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.

222 POSTER

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.

223 POSTER

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.

224 POSTER

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

JNK substrate. Using jnk1-deficient MEFs and the inhibitor SP600125, we also show that specifically JNK1 is required for cisplatin-induced apoptosis. However, although Bak is required for apoptosis, JNK1 was not required for the conformational activation of Bak. Furthermore, a dominant-positive mutant of MEKK1 could activate Bak also in the absence of JNK1. We demonstrate that the role of JNK1 is instead to provide the signal for already activated Bak to form the high-molecular weight complexes likely involved in cytochrome c release. Thus, in the absence of JNK1 activity, Bak activation is not necessarily a marker for apoptosis. Finally, we show that apoptosis correlates with Bak complexes in the range of 80–170 kDa, but not with larger complexes which are formed also in the absence of JNK activity and apoptosis.

225 POSTER

Variable distribution of TRAIL Receptor 1 in primary human tumor and normal tissues

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Background: TRAIL-Receptor 1 (TRAIL-R1/Death-receptor 4) has been reported to be selectively and generally expressed in tumors. The objective of this study was to identify specific malignancies that may be most likely to express this receptor. Such malignancies could be strong candidate indications for TRAIL-R1 specific therapeutics in development, such as HGS-ETR1, a fully human agonistic TRAIL-R1 mAb. The current study utilizes a highly specific immunohistochemical assay to evaluate TRAIL-R1 in human tumor and normal tissues.
Methods: Samples of 160 formalin-fixed tumors and 17 normal tissues were evaluated by immunohistochemistry for TRAIL-R1 expression. Tumor types included at least 10 samples each of 7 types of carcinoma: colon, breast, pancreas, ovary, prostate, hepatocellular, and non-small cell lung. For neoplasms of the colon, the full continuum of hyperplastic polyp through invasive carcinoma was evaluated. Receptor expression was also studied in 15 other malignancies. Each sample was scored for TRAIL-R1 expression according to intensity of cytoplasmic and membrane staining distribution throughout the tumor.
Results: Of the first 134 malignancies evaluated, 57 (43%) were positive for TRAIL-R1, and 47 were negative (35%). Staining was weak or limited to individual and small clusters of tumor cells in the remaining 30 samples (22%). Thus, a total of 65% of tumors showed some degree of TRAIL-R1 specific staining. TRAIL-R1 staining was consistently weak or absent in all 17 normal tissues. Tumors of the colon, lung and pancreas were most likely to be positive for TRAIL-R1. The level of TRAIL-R1 in the colon was explored further in 26 additional samples representative of typical neoplastic progression. Of these, 10 of 15 adenocarcinomas were positive, while only 1 of 11 benign or focal carcinoma in situ samples were positive. Staining was predominantly membrane and/or cytoplasmic for each individual positive tumor; this pattern, as well as intensity and distribution of positive cells, varied between tumor samples of a single histologic type.
Conclusions: TRAIL-R1 is expressed with variable frequency for different tumor types. Tumors of the colon, lung and pancreas were most likely to express TRAIL-R1. Furthermore, in the colon samples, TRAIL-R1 expression appeared to be increased in malignancies as compared to less advanced neoplasms. All normal tissues evaluated were weak or negative for TRAIL-R1.

226 POSTER

Sulphamoylated 2-substituted oestrogens induce apoptosis through G2-M checkpoint arrest

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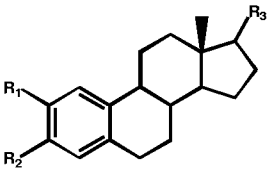
2-Methoxyestradiol (2-MeOE2), an endogenous metabolite of 17β-estradiol, has been found to inhibit cancer cell proliferation *in vitro*, and to inhibit tumour growth *in vivo*. It mediates these effects through microtubule interactions by an oestrogen receptor-independent mechanism. Previously, we have demonstrated that sulphamoylated derivatives of 2-MeOE2 induce p53 expression, cell cycle arrest and apoptosis in human breast cancer cell lines. As an extension of this research, two compounds, 2-MeOE2bisMATE (Compound A) and a D ring modified analogue of compound A (Compound B), were investigated to determine their ability to induce G_2-M cell cycle arrest and apoptosis, at a dose of 1 μM, in a panel of cell lines (A2780, LNCaP, PC3, and MCF-7) using flow cytometric techniques. In all cell lines tested, drug-induced G_2-M cell cycle arrest was seen at 12–18h. In A2780 cells, the percentage of cells arrested in G_2-M were 49% and 47%, treated with Compound A and Compound B respectively. In LNCaP cells, the percentages were, 37% and 33%; in MCF-7 cells, 81% and 37%; and in PC3 cells, 73% and 77%, respectively. However, Annexin V flip-out onto the outer leaflet of the plasma membrane (an indicator of early apoptosis) reveals that the time to resultant apoptosis varies for different cell lines. For A2780 cells, both compounds induced apoptosis at 48h, LNCaP at 72h, MCF-7 at 120h and PC-3 at 96h post-treatment. Western blot analysis of cyclin B1, an essential component of the G_2-M checkpoint whose activity is crucial for progression from prophase to metaphase, indicates upregulation and degradation of this protein occurs at different time points for individual cell lines. The induction of cyclin B1 correlates with entry into mitosis and its degradation with passage through the G_2-M checkpoint and subsequent apoptosis, for all cell lines tested. Taken together, these results suggest that sulphamoylated 2-substituted oestrogens act through induction of G_2-M blockade and bring about apoptosis in cells, which have undergone mitotic slippage or mitotic catastrophe.



Compound	R_1	R_2	R_3
2-MeOE2	MeO	OH	OH
Compound A	MeO	OSO_2NH_2	OSO_2NH_2

227 POSTER

NF-kappa-B: a differential modulator of chemotherapy induced apoptosis

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Members of the NFkB (nuclear factor kappa B) family of transcription factors play key roles in mediating immune, inflammatory and apoptotic responses. Distinct homo and heterodimeric forms are activated in response to a wide range of stimuli including cytokines (e.g. TNF, IL-1), oxidative stress, and DNA damage. Dimers are normally retained in the cytosol in a latent form sequestered by an inhibitor protein (IkB). Despite their diversity, many stimuli lead to the phosphorylation and degradation of IkB allowing nuclear translocation of NFkB and the induction of target genes. Previously we found that a subset of anti-cancer drugs could activate NFkB in cancer cells and that this was dependent on their ability to induce DNA damage. Given the diversity of literature, which has described NFkB's role in both inhibiting and promoting apoptosis, our observations and those of others seem to suggest an anti-apoptotic function in response to DNA damaging chemotherapy. We generated a stably transfected cell-line using cancer cells (MDA-MB-435) expressing an NFkB-GFP responsive promoter. Using this model we confirmed that a range of topoisomerase-targeted drugs (doxorubicin, mitoxantrone, camptothecin) could stimulate the expression of GFP, a marker of NFkB activation, using both Western blotting and immunofluorescent detection. This correlated with IkB degradation and NFkB activation, the latter revealed by EMSA analysis using radioactive labelled NFkB oligonucleotide. However, closer examination revealed distinct patterns of NFkB sub-unit activation, which may reflect the differential response of these cells to the apoptotic potential of individual topoisomerase-targeted drugs. This was determined by supershift analysis and ELISA based detection. Finally, using dual immunofluorescence the temporal pattern of NFkB activation has been analysed in conjunction with Annexin V binding, a marker of phosphatidylserine expression during the apoptotic process. Analysis of individual cells revealed that strong GFP expression did not correlate with apoptotic induction in response to select drugs. Moreover this pattern of activation was influenced by the presence and absence of p53, as revealed through analysis of samples following drug treatment of the HCT116 somatic knockout cell culture model A recent gene-chip experiment has also revealed several potential NFkB regulated genes in response to doxorubicin, which further highlight the role of NFkB in regulating drug-induced apoptosis.