

and their significant role in cancer progression, *CCR5* and *TNF* gene expression studies are being investigated.

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POSTER

Reolysin induces endoplasmic reticular stress in multiple myeloma and enhances the activity of bortezomib

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Background: While the approvals of bortezomib (BZ) and lenalidomide have significantly improved the treatment of multiple myeloma (MM), it remains an incurable disease. MM cells have high protein synthesis rates due to their heavy engagement in immunoglobulin production, which renders them hypersensitive to endoplasmic reticulum (ER) stress-mediated apoptosis. Reolysin is a proprietary formulation of the human reovirus that has shown anticancer efficacy in clinical studies. We hypothesized that Reolysin treatment would lead to the accumulation of viral products in MM cells, stimulate ER stress, and significantly enhance BZ-induced apoptosis.

Materials and Methods: The antimyeloma activity of Reolysin was assessed by MTT assay, propidium iodide staining followed by flow cytometry, and measuring active caspase-3 levels in a panel of MM cell lines. Dual reovirus and ubiquitin accumulation were visualized by confocal microscopy. Intracellular Ca²⁺ levels were quantified by flow cytometry and increases in ER stress-related genes were determined by quantitative real-time PCR and immunoblotting. Xenograft (RPMI-8226) and syngeneic (5TGM1) models of MM were used to evaluate the effects of Reolysin and bortezomib in vivo.

Results: Reolysin induced viral replication and apoptosis selectively in MM cell lines and not in normal peripheral blood mononuclear cells (PBMCs). Reolysin treatment stimulated ER stress as measured by increased expression of GADD153, GADD34, and XBP-1s, ER swelling visualized by electron microscopy, and an elevation of intracellular Ca²⁺ levels. Co-treatment with Reolysin and BZ promoted the simultaneous accumulation of viral and ubiquitin-conjugated proteins, which resulted in enhanced levels of ER stress and cell death. Importantly, the Reolysin and bortezomib combination significantly reduced tumor burden in both xenograft and syngeneic MM mouse models.

Conclusion: Reovirus replication in MM cells induces an accumulation of viral products that stimulates ER stress and apoptosis. Co-treatment with Reolysin and bortezomib stimulated a simultaneous accrual of viral and ubiquitinated proteins leading to enhanced ER stress-mediated apoptosis. Reolysin is a promising anticancer agent that displays activity against MM alone and in combination with bortezomib and warrants further investigation for the treatment of MM and other malignancies.

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POSTER DISCUSSION

Use of functional human cancer cell line mitochondria to explore the mechanisms of ABT-737-induced mitochondrial membrane permeabilization

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Current limitations of chemotherapy include toxicity on healthy tissues and multidrug resistance of malignant cells. A number of recent anti-cancer strategies aim at targeting the mitochondrial apoptotic machinery to induce tumor cell death. In this study, we set up protocols to purify functional mitochondria from various human cell lines to analyze the effect of peptidic and xenobiotic compounds described to harbour either Bcl-2 inhibition properties or toxic effects related to mitochondria. Mitochondrial inner and outer membrane permeabilization were systematically investigated in cancer cell mitochondria versus non-cancerous mitochondria. The truncated (t-) Bid protein, synthetic BH3 peptides from Bim and Bak, and the small molecule ABT-737 induced a tumor-specific and OMP-restricted mitochondrio-toxicity, while compounds like HA-14.1, YC-137, Chelerythrine, Gossypol, TW-37 or EM20-25 did not. We found that ABT-737 can induce the Bax-dependent release of apoptotic proteins (cytochrome c, Smac/Diablo and Omi/HtrA2 but not AIF) from various but not all cancer cell mitochondria. Furthermore, ABT-737 addition to isolated cancer cell mitochondria induced oligomerization of Bax and/or Bak monomers already inserted in the mitochondrial membrane. Finally immunoprecipitations indicated that ABT-737 induces Bax, Bak and Bim sequestration from Bcl-2 and Bcl-xL but not from Mcl-1L. This study investigates for the first time the mechanism of action of ABT-737 as a single agent on isolated cancer cell mitochondria. Hence, this method

based on MOMP (mitochondrial outer membrane permeabilization) is an interesting screening tool, tailored for identifying Bcl-2 antagonists with selective toxicity profile against cancer cell mitochondria but devoid of toxicity against healthy mitochondria.

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POSTER

Disruption of autophagic and autolysosomal signaling pathways leads to synergistic augmentation of erlotinib-induced apoptosis in wild type EGFR human non-small cell lung cancer cell lines

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Background: autophagy is a tightly regulated lysosomal self-digestion process that may promote cell survival and/or be involved in type II cell death. Erlotinib, an orally bioavailable EGFR TK inhibitor, has modest but real efficacy in wild type EGFR NSCLC tumors. The potential role of autophagy in erlotinib-induced cytotoxicity has not been previously investigated.

Materials and Methods: four wild type EGFR NSCLC cell lines were used: erlotinib-sensitive H322 and H358, and erlotinib-resistant H460 and A549.

Results: erlotinib at a clinically achievable concentration (2 mM) induced autophagic features including an increase in the formation of AVO and MDM staining, conversion of LC3-I (the cytosolic form) to LC3-II (the lipidated form associated with autophagosome), and the formation of autophagic vacuoles in both erlotinib-sensitive and -resistant cell lines. The combination of erlotinib with chloroquine, an autophagy inhibitor, was synergistic in all tested human NSCLC cell lines. Co-treatment with 3-methyladenine (3-MA), an inhibitor of early stage autophagy or with bafilomycin A1, an inhibitor of late stage autophagy, and down-regulation of Atg-5 and Beclin-1 gene expression by siRNA resulted in enhanced apoptotic cell death and cytotoxicity as compared with erlotinib alone in H460 and A549 cells. Moreover, co-treatment with lysosomal inhibitors, ammonium chloride, E-64, Pepstatin-A and Z-LA-fmk also resulted in the enhancement of erlotinib-induced cytotoxicity. The combined treatment with chloroquine did not alter erlotinib-induced EGFR pathway inhibition or G1 cell cycle arrest, but significantly induced apoptosis associated with the activation of caspase-9 and caspase-3, and increase in cleavage of PARP protein in all tested cells. Interestingly, the combination of erlotinib and chloroquine induced up-regulation of Bim protein expression, and down-regulation of Bim gene expression by siRNA attenuated cell death induced by the drug combination. In addition, inhibition of autophagy by chloroquine treatment enhanced erlotinib-induced ROS generation, and activation of p38 but not JNK signaling. Inhibition of ROS generation by NAC, or inhibition of p38 by SB202190 resulted in the attenuation of the potency of the combination, suggesting that ROS generation and p38 activation are potential mediators of the effect of this combination.

Conclusion: inhibition of autophagy by chloroquine represents a novel strategy to enhance erlotinib efficacy in wild type EGFR NSCLC tumors. This work was supported in part by NIH grant CA84119 and CA96515.

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POSTER

PI3-kinase inhibition enhances ABT-737 induced apoptosis in colorectal cancer cell lines

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Background: Although context dependent, the PI3-kinase signalling pathway is widely reported to promote cell survival, proliferation and migration. This pathway is up-regulated in several solid tumours via mechanisms including PTEN deletion, RAS or PIK3CA mutation and aberrant activation of receptor tyrosine kinases. As such, inhibitors of PI3-kinase and its down-stream effectors are of great interest as tractable anti-cancer agents. In colorectal cancer (CRC) cells inhibition of the PI3-kinase pathway did not induce apoptotic cell death. Moreover, CRC cells were relatively resistant to the pro-apoptotic BH-3 mimetic ABT-737. This study examined whether PI-3K inhibition and ABT-737 combined reduced the threshold of apoptosis in CRC cells.

Methods: The PI3-kinase pathway was inhibited with PI-103 (PI3-kinase inhibitor), AKT1/2 (AKT inhibitor) or rapamycin (mTORC1 inhibitor). Apoptosis was assessed by activating conformational changes in BAK, caspase 3 cleavage, and phosphatidylserine exposure. Interactions between Bcl-2 BH3 family members were assessed by co-immunoprecipitation. Expression of the ABT-737 resistance factor Mcl-1 was knocked down by transfection of siRNA.

Results: PI3-kinase inhibition enhanced apoptosis induced by ABT-737 upstream of cytochrome c release in CRC cells. PI3-kinase inhibition reduced the levels of Mcl-1. However, PI3-kinase inhibition further enhanced ABT-737 induced apoptosis in CRC cells were Mcl-1 was

depleted by siRNA or in Mcl-1 $-/-$ MEFs suggesting mechanisms in addition to Mcl-1 downregulation contributed to ABT-737 sensitisation. Co-immunoprecipitation revealed that PI3-kinase inhibition increased Bcl-x_L-bound Bim which also occurred in Mcl-1 depleted CRC or Mcl-1 $-/-$ MEFs. ABT-737-induced apoptosis was independent of AKT and mTOR in CRC cells as it was unaffected by PI-3K pathway inhibition using AKT1/2 and rapamycin.

Conclusion: PI3-kinase inhibition enhanced ABT-737 induced apoptosis via a Mcl-1 independent mechanism, consistent with a mechanism that altered the affinity for Bim to Bcl-x_L. ABT-737 sensitisation by PI3-kinase inhibition occurred via an Akt/mTORC1 independent arm of the PI3-kinase signalling pathway.

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POSTER

c-FLIP, a critical target for histone deacetylase inhibitors in mesothelioma

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Background: Mesothelioma typically presents at a late stage and therefore carries a poor prognosis in the majority of cases. Chemotherapy is the mainstay of treatment, but only one quarter of patients respond to first line treatment (cisplatin/pemetrexed). Resistance to apoptosis is a key mechanism underlying the failure of anti-cancer therapies, and targeting the death receptor apoptotic pathway is a novel strategy to overcome this problem in mesothelioma. The caspase 8 inhibitor c-FLIP is a key inhibitor of death receptor signalling, which has previously been shown to be important in regulating apoptosis and drug resistance in several cancers. Here, we investigated the role of c-FLIP in regulating the response of mesothelioma cells to the histone deacetylase (HDAC) inhibitor Vorinostat, which has been found to be active in mesothelioma in early phase clinical trials.

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Materials and Methods: Four mesothelioma cell lines were studied: REN, E58, H28 and MSTO. Cells were maintained in 5% CO₂ at 37° in F-12 or RPMI medium supplemented with 10% FCS, 1% penicillin/streptomycin. Stably transfected clones were maintained in selection medium containing 600µg/ml G418. c-FLIP expression was analysed by Western blot. Apoptosis was measured by PARP cleavage and flow cytometry (propidium iodide, PI staining). Silencing of caspase 8 and c-FLIP was achieved by caspase 8 and c-FLIP specific siRNA. Expression of c-FLIP mRNA was measured by QRT-PCR.

Results: The IC₅₀ doses of Vorinostat were found to be in the 5µM range, as determined by MTT assay. We found that c-FLIP was down-regulated at the protein and mRNA level, 6–12 hours after exposure to Vorinostat in a dose-dependent manner, with potent down-regulation observed at the IC₅₀ dose. Vorinostat-induced down-regulation of c-FLIP correlated with caspase 8 activation and induction of apoptosis. Importantly, apoptosis induced by Vorinostat was significantly reduced in cells in which caspase 8 and the key death receptor adapter protein FADD were silenced. Furthermore, siRNA-mediated silencing of c-FLIP was found to be sufficient to activate caspase 8 and induce apoptosis in the mesothelioma cell lines. These results are consistent with c-FLIP down-regulation being a major mechanism of Vorinostat-induced apoptosis in mesothelioma. Moreover, Vorinostat was not found to affect expression of other proteins involved in apoptotic pathway, such as Mcl-1, Bcl-2, Bcl-XL, BAK, and XIAP.

Conclusions: c-FLIP is down-regulated at both a transcriptional and post-transcriptional level in response to Vorinostat. This appears to be a major mechanism leading to apoptosis induction by this agent in mesothelioma cell lines and suggests that c-FLIP, caspase 8 and other death receptor signalling molecules may be potential biomarkers of response to Vorinostat in mesothelioma.

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POSTER

NOXA as mediator for drug-induced apoptosis – molecular studies in patient-derived ALL cells

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Background: Chemotherapy is given as polychemotherapy, where drugs are combined to yield super-additive anti-tumor effects. Betulinic acid is a natural compound which is intensively studied as potent novel anti-cancer drug. We were first to show that Betulinic acid induced apoptosis in leukemia cells (Ehrhardt et al, Leukemia 2004). Here, we studied which conventional cytotoxic drugs cooperate with Betulinic acid and which signaling pathways are responsible for positive interaction.

Materials and Methods: Leukemic cell lines and primary tumor cells from 35 children with acute leukemia were studied in vitro. Classical cell death assays were performed. For release of mitochondrial factors, subcellular compartments were isolated and subjected to Western Blot analysis. Primary acute leukemia cells were amplified in NOD/SCID mice. As a completely new method, xenograft cells were transiently transfected by electroporation.

Results: Betulinic acid synergized for apoptosis induction with doxorubicin, asparaginase and vincristine, but not other conventional cytotoxic drugs. Clinically most important, these drugs induced cooperative apoptosis also in primary tumor cells freshly obtained from children with acute lymphoblastic leukemia (ALL). To characterize the mechanism responsible for effective apoptosis induction by drug combinations with Betulinic acid, we found that apoptogenic factors like Cytochrome c, Smac and OMI/HtrA2 were released from mitochondria and enhanced caspase activation and DNA-fragmentation. Upon combinatorial treatment, p53 was activated which lead to the upregulation of the pro-apoptotic Bcl-2 family member NOXA. Knockdown of either p53 or NOXA disabled synergistic apoptosis induction proving that both proteins mediate the cooperative pro-apoptotic effect. Most importantly and beyond tumor cell lines, we tested this signaling mechanism in primary leukemia cells derived from children with acute leukemia after amplification of the cells in NOD/SCID mice. These data represent the first data ever obtained in patient-derived ALL cells. Knockdown of either p53 or NOXA in patient-derived xenograft leukemia cells disabled both upregulation of NOXA as well as synergistic apoptosis induction by Betulinic acid and conventional cytotoxic drugs.

Conclusion: Our data suggest that NOXA represents an important, p53-regulated molecular target for the combination therapy of Betulinic acid and conventional cytotoxic drugs which enables apoptosis induction, e.g., in primary, patient-derived leukemia cells. If incorporated into polychemotherapy protocols for treatment of ALL, Betulinic acid should be applied in close proximity to doxorubicin, asparaginase or vincristine in order to take advantage of the favorable regulation of p53 and NOXA.

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POSTER

Lucanthone: A novel inhibitor of autophagy that induces cathepsin D-mediated apoptosis

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Background: Cellular stress induced by nutrient deprivation, hypoxia, and exposure to many chemotherapeutic agents activates an evolutionarily conserved cell survival pathway termed autophagy. This pathway enables cancer cells to undergo self-digestion to generate ATP and other essential biosynthetic molecules to temporarily avoid cell death. Therefore, disruption of autophagy may sensitize cancer cells to cell death and augment chemotherapy-induced apoptosis. Chloroquine and its analog hydroxychloroquine are the only clinically relevant autophagy inhibitors. Since both of these agents induce ocular toxicity, novel inhibitors of autophagy with a better therapeutic index are needed. Here we demonstrate that the small molecule lucanthone inhibits autophagy and induces apoptosis in breast cancer models.

Materials and Methods: Inhibition of autophagy was visualized by electron microscopy and lysosomal membrane permeabilization was measured by quantification of acridine orange fluorescence. The anticancer efficacy of lucanthone was determined by MTT assay and propidium iodide staining followed by flow cytometry. Gene expression arrays, quantitative real-time PCR, immunocytochemistry, and immunoblotting were used to measure cathepsin D expression. siRNA targeting cathepsin D was used to evaluate its contribution to lucanthone-mediated apoptosis.

Results: Lucanthone inhibits autophagy, induces lysosomal membrane permeabilization, and possesses significantly more potent anticancer activity compared to chloroquine. Exposure to lucanthone resulted in processing and recruitment of microtubule-associated protein 1 light