activated telomerease, as shown by immuno-detection of telomerase and TRAP assays. Furthermore, inhibition of BARD1 expression by siRNA transfection leads to loss of telomeric sequences, telomeric fusions, and chromosomal translocations in mouse mammary gland cells TAC-2, primary rat mesothelial cells, and Hela, or MCF-7 cells. This indicates that BARD1 controls telomere structures independently of telomerase activity. A fraction of BARD1 co-localizes with telomeres, as well as with the bona fide telomere binding protein TRF2, shown by FISH and immuno-staining and DNA chip assay. Our data are consistent with the following model: i) BARD1 might be sequestered on telomeres, ii) critically short telomeres would lead to an increase of unbound BARD1, iii) free BARD1 could act in apoptosis induction by binding and stabilizing p53 as demonstrated previously (Irminger-Finger Mol Cell 2001). Repression of BARD1 allows cells with critically short telomeres continue to divide. Due to genomic instability, eventually, some of these cells can activate telomerase and therefore pass crisis.

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Both RIP and c-FLIP are required for inhibition of caspase-8 cleavage in TRAIL-DISC in human cancer cells

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Background: Many cancer cells express the death receptors DR4 and DR5 for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) yet are resistant to TRAIL-induced apoptosis. TRAIL induces apoptosis through the recruitment of Fas-associated death domain (FADD) and caspase-8 to DR4/DR5, leading to assembly of death-inducing signaling complex (DISC) where caspase-8 is cleaved and initiates apoptosis. To explore the mechanisms in TRAIL resistance, we analyzed TRAIL-DISC in resistant cancer cells.
Methods: Non-small cell lung carcinoma A549, H596 and H1792, colon carcinoma Caco-2 and Colo320, breast carcinoma MB456, and pancreatic carcinoma Panc-1 cell lines were included in the study. TRAIL-DISC was immunoprecipitated using Flag-tagged TRAIL and anti-Flag M2 antibody and examined on Western blots. Cell death was analyzed by acid phosphatase assay and caspase cleavage was examined on Western blots. Synthetic small interfering RNA (siRNA) was generated by Qiagen Inc. and transfected with TransMessenger transfection reagent.
Results: Receptor-interacting protein (RIP) and cellular Fas-associate death domain-like, IL-1β-converting enzyme-inhibitory protein (c-FLIP) were reported in TRAIL-DISC. Western blot analysis of TRAIL-DISC revealed RIP and c-FLIP in TRAIL resistant A549 and H596, but not sensitive H1792 cells. Western blots detected cleavage products of caspase-8, caspase-3 and DNA fragmentation factor 45 (DFF45) in the cytoplasm in the sensitive, but not in the resistant cells. Transfection of siRNA targeting *c-FLIP* gene inhibits c-FLIP expression and sensitized the resistant cells to TRAIL-induced apoptosis through caspase-8-mediated caspase cascade. The results suggested that c-FLIP inhibited caspase-8 cleavage in the DISC in the resistant cells. Detection of RIP-mediated inhibitor of κB kinase (IKK) complex in the DISC suggested that RIP mediated IKK-mediated nuclear factor-κB (NF-κB) activation in resistant cells. Transfection of siRNA specific to *RIP* gene, however, sensitized the resistant cells to TRAIL-induced apoptosis through caspase-8-mediated caspase cascade. Transfection of either RIP or c-FLIP siRNA in TRAIL resistant Caco-2, Colo320, MB456 and Panc-1 cell lines resulted in the cell sensitivity to TRAIL-induced apoptosis.
Conclusion: The results indicate that both RIP and c-FLIP are required for inhibition of caspase-8 cleavage in TRAIL-DISC and thus targeting either RIP or c-FLIP may provide novel therapeutic strategies in cancer therapies.

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Mutant p53 reactivation by PRIMA-1: a novel strategy for cancer therapy

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Mutant p53 reactivation in tumors should trigger massive apoptosis and thus eliminate the tumor. We previously identified PRIMA-1, a low molecular weight compound that reactivates mutant p53 and induces robust apoptosis in human tumor cells. Intravenous adminstration of PRIMA-1 inhibited human tumor xenograft growth in mice (1). These results were corroborated by our statistical analysis of available information in the National Cancer Institute database (2). Growth inhibition profiles for PRIMA-1 and known anticancer agents were analyzed. PRIMA-1 was consistently more efficient in inhibiting growth of mutant p53-carrying tumor cell lines compared to wild type p53-carrying lines, and sensitivity to PRIMA-1 was correlated to mutant p53 expression levels. This distinguishes PRIMA-1 from most known anticancer drugs which preferentially affect tumor cells carrying wild type p53. We have found that PRIMA-1 acts synergistically with several anticancer drugs to inhibit tumor cell growth. Our further studies revealed that PRIMA-1 and cisplatin showed synergistic induction of apoptosis in a mutant p53-dependent manner. Combined systemic treatment with low doses of PRIMA-1 and cisplatin produced a significant synergistic antitumor effect in mice carrying human tumor xenografts. Enhancement of mutant p53 expression levels by DNA-damaging chemotherapeutic drugs may increase sensitivity to PRIMA-1-induced apoptosis. The anticancer efficacy of PRIMA-1 will be tested in clinical trials. Reactivation of mutant p53 by PRIMA-1 alone or in combination with conventional chemotherapeutic drugs is a novel strategy for cancer therapy that should allow efficient elimination of mutant p53-carrying tumors.

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201 POSTER

Analysis of survivin splice variant transcripts in human breast tumor cells

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Background: Survivin, is a member of the apoptosis inhibitors family, and is expressed in several human tumors. Two alternative splice variants of survivin (survivin-DEx3, and survivin-2B) differing in their anti-apoptotic properties were recently identified. While the anti-apoptotic effect of survivin-DEx3 is preserved, survivin-2B has lost its anti-apoptotic potential and may act as a naturally occurring antagonist of survivin and survivin-DEx3. Because in vivo studies have reported absence of survivin-2B in some cases of tumor progression, we analyzed the expression of these transcripts in breast cancer.
Material and Methods: reverse transcriptase polymerase chain reaction was performed using RNA samples obtained from 2 groups of breast cancer: node-negative (N−) and locally advanced (LA) tumors.
Results: all survivin variants were expressed in a majority of tumor samples, with survivin variant being the most dominant. In contrast, survivin-2B expression frequency was higher in N− (37/40: 93%) than in LA tumors (56/84: 67%; p=0.002).
Conclusions: These results demonstrated the expression of survivin splice variants in breast tumors and strongly suggested that the absence of expression of survivin-2B could be related to tumor progression in this disease.

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The proteasome inhibitor bortezomib (VELCADE™) sensitizes human tumor cells to TRAIL-mediated apoptosis by reduction of c-FLIP

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We have previously reported that the proteasome inhibitor bortezomib (PS-341, VELCADE™) can sensitize a murine acute myeloid leukemia (C1498) and a murine renal cancer (Renca) to TRAIL-mediated apoptosis. The effects of bortezomid and TRAIL were selective, since this combination could purge C1498 tumor cells from bone marrow, without major effects on normal bone marrow cells. Surprisingly, sensitization of these murine tumor cells was independent of any effect of bortezomib on NF-kB activation, yet did correlate with a reduction in levels of the anti-apoptotic protein c-FLIP. Utilizing the NCI panel of 60 human tumor cell lines, we assessed the sensitivity of a wide variety of different human cancer cell lines to the combination of bortezomid and TRAIL. A significant number of the tumor cell lines (20–30%) were dramatically sensitized to TRAIL-mediated apoptosis by treatment with bortezomib (20nM). However, for the remainder of the tumor cell lines, no such sensitization occurred. No

obvious pattern emerged to account for these differences. There was no apparent correlation for bortezomid sensitization with the tissue of origin of the tumor cells, their sensitivity to TRAIL as a single agent, or their p53 status. A more detailed analysis of 7 human renal cancer cell lines demonstrated a clear increase in the sensitivity of 3 renal lines to TRAIL following bortezomib treatment. For 2 of 3 sensitized renal lines, c-FLIP levels were significantly reduced by bortezomib as assessed by western blotting. No changes in c-FLIP were seen in the remaining 4 renal cancer lines. In contrast, cycloheximide (CHX) sensitized all 7 human renal cancers to TRAIL, and dramatically reduced the levels of c-FLIP in all cases. Also CHX did not affect cell surface levels of the TRAIL death receptors. In addition, siRNA to c-FLIP reduced concentrations of the protein by western blotting and sensitized the human renal cancers to TRAIL This data suggests that can sensitize some human tumor cells to TRAIL-mediated apoptosis, and reduction in the levels of c-FLIP may be an important component of the molecular mechanism. Funded in part by DHSS #NO1-CO-12400

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Modulation of DNA damage induced apoptosis by the proteasome inhibitor bortezomib (PS) in human colorectal and non-small cell lung cancer cells is p53-dependent and NF-kB-independent

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Inhibition of anti-apoptotic signaling via NF-κB activation is a major mechanism of action of proteasome inhibition. However, our data in human non-small cell lung carcinoma cells (NSCLC-3) suggest that synergistic enhancement of DNA damage induced apoptosis by the proteasome inhibitors, MG-132 (J. Biol. Chem. 276: 8029, 2001) and PS is NF-κB independent. To determine the mechanism by which PS modulates DNA damage (γ-radiation or topoisomerase I inhibitor, SN-38) induced apoptosis, we evaluated the role of p53, another important signaling target of the 26S proteasome. Treatment of human colorectal carcinoma cells, either wild type, HCT-116 (p53 +/+), or p53 null, HCT-116 (p53 −/−), with 40–200 nM SN-38 for 60 min followed by 1 μM PS for 30 min (SN-38 → PS) led to a synergistic increase in SN-38-induced apoptosis only in HCT-116 (p53 +/+) cells. This result suggests that enhancement of SN-38 induced apoptosis by PS involves a p53-dependent pathway. The functional role of p53 was confirmed in HCT-116 (p53 +/+) cells or NSCLC-3 cells transfected with p53 targeted siRNA in which SN-38 → PS treatment did not lead to enhanced apoptosis. Interestingly, treatment with PS followed by SN-38 (PS → SN-38) led to an antagonistic effect. Enhanced apoptosis was accompanied by increased accumulation of p53, including higher molecular weight ubiquitinated species, in SN-38 → PS compared to PS → SN-38 treated cells. Analysis of sub-cellular distribution revealed significantly higher levels of p53 in the cytosolic fraction in SN-38 → PS treated cells. In contrast, p53 was primarily localized to the nucleus in PS → SN-38 treated cells. Increased accumulation of p53 in SN-38 → PS treated cells correlated with persistent inhibition of proteasome activity (up to 16 h) in these cells compared to PS → SN-38 treated cells in which proteasome inhibition was observed only for 3h. Analysis of p53 dependent downstream effectors of the synergistic apoptotic response revealed the down-regulation of survivin transcript and protein in HCT-116 or NSCLC-3 cells, only following SN-38 → PS treatment. The role of survivin in the apoptotic response was confirmed in cells transfected with dominant-negative threonine34alanine mutant survivin or survivin targeted siRNA in which significantly ($P<0.05$) higher apoptosis was observed with SN-38 → PS compared to PS → SN-38 treatment. These results demonstrate that p53 is a key regulator in NF-κB independent sensitization of DNA damage-induced apoptosis by bortezomib.

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HGS-TR2J, a human, agonistic, TRAIL receptor 2 monoclonal antibody, induces apoptosis, tumor regression and growth inhibition as a single agent in diverse human solid tumor cell lines

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Introduction: Tumor necrosis factor related apoptosis-inducing ligand, (TRAIL) death receptors are expressed on the cell surface of many human tumor cells and activation by the ligand, TRAIL, induces programmed cell death. We have developed, in collaboration with Kirin Brewery Co., HGS-TR2J (also known as KMTR-2), a human, agonistic, TRAIL receptor 2 (TRAIL-R2) monoclonal antibody (mAb), for use as therapeutic treatment for human cancer.

Methods: We assessed the in vitro and in vivo efficacy of this human mAb in NSCLC (non-small cell lung cancer), ovarian and colon tumor cell lines in cytotoxicity assays and xenograft tumor models. Several NSCLC, ovarian and colon tumor cell lines were examined for TRAIL receptor expression by flow cytometry and sensitivity to HGS-TR2J in vitro. Multiple in vivo xenograft experiments were used to evaluate the activity of HGS-TR2J as a single agent in these diverse solid tumor types. Immunohistochemical analysis for apoptotic cells was performed on tumors from HGS-TR2J treated animals to confirm the induction of programmed cell death in xenograft tumors.
Results: A majority of the cell lines in each tumor type expressed high levels of TRAIL-R2 on the cell surface and displayed moderate to significant sensitivity (50–90% cell death) to HGS-TR2J in vitro. Two NSCLC (H2122 and H460), one ovarian (A2780) and one colon (COLO205) cell line, that all possessed equivalent TRAIL-R2 cell surface expression, were used to evaluate efficacy of HGS-TR2J in xenograft tumor models. In subcutaneous COLO205 and H2122 xenograft models, HGS-TR2J induced significant ($p<0.0001$) and rapid tumor regression (~80% decrease in tumor volume in 4 days) after a single 2.5 mg/kg IV dose, and persistent inhibition of tumor growth with continual weekly treatment. In contrast, in H460 and A2780 xenograft models, HGS-TR2J significantly ($p<0.001$) inhibited tumor growth but did not induce tumor regression. The difference in observed in vivo responsiveness did not correlate with in vitro TRAIL-R2 expression. In the COLO205 xenograft model the rapid decrease in tumor volume after HGS-TR2J administration was associated with a dramatic increase in intra-tumor apoptosis within 12 hours of treatment.
Conclusions: These data reveal that HGS-TR2J has significant anti-tumor activity that is associated with increased apoptosis in a range of human tumor types and settings. This demonstrates the potential of HGS-TR2J as a therapeutic for the treatment of human cancer.

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Erucylphosphocholine: molecular requirements for apoptosis induction by a membrane targeted drug

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Alkylphosphocholines (APC) have been identified as promising membrane targeted drugs with antineoplastic activity *in vitro* and in animal models. We have shown earlier that erucylphosphocholine (ErPC), the prototype of intravenously applicable APC derivatives induces apoptosis independently from caspase-8 and FADD via a mitochondrial pathway that is inhibited by over-expression of Bcl-2.
The aim of the present study was to analyse further molecular requirements for ErPC induced apoptosis and to define the role of the subcellular localisation of Bcl-2 for its inhibitory action in this process. To this end, caspase activation, alteration of mitochondrial functions and apoptosis induction was tested in cellular systems with dysfunctions of the mitochondrial death pathway and deficiency of Bax as well as in Jurkat T-lymphoma cells with over-expression of Bcl-2 in defined subcellular compartments. Expression of Bcl-2 was restricted to the outer membrane of the mitochondria or the ER by replacing its membrane anchor with the mitochondrial insertion sequence of ActA (Bcl-2/MT) or the ER-specific sequence of cytochrome b5 (Bcl-2/ER), respectively. Additionally, Jurkat cells expressing wild-type Bcl-2 (Bcl-2/WT) or a transmembrane domain-lacking mutant (Bcl-2/deltaTM) were used.
Our results show that apart from the requirement of caspase-9 ErPC-induced apoptosis was dependent on the expression of Apaf-1 and Bax. Furthermore, over-expression of Bcl-2 in the outer membranes of the ER or in the outer membranes of the mitochondria, as well as over-expression of Bcl-2 in the outer membranes of both compartments strongly inhibited ErPC-induced mitochondrial alterations, caspase activation, and apoptosis, while cytosolic Bcl-2/deltaTM was inactive. However, for efficient long-term inhibition Bcl-2 had to be present in both, the outer membrane of the ER as well as of the mitochondria.
In conclusion, our data support our previous findings that ErPC induces apoptosis via a mitochondrial death pathway and demonstrate that membrane localisation of Bcl-2 either in the mitochondria or the ER is a prerequisite for its inhibitory action on ErPC-induced apoptosis. The finding that ER-targeted Bcl-2 can strongly interfere with the ErPC-induced mitochondrial death pathway points to a cross-talk between mitochondria and ER during apoptosis signaling which is reminiscent of our recent results obtained with radiation-induced apoptosis.
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