43 (dd, J = 6.6, 2.7 Hz, 1H), 7.25 (m, 3H), 6.90 (ddd, J = 6.8, 6.6, 1.2 Hz, 1H); ^{13}C NMR (400 MHz, DMSO-d_6) δ 160.43, 159.96, 147.87, 144.77, 144.52, 143.85, 142.54, 138.38, 134.69, 129.42, 126.92, 125.25, 125.06, 121.50, 120.33, 120.11, 119.68, 119.43, 119.10, 116.93, 116.64, 112.33, 109.39; LCMS (ESI) *m/z* 412 (MH^+).

Synthesis of 6-Bromo-2-oxo-2H-chromene-3-carboxylic acid (3-imidazo[1,2-a]pyridin-2-yl-phenyl)-amide (1541C): DIEA (35.6 μL, 0.2 mmol) was added to 6-bromo-3-carboxy-coumarin (50.0 mg, 0.19 mmol) and HATU (77.7 mg, 0.2 mmol) in 2 ml of DMF with constant stirring at room temperature until a clear solution resulted. 3-Imidazo[1,2-a]pyridin-2-yl-phenylamine (38.9 mg, 0.19 mmol) was then added and allowed to react overnight with a resulting solid precipitation. The precipitate was filtered, dried under suction and *in vacuo* to give 0.061 g of product in 68 % yield: ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.71 (s, 1H), 8.87 (s, 1H), 8.54 (d, *J* = 6.6 Hz, 1H), 8.43 (s, 1H), 8.32 (dd, *J* = 1.7, 1.8 Hz, 1H), 8.30 (d, *J* = 2.3 Hz, 1H), 7.93 (dd, *J* = 8.8, 2.1 Hz, 1H), 7.72 (m, 2H), 7.59 (d, *J* = 9.2 Hz, 1H), 7.54 (d, *J* = 8.8 Hz, 1H), 7.45 (dd, *J* = 7.9, 7.9 Hz, 1H), 7.25 (ddd, *J* = 8.1, 6.7, 0.8 Hz, 1H), 6.90 (dd, *J* = 6.7, 6.8 Hz, 1H); ^{13}C NMR (400 MHz, DMSO-d_6) δ 159.91, 159.64, 152.90, 145.89, 144.79, 143.86, 138.29, 136.38, 134.72, 132.10, 129.42, 126.91, 125.04, 121.60, 121.29, 120.34, 119.12, 118.54, 116.96, 116.77, 116.66, 112.32, 109.39; LCMS (ESI) *m/z* 461 (MH^+).

8-Methoxy-2-oxo-2H-chromene-3-carboxylic acid phenylamide (1541D): DIEA (0.044 ml, 0.25 mmol) was added to 8-methoxy-2-oxo-2H-chromene-3-carboxylic acid (0.050 g, 0.23 mmol) and HATU (0.095 g, 0.25 mmol) in 1 ml of DMF with constant stirring at room temperature until a clear solution resulted. Aniline (0.021 g, 0.23 mmol) was then added and allowed to react overnight with a resulting yellow solid precipitation. The precipitate was filtered and dried under suction and *in vacuo* to give 0.055 g of 1541D in an 82% yield: ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.65 (s, 1H), 8.88 (s, 1H), 7.72 (m, 2H), 7.54 (dd, *J* = 7.7, 1.5 Hz, 1H), 7.46 (dd, *J* = 8.2, 1.5 Hz, 1H), 7.39 (m, 3H), 7.14 (ddd, *J* = 7.4, 7.3, 1.0 Hz, 1H), 3.95 (s, 3H); ^{13}C NMR (400 MHz, DMSO-d_6) δ 160.13, 159.80, 147.58, 146.30, 143.16, 137.89, 129.00, 125.20, 124.29, 121.16, 120.06, 119.87, 119.03, 116.21, 56.23; LCMS (ESI) *m/z* 296 (MH^+).

Cellular procaspase activator characterization. All cells were maintained in the optimal media as suggested by ATCC.

1541 and 1541B EC_{50} determination with panel of cancer cell lines: 2,000 BT549, MDA-MB361, HeLa, HCC1954, 600MPE and HEK293 cells were incubated with 1541 or 1541B (0.39 to 50 μM) or DMSO (final DMSO 0.5%) for 24 hours in a final volume of 50 μl. Incubations were assayed for cell viability by addition of 50 μl of the CellTiter-Glo® (Promega).

Caspase activation and cell death time course: 2,000 BT549, MDA-MB361 or HEK293 cells were plated in 96-well plates for incubation with 25 μM 1541, 25 μM 1541C, 25 μM 1541D, 1 μM staurosporine (STS, Cayman Chemicals), 100 μM Etoposide (Sigma), or DMSO (final DMSO 0.5%). Plates were assayed for executioner caspase activity and cell viability with Caspase-Glo® 3/7 and

CellTiter-Glo® Luminescence Assay Kits (Promega), respectively, at 2, 4, 6, 8, 12 and 24 hours according to manufacturer's protocol.

Protection against apoptosis by 1541 with irreversible caspase peptide inhibitors: 2,500 BT549 cells were plated in 96-well plates and incubated overnight with Biotin-DEVD-FMK or Biotin-VAD-FMK (0.78 to 100 μM). Cells were exchanged into new media containing 25 μM 1541 and respective concentration of peptide inhibitor. Cells were incubated for 24 hours in a final volume of 50 μl. Incubations were assayed for cell viability by addition of 50 μl of the CellTiter-Glo® with luminescence read on an Analyst HT plate reader.

Whole cell lysate western blot analysis of apoptotic BT549 cells: One million BT549 cells were plated in 75 cm^2 T flasks for incubation with 25 μM 1541, 1 μM STS, 100 μM Etoposide or DMSO (final DMSO 0.5%) for 8 hours. In all cases, medium from individual wells, which might contain floating dead cells, was collected and mixed with the cell pellet from the same well. Cells were resuspended in 200 μl of PBS containing 1% SDS for cellular lysis and subjected to sonication. Whole cell lysates were quantified with BCA Protein Assay reagent (Pierce) to standardize protein levels, boiled for 5 minutes in the presence of reducing SDS-PAGE loading buffer and subjected to Western blot analysis probing for the presence of active caspase-3 (Cell Signaling 9664), caspase-6 (AbCam 32366), caspase-7 (Cell Signaling 9492) and PARP (Cell Signaling 9542). Western blots were stripped with methanol and re-probed for GAPDH (Cell Signaling 2118) as a loading control. For BT549 cytoplasmic extracts, cells were treated as described, harvested into PBS containing 0.025% digitonin, incubated for 30 minutes on ice and clarified by centrifugation at 4 °C. Western blots were probed for presence of active caspase-3 and cytochrome C (Cell Signaling 4272).

1541-induced cellular death of wild type and caspase-8 deficient human Jurkat: 100,000 wild type A3 or caspase-8 deficient I9.2 human Jurkat cells (*14*) were incubated with 25 μM 1541, 1 μM STS or 100 ng/ml Super FasL (Axorra) in 100 μl of optimal media. Incubations were assayed for cell viability every 2 hours by addition of 100 μl of CellTiter-Glo®.

Apoptosis of wild type and $Bak^{-/-}/Bax^{-/-}$ MEFs: 5,000 wild type and $Bak^{-/-}/Bax^{-/-}$ double knockout MEFs (kind gift from Dr. Scott Oaks, UCSF) were incubated with 1 μM STS, 25 μM 1541 or DMSO (final DMSO 0.5%) at 37 °C for 12 hours and assessed for apoptosis with the Cell Death Detection Elisa kit (Roche) according to manufacturer's protocol. The histone ELISA was used instead of the simpler CellTiter-Glo® viability assay to measure ATP levels because the mitochondria, and hence ATP levels, are preserved in the DKO MEFs.

Whole cell lysate western blot analysis of apoptotic wild type and Bak$^{-/-}$/Bax$^{-/-}$ MEFs: One million cells of both wild type and Bak$^{-/-}$/Bax$^{-/-}$ DKO MEFs were plated and incubated with 1 μM STS, 25 μM 1541 or DMSO (final DMSO 0.5%) at 37 °C for 12 hours. After harvesting both cells and media, cell pellets were resuspended in 200 μl of PBS containing 1% SDS for cellular lysis and subjected to sonication. Whole cell lysates were treated quantified and probed as described for the presence of active caspase-3 and PARP cleavage. For all cellular experiments, assays were performed in 96-well plates with luminescence read on an Analyst HT plate reader. IC_{50} values were determined using GraphPad Prism software.

Cell death comparison among cancerous and immortalized normal B cells: 25,000 normal (Epstein Barr Virus (EBV)-transformed B cells and cancerous large B cell lymphoma (DB) cells were incubated with 12.5 μM 1541B (final DMSO 0.5%). Plates were assayed for cell viability with CellTiter-Glo® at 2, 4, 6, 8, 12 and 30 hours according to manufacturer's protocol.

Mutations of procaspase-3 that confer resistance to activation by 1541. Sequence alignment of procaspase-3, -6 and -7 revealed residue conservation in areas near the active site with large conformational rearrangement upon procaspase activation (*15*) and were subjected to point mutational analysis. Point mutations of procaspase-3 were performed using QuickChange (Qiagen) mutagenesis. Procaspase-3 mutants were constructed using forward primer 5'-CGA CTT CTT GTA TGC ATA CGC CAC AGC ACC TGG-3' and complement for S198A, forward primer 5'-CGA CTT CTT GTA TGC ATA CTC CGC AGC ACC TGG-3' and complement for T199A and forward primer 5'-CCT GGT TAT TAT GCT TGG CGA AAT TCA AAG GAT GGC-3' and complement for S205A. Purification was performed exactly as described for procaspases-3, -6 and -7. Procaspase-3 mutants were incubated at 100 nM with 25 μM 1541 in 50 μL of 50 mM HEPES, pH 7.4, 0.01% Triton-X100, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT at 37 °C and sampled for activation via kinetic analysis after 5 hours. Kinetic assays were initiated by addition of substrate Ac-DEVD-AFC to a final concentration of 50 μM. The activity was measured for 20 minutes in 96-well plates on a SpectraMax M5 microplate reader.

Preparation of stable MCF-7 transfections with wild type and mutant procaspase-3: Wild-type and 1541-resistant (S198A) procaspase-3 variants were PCR subcloned from the corresponding pET23b vectors into the NotI and EcoRI sites of the bicistronic retroviral expression vector pQCXIP (Clontech) using forward primer 5'-AAA GGG AAA GCG GCC GCC ACC ATG GAG AAC ACT GAA AAC TCA GTG-3' and reverse primer 5'-AAA GGG AAA GAA TTC TCA TTT GTC GTC GTC ATC TTT GTA ATC GTG ATG GTG GTG ATG ATG GTG ATA AAA ATA GAG TTC TTT TGT GAG CAT-3'. A 6x-His-FLAG was appended in-frame to the C-terminus of procaspase-3 to facilitate detection by Western

blotting. Expression clones were sequence-verified and transfected into the GPG retroviral producer cell line (a kind gift from Orion Weiner, UCSF) using Lipofectamine 2000 (Invitrogen) to generate retroviral supernatants, which were clarified by centrifugation, supplemented with 8 μg/ml polybrene and used to transduce early-passage MCF-7 cells for 12 hours. Infections were repeated three times over the course of 72 hours and stable pools of procaspase-3-expressing MCF-7 cells were selected in 0.5 μg/ml puromycin (Invivogen) for 12 days. *MCF-7 transfection cell viability assays:* Stable procaspase-3 variant transfection MCF-7 cell lines (wild type, S198A, empty pQCXIP vector) were plated at 6,000 cells per well in a 96-well plate and incubated in the presence of 2 μM STS or 7.5 μM 1541 at 37 °C for 24 hours. Incubations were assayed for cell viability by addition of CellTiter-Glo® Luminescence Assay. Plates were analyzed for luminescence on an Analyst HT plate reader.

Figure Legends

Fig. S1. HTS scatter plot identifies 1541 as a procaspase-3 activator. A total of 62,000 compounds were screened for their ability to activate procaspase-3 after a 3-hour incubation at 37 °C. Granzyme B-activated caspase-3 fluorescence averaged 100-fold greater than the background procaspase-3 fluorescence. The assay was very reproducible with Z' ranging from 0.85 to 0.95. Blue lines indicate 3 standard deviations above or below the mean. All compounds that induced procaspase-3 activity above 3 standard deviations were considered potential "hits". Compound 1541 is circled in red.

Fig. S2. Specificity of 1541 and time course. Time course of executioner procaspase activation by 1541 facilitates full self-cleavage of procaspases-3 and -6. Procaspases-3, -6 and -7 were incubated at 100 nM with 1 nM granzyme B, DMSO, 100 μM PAC-1 or 25 μM 1541 at 37 °C. All procaspase incubations were harvested at 4 hours and subjected to silver-stain SDS-PAGE to determine the extent of auto-cleavage to the mature, active caspases. Schematic representations of the executioner procaspases-3, -6 and -7 sequences depict aspartic acid residues cleaved during activation. At 4 hours, 1541 induces full processing of procaspases-3 and -6 as seen by cleavage of both the prodomain and the intradomain junction between the large (17 kDa) and small (12 kDa) subunits. Procaspase-7 is robustly activated by granzyme B, but not by 1541 as indicated by only ~10% auto-proteolysis of the prodomain (residues 1-23). Previous studies show removal of the prodomain alone does not activate caspase-7 (*16*). Under our assay conditions, PAC-1 does not detectably increase the activity nor promote self-proteolysis of any of the executioner procaspases.

Fig. S3. Procaspase-1 is insensitive to 1541 activation. Procaspase-1 (expressed and purified in the absence of the N-terminal prodomain residues 1-120) was incubated at 1 μM for 24 hours at 37 °C with

25 μM 1541 in an optimal reaction buffer. SDS-PAGE reveals procaspase-1 does not self-activate in the presence of 1541.

Fig. S4. Schematic of general chemical synthesis of 1541 derivatives. The 3-carboxycoumarin ring is constructed from commercially available salicylaldehydes. The salicylaldehyde is reacted with Meldrum's acid in an aqueous solution (50-100 °C) to assemble the 3-carboxycoumarin (*13*). The carboxylic acid substituent on the coumarin scaffold is subsequently converted to amide derivatives using well-known coupling reactions with the final products collected by filtration and dried under vacuum.

Fig. S5. Initial activity constants of procaspase-3 in presence of 1541B. (**A**) Procaspase-3 was incubated at 100 nM in the presence of 1541B (0.78 to 50 μM) and immediately assessed for kinetic activity by addition of Ac-IETD-AFC (1.2 to 300 μM) at 37 °C. Ac-IETD-AFC represents the intradomain sequence procaspase-3 recognizes and cleaves to become active. Incubation with 1541 increases the catalytic efficiency of procaspase-3 by 57-fold over the unstimulated protein in the absence of self-proteolysis. Importantly, the K_M for Ac-IETD-AFC of mature caspase-3 is 10-fold greater (~215 μM) and, even in the presence of 50 μM 1541B, remains nearly unchanged (~140 μM). For amide bond hydrolysis by serine and cysteine proteases, K_M is a reasonable approximation of K_d (*17, 18*). Therefore, the Ac-IETD-AFC substrate binds more tightly to the 1541B-stimulated procaspase-3 than to mature caspase-3. (**B**) Plot of k_{cat}/K_M of procaspase-3 versus the concentration of 1541B. Procaspase-3 (100 nM) was incubated with 1541B (0.78 to 50 μM) and initial rates of activity were measured over ~10 min by addition of Ac-IETD-AFC (1.2 to 300 μM) at 37 °C. The k_{cat}/K_M inflection point matches the EC_{50} of 1541-induced autoproteolysis of procaspase-3 (Fig. 1A).

Fig. S6. Procaspase-3 self-cleavage in the presence of 1541. Procaspase-3 was incubated at 100 nM with increasing concentrations of 1541 (0-100 μM) for 4 hours at 37 °C. Aliquots were quenched with addition of 1% SDS reducing gel loading buffer and boiled. Samples were subjected to silver stain SDS-PAGE electrophoresis to determine extent of autocatalysis. A diagram of the procaspase-3 protein with cleavage sites is indicated. At intermediate concentrations, mixed populations of all possible cleaved species are present. Procaspase-3 becomes fully activated at higher 1541 concentrations, despite a reduced activity, suggesting 1541 inhibits the activated caspase species.

Fig. S7. Michaelis-Menten analysis shows competitive inhibition of executioner caspases-3 and -6 by 1541. (**A**) Caspases-3, -6 and -7 were incubated with increasing concentrations of 1541 (0 μM – red, 3.125 – blue, 6.25 – purple, 12.5 – light blue, 25 – green and 50 – magenta) and assayed for 10

minutes at room temperature by addition of various concentrations of fluorogenic peptides Ac-DEVD-AFC (caspases-3 and -7) or Ac-VEID-AFC (caspase-6). (**B**) In the presence of 50 μM 1541, the strongest effect by the compound is reflected in binding affinity of the substrate as K_M decreases by up to 30% for active caspase-3 and k_{cat} decreases by only a marginal amount of 8% (apparent K_i is ~98 μM). A similar effect occurs with active caspase-6, as the majority of 1541 influence is exerted on K_M (decrease ~50%) as k_{cat} decreases by only 7% (apparent K_i is ~63 μM). Unlike the other two executioner caspases, caspase-7 remains essentially unaffected with 1541 incubation.

Fig. S8. Traditional apoptotic hallmarks. One million BT549 cells were incubated for 8 hours in the presence of 25 μM 1541, 1 μM STS, 100 μM etoposide or DMSO (final 0.5%). Whole cell lysates were subjected to Western blot analysis probing for presence of active caspases-3, -6 and -7 and PARP cleavage. Large subunits of active caspases-3, -6 and -7 are clearly observable for the cells incubated with compound 1541; however, cleavage is more pronounced when incubated with 1 μM STS and is consistent with the higher overall DEVDase activity, as shown in **Fig. 3B**.

Fig. S9. DEVDase activity and cellular viability time courses with HEK293 and MDA-MB361. 2,500 cells were incubated with 25 μM 1541 (red), 25 μM 1541C (green), 25 μM 1541D (orange), 1 μM STS (blue), 100 μM etoposide (light blue), or DMSO (final 0.5%) and assayed for executioner caspase DEVDase activity and cell viability at 2, 4, 6, 8, 12 and 24 hours. Similar to BT549, cell death was achieved rapidly for both cell lines despite having an overall lower executioner caspase activity in the presence of 1541 compared to STS. Compounds 1541C and D, respectively, induce limited to no apoptosis or DEVDase activity in these cell lines. The cellular results for these 1541 analogs correlate with their *in vitro* activity against procaspase-3.

Fig. S10. Induction of apoptosis by 1541 in cancer cell lines. 2,000 cells from BT549 (blue), MDA-MB361 (green), HeLa (red), HCC1954 (light blue), 600MPE (magenta) and HEK293 (orange) were incubated with 1541 or 1541B (0.39 to 50 μM) (final DMSO 0.5%) for 24 hours. Cell viability was determined by the reduction in ATP levels using CellTiter-Glo®. The EC_{50} values range between 4 and 9 μM and are consistent with the *in vitro* EC_{50} of 1541 and 1541B for procaspases-3 and -6 activation. Compound 1541B, which is comparably active toward procaspase-3, but inactive against procaspase-6, shows virtually the same EC_{50} for inducing apoptosis. This implies that procaspase-3 activation is much more important for driving cellular apoptosis than procaspase-6.

Fig. S11. Cellular viability time course with normal and cancerous B cells. 25,000 normal EBV-transformed B cells (blue) and large B cell lymphoma (DB) cells (red) were incubated with 12.5 μM

1541B and assayed for cell viability at 2, 4, 6, 8, 12 and 30 hours. Preferential cell death was rapidly achieved for the B cell lymphoma in comparison to the immortalized normal B cell line. These preliminary results suggest that the cancerous cells were more sensitive to apoptosis induced by 1541.

References

1. H. Edelhoch, *Biochemistry* **6**, 1948 (1967).
2. C. Pop *et al.*, *Biochemistry* **40**, 14224 (2001).
3. P. A. Svingen *et al.*, *Clin Cancer Res.* **10**, 6807 (2004).
4. B. Y. Feng, B. K. Shoichet, *Nat. Protoc.* **1**, 550 (2006).
5. S. L. McGovern, E. Caselli, N. Grigorieff, B. K. Shoichet, *J. Med. Chem.* **45**, 1712 (2002).
6. M. Garcia-Calvo *et al.*, *Cell Death Differ.* **6**, 362 (1999).
7. K. S. Putt *et al.*, *Nat. Chem. Biol.* **2**, 543 (2006).
8. H. R. Stennicke, G. S. Salvesen, *J. Biol. Chem.* **272**, 25719 (1997).
9. H. R. Stennicke, G. S. Salvesen, *Biochim. Biophys. Acta* **1387**, 17 (1998).
10. H. R. Stennicke, G. S. Salvesen, *Cell Death Differ.* **6**, 1054 (1999).
11. C. Pop, J. Timmer, S. Sperandio, G. S. Salvesen, *Mol. Cell* **22**, 269 (2006).
12. E. N. Shiozaki, J. Chai, Y. Shi, *Proc. Natl. Acad. Sci. U.S.A.* **99**, 4197 (2002).
13. M. N. Deshmukh, R. Burud, C. Baldino, P. C. M. Chan, J. F. Liu, *Synthetic Commun.* **33**, 3299 (2003).
14. P. Juo, C. J. Kuo, J. Yuan, J. Blenis, *Curr. Biol.* **8**, 1001 (1998).
15. J. Chai *et al.*, *Cell* **107**, 399 (2001).
16. M. Van de Craen, W. Declercq, I. Van den brande, W. Fiers, P. Vandenabeele, *Cell Death Differ.* **6**, 1117 (1999).
17. B. Asboth, E. Stokum, I. U. Khan, L. Polgar, *Biochemistry* **24**, 606 (1985).
18. L. Hedstrom, *Chem. Rev.* **102**, 4501 (2002).

Fig. S1

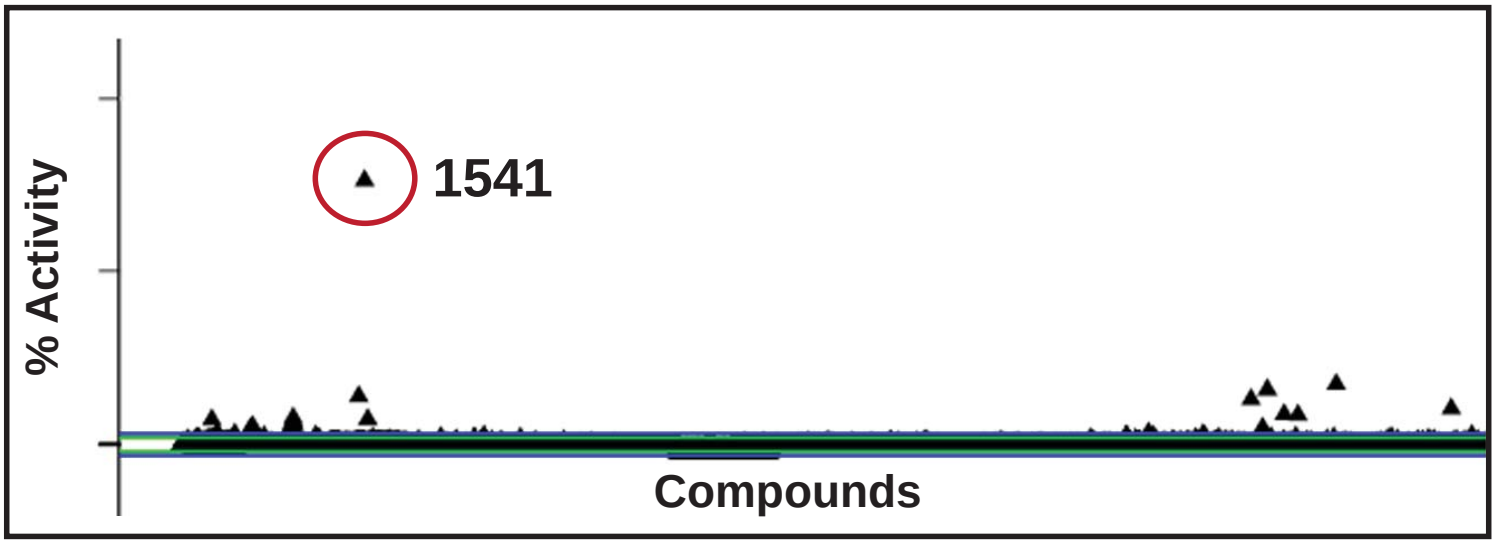


Fig. S2

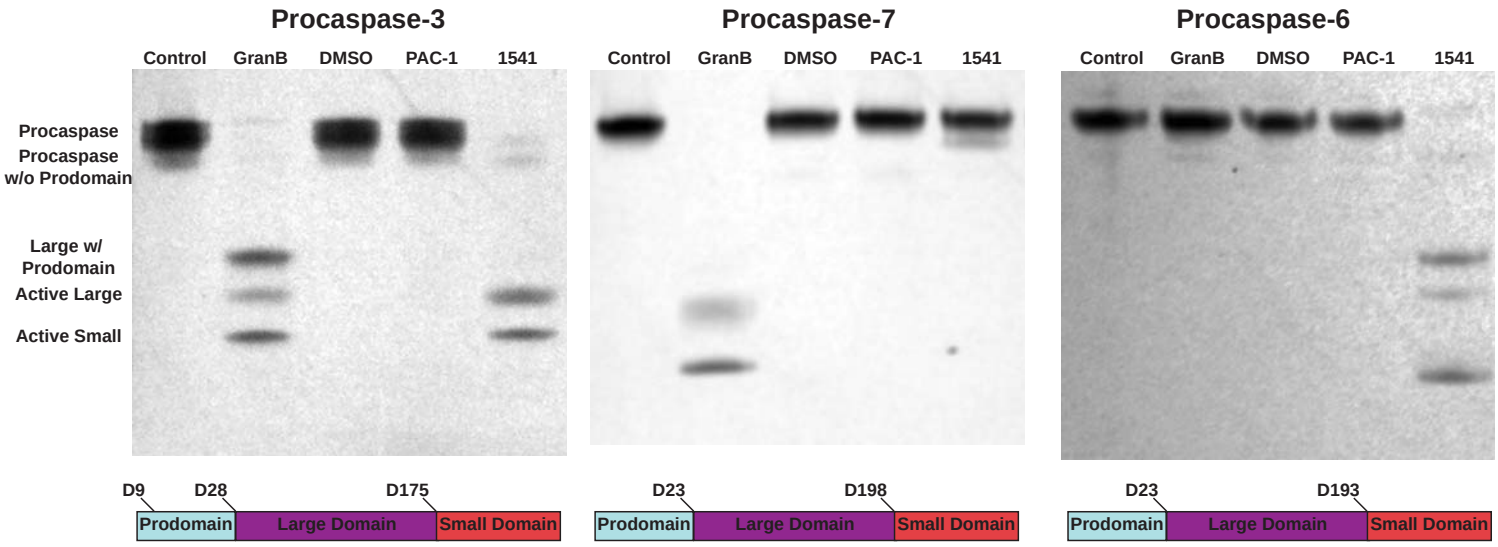

Procaspase-3
Control
GranB
DMSO
PAC-1
1541
Procaspase
Procaspase
w/o Prodomain
Large w/
Prodomain
Active Large
Active Small
D9
D28
D175
Prodomain
Large Domain
Small Domain
Procaspase-7
Control
GranB
DMSO
PAC-1
1541
D23
D198
Prodomain
Large Domain
Small Domain
Procaspase-6
Control
GranB
DMSO
PAC-1
1541
D23
D193
Prodomain
Large Domain
Small Domain

Fig. S3

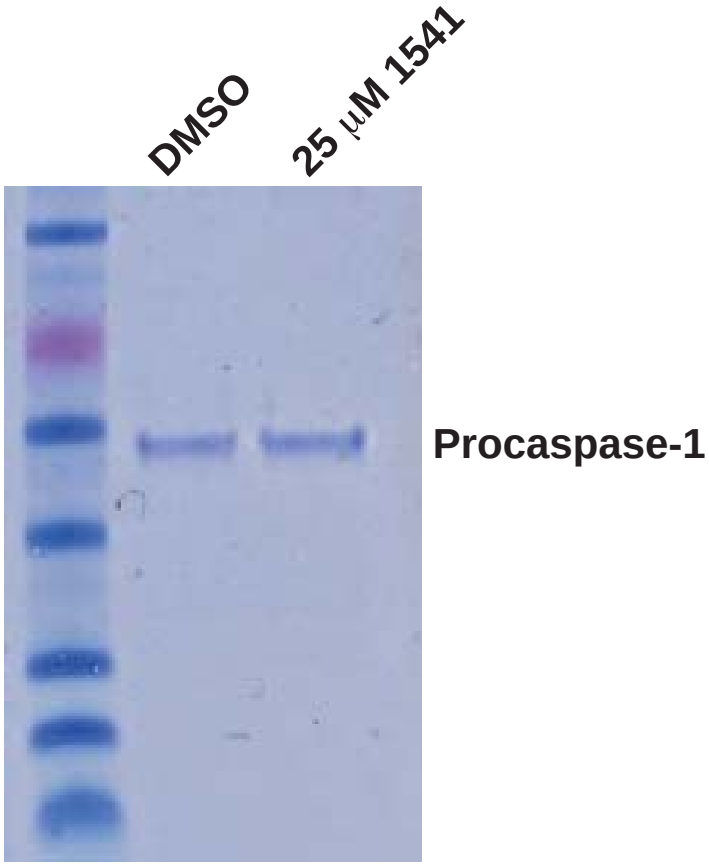


Fig. S4

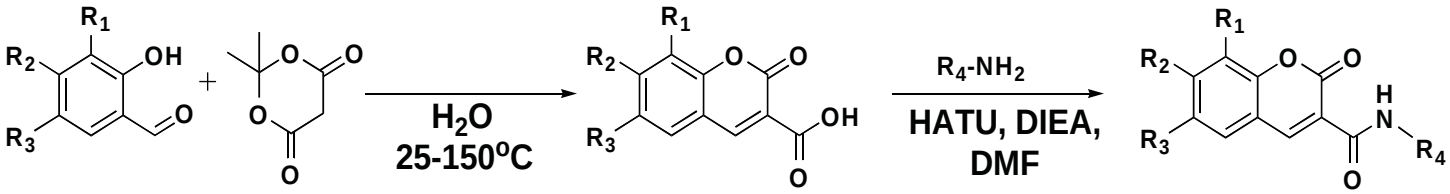

R1
R2
R3
OH
O
O
O
O
O
H2O
25-150°C
R1
R2
R3
O
O
OH
O
R4-NH2
HATU, DIEA,
DMF
R1
R2
R3
O
O
H
N
R4
O

Fig. S5

A

1541B (μM)	K_M (μM)	k_{cat} (min^{-1}) Ac-IETD-AFC	k_{cat}/K_M ($min^{-1}\mu M^{-1}$)
50.0	19	1.2	0.063
25.0	14	0.93	0.068
12.5	15	0.79	0.053
6.3	15	0.58	0.039
3.1	23	0.96	0.043
1.6	54	0.65	0.012
0.8	92	0.43	0.0047
0	101	0.12	0.0012
Active Casp-3 50 μM 1541B	140	300	2.1
Active Casp-3 Alone	212	530	2.5

B

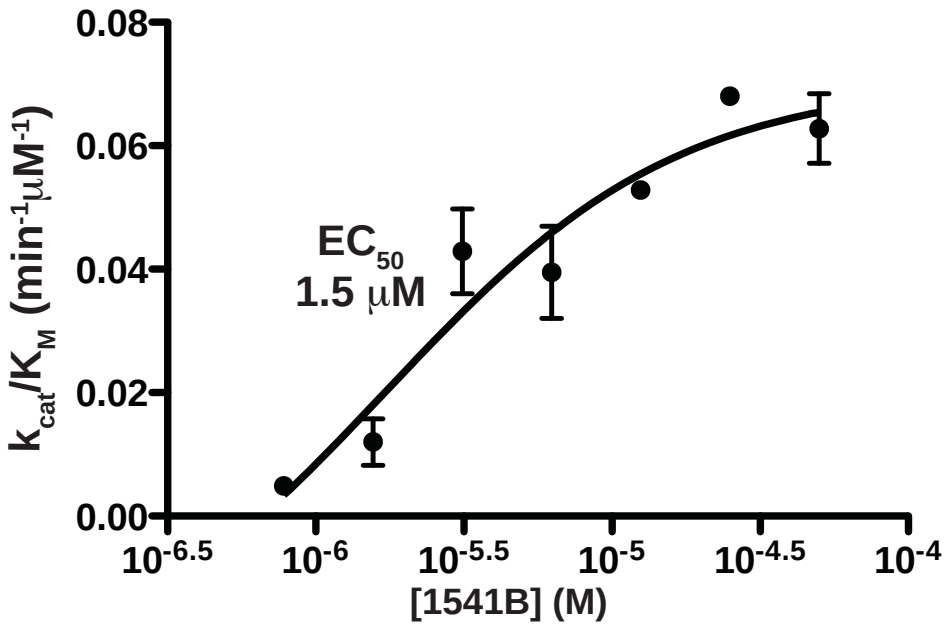


Fig. S6

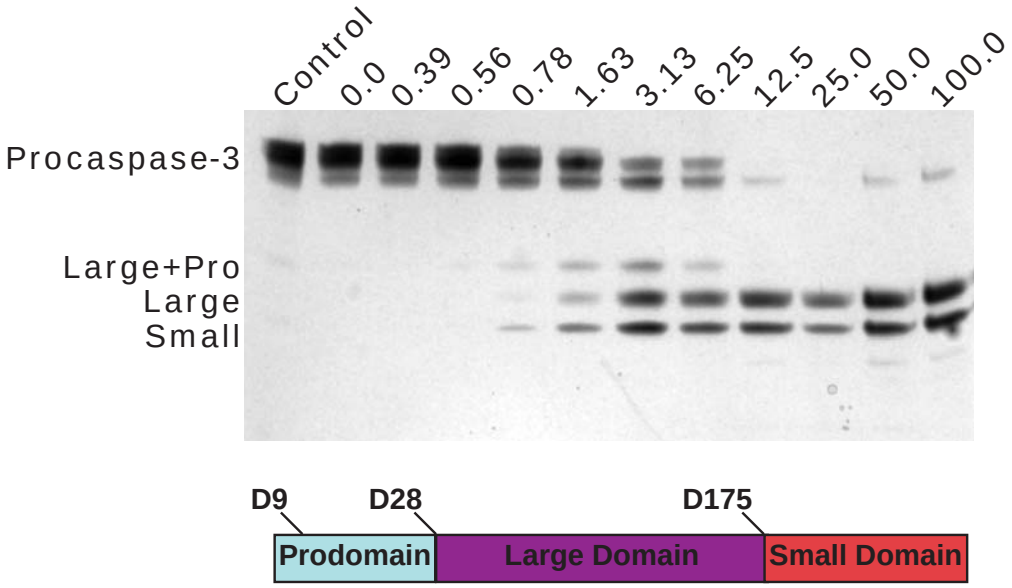
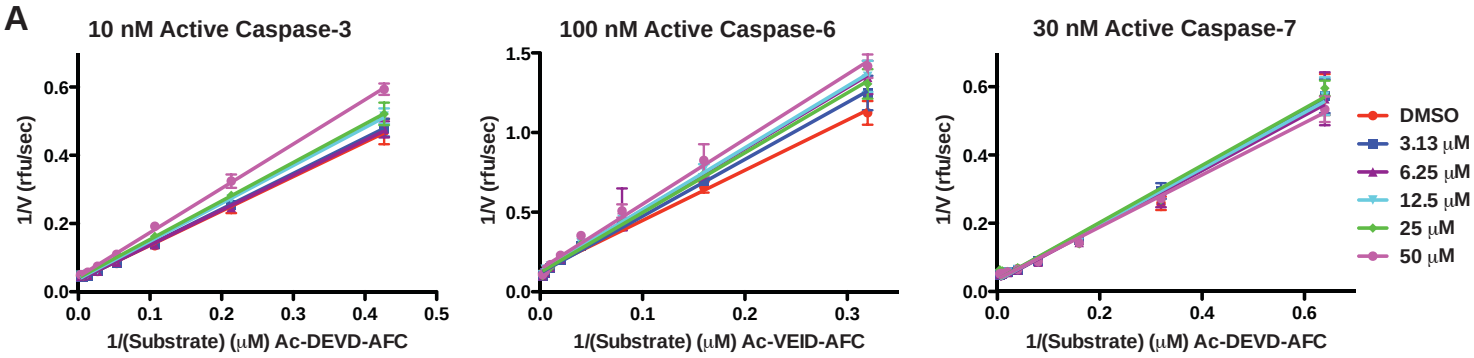

Control
0.0
0.39
0.56
0.78
1.63
3.13
6.25
12.5
25.0
50.0
100.0
Procaspase-3
Large+Pro
Large
Small
D9
D28
D175
Prodomain
Large Domain
Small Domain

Fig. S7

A



B

Protein Species	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\mu M^{-1}s^{-1}$)	Decrease in Catalytic Efficiency
10 nM Active Caspase-3 Alone	19.0	11.4	0.6	1.5
10 nM Active Caspase-3 50 μM 1541	27.0	10.7	0.4	
100 nM Active Caspase-6 Alone	38.4	0.4	1.1X10-2	1.6
100 nM Active Caspase-6 50 μM 1541	68.8	0.5	7.0X10-3	
30 nM Active Caspase-7 Alone	11.9	3.3	0.28	0.9
30 nM Active Caspase-7 50 μM 1541	11.4	3.3	0.29	

Fig. S8

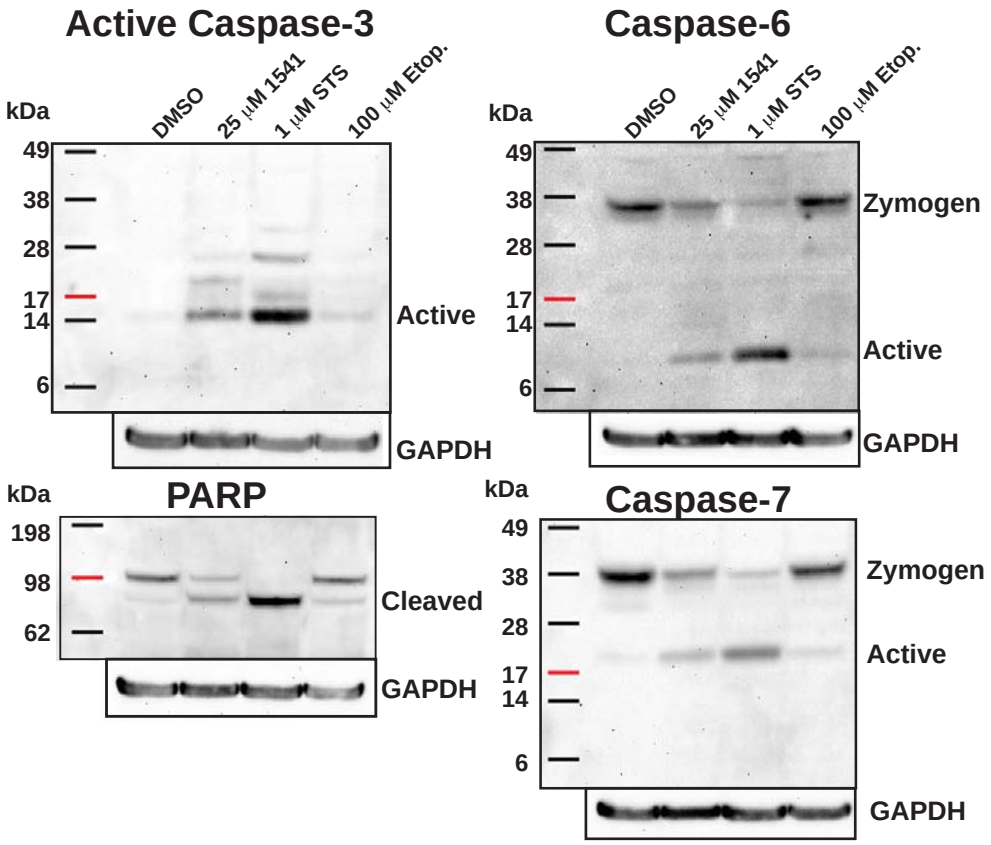

Active Caspase-3
kDa
DMSO
25 μM 1541
1 μM STS
100 μM Etop.
49
38
28
17
14
6
Active
GAPDH
Caspase-6
kDa
DMSO
25 μM 1541
1 μM STS
100 μM Etop.
49
38
28
17
14
6
Zymogen
Active
GAPDH
kDa
PARP
198
98
62
Cleaved
GAPDH
kDa
Caspase-7
49
38
28
17
14
6
Zymogen
Active
GAPDH

Fig. S9

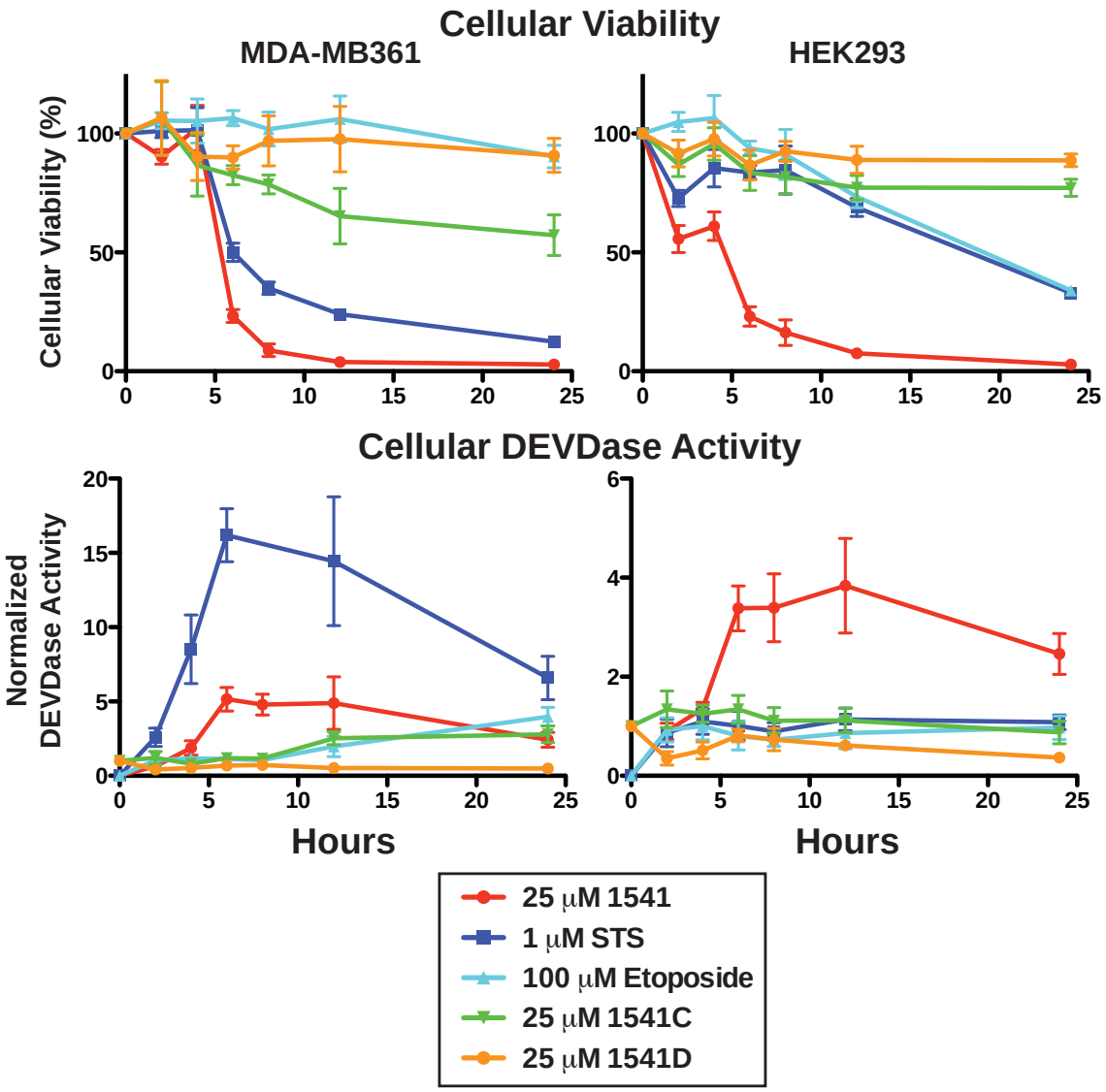
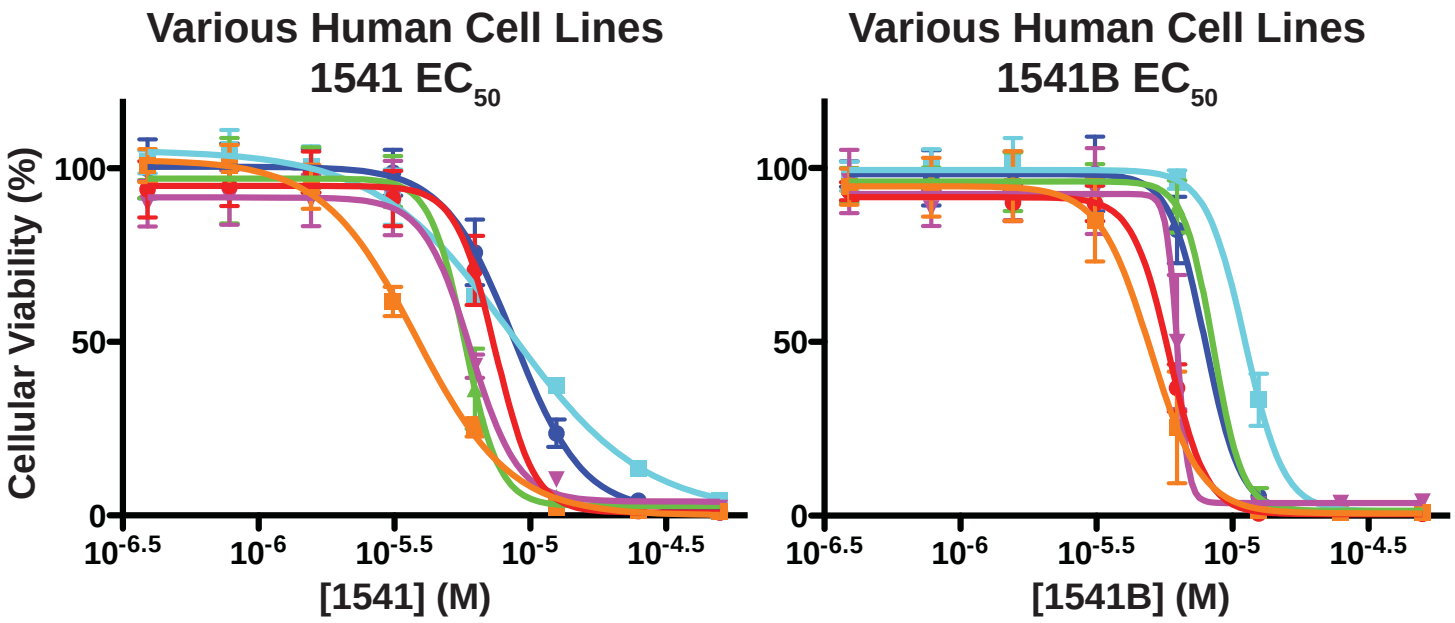
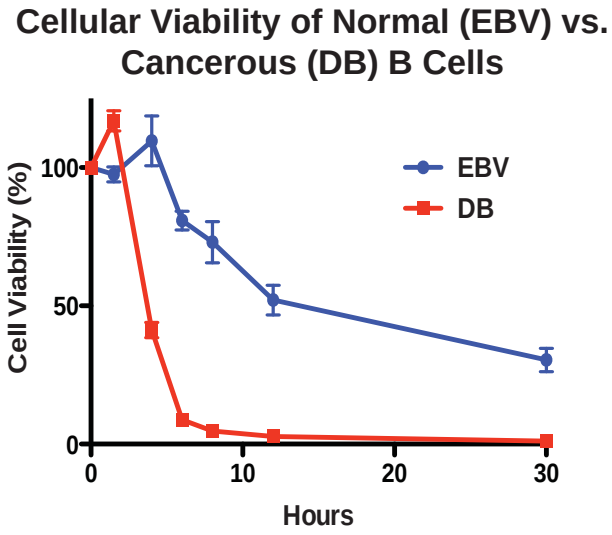

Cellular Viability
MDA-MB361
HEK293
Cellular Viability (%)
100
50
0
0
5
10
15
20
25
Cellular DEVDase Activity
Normalized DEVDase Activity
20
15
10
5
0
6
4
2
Hours
Hours
25 μM 1541
1 μM STS
100 μM Etoposide
25 μM 1541C
25 μM 1541D

Fig. S10



Cell Line	1541 EC_{50} (μM)	1541B EC_{50} (μM)
BT549	8.7	8.0
HCC1954	8.4	11.1
MB361	5.7	8.5
600MPE	6.0	6.3
HeLa	7.4	6.2
HEK293	3.8	5.0

Fig. S11


Cellular Viability of Normal (EBV) vs. Cancerous (DB) B Cells
Cell Viability (%)
100
50
0
0
10
20
30
Hours
EBV
DB