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Srp20, the first transcriptionally up-regulated gene by flavopiridol (flavo), is a pre-mRNA splicing factor that induces apoptosis in human colon cancer cells

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Alternate pre-mRNA splicing contributes significantly to developmental regulation of gene expression and various cellular processes. Changes in pre-mRNA splicing can have profound effects on cellular behavior including sensitivity to chemical stimuli. We identified Srp20 as a gene that is transcriptionally induced during apoptosis by combination treatment of SN-38 (the active metabolite of CPT-11) followed by flavo in human colon cancer Hct116 cells. Srp20 has profound effects on sensitivity to flavo. Inhibition of endogenous Srp20 expression in Hct116 cells by stable expression of an antisense (AS) Srp20 cDNA decreased the sensitivity of cells to flavo as measured by DNA condensation and PARP cleavage. The clonogenic assays showed a 2-fold increase in the IC50 of AS-Srp20 with flavo as compared with vector transfected cells. Transient expression of Srp20 induces apoptosis with significant PARP cleavage and Caspase-8 activation after 72 h in Hct116 cells. Additionally, when xenografted in mice, AS-Srp20-expressing Hct116 cells were significantly more resistant to CPT-11 followed by flavo as compared with vector transfected Hct116 cells. Flavo induces Srp20 mRNA in a time dependent manner. To investigate the regulation of Srp20 gene, we cloned 2.7 kb fragment of human genomic DNA immediately 5′ of the Srp20 coding region. This 5′-flanking region contain a TATA box and consensus binding sequences for CREB and E2F-1 transcription factors. To identify the DNA elements in the Srp20 promoter region responsible for transcriptional up-regulation by flavo, we fused the 2.7 kb and serially truncated human Srp20 5′-flanking region to a luciferase reporter gene. The transient transfection of Srp20 promoter vectors indicated that the −137 bp Srp20 promoter (Srp137) was the minimal region required to show enhancement in activity by flavo treatment in Hct116 cells. This region of promoter contains a consensus sequence for binding site for transcription factor CREB. Site directed mutagenesis of CREB binding sequences in the Srp20 promoter completely abrogated the induction of its activity by flavopridol. Furthermore, chromatin immunoprecipitation analysis indicates higher binding of CREB protein to Srp20 promoter following flavo treatment. Our studies suggest that transcriptional up-regulation of pre-m-RNA splicing factor, Srp20, may be a mechanism by which flavo induces apoptosis or augments the CPT-11/SN-38 effect. The increased Srp20 expression may result in altered splicing of genes that mediate the process of apoptosis. Flavo is a drug that suppresses transcription of numerous genes. Srp20 represents the first gene to be transcriptionally induced by flavopridol and induce apoptosis. Plans are underway in both the laboratory and the clinic to investigate the importance of this gene relative to response to flavo based therapies.

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A phase I and pharmacokinetic trial ARQ 501, an Activated Checkpoint Therapy (TM) agent, in patients with advanced solid tumors

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ARQ 501 is an investigational anticancer agent that works through the novel process of activation of cellular checkpoints, termed Activated Checkpoint Therapy (ACT). ARQ 501 selectively induces sustained elevation of E2F-1 followed by activation of the S phase checkpoint and apoptosis in cancer cells. Potent and highly selective anticancer activity has been demonstrated in a broad range of tumor models.

To test the anticancer activity of ARQ 501 in human disease, a phase I dose escalation study was commenced in September 2003 with the aim of defining the Maximum Tolerated Dose (MTD) and to characterize the pharmacokinetic parameters of a weekly one hour infusion of ARQ 501 given for a minimum of 4 weeks (one cycle). Single subject cohorts were evaluated between 10 and 140 mg/m^2; 3 subject cohorts have been enrolled at doses beginning at 200 mg/m^2. To date, 11 pts have been enrolled and have received a total of 25 cycles of treatment between the dose range of 10 to 280 mg/m^2. Provisional data on these patients is reported here. The patient characteristics are as follows: 5M/6F; median age: 57 years (range 38–73). Tumor types are pancreatic (1), breast (1), NSCLC (1), sarcoma (5), adenocarcinoma of unknown primary (2) and papillary thyroid (1). All patients had received prior chemotherapy.

Pharmacokinetic data showed a dose proportional increase in c_{max} and AUC for ARQ 501, with no evidence of drug accumulation. No objective responses have been observed to date, although stable disease greater than 6 months has been observed in a sarcoma patient treated at a dose of 20 mg/m^2 (dose was escalated from 10 mg/m^2 after 8 weeks). Six patients were taken off study for disease progression after 5 to 16 weeks of therapy. Only one serious adverse event has been reported, a disease related pulmonary embolism in a pancreatic carcinoma patient. Adverse events have been minimal and have included pruritis, rash, injection site reaction, anaemia, myalgia, fatigue, sweating and loss of appetite.

In conclusion, rapid dose escalation of ARQ 501 has been possible with minimal toxicity. Dose escalation of ARQ 501 continues, with a 1.4-fold increment for each dose level. Additional pharmacokinetic and safety data will be presented.

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FasL, but not TRAIL, induces apoptosis in human hepatocytes in chimeric mice

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Introduction: While tumor necrosis factor (TNF) family ligands TNFα and FasL can kill solid tumors, their clinical usage has been limited by their hepatotoxicity. TNF-related apoptosis-inducing ligand (TRAIL), a new TNF member, is currently in development as a potential antitumor agent because it kills tumor cells but spares normal cells in cultures and animals. However, a polyhistidine-tagged human TRAIL was reported to kill isolated human hepatocytes. In this study, we examined a recombinant non-tagged native sequence of human TRAIL for its toxicity and antitumor effects in the chimeric mice with human hepatocytes.

Methods: The chimeric mice were generated through a crossing homozygous Alb-*uPA*transgenic mouse with homozygous SCID/*bg* mouse. The litters with Alb-*uPA* homozygosity were injected with freshly isolated human hepatocytes.

Results: To test the TRAIL toxicity, two-month-old chimeric mice were injected intravenously either with 500 μg of the TRAIL or 30 μg of the antibody cross-linked Flag-FasL (30 μg of Flag-FasL mixed with 12 mg antibody). The chimeric mice that received the FasL injection succumbed within 90 minutes whereas the chimeric mice injected with 500 μg TRAIL remained healthy. α 1-antitrypsin (hAAT) concentrations before and after TRAIL injection in the mice. Histologic examination revealed extensive necrosis, severe edema, hemorrhage, and caspase-3 cleavage in the livers of the chimeric mice treated with FasL, but not TRAIL. Western blots detected caspase-8, -3 and DFF45 cleavage products in the liver tissues from the mice injected with FasL, but not the TRAIL. To show TRAIL selective antitumor activity, we injected two-month-old chimeric mice with 8×10^6 tumor cells either intraperitoneally or subcutaneously and then treated the mice with intraperitoneal injections of 100 μg TRAIL or 100 μl normal saline, twice per day for consecutive 10 days. Analysis of the peritoneal and subcutaneous tumor sizes indicated TRAIL treatment either eliminated or inhibited the tumor growth. Serum tests showed no difference in human α1-antitrypsin concentrations between the TRAIL treated and untreated mice. Histologically, the human hepatocytes appeared to be normal. Biochemically, caspase-8, -3 and DFF45 cleavage was not detected in the liver tissues from the TRAIL treated or untreated mice.

Conclusion: The evidence presented here demonstrates that the recombinant non-tagged soluble human TRAIL (amino acids 114–281) has a profound apoptotic effect on tumors but is non-toxic to human hepatocytes *in vivo*. This form of TRAIL may prove to be a safe and effective biological agent for cancer therapy in future human clinical trials.

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Identification of the molecular target for MX2167, a novel anticancer agent

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We have previously reported that derivatives of gambogic acid (GA) demonstrate good pharmacokinetic properties and anti-tumor efficacy in several rodent tumor models with tumor growth inhibition ranging from 60 to 90% using various dosing schedules. MX2167 is our lead drug candidate derived from GA and is a novel inducer of apoptosis with demonstrated activity in different cancer cell lines including breast, prostate

and colorectal. An unique feature of MX2167 includes rapid induction of apoptosis (<1 h) mediated through a cell-surface receptor. We have now identified the molecular target for MX2167 through different affinity procedures and LC/MS/MS sequencing and have validated its identification through different studies that include RNA interference and protein binding assays. We will describe the target for MX2167 and its validation as it relates to the induction of apoptosis. These results suggest the potential for MX2167 to be developed as a potential anticancer agent and MX2167 represents a molecular mechanism of action uniquely different from known cancer drugs.

221 POSTER

Ceramide promotes JNK translocation and Bim phosphorylation in lung cancer derived A549 cells

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The sphingolipid, ceramide, is an important second signal molecule and potent apoptotic agent. The production of ceramide is associated with virtually every known stress stimuli, and thus, generation of this sphingolipid has been suggested as a universal feature of apoptosis. Ceramide regulates diverse signaling pathways involving cell senescence, the cell cycle, and apoptosis. Ceramide is known to potently activate a number of stress-regulated enzymes including the c-Jun N-terminal kinase (JNK). Though ceramide promotes apoptosis in human lung cancer derived A549 cells, a role for JNK in this process is unknown. Here, we report that ceramide promotes apoptosis in A549 cells by a mechanism involving the translocation of JNK. A role for JNK in ceramide-induced apoptosis in A549 cells became apparent when it was found that cells pretreated with the JNK inhibitor SP600125 became resistant to killing by ceramide. A similar role for the p38 kinase is not likely since the p38 inhibitor, SB 203580, failed to effectively protect A549 cells from ceramide. To understand which JNK-mediated pathway may be involved, a number of JNK target proteins were examined including the transcription factor, c-Jun, and the apoptotic regulatory proteins Bcl2, Bcl-X_L, and Bim. A549 cells exhibited basal levels of phosphorylated c-Jun in nuclear fractions revealing active c-Jun is present in these cells. Ceramide was found to inhibit c-Jun phosphorylation suggesting that JNK-mediated phosphorylation of c-Jun is not likely involved in ceramide-induced apoptosis. Likewise, ceramide suppressed phosphorylation of Bcl-X_L, suggesting that dephosphorylation of this Bcl2 family member is not involved in the apoptotic process. Little if any Bcl2 was detected in A549 cells. Thus Bcl2 also appears not to be involved in ceramide-induced killing. On the other hand, ceramide promoted phosphorylation of Bim and promoted JNK translocation from the nucleus to the cytosol and the mitochondria. Ceramide-mediated changes in localization of JNK were consistent with the observed changes in phosphorylation status of c-Jun, Bcl-X_L, and Bim. Furthermore, ceramide promoted Bim translocation to the mitochondria. Mitochondrial localization of Bim has recently been shown to promote apoptosis. These results suggest that JNK may participate in ceramide-induced apoptosis in A549 cells by a mechanism involving Bim.

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P53-mediated apoptosis induced by NCX 4040, a nitric oxide-releasing aspirin derivative, in human colon cancer cell lines

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Background: Nitric oxide-releasing non-steroidal anti-inflammatory drugs (NO-NSAIDs) are reported to be safer than NSAIDs because of their ability to decrease gastric toxicity. In our work, we assessed the cytotoxic activity of a new aspirin derivative, NCX 4040, and of its parental compound, aspirin, in *in vitro* and *in vivo* human colon cancer models.
Material and Methods: *In vitro* cytotoxicity was evaluated on a panel of colon cancer lines (LoVo, LoVoDX, WiDr and LRWZ) by sulforhodamine B assay and data were elaborated according to Monk's model. Cell cycle perturbations and apoptosis were evaluated by flow cytometry. P rotein expression and mRNA content were detected by Western blot and RT-PCR. In the i*n vivo* experiments, tumor-bearing mice were treated with 10 mg/kg/die of NCX 4040, five times a week and treatment was repeated for six consecutive weeks. Treatment was begun on day six after tumor cell injection when the tumor mass weighed about 300 mg.
Results: In the *in vitro* studies, the parental compound, aspirin, did not induce an effect on any of the cell lines used, whereas NCX 4040 produced a marked cytostatic dose-related effect, with GI_{50} values already reached after a 24-h drug exposure in all lines. A significant cell killing was observed at the highest concentrations in all but LoVo DX cells, which showed the lowest sensitivity. NCX 4040 induced an accumulation of cells in S phase in all four cell lines. Furthermore, in LoVo and LRWZ cell lines, which basally express p53 wild type, we observed Caspase-9- and 3-mediated apoptosis with a maximal peak after 20-h and 48-h drug exposures, respectively, and also an increased level of the p53-target protein, NAG-1. Conversely, no apoptotic effect was observed after NCX 4040 exposure in WiDr or LoVoDx cell lines, which harbored p53 mutations and also expressed COX-2. In *in vivo* studies, both NCX 4040 and its parental compound were administered *per os*. At a non toxic dose of 10 mg/kg, NCX 4040 exhibited a half-life of about 6 h and induced a 40% reduction in tumor weight. This antitumor effect is important, especially if we consider that antitumor drugs widely used in clinical practice are ineffective on this colon cancer model. Conversely, aspirin did not influence tumor growth at all.
Conclusion: NCX 4040, but not its parental compound, aspirin, showed an *in vitro* and *in vivo* antiproliferative activity, indicating its potential usefulness alone or in combination with conventional cytotoxic drugs to treat colon cancer.

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Evaluation of 2 new Rhodium ferrocene complexes for cytotoxicity, and apoptotic propensity to invoke alternative cell death pathways in prostate tumour cell lines

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Background: Drug induced cytotoxicity may invoke apoptosis, necrosis, micronucleation, abnormal nuclear morphology and intermitotic failure. The operation of these cell death pathways has now become an important criterion in the mechanistic distinction of cytotoxic drugs. This investigation explores the onset of apoptosis and abnormal morphology in response to 3 drugs i.e. cisplatin, a novel ferrocene (fctfa) and a novel Rhodium-ferrocene [Rh(fctfa)(cod)] complex.
Materials and Methods: A pair of prostate cell lines from normal human prostate epithelium (1542N) and malignant human prostate epithelium (1542T) were exposed to increasing concentrations of the drugs for 24 hours, double stained with FITC-AnnexinV and with Propidium Iodide and analysed by dual parameter flow cytometry to quantitate viable cells in quadrant **I**, early apoptotic cells in quadrant IV and late apoptotic/necrotic cells in quadrant **III**. Apoptosis was also scored by microscopy after Acridine Orange staining, by Western Blots for caspase 3 induction and for caspase 8 induction using a colorimetric assay.
Results: The toxicity of cisplatin and the ferrocene and Rhodium-ferrocene complexes was found to be 0.9–1.3 μM; 4.1–4.5 μM and 10.1–13.2 μM, respectively. Apoptotic propensity scored after 24 hours was found to be dose dependent and in the range of 7–19% for cisplatin and 1–4.1% for the ferrocene and Rhodium-ferrocene complexes. Cisplatin produces a distinct apoptotic response followed by a necrotic response, whereas the ferrocene and the Rhodium-ferrocene complexes produce a massive necrotic reaction in the region of 3–19% and very little if any apoptosis. Absence of apoptosis was corroborated by lack of caspase 3 activation, absence of typical apoptotic morphology and by lack of caspase 8 activation.
Conclusions: The 3 drugs cisplatin, the novel ferrocene and the novel Rhodium-ferrocene complexes show similar toxicities in the 1–10 micromolar range in prostate cell lines. However the drugs differ significantly in the activation of death pathways. While cisplatin predominantly induces apoptosis documented by morphology, Annexin V staining and caspase 8 activation, the ferrocene and Rhodium-ferrocene complexes induce late necrosis and abnormal nuclear morphology. Unlike cisplatin-treated cells which enter apoptosis and necrosis sequentially the 2 Ferrocene drugs invoke direct entry of cells into late necrosis without first entering the early apoptotic compartment.

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Two distinct pathways regulate Bak function in apoptosis: a requirement for JNK1 in Bak 80–170 kDa complex formation but not in Bak activation

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The stress-activated protein kinase JNK1 is required for apoptosis induced by many cell death stimuli, likely by regulating Bcl-2 protein family members. Of the two Bcl-2 family members Bim and Bid, which are both known to be upstream regulators of Bak, Bim rather than Bid is here shown to be required for cisplatin-induced apoptosis, and to be a cisplatin-induced