conclusion, both in vivo imaging methods were able to detect and quantify induction of apoptosis in response to Plk1 inhibition early in the course of treatment and prior to detectable changes in tumor volume. These non-invasive approaches may be useful in further preclinical profiling and clinical development of Plk1 inhibitors.

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Detection of surrogate markers of apoptosis in the peripheral blood of a preclinical tumour model treated with a selective inhibitor of Aurora B kinase (AZD1152)

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AZD1152 is a specific aurora kinase inhibitor with selectivity for Aurora B kinase, targeting proliferating tumour cells. Inhibition of Aurora B reduces histone H3 phosphorylation and inhibits cytokinesis, inducing multi-nucleation and polyploidy, leading to cell death and apoptosis. AZD1152 shows antitumour activity against a number of human tumour cell lines and preclinical xenograft models, and is being evaluated in early clinical studies. It has been suggested that cytokeratins released from dying carcinoma cells into the peripheral blood could serve as useful surrogate markers in the treatment of epithelial malignancies. The neo-epitope M30, revealed after cleavage of cytokeratin 18 (CK18) by activated effector caspases may provide an indirect marker for apoptosis. In contrast, the M65 epitope, present on both cleaved and intact CK18, should provide a measure of overall cell death.
We studied the correlation between cell death in tumour tissue and the level of M30/M65 in peripheral blood of nude rats bearing established human SW620 colon cell xenografts. Rats were dosed with either vehicle or AZD1152 (iv 25 mg/kg/day for 4 days), which leads to significant antitumour effects (maximum tumour growth inhibition >90%). Flow cytometric analysis of disaggregated xenograft tissue indicated a sequence of phenotypic events in tumours treated with AZD1152 including suppression in phosphorylation of histone H3 followed by an increase in polyploidy. Histological analysis in AZD1152-treated tumours confirmed the aberrant cell division phenotype and indicated an increase in apoptosis (by cleaved caspase 3 immunoreactivity) versus control treated tumours.
Dynamic changes in M30 and M65 levels were detected using ELISA. A rapid elevation in M30 plasma levels was observed in the AZD1152-treated group compared with the control group. Compared with M30, higher levels of M65 antigen were observed in both groups. Interestingly, at later timepoints higher M65 levels were observed in the control group, possibly reflecting higher tumour burden associated with more cell death at the core of the larger tumours.
These findings demonstrate that AZD1152 induces apoptotic cell death in a tumour xenograft model and this effect correlates with an increase in M30 and M65 detection in the peripheral blood. We are currently validating these findings with different dosing schedules of AZD1152 to further assess the utility of such non-invasive 'signals of efficacy'.

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Molecular characterization of cell death signaling by mercaptopyridine oxide and its analogs in human tumor cells-evidence for p53-independent growth arrest

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Efficient execution of cell death signaling is the desirable fate of cancer chemotherapy, and therefore the need to identify novel compounds with better apoptotic potential. Over the years, our laboratory has been studying the mechanism(s) underlying resistance to death signaling in tumor cell with the ultimate goal of identifying druggable targets for enhancing the efficacy of cancer chemotherapy. Among the various small molecules under investigation for their growth inhibitory activity is mercaptopyridine oxide (MPO) and its two synthetic analogs, MPO-Na and MPO-Zn. Using conventional biochemical approaches and laser scanning cytometry we investigated the mechanism of action of these small cell permeable compounds. Results show that all three compounds possess promising cell death inducing activity against a host of human tumor cell lines, however interestingly with diverse mechanisms. Whereas MPO and MPO-Na activated classical apoptotic pathway, characterized by robust caspase activation, H2gX phosphorylation (DNA damage), and mitochondrial outer membrane permeablization (MOMP), the mode of action of MPO-Zn appeared distinctly different with minimal involvement of the caspase proteases, but inhibitable by the necro-apoptosis inhibitor, necrostatin. Interestingly, exposure of human colorectal carcinoma cells expressing wild type p53 (HCT116 p53 +/−) to MPO resulted in early cell cycle arrest in the G2/M phase and later in G1 phase, mediated by ser15 phosphorylation of p53 and upregulation of the p53 inducible cell cycle inhibitor p21. In addition, these cells stained positive for senescence associated beta galactosidase (SA-b gal), thus strongly suggesting the acquisition of senescent phenotype. In comparison, p53 −/− variant of the cell line underwent a late S phase arrest and exhibited morphological features consistent with mitotic arrest. Furthermore, MPO compounds were excellent sensitizers when used alongside TRAIL in TRAIL responsive tumor cells. Finally, we tested these compounds against human B cell lymphomas derived from biopsies. Indeed, MPO and its analogs showed excellent growth inhibitory and death inducing activities against clinical lymphoma cells. Taken together, these data highlight the tremendous potential of these compounds as anti-cancer agents with the ability to either induce cell cycle arrest or apoptosis or caspase-independent cell death.

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Functional assessment of Bcl-2 disordered loop through plasmon surface resonance technology

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Bcl-2 family is divided in proapoptotic and antiapoptotic members. Protein function within the family is regulated at several levels, but a prominent mechanism consists in the dimerization of the proteins and the consequent modulation of the apoptotic threshold at the mitochondrial level. Within the family, there are conserved regions referred to as Bcl-2 homology domain (BH1–4). In some antiapoptotic members like Bcl-2 and Bcl-X_L, domains BH4 and BH3 are separated with a disordered loop. In this work, we used surface plasmon resonance technology to characterise the loop as the most important site for the protein-protein interactions within the Bcl-2 family. Bcl-2 strongly interacts with Bcl-2 itself and Bcl-X_L. Kinetics parameters were obtained using the heterogeneous ligand model, since dimers of Bcl-2 (KD 8.1×10^{-7} and 2.7×10^{-6} for Bcl-2 and Bcl-X_L, respectively) revealed minor affinity of binding with respect to the monomers (KD 2.3×10^{-9} and 6.2×10^{-9} for Bcl-2 and Bcl-X_L, respectively). To analyse the relevance of the loop in this binding, we replaced the disordered loop with a linker sequence of 4 alanines (Bcl-2 Δ), thereby demonstrating that without the loop the ability of Bcl-2 to homodimerize is completely abrogated. Computer assisted modelling helped us to design a Bcl-2 mutant in which Pro-39 was replaced with a Gly. As predicted, this mutation disrupted the structure of the loop and consequently the ability to homodimerize. Recently, also tubulin has been reported as a protein able to interact with Bcl-2. Upon the activity of microtubule polymerizing agents, it could occur Bcl-2 sequestering and consequently the induction of apoptosis. Using this technology, we measured the binding of tubulin to Bcl-2. Kinetic analysis showed that heterogeneous ligand model does not fit experimental data, thereby suggesting that both monomers and dimers of Bcl-2 equally bind to tubulin (KD 3.2×10^{-7}). As a first approach with three monoclonal antibodies specific for BH3, N terminal and the loop we performed the epitope mapping upon Bcl-2/tubulin interaction. The results pointed out that only the monoclonal antibody specific for the loop domain was relevantly affected upon binding, thereby indicating the involvement of the loop in this interaction. The same experiments was repeated with two antibodies specific for α and β-tubulin, thereby demonstrating that only this latter was affected in this interaction. These findings point out that the disordered loop plays a prominent role in interactions of Bcl-2 within the family and with tubulin.

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Development of a 300,000 base pair custom sequencing microarray for biomarker discovery and anticancer drug development

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Biomarker discovery is increasingly recognized as an important strategy for improving the efficiency of anticancer drug development and for optimizing cancer therapy in individual patients. For example, the presence of c-Kit in GIST or the Philadelphia chromosome in CML can be thought of as biomarkers of response to imatinib in patients with these diseases [1,2]. Also, specific mutations in the active site of the EGFR receptor were found in tumors from patients that were highly responsive to gefitinib therapy [3,4]. Because of the economy of scale and the immense quantity

of information that can be obtained from a single assay, microarrays are an extremely useful tool for candidate biomarker discovery. In addition to gene expression profiling, microarrays can also provide a powerful platform for high-throughput custom sequencing analysis. We designed an Affymetrix CustomSeq® microarray that reads over 300,000 base pairs spanning the entire coding regions of 81 genes. The genes selected for this CustomSeq® microarray regulate important tumor progression processes such as cell-cycle, apoptosis (intrinsic & extrinsic), drug-resistance, pro-survival, proliferation, metastasis, and angiogenesis. Also included in the microarray design are many of the molecular targets for the promising new anti-apoptotic therapies. To validate this new drug development microarray, we PCR amplified EGFR and members of the PI3K/AKT pathway from whole blood and HT29 colorectal tumor cells. After the PCR products were pooled and quantified, the samples were chemically fragmented, hybridized, washed, stained and scanned for fluorescent hybridization signatures indicating the sequence of the amplified genes. We demonstrated the ability of this array to perform high-throughput mutation and SNP screening. Information from this new molecular tool integrates well with gene expression profiling data and will help to implicate important genes, molecular pathways and host-drug interactions that influence cancer growth, development, disease susceptibility, drug resistance and drug response.

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473 POSTER

p53-dependent repression of CHEK1 contributes to apoptosis in colorectal cancer cells: an in vitro and in vivo study

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Background: There are only a few reports on the role of p53-dependent gene repression in apoptotic cell death. Here, we used the plant drug thymoquinone (TQ), to identify potential targets of p53.
Material and Methods: Human colon cancer cells HCT116 p53 +/+ and p53 −/− were treated with 60 μM TQ, and apoptosis-associated genes were analyzed by a cDNA microarray. Drug-induced DNA-damage was characterized by H2A.X foci and H_2O_2 formation. Apoptosis induction was analyzed by flow cytometry, caspase 3- and DASH assay. mRNA and protein expression were determined by *real-time* RT-PCR and Western Blotting, respectively. Using chromatin immunoprecipitation (ChIP), we studied p53 binding at the CHEK1 promoter. HCT116 p53 −/− cells were transfected with a wt-p53 vector in order to restore the p53 function and CHEK1 binding. Furthermore, we investigated the p53, CHEK1 and apoptosis status in a panel of human colon cancer tissues with known p53 mutation status.
Results: Only 17% of genes were dysregulated and might contribute to a significant portion of the TQ response. Strikingly, CHEK1 mRNA and protein were significantly induced in TQ-treated HCT-116 p53 (−/−) cells. Using ChIP, we verified a transcriptional repression of p53 at the CHEK1 promoter. Apoptosis was induced in response to TQ treatment in HCT-116 p53 (+/+) cells but to a much lower extent in HCT-116 p53 (−/−) cells, which fits with the drug-induced, significantly higher DNA damage signal in p53 +/+ cells. Transfection of p53 (−/−) cells with a p53-wt vector decreased the CHEK1 mRNA and protein levels and restored the apoptosis to the level of the p53 (+/+) cells. P53 (−/−) cells transplanted to nude mice intraperitoneally treated with 20 μM TQ also highly up-regulated CHEK1 expression and did not undergo apoptosis in contrast to p53 (+/+) cells. Colon carcinomas with p53 deletions resulting in a truncated non-functional p53 protein had significantly higher CHEK1 mRNA and protein expression levels which were accompanied by poor apoptosis compared to p53 wt-expressing tumors.
Conclusions: CHEK1 repression by p53 in HCT-116 p53 (+/+) cells could be responsible for drug-induced apoptosis, supporting recent findings that transcriptional repression by p53 rather than activation and selective blockade of p53-dependent gene repression accounts for DNA damage-induced apoptosis. In cancer therapy of colorectal cancer, the inactivation of CHEK1 might contribute to the anti-tumor activity of specific DNA-damage-inducing drugs.

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Inhibition of PDGFR-beta increases sarcoma cell sensitivity to tumor necrosis factor related apoptosis inducing ligand, TRAIL and promotes inhibition of tumor growth in dual therapy using imantanib (Gleevac) and TRAIL

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Background: The activation of the intrinsic and extrinsic apoptotic pathways have been found to sensitize cells to TRAIL. However, the interaction between antiangiogenic agents and TRAIL induced apoptosis has not been well studied. We hypothesize that inhibition of PDGFR-beta will sensitize sarcoma cells to TRAIL and therefore promote synergistic cytotoxicity, and inhibition of tumor growth using antiangiogenic and apoptotic therapy.
Methods: si RNA technology was used to 'knock down' PDGFR-beta in human Ewing's sarcoma cells, TC-71. More than 90% inhibition was achieved in the TC-71si cell line. The expression of death receptors-4 and 5 in the TC-71 versus TC-71si cell lines was then compared. TRAIL induced cytotoxicity in the 'knockdown' versus the wild type cell line was evaluated. The TC-71w cells were then injected into an orthotopic xenograft model of Ewing's sarcoma. An Imantanib (Gleevac) and TRAIL combination was then used to treat mice with either locally advanced chest wall Ewing's sarcoma, or spontaneous pulmonary metastasis secondary to Ewing's sarcoma. Combination treatment was compared to single therapy.
Results: The TC-71si cell line showed increased expression of DR-5 receptors (78% vs 48%) and DR-4 receptor expression (58% vs 35%) compared to the TC-71 cell line. Also, dose dependent TRAIL cytotoxicity was significantly more profound in the TC-71si cells (0% viability) compared to the TC-71 (35% viability) cells at 1500 ng/ml of TRAIL. In our locally advanced chest wall Ewing's model, maximal growth inhibition was seen using the Gleevac and IP TRAIL combination. Ewing's sarcoma chest wall tumors treated with Gleevac plus IP trail grew to an average of 100 mm^3 compared to an average of 1300 mm^3 in the control group without treatment. However, there was no significant difference in the inhibition of tumor growth seen in the the Gleevac and IP versus Gleevac and IN TRAIL. When using the Gleevac and IN TRAIL combination in mice with pulmonary metastasis, only 1 of 6 (16%) mice developed gross pulmonary metastasis versus 4 of 5 (80%) in the control group compared to Gleevac alone (3/6, 50%) versus IN TRAIL (2/6, 33%) or IP TRAIL alone 3/6 (50%).
Conclusion: A combination of TRAIL and Gleevac causes significant inhibition of pulmonary metastasis and primary tumor growth in Ewing's sarcoma. This is the first known report of synergy between apoptosis and antiangiogeneic therapy in a preclinical model.

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Involvement of the multi-ligand cell surface receptor RAGE in Tumor Necrosis Factor-induced cell death

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Tumor Necrosis Factor (TNF) is a potent anti-tumor agent and therefore an ideal tool to identify the signaling pathways that are required to kill a cancer cell efficiently. Our work is focused on a caspase-independent cell death pathway that is characterized by a necrosis phenotype and that requires the increased production of reactive oxygen species (ROS) in the mitochondria.
We have recently shown that the TNF induces increased concentrations of methylglyoxal, a cytotoxic metabolite derived from glycolysis that been considered for a long time as a natural anti-cancer agent. This, together with the TNF-induced phosphorylation of glyoxalase I, leads to the formation of specific methylglyoxal-derived Advanced Glycation End products (AGEs). The effect of AGEs are mediated via cell surface receptors of which the Receptor for AGEs (RAGE) is the best known. RAGE is a multi-ligand receptor involved in tumor growth, invasion and metastasis. Here we report that TNF-induced cell death is mediated via RAGE. Induced overexpression of the ligand-binding domain of RAGE [soluble (s)RAGE] strongly inhibits TNF-induced cell death, as did overexpression of WT RAGE but to a lesser extent than sRAGE. However, overexpression of a mutant of RAGE that lacks the intracellular domain has no significant effect on TNF-induced cell death. We found that TNF induces rapidly nucleocytoplasmic translocations of endogeneous full-length RAGE, which is followed by a considerable reduction in the amount of FL-RAGE and its higher molecular weight complexes. These TNF-induced nucleocytoplasmic translocations of endogeneous FL-RAGE are disrupted by overexpression of sRAGE. This implies that sRAGE may sequester a ligand that is required for TNF-induced nucleocytoplasmic translocations of RAGE and cell death. We further demonstrate that inhibition of a secretory pathway by Brefeldin A completely inhibits TNF-induced cell death as well as the nucleocytoplasmic translocation of FL-RAGE and the subsequent