Figure S1. The IAP antagonist leads to down-regulation of cIAP1 and -2 in HaCaT and SCC cell lines, whereas DR expression is unchanged. (A) HaCaT, ASRT3, and MET1 cells were treated with 100 nM of the IAP antagonist or were co-stimulated with 10 µg/ml TNF-R2-Fc for the indicated time. Expression levels of cIAP1 and -2 and XIAP (in MET1 cells) were examined by Western blot analysis with specific antibodies to the respective proteins. Caspase-3 cleavage was monitored over time in MET1 cells accordingly. β-Tubulin served as an internal control. One of four representative results is shown. MM, molecular mass. Asterisks indicate nonspecific bands. (B) HaCaT and MET1 cells were stimulated with 100 nM of the IAP antagonist for 4 h or treated with diluent alone. Surface expression of CD95 and TRAIL-R1 and -R2 were examined with the respective antibodies to DRs and visualized by FACS. One of two independent experiments is shown. PE, phycoerythrin.
Figure S2. Resistance of ASRT3 cells to CD95L/IAP antagonist treatment is independent of TRAF2 or cIAP2. (A) Protein expression of FADD, cFLIP, caspase-8, TRAF2, RIP1, cIAP1 and -2, and XIAP was analyzed by Western blotting of 5 µg of total cellular lysates of HaCaT, ASRT3, and MET1 cells. β-Tubulin served as an internal control for even loading. One of three representative results is shown. (B and C) ASRT3 cell were transduced with TRAF2 cDNA containing retrovirus (B) or with cIAP2-specific shRNA containing retrovirus (C) or with the respective vector controls. Overexpression of TRAF2 (B; inset) or expression of cIAP2 and -1 (C; inset) was analyzed by Western blotting of 5 µg of total cellular lysates of transduced ASRT3. β-Tubulin (B) or β-actin (C) served as an internal control for even loading of protein lysates. One of two representative results is shown. Transduced ASRT3, as shown in C, was prestimulated for 30 min with 100 nM of the IAP antagonist (Ant) and subsequently treated with the indicated concentrations of CD95L for 18–24 h. The viability of cells was assayed by crystal violet assay. Mean and SEM of three independent experiments are shown. The inset of C also shows transduced ASRT3 cells that were stimulated for 30 min with 100 nM of the IAP antagonist, and expression of cIAP1 and -2 was determined by Western blotting. MM, molecular mass.
**Figure S3.** cFLIP regulates sensitivity to the combination of DL and IAP antagonist in ASRT3 cells. (A) ASRT3 cells were retrovirally transduced with either HRS shRNA or cFLIP-specific shRNA and selected for 3 d with 3 µg/ml puromycin. Knockdown efficiency of cFLIP<sub>L</sub> and cFLIP<sub>S</sub> was controlled by Western blot analysis. Reprobing of the membrane with antibodies to RIP1, FADD, caspase-8, and β-tubulin served as an internal control for protein loading. A representative of three independent experiments is shown. MM, molecular mass. (B) Transduced ASRT3 cells as shown in A were prestimulated for 30 min with 100 nM of the IAP antagonist (Ant) and 10 µg/ml TNF-R2-Fc and subsequently stimulated with the indicated concentrations of TRAIL (left) or CD95L (right) for 18–24 h and assayed by crystal violet assay. The mean ± SEM of three independent experiments is shown. (C) Inhibition of caspase activity (10 µM zVAD-fmk) and RIP1K activity by 50 µM Necrostatin-1 completely protects ASRT3 cells from DL-mediated cell death in the presence of the IAP antagonist. Transduced ASRT3 cells were separately prestimulated with 10 µM zVAD-fmk for 1 h, 50 µM Necrostatin-1 for 1 h, and 100 nM of the IAP antagonist for 30 min, followed by stimulation with 25 U/ml CD95L in triplicate wells. The viability of cells was analyzed by crystal violet assay. SEM of three independent experiments is shown.
Figure S4. The IAP antagonist sensitizes HaCaT cells to apoptotic and nonapoptotic cell death. (A) For the detection of phosphatidylserine externalization, HaCaT cells were pre- or co-stimulated with 10 µM zVAD-fmk for 1 h, 50 µM Necrostatin-1 for 1 h, and 100 nM of the IAP antagonist (Ant) for 30 min and subsequently treated with the indicated concentration of CD95L for 24 h. Cells were stained with annexin V–Cy5 and PI and then analyzed by FACS. One of two independent experiments is representatively shown. (B) For analysis of the release of HMGB-1 protein into the supernatant, HaCaT cells were pre- or co-stimulated with 10 µM zVAD-fmk for 1 h and 100 nM of the IAP antagonist for 30 min and subsequently stimulated with 5 U/ml CD95L for 24 h. Cell-free supernatants as well as total cellular lysates were analyzed for HMGB-1 protein expression. β-Tubulin was used as a loading control. One of two representative experiments is shown. (C) HaCaT cells were cotransduced with GEV16- and cIAP1-Flag cDNA containing lentivirus and selected for 5 d with 3 µg/ml puromycin and 150 µg/ml hygromycin. Inducible expression of cIAP1-Flag protein and IAP antagonist–mediated degradation of cIAP1 and -2 were controlled by Western blot analysis after prestimulation with 100 nM 4-HT for 24 h or co-stimulation with 100 nM of the IAP antagonist for 1 h. Reprobing of the membrane with antibody to β-tubulin served as an internal control for protein loading. A representative of two independent experiments is shown. (D) Transduced HaCaT cells as shown in C were prestimulated for 24 h with 100 nM 4-HT, co-stimulated for 1 h with 10 µM zVAD-fmk, co-stimulated for 30 min with 100 nM of the IAP antagonist, and subsequently stimulated with 2.5 U/ml CD95L for 18–24 h and assayed by crystal violet assay. SEM of three independent experiments is shown. All samples prestimulated with 4-HT for 24 h were normalized to control cells (e.g., set as 100%) in the presence of 4-HT because of the inhibitory effect of 4-HT on cellular growth under control conditions seen in all experiments. MM, molecular mass.
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Figure S5.  cFLIP, but not cFLIP, blocks IAP antagonist–DI-induced cell death in MET1 cells. (A) MET1 cells were retrovirally transduced with cFLIP, or cFLIP, or control vector. Total cellular lysates were analyzed for cFLIP and caspase-8. β-Tubulin served as an internal control for protein loading. MM, molecular mass. (B and C) Cells were prestimulated with 10 µM zVAD-fmk for 1 h, 50 µM Necrostatin-1 for 1 h, and 100 nM of the IAP antagonist for 30 min or diluent alone. Subsequently, cells were stimulated with 2.5 U/ml CD95L in triplicate wells. The viability of cells was analyzed by crystal violet assay after 18–24 h. The mean and SEM of three independent experiments are shown.