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MKP-1/CL100 activity modulates cisplatin responses in non-small cell lung carcinoma

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Non-small cell lung carcinoma (NSCLC) represents the most abundant and therapy refractory subclass of lung cancer. Improving apoptosis induction in NSCLC represents a logical way to advance in treatment is this tumor. In NSCLC derived cell lines H-460 and H-1299, with different degrees of sensitivity to cisplatin, this drug differentially induces activation of N-terminal c-Jun Kinase, that in turn mediates induction of apoptosis. MKP-1/CL100 a negative regulator of JNK, is up-regulated in H-460 cells. By expressing a SiRNA vector for MKP-1 we found that in H-460 MKP-1/SiRNA expressing cells, cisplatin induces more efficiently activation of JNK and p38 and that correlated with an increase in 10-fold sensitivity to cisplatin. By contrast to H-460, no differences in survival were observed in H-1299 expressing the MKP-1SiRNA. Moreover, expression of a SiRNA to MKP-2, a CL100 related phosphatase, showed no effect in H-460 cell viability to cisplatin treatment. MKP-1SiRNA/H-460 cells grow both slower in nu/nu mice and also show more susceptibility to cisplatin than parental cells, hence resulting in an impaired growth of the tumor in mice. When analyzing surgical samples of NSCLC patients we have found that expression of MKP-1 showed a strong nuclear staining for tumor cells while in normal bronchial epithelia, MKP-1 was localized both in cytoplasm and nuclei. Altogether, the results showed that inhibition of MKP-1 expression contributes to a slower growth of cell in mice and also to an increase of cisplatin induced cell death in NSCLC, suggesting that MKP-1 constitutes an attractive target for sensitizing cells to cisplatin in human tumors.

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ZD4054 specifically inhibits endothelin A receptor-mediated anti-apoptotic effects, but not endothelin B receptor-mediated pro-apoptotic effects

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The clinical use of endothelin antagonists in oncology is a new and interesting area of study. In most oncology settings, blockade of only one of the two endothelin receptor subtypes (endothelin A receptor [ET_A]) infers benefit, while blockade of the other (endothelin B receptor [ET_B]) may lead to undesirable effects. Evidence suggests that stimulation of the ET_B receptor increases apoptosis, therefore antagonism of the ET_B receptor could inhibit apoptosis of tumour cells. We and others have shown that ET_B receptor expression is reduced, but still present, in clinical samples from a number of solid tumours (but not in melanoma and glioma where the ET_A and ET_B receptor seem to have opposite roles); a finding which is consistent with its pro-apoptotic role. We have also shown previously that ZD4054 is a specific ET_A receptor antagonist because it binds to the ET_A receptor with high affinity *in vitro,* but has no measurable affinity for the ET_B receptor (Curwen and Wilson. Eur J Cancer 2002;38[Suppl 7]: S102). Here we demonstrate that ZD4054 inhibits ET_A-mediated anti-apoptotic events while allowing pro-apoptotic signalling via ET_B in both human and rat epithelial cell lines *in vitro*.

We confirm the findings of Wu-Wong et al (Biochem J 1997;328:733–7) showing that endothelin 1 (ET-1) inhibits apoptosis induced by serum starvation and show that a specific ET_A receptor antagonist BQ123 inhibits this response. We also demonstrate that ZD4054 inhibits this ET_A-mediated process in the rat A10 and the human VLTR 16 smooth muscle cell lines in a dose-responsive manner. In contrast, selective activation of the ET_B receptor by the peptide agonist BQ3020 induced pro-apoptotic signalling in the same lines, a response which was not reversed by ZD4054.

The data presented here show that ZD4054, a specific ET_A receptor antagonist, blocks ET_A receptor-mediated anti-apoptotic signals leading to apoptosis, while at the same time, allows pro-apoptotic signalling to continue via the ET_B receptor. The specificity of ZD4054, together with the opposing roles of ET_A and ET_B receptors in mediating apoptosis, suggests that ZD4054 has the potential to block the pathological processes mediated by the ET_A receptor, but allow the beneficial processes mediated by the ET_B receptor to proceed.

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Characterization of novel therapeutic receptor target candidates for treatment of small cell lung cancer

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An oligonucleotide microarray analysis on a panel of 21 small cell lung cancer (SCLC) cell lines and 8 xenografts, as well as data from 5 resected tumors (Bhattacharjee A, et al. (2001) Proc. Natl. Acad. Sci. USA **98**:13790) revealed expression of a large number of highly expressed genes, including genes encoding cell surface receptors. Aiming to identify novel receptor targets for treatment of SCLC, some of these receptors were selected for assessment of therapeutic potential. Receptor selection criteria included high mRNA expression in SCLC (cell lines, xenografts and tumors) and low or absent expression in all or most adult normal human tissues. The analysis resulted in identification of approximately 120 genes of interest. From this selection a small number of genes were chosen for further studies on the basis of availability of identified ligands.

Selected receptors include the ionotropic Glutamate Receptor Subunit 2 (GRIA2), the metabotropic Glutamate Receptor 8 (GRM8) and the Neuronal Pentraxin Receptor (NPTXR). GRIA2, GRM8 and NPTXR mRNA expression has been confirmed by semi-quantitative RT-PCR and protein expression was verified by western blot analysis of total cell lysates from all the cell lines and xenografts used for the microarray analysis. Western blotting of membrane fractions from a selection of SCLC cell lines confirmed the localization of the three receptors to the cell membrane.

For GRIA2 no specific binding could be detected using the radio ligand ^{3}H-AMPA. However preliminary results showed a concentration dependent antiproliferative response for SCLC cell lines expressing GRIA2 upon exposure to the ionotropic glutamate receptor antagonist GYKI-52466.

A recombinantly produced ligand of the NPTXR was found to associate with the cell surface in a NPTXR expressing SCLC cell line, but not to a fibroblast cell line without expression of NPTXR.

These results indicate that the ligand binding functionality of GRIA2 and NPTXR is retained in SCLC and these receptors may therefore candidate as novel targets for treatment of SCLC.

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Membrane gamma-glutamyltransferase as a target for intervention on several redox-modulated functions of the cancer cell

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Background: Biomolecules involved in signal transduction and gene expression are sensitive to prooxidants, and a "redox regulation" has been described for many of them. Prooxidants originate from several sources; our previous work identified membrane gamma-glutamyltransferase (GGT) as one such source (Biochem. Pharm. 64, 1029, 2002). ROS and other free radicals are in fact produced during GGT-mediated metabolism of glutathione (GSH).

Methods: Several tumor cell lines are being used. Clones transfected for increased GGT expression have been obtained. Cells are studied in conditions of GGT stimulation vs. GGT inhibition. Determinations are aimed to investigate the effects of GGT activity on: i) cellular low mol. wt. thiols (HPLC studies); ii) the cellular balance of critical redox-active compounds, e.g. vitamin C (HPLC); iii) thiol redox status of selected membrane proteins (immunoblot, Elisa and FACS studies); iv) ligand binding affinity of membrane receptors (125I-labelled agonists); v) selected cell functions (proliferation; viability and apoptosis; adhesion); vi) resistance of cells to platinum-based drugs.

Results: i) Cysteinyl-glycine (CG) produced by GGT forms mixed disulfide bonds with cellular protein; ii) extracellular ascorbic acid is easily oxidized by GGT to dehydroascorbate, i.e. a form of the vitamin that is promptly absorbed; thus, GGT expression facilitates the supply of vitamin C; iii) at least five distinct redox forms of the TNFR1 receptor were identified, whose relative abundance is dependent on the GGT activation status; iv) TNFR1 redox status is reflected in changes of its ligand binding affinity for TNF-alpha; v) redox activity of GGT affects the basal levels of poly(ADP-ribose) polymerase activity; vi) GGT expression increases cell resistance to cisplatin toxicity, likely due to formation of complexes with CG outside cells.

Conclusions: Prooxidant reactions initiated at the cell surface during GGT-mediated catabolism can have profound effects on a series of cellular parameters connected with viability, apoptosis and drug resistance. As expression of GGT is frequent in human neoplasms, and often

increases during progression, the potential role of GGT as candidate target for antineoplastic treatments is suggested. The development of GGT inhibitors of pharmacological significance would likely enrich the therapeutic spectrum with an additional tool, to be exploited in selected situations. Supported by FIRB 2001 and AIRC 2001–03 funds.

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Synergistic effects of Apo-2L/TRAIL and ionizing irradiation in human tumor cell lines without relevant damage of normal tissue cells

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Background: An outstanding feature of tumour necrosis factor related apoptosis inducing ligand (Apo-2L/TRAIL) is the pronounced tumour cell specificity. Thus APO-2L/TRAIL is now tested in clinical settings as new anti-cancer agent. Up to now, little is known about the effects of a combined therapy using Apo-2L/TRAIL and ionising radiation. In order to examine the efficacy of a combined treatment several malignant cell lines and diverse normal tissue systems were treated with various combinations of APO-2L/TRAIL and radiation.
Material and Methods: Colo 205 and HCT-15 (colorectal carcinoma), NCI H460 (lung adenocarcinoma), MDA MB231 (breast cancer), two squamous cell carcinoma cell lines (FaDu and SCC-4) as well as normal tissue cell system derived from prostate, mammal, renal and bronchial epithelia, fibroblasts and hepatocytes were treated with a combination of Apo-2L/TRAIL and irradiation. Apoptosis was quantified by fluorescence microscopy after Hoechst-staining. The degree of interaction was evaluated by isobologramm-analysis. Regulation of the surface expression of the APO-2L/TRAIL receptors R1/DR4 and R2/DR5 was determined by flow cytometry (Quantibrite^{TM}).
Results: The combination of APO-2L/TRAIL and radiation was associated with pronounced additive effects on apoptosis induction in tumour cell systems when APO-2L/TRAIL and radiation were applied simultaneously. In contrast a striking synergy occurred when APO-2L/TRAIL was added 14 hours after irradiation in all cell lines except the NCI H460 cells. Ionising radiation triggered an upregulation of DR5 in most cell systems. However, no straight correlation with the induction of synergistic cell death was observed. In contrast, the combined treatment of normal tissue cell systems was not associated with additive or synergistic effects regarding apoptosis induction.
Conclusion: Preirradiation sensitises several tumour cell systems towards APO-2L/TRAIL induced apoptosis. The concurrent application is less effective. Regardless of any preirradiation APO-2L/TRAIL did not induce apoptosis in any of the tested normal cell systems. Thus, the in vitro data do not suggest any increased toxicity of the combined treatment. Although DR5 is clearly upregulated in response to irradation this mechanism might not represent an exclusive regulatory mechanism responsible for the observed synergistic effects.

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MS-275, a potent orally active inhibitor of histone deacetylases is highly active in experimental tumor models of melanoma and prostate cancer

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Background: Histone deacetylases (HDACs) are a family of enzymes that are involved in the epigenetic regulation of gene expression. HDACs keep histones in a hypoacetylated, positively charged state that tightly binds the negatively charged phosphate backbone of DNA, so preventing gene transcription. Transcription factor complexes must get access to the DNA to allow gene expression, which is normally achieved by histone acetyl-transferases, the natural counterparts of HDACs. The balance between transcriptional activity and gene silencing is often disturbed in tumors and the inhibition of HDACs may activate tumor suppressor genes such as the cell cycle inhibitor p21$^{WAF1/CIP1}$. HDAC inhibition may be antiproliferative, and induce differentiation and/or apoptosis. There is growing experimental evidence for this hypothesis and a number of HDAC inhibitors are currently in phase **I/II** clinical trials. Here, we summarize experimental results obtained with MS-275, an orally active synthetic pyridylcarbamate, in a number of melanoma and prostate cancer tumor models.
Material and Methods: Melanoma (A375, SK-Mel28, B16F10) and prostate carcinoma (DU145, PC3) cell lines were grown as xenografts in nude mice. After establishment, tumors were treated with MS-275 daily p.o. Tumor area and body weight was determined during treatment, and final tumor weight after sacrifice used to calculate the tumor/control ratio (T/C).
Results: MS-275 showed a dose-dependent efficacy in almost all experiments. Lower doses (5 and 10 mg/kg) revealed a slight response whereas higher doses (25 and 50 mg/kg) showed a marked antitumor efficacy. The highest dose of MS-275 (50 mg/kg) showed a very high efficacy in the SK-Mel28 model (T/C 0.1, i.e. 90% inhibition). A transient decrease in body weight was noted at higher doses, but this recovered within a few days without disrupting treatment. At lower doses the compound was very well tolerated. MS-275 exhibited a higher efficacy in the SK-Mel28 model compared with dacarbacine. These data support preliminary results from an ongoing phase **I** clinical trial with MS-275. Nine patients with melanoma have been treated so far, with one patient showing a partial response for 78 weeks and is still on treatment, and 5 patients having disease stabilization for $\leqslant$38 weeks. MS-275 exhibited a marked antitumor efficacy in the prostate carcinoma models, where even the lower doses of 5 and 10 mg/kg showed a significant effect in the DU145 model.
Conclusion: These results indicate that MS-275 exhibited a marked, and in most cases dose-dependent, antitumor efficacy. These data are in agreement with preliminary findings from a phase **I** clinical trial where the majority of pretreated melanoma patients showed disease stabilization. Thus, highly chemotherapeutic resistant tumors such as melanoma and prostate carcinoma may be suitable indications for phase **II** clinical trials with MS-275.

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Antitumor activities of MGCD0103, a novel isotype-selective histone deacetylase inhibitor

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Targeting Histone deacetylases (HDACs) is a new approach in human cancer therapy in recent years. Several HDAC inhibitors had been advanced into human clinical trials. We have rationally designed MGCD0103, a non-hydroxamate small molecule HDAC inhibitor, as a novel anti-cancer therapeutic. MGCD0103 selectively targets certain specific class **I** HDAC enzymes at IC50's of submicromolar concentrations *in vitro* and induces hyperacetylation of histones in cultured human cancer cells. MGCD0103, but not its inactive analog, selectively and potently inhibits proliferation of human cancer but not normal cells. It causes G2/M cell cycle block and induces apoptosis in human cancer cells in a dose-dependent manner. By using cDNA expression array analysis of human cancer cells treated with either MGCD0103 or other HDAC inhibitors in clinical development, we found MGCD0103 regulates transcription of a smaller subset of downstream genes, reflecting its inhibitory specificity. *In vivo*, MGCD0103 significantly inhibits growth of human tumors in various xenograft models in nude mice in a dose-dependent manner with minimal toxicity. In correlation with its antitumor activities, MGCD0103 induces hyperacetylation of both white blood cells and tumors in tested animals. We conclude that MGCD0103 appears to have a favorable therapeutic index *in vivo*. MGCD0103 is now under investigation in Phase **I** clinical trials.

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Regulation of the oncogenic x-protein of hepatitis B by cellular chaperones

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HBx, the x-protein of the Hepatitis B virus, has been shown in cells and animal models to promote the development of hepatocellular carcinoma, probably due to its transactivating function. It is thus of great interest to understand the regulation of HBx in the host cell. Here we describe a novel mechanism by which HBx is regulated by cellular chaperones, and discuss its pathophysiological implications. HBx was previously shown to interact with XAP-2, an immunophilin that can serve as a co-chaperone for Hsp90 or Hsp70, implicating these chaperones in the regulation of HBx. To determine the functional role of Hsp90, we treated the hepatoma cell line HepG2 transiently expressing HBx with the antibiotic geldanamycin (GA), an inhibitor of Hsp90. GA induces the degradation of diverse Hsp90 client proteins. To our surprise, instead of reducing HBx levels, GA treatment increased the expression of HBx in HepG2 cells. Interestingly, differential lysis and western blotting indicated that the increase occurred mainly in the cytosol. In contrast, the nuclear fraction showed a modest decrease in HBx level. These observations were confirmed by immunofluorescence experiments which showed increased appearance of HBx in the cytosol of GA-treated cells. These data suggest that Hsp90 is involved in cellular distribution of HBx. Given that the major effect of HBx occurs in the nucleus, one may be able to inhibit HBx function by targeting its interaction with Hsp90, thereby inhibiting HBx nuclear entry. We also observed that GA induced HBx binding to Hsp70, and that a dominant negative CHIP protein, a co-chaperone of Hsp70, demonstrated GA-like effects on HBx expression. Taken together, these data suggest that cooperation between