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 AKTi1/2 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.

584 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.
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.
Materials and Methods: Four mesothelioma cell lines were studied: REN, E58, H28 and MSTO. Cells were maintained in 5% CO2 at 37^0 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 (protium 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 IC_{50} 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 IC_{50} 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.

585 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.

586 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

chain 3 (LC3) to autophagosomes, but impaired autophagic degradation as revealed by electron microscopy and the accumulation of p62/SQSTM1. Lucanthone stimulated a large induction in cathepsin D expression, which correlated with cell death. Accordingly, knockdown of cathepsin D reduced lucanthone-mediated apoptosis. Subsequent studies using p53+/+ and p53−/− HCT116 cells established that lucanthone induced cathepsin D expression and reduced cancer cell viability independently of p53 status.
Conclusion: Our results demonstrate that lucanthone is a novel autophagic inhibitor that induces apoptosis via cathepsin D accumulation in breast cancer models.

587 POSTER

Down-regulation of FLT3 kinase phosphorylation and survivin expression contributes to the activity of imidazoacridinone C-1311 against leukemia cells with FLT3 ITD mutation

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Background: Antitumor imidazoacridinone C-1311, is a DNA-reactive inhibitor of topoisomerase II, undergoing phase II clinical trials. Recent studies against recombinant receptor tyrosine kinases (RTKs) showed that C-1311 is a potent and selective inhibitor of Fms-like tyrosine kinase 3 (FLT3). Since activating mutations of FLT3 occur in one third of acute myeloid leukemias (AML) and are associated with poor prognosis, we studied the effect of C-1311 on proliferation, cell cycle and mode of cell death in human AML models.
Materials and Methods: C-1311 was tested on two leukemic cell lines with contrasting FLT3 status. MV4;11 cells harbor internal tandem duplications (ITD) resulting in constitutively activated FLT3 while RS4;11 cells express wild type FLT3. The direct effect of C-1311 on FLT3 was examined based on its phosphorylation status using ELISA. Apoptosis was determined based on DAPI staining, annexin-V assay and caspase-3 activation. RNA levels of 84 different apoptosis-related genes were quantified using real-time PCR.
Results: Cytotoxic activity of C-1311 against MV4;11 cells was 4-fold greater compared with RS4;11, indicating more potent inhibition against cells with constitutively activated FLT3 ITD. In MV4;11 cells, C-1311 strongly reduced FLT3 autophosphorylation in a dose- and time-dependent manner with IC_{50} values of 14 μM and 0.7 μM after 3 h and 24 h of treatment, respectively. Importantly, down-regulation of FLT3 phosphorylation was achieved at biologically active doses of C-1311, indicating the inhibition of FLT3 activity crucial for cytotoxic effect of the drug. Sequence of cellular responses in MV4;11 cells treated with 1 μM C-1311 (IC_{90} concentration) included transient accumulation of cells in G1 phase followed by caspase-3 activation and cell death via apoptosis. The recruitment of the mitochondrial apoptosis pathway was suggested by the moderate up-regulation of proapototic genes e.g., Bax, BBC3, and APAF-1. Surprisingly, C-1311 markedly down-regulated antiapototic survivin gene expression (>30 difference over control), suggesting survivin as a new target for C-1311 action.
Conclusions: Inhibition of FLT3 by C-1311 resulted in reduced proliferation and induction of apoptosis in FLT3 ITD cells. Our finding that C-1311 decreases survivin expression is of particular interest since survivin regulates development of ITD FLT3-positive AML. How dual targeting of survivin and FLT3 contributes to the activity of C-1311 needs further studies.

588 POSTER

Trans-membrane-p53 peptide therapy for malignant glioma

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Background: Malignant gliomas are the most aggressive primary brain tumor in adults. The prognosis of patients with malignant gliomas remains poor despite decades of basic and clinical research. Malignant gliomas are still fatal disease.
It is known that glioblastoma cells have multiple genetic alterations associated with the p53/MDM2/ERK pathway, which is related to cell-cycle regulation and apoptosis. The goal of therapies such as p53 protein transfer is to recover the function of this tumor suppressor gene in cancer cells. Recent studies have reported the efficiency of protein transduction therapy for several cancers. In our present study, we use p53 peptide including Mdm2 binding site and examined its effects on the proliferation of three different glioblastoma cell lines.
Materials and Methods: We use p53 peptide including Mdm2 binding site with the 11 polyarginine PTD (protein transfer domain) and the SV40 large T-antigen NLS (nuclear localization signal) (p53-NLS-11R).We examined effects on the proliferation of three different glioblastoma (YKG, T98, U87) cell lines and peptide delivery into the nucleus was also confirmed using a fluorescently labeled peptide.
Results: p53-NLS-11R peptide was delivered into cells within 30 minutes and showed growth inhibitory effect in 1−10 μM on three cell lines. The growth inhibitory effect was achieved by the apoptosis with p53-NSL-11R peptide.
Conclusion: Direct intracellular delivery of p53-NSL-11R peptide is a possible useful method as a glioma therapy.

589 POSTER

HDAC inhibitors induce FLIP down-regulation and caspase 8 dependent apoptosis in colorectal cancer cells

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Introduction: c-FLIP is an anti-apoptotic protein that blocks death receptor mediated apoptosis by inhibiting Caspase 8 activation. FLIP is often over-expressed in colorectal (CRC) cells, and it has previously been shown that high FLIP expression limits the effectiveness of chemotherapy treatment. HDAC inhibitors (HDACi) such as SAHA have been shown to induce apoptosis via the intrinsic apoptotic pathway, and are currently in phase I trials. We have shown that the HDAC inhibitors SAHA and CMPHB down regulate the expression of c-FLIP at the protein level in a panel of cell lines. Our aims are to assess the effect of HDACi on the extrinsic apoptotic pathway, elucidate the mechanism of c-FLIP down-regulation and investigate the potential of combination therapies with TRAIL and chemotherapy.
Methods: HCT116 parental and HCT116 $FLIP_L$ over-expressing colorectal cancer (CRC) cell lines were utilised. Levels of FLIP expression were analysed by Western Blot. Apoptosis was measured by PARP cleavage and Flow cytometry (PI staining). Inhibition of Caspase 8 activation was achieved by siRNA and the Caspase 8 inhibitor IETD. Death Receptor expression was inhibited using DR4/5 targeted siRNA transfection. Expression of FLIP mRNA was analysed by RT-PCR. Proteosomal inhibition was achieved using MG132.
Results: It was found that SAHA and CMPHB induce apoptosis in HCT116 cells in a manner that is dependent on Caspase 8 and the ability of the compound to reduce c-FLIP protein expression. Furthermore, the apoptosis induced by both HDAC inhibitors was found to be dependent on the TRAIL receptors DR4 and DR5. Further analysis revealed that SAHA and CMPHB down regulate FLIP expression post-transcriptionally by triggering its degradation via the ubiquitin- proteasome system. Combination of SAHA and CMPHB with TRAIL indicates that treatment with HDAC inhibitors synergistically sensitise CRC cells to TRAIL and chemotherapy induced apoptosis.
Conclusions: We have previously shown that reduction of FLIP expression using siRNA synergistically enhances the response of CRC cells to chemotherapy or TRAIL. The results presented here indicate that HDAC inhibitors induce apoptosis in CRC cells by down regulating c-FLIP and thereby activating DR4/5-dependent Caspase 8-mediated apoptosis. Since the clinical down regulation of FLIP expression by siRNA is not yet feasible, the use of small molecules such as HDAC inhibitors which have a profound effect on c-FLIP expression may have important therapeutic implications.

590 POSTER

Transcription factor, Id4, induced cellular reprogramming of cancer cells using an advanced prostate cancer cell model

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Id4 belongs to a family of genes that are dominant negative regulators of basic helix-loop-helix (bHLH) transcription factors. Id1−3 expression correlates with various advanced metastatic cancers and act as tumor promoter genes. Many studies have suggested that loss of Id4 expression in many types of metastatic cancers is the result of promoter hypermethylation. The Id genes have been linked to a number of cellular responses including differentiation and proliferation. Id4 may act as a tumor suppressor by linking several cellular events. In the DU145 prostate cancer cell line, Id4 down regulation is responsible for increased cell survival, metastasis and decreased apoptosis. In this study we used advanced prostate cancer cell line DU145, negative for Id4 to observe the effects that ectopic Id4 expression plays in cellular reprogramming. Therefore, the role Id4 in cell cycle control, apoptosis, senescence, proliferation and hormone regulation were all aspects of the focus of this experiment. FACS analysis highlighted the ability of Id4 to regulate key stages of the cell cycle. Apoptosis assays, and observation of key apoptotic genes support the tumor suppressor role of Id4. Induction of senescence was a major characteristic of Id4 expressing DU145 cells, which simultaneously decreased proliferation. RT-PCR and western blot analysis were used to study gene expression of downstream apoptosis and cell cycle control genes E-cadherin, p21,