ZIO-101 is clinically active. MTD, 500 mg/me2/d, is >50-fold higher than the dose of As_2O_3. ZIO-101 is a promising drug for further development: phase-2 trials are starting.

480 POSTER

Bcl-2 nineteen kilodalton interacting protein (BNIP3) is a transcriptional regulator in glioma cells that acts as a survival factor, silencing the expression of pro-apoptotic genes

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Background: Novel mechanisms have recently been proposed for the Bcl-2 family members Bok, Bcl-2 and BID in the nucleus. They have been found to play a role in induction of apoptosis, alteration of gene transcription, and the DNA damage response. We have found that BNIP3 (a BH3-only member of the Bcl-2 family) is expressed in the nucleus of astrocytic cells under normal conditions, and in a subset of glioblastoma multiforme tumors (GBMs). We present that BNIP3 plays a novel role in the nucleus of glial cells by binding to the promoter/silencer regions of genes involved in induction of cell death or apoptosis, and silences these genes. If low expression of these genes results in a survival advantage, this may explain why expression of nuclear BNIP3 is selected for in GBMs.
Materials and Methods: Formaldehyde and cisplatin crosslinking of proteins to DNA was completed and: 1) protein was extracted and analyzed by western, 2) DNA was extracted by chromatin immunoprecipitation (ChIP) with the BNIP3 antibody. A gel shift assay was completed with probes specific for genes identified in the ChIP. Proteins isolated from a His-tag pull down with a His-BNIP3 construct were separated by 2-d gel electorophoresis. Spots were picked, sent for mass spectrometric analysis and confirmed by co-immunoprecipitation (co-IP). Stable transfection of nls-BNIP3 and shRNA-BNIP3 constructs were completed in U251 cells and these cells were treated with temozolomide (TMZ) and hypoxia.
Results: We have determined that the over-expression of BNIP3 in the nucleus in glioma cells provides a survival advantage against hypoxic stress as well as TMZ treatment. BNIP3 binds to a consensus sequence in the promoter/enhancer regions of genes involved in apoptosis and cell death. One of these genes is the PDCD8 gene, which codes for the AIF (apoptosis inducing factor) protein. We have confirmed in U251 cells that overexpression of BNIP3 in the nucleus decreases the level of protein expression of AIF, and concurrently stable expression siRNA for BNIP3 leads to an increase in AIF expression. Also, we have identified a subset of DNA/RNA binding proteins that interact with BNIP3 in the nucleus of glioma cells. PSF (polypyrimidine tract associated splicing factor) has been confirmed to interact with BNIP3 by co-IP.
Conclusions: We have found that nuclear BNIP3 downregulates AIF expression in astrocytes leading to resistance to TMZ and hypoxia-induced cell death. The interaction of BNIP3 with PSF indicates that BNIP3 may also regulate specific genes by alternative splicing. Nuclear BNIP3 therefore would be selected for in GBM tumors because it would provide a survival advantage in hypoxic conditions created in the interior of the tumor.

481 POSTER

Hyaluronan induces apoptosis through CD44 in activated T lymphoma cells

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Altered expression of the cell adhesion molecule CD44 is associated with metastasis in several human cancers and numerous studies have implicated the binding of CD44 to its primary ligand hyaluronan (HA) as being responsible. The CD44-HA interaction may also be important in regulating cell survival as binding to HA promotes anchorage-independent cell growth and mediates resistance to drug-induced apoptosis in human lung carcinoma cells. In contrast, anti-CD44 antibodies can inhibit proliferation and induce apoptosis in human leukemia cells, while in mouse T lymphoma cells, HA both enhances and protects from apoptosis depending on the type of drug used. Together, these findings suggest that the effect of CD44 on apoptosis may be cell type and condition specific. To better understand the role of the CD44-HA interaction in the induction of apoptosis in T cells, human Jurkat T lymphoma cells were transfected with CD44 or CD44 containing mutations that either increase or prevent binding to HA. Jurkat cells were stained with Annexin V-FITC and propidium iodide and analyzed by flow cytometry to measure apoptosis. Cells were found to be equally sensitive to apoptosis induced by treatment with staurosporine or an anti-CD95 antibody, suggesting that CD44 expression alone did not affect apoptosis. However, the activation of CD44 transfected Jurkat cells with immobilized anti-T cell receptor (TCR) antibody or phorbol myristate acetate (PMA) increased binding to HA and resulted in apoptosis in the presence of HA. Apoptosis was enhanced during activation in cells expressing high HA-binding mutant CD44, while it did not occur in cells transfected with mutant CD44 incapable of binding HA. Similarly, incubation with an HA blocking anti-CD44 antibody or hyaluronidase prior to activation prevented apoptosis. While it has been previously shown that CD44-deficient mouse T cells are resistant to activation-induced cell death, our data are the first to demonstrate that binding of CD44 to HA can induce apoptosis in activated T cells.

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c-FLIP regulates the interaction between interferon-gamma and doxorubicin in breast cancer cells

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Combination treatment regimens that include the topoisomerase-II (topo-II)-targeted drugs, such as doxorubicin, are widely used in the treatment of both early and metastatic breast cancers. Previously we demonstrated that combinations of these drugs with IFN-γ potentiated apoptosis in breast cancer cells in a STAT-1-dependent manner. In this study we found that this synergy was caspase-8-dependent. Furthermore, we found the enhanced apoptosis was mediated by the death receptors Fas and DR5. However, the cognate ligands of these receptors were not constitutively expressed or up-regulated by either IFN-γ or doxorubicin in these cells, suggesting that a ligand-independent signalling mechanism was stimulating the activation of these receptors. In addition, we found that IFN-γ dramatically down-regulated the expression of the caspase-8 inhibitor, cellular-FLICE-like inhibitory protein (c-FLIP), in MDA-435 cells, in a STAT1 and IRF-1-dependent manner. Characterisation of the functional significance of c-FLIP modulation by siRNA gene silencing and stable over-expression studies, revealed it to be a key regulator of IFN-γ and doxorubicin-induced apoptosis in MDA-435 cells. Analysis of a wider panel of breast cancer cell lines also indicated that c-FLIP was a key regulator of IFN-γ/doxorubicin-induced cell death. Furthermore, *c-FLIP* gene silencing also sensitised MDA-435 cells to the other topo-II inhibitors, etoposide and mitoxantrone, as well as the topo-I inhibitor, SN-38. These results indicate that c-FLIP plays a pivotal role in the modulation of drug-induced apoptosis in breast cancer cells and may have important clinical applications as a therapeutic target and/or a marker of chemosensitivity in tumour cells.

483 POSTER

Kinetic modelling of R8BH3BID induced BAX/BAK activation dynamics in Non-Small Cell Lung Cancer cells

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Non-small cell lung cancer (NSCLC) exhibits de novo resistance to chemotherapy. Suppression of apoptosis, a hallmark of NSCLC may contribute significantly to the chemoresistant phenotype. Activation of proapoptotic BCL-2 family proteins BAX and BAK constitutes a critical switch that initiates mitochondrial outer membrane permeabilization (MOMP) and inner membrane permeabilization (MIMP). Regulation of BAX/BAK oligomerization kinetics in NSCLC may impact susceptibility to chemotherapy induced apoptosis, however robust quantitative methods for direct estimation of MOMP/MIMP kinetics have not been explored. R8BH3BID peptide, a direct activator of BAX/BAK conformation change, was synthesized and as an N-terminal D-octoargarginine conjugate (R8), validated by electrospray mass spectroscopy and purified by high performance liquid chromatography. Analogues containing negative control point mutant, hexanoic acid spacing between R8 and BH3BID and N-terminal acetyl capping were equipotent. Rapid cell uptake was verified using carboxyfluorescein conjugated analogue which localized to mitochondria, and alpha-helical secondary structure confirmed by circular dichronism spectroscopy. Exogenous R8BIDBH3 (50microM) mediated rapid BAX conformation change, MOMP (cytochrome C, SMAC release), and MIMP measured by tetramethylrhodamine ester (TMRE) within 3 hours. At single cell resolution, MIMP exhibited stochastic behaviour. A machine vision algorithm developed to detect loss of TMRE fluorescence by live cell microscopy, enabled modelling of the survival function by the product limit estimator. NSCLC cells (H460) co-expressing BCL-2, BCL-XL, MCL-1 and BCL-W by RTPCR and western analysis, exhibited significantly faster R8BIDBH3 induced MIMP kinetics compared with human bronchial epithelial cells (BAES2B) lacking these antiapoptotic proteins, suggesting

differential BAX/BAK regulation despite equivalent R8BIDBH3 uptake kinetics. Subcellular fractionation confirmed constitutive BAX localization to the mitochondrial membrane in H460 cells in the absence of cytochrome/SMAC release, suggesting priming for death, and enhanced susceptibility to R8BIDBH3. In summary, machine vision based modelling of BAX/BAK dependent mitochondrial permeabilization is a useful tool for quantitative study of differential apoptosis dynamics in living cancer cells.

484 POSTER

Growth prevention of cancer cells by naphtoquinone derivatives

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The mitogen activated protein kinases (MAPKs) family consists of serine/threonine kinases. Extracellular signal regulated protein kinases (ERK1/2), c-Jun amino-terminal kinases (JNKs) and p38 MAPKs are the major members in this family. ERK1/2 are generally associated with cell proliferation and survival, whereas JNKs and p38 are usually related to apoptotic response. Activated MAPKs pathways have been detected in carcinoma of the colon. In this research, we focused on the EGFR-induced signal transduction in HT29 cells, which are colorectal adenocarcinoma cells.

The purpose of this research is to examine the effects of naphtoquinones derivatives (NQs) on cell growth, apoptosis and cell signaling. Furthermore, we intend to clarify the mechanism by which the NQs prevent growth of HT-29 cancer cells.

In order to examine the effect of the NQs on biological activities we preformed the following assays:

1. XTT assay – to measure cell viability.
2. DNA fragmentation and FACS analysis – to determine cell apoptosis.
3. Western blot – to examine cell signaling, using specific antibodies against phosphorylated and thus activated ERK1/2 and p38 MAPKs.

Among the various NQs that we screened, TW69 was the first that had a significant effect on cell proliferation. Using DNA fragmentation assay, we demonstrated that extended incubation with TW69 (25 μM for 48h) leads to apoptotic cell death. In addition, FACS analysis of cells treated with TW69 (25 μM for 48h), showed a high percent of apoptotic cells (33.97%). These preliminary results have led to synthesis of a new derivative, TW96, which is based on TW69 structure. Cell viability assays revealed that TW96 induces cell death with IC_{50} values of 0.3 μM. Furthermore, FACS analysis showed a high percent of apoptotic cells (78.75%) after treatment with 5 μM of TW96. In addition, cell signaling analysis demonstrates an increased activation of ERK1/2 and p38 in response to TW96 in a time dependent manner. Recent reports indicate that NQs cause an increase in reactive oxygen species (ROS) formation. It is well known that high levels of ROS lead to apoptosis. Experiments with N-acetyl-L-cysteine, (NAC), which is a ROS scavenger, showed inhibition of both ERK1/2 and p38 activation in response to TW96, suggesting that TW96-induced p38 activation is ROS-dependent. Further research is underway in order to determine whether ROS formation is involved in TW96-induced cell death.

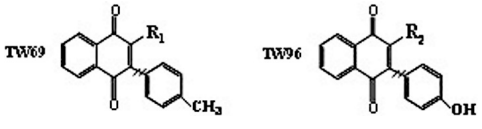

Fig. 1. Chemical structure of naphthoquinone derivatives.

Our study reveals newly synthesized compounds which affect cell viability and mediate apoptosis of HT29 cells. We suggest that these NQs may serve as lead compounds for the development of potential anti-cancer drugs.

485 POSTER

The inhibition of PI3K/Akt pathway as a major molecular determinant of bortezomib-induced apoptosis in hepatocellular carcinoma cells

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Background: Hepatocellular carcinoma (HCC) is one of the most common and aggressive human malignancies. Bortezomib, a proteasome inhibitor, has been shown good clinical responses in hematological malignancies and has been approved by FDA in refractory multiple myeloma in 2003. In this study, we examined the efficacy of bortezomib in HCC cells and identified the molecular change responsible for the resistance of bortezomib.
Methods: Five human HCC cells were used, including HuH7, HepG2, HepG2.2.15, Hep3B and PLC/PRF/5. Cells were exposed at various concentrations of bortezomib for 72 hours and cell viability was assessed by using the MTT assay. Cell cycle assay and apoptosis assay were done by flow cytometry. Protein expressions were assessed by western blot assay.
Results: Our data indicated bortezomib has biphasic effects in HCC cells. Bortezomib induced cell cycle arrest in G2/M phase starting at the lower concentration in all types of HCC cells. At the higher concentrations, bortezomib would induce massive apoptosis in HCC cells within 24 hours except PLC/PRF/5 cells. Bortezomib was unable to induced apoptosis at the clinical relevant concentrations (below 1000 nM) in PLC/PRF/5 cells. Compared with the molecular change between PLC/PRF/5 and other cells, we found PI3K/Akt pathway played a very important role in mediating the resistance of bortezomib-induced apoptosis. Our first evidence showed that bortezomib downregulated phospho-Akt in a dose- and time-dependent manner in sensitive HCC cells. However, in PLC/PRF/5 cell, bortezomib could not inhibit the activity of Akt even at the concentration of 1000 nM. Secondly, we applied a well-known PI3K inhibitor, LY294002, in our study and found that the combination of bortezomib and LY294002 induced apoptosis in PLC/PRF/5 cells in a dose- and time-dependent manner, indicating the importance of PI3K/Akt pathway in bortezomib-induced apoptosis in HCC cells.
Conclusions: Bortezomib could induce apoptosis at the clinical relevant concentration in most of HCC cells except PLC/PRF/5. Bortezomib downregulated phospho-akt in sensitive HCC cells but not in PLC/PRF/5 cells. Combination of bortezomib and PI3K inhibitor, LY294002 abrogated the resistance of bortezomib in PLC/PRF/5 cells. The inhibition of PI3K/Akt signaling pathway might improve the efficacy of bortezomib and overcome the drug resistance in the treatment of HCC.
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486 POSTER

Imidazoacridinone derivative C-1311 (SymadexTM) induces apoptosis, mitotic catastrophe or senescence in human colon carcinoma HCT116 cells depending on p53 status

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Antitumor imidazoacridinone C-1311 (Symadex^{TM}), active especially against colon carcinomas, has recently entered phase II clinical trials. C-1311 has been shown to inhibit catalytic activity of DNA topoisomerase II and to induce interstrand DNA crosslinking after metabolic activation. Here, we studied the cellular response of colon carcinoma HCT116 cells to C-1311 treatment in relation to p53 function.

HCT116 $p53^{-/-}$ and HCT116 $p53^{+/+}$ cells were treated with C-1311 at EC_{80} concentration for different time periods varying from 24 to 144 h. Cell cycle distribution was analysed by flow cytometry. Phosphorylation status of Tyr15 on cdc2 was studied to monitor mitotic progression. DAPI staining was used to identify micronucleated cells. Annexin V/PI, TUNEL and caspase-3 activity assays were used for studies on apoptosis. Analysis of cellular morphology and expression of SA-β-galactosidase were performed to identify cells with senescence-like phenotype.

HCT116 $p53^{-/-}$ cells underwent a short-term G2M arrest after 48 h of C-1311 treatment. The arrest however, could not be sustained and we observed the reduction in G2M arrested cells which was associated with gradual increase in cells with a subG1 and a slight increase in cells with >4n DNA content. Tyr15-phospho-Cdc2 was initially up-regulated but decreased after 48 h, which indicated that G2 arrested cells progressed into mitosis. Enlarged cells with multiple micronuclei, typical for mitotic catastrophe, appeared after 48 h. Early apoptotic cells occurred after 48 h of C-1311 exposure, as demonstrated by Annexin V/PI assay. Prolonged incubation with C-1311 led to increase in apoptotic population (50% of cells were TUNEL-positive and had active caspase-3 after 144 h of treatment). In contrast, HCT116 $p53^{+/+}$ cells showed an apparent cell growth arrest at G1 and G2M phases. This growth arrest was accompanied by apoptosis of only 20% of treated cells at the 144 h time point. Surviving HCT116 $p53^{+/+}$ cells, starting from 96 h, developed features of drug-induced senescence with flattened, enlarged morphology and increasing degree of SA-β-galactosidase staining. Such effect was not observed in p53-null cells.

The overall results suggest that in HCT116 $p53^{-/-}$ cells, C-1311 induced transient G2M arrest followed by mitotic catastrophe and p53-independent apoptosis. In HCT116 $p53^{+}/^{+}$ senescence-like arrest appears to be a major p53-induced cellular response to C-1311 treatment.