that other member(s) of programmed cell death participate in the completion of EGFR-TKI induced cell death in NSCLC.

In about half of the patients, the acquired resistance to EGFR-TKI monotherapy upon prolonged treatment is due to T790M secondary mutation in the kinase domain of EGFR. However, a significant portion of patients develop acquired resistance without alterations in the primary sequence of EGFR kinase domain. A few mechanisms, including receptor cross-talks and transphosphorylation by non-ErbB family members, were suggested as possible explanations of acquired EGFR independence, though the physiological relevance of these results obtained from in vitro experiments still remains unclear. As an alternative pre-clinical model system of the acquired resistance to the current reversible TKI therapy, orthotopic tumor xenografts mice were treated with EGFR-TKI for a prolonged period of time. Refractory clones obtained from the lung of such mice display a series of histological and cellular phenotypes distinct from its parental cells. A subgroup of mice developed acquired resistance through T790M mutation. Other mice, however, developed resistance independent of T790M mutations. The molecular mechanisms leading to the acquired resistance are discussed.

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Specific inhibition of hypoxia-induced vascular endothelial growth factor expression by flavonoids in human lung cancer cells

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Flavonoids are a group of polyphenolic secondary metabolites important for plant biology and human nutrition. Epidemiological studies have shown that these compounds may have an important role in explaining the favorable effects of vegetables and fruits against cancer, in special lung cancer. Previous studies have reported that some flavonoids, such as quercetin, luteolin or fisetin, induced apoptosis in several cancer cell types. Another important property related to cancer chemoprevention may be their ability to inhibit tumor angiogenesis. Cells exposed to hypoxia up regulate the expression of several transcription factors, including the hypoxia inducible transcription factor (HIF), which induces the coordinated expression of many genes involved in glucose metabolism, pH regulation and angiogenesis. Among these genes, the vascular endothelium growth factor (VEGF) plays a key role in the stimulation of tumor angio and lymphangiogenesis. We investigated the effects of a group of 40 structurally related flavonoids on the expression of VEGF and HIF-1 alpha in human lung cancer cells. We found that several of these compounds inhibited VEGF production under hypoxic conditions at non cytotoxic concentrations. We also investigated the molecular pathways involved in the inhibition of VEGF expression, including MAP kinase and PI3-kinase-signaling pathways, and the expression and transactivation of HIF-1 factor. This research leads us to analyze the structure-activity relationships for these compounds and the relevance of each pathway in the inhibition of VEGF production. Overall, it is concluded that among the wide range of biological effects that flavonoids may exert, inhibition of angiogenesis may be of great relevance in their anticarcinogenic properties.

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The combined effect of a non selective and a selective cyclooxygenase-2 inhibitor and 5- fluorouracil treatment on HCA-7 human colorectal carcinoma cell line

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Background: 5-FU is included in many major chemotherapeutic regimens which have been statistically judged to be effective adjuvant therapy for patients with colorectal cancer. However, 5-FU itself does not substantially improve survival rates. Several NSAIDs have been tested in combination with a number of cytotoxic drugs. We hypothesized that treatment of cancer cells with 5-FU combined with indomethacin (INDO) or NS-398 might have a synergistic antiproliferative effect.

The aim of the study was to investigate whether INDO a nonselective cyclooxygenase (COX) inhibitor or NS-398, a COX-2-selective inhibitor, influence the cytotoxic effect of 5-fluorouracil on high COX-2 protein expressing HCA-7 colorectal cells. Considerable research effort was directed towards understanding the mechanism how these COX inhibitors modify the cytotoxicity of 5-FU.

Materials and methods: Sulphorhodamine B proliferation assay was used to measure the effect of 48 h 5-FU±INDO or 5-FU±NS-398 treatments on HCA-7 cells. COX-2 protein levels were analysed by Western blot and immunofluorescent method. PGE2 production was measured by ELISA. To investigate the cell cycle and apoptotic cells FACS analysis was used.

In order to understand the relative insensitivity of HCA-7 cells against 5-FU (IC50 value: 1mM) we studied the rate limiting enzyme of 5-FU catabolism dihydropyrimidine dehydrogenase (DPD)

Results: INDO or NS-398 treatment alone did not influence the proliferation of HCA-7 cells. INDO or NS-398 combinated with 5-FU significantly enhanced the proliferation inhibition caused by 5-FU alone. ($p<0.01$). The PGE2 production was reduced by 90% after 48 hours treatment with INDO or NS-398 which was similar range in case of 5-FU combinations as well. COX-2 protein levels were relatively unchanged. FACS analysis showed a delay in S phase progression and a marked decrease of G2/M fraction after treatment with 5-FU + INDO or 5-FU + NS-398.

The combined treatments also caused a significant increase in the number of apoptotic cells compared to 5-FU alone ($p<0.01$). High DPD enzyme activity was demonstrated in HCA-7 cells which was strongly reduced by both INDO and NS-398 as well.

Conclusion: 5-FU cytotoxicity against HCA-7 cells was augmented by combination with COX inhibitors which could be due at least partly to the increase of apoptotic cells and increase of the amount of 5-FU available for the anabolism as a consequence of the reduction of DPD activity.

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NSC-mediated tumor selective therapy - towards glioma clinical trials

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Neural stem cells (NSCs) display inherent tumor-tropic properties that can be exploited for targeted delivery of anti-cancer agents to invasive and metastatic tumors. Malignant gliomas are the most common primary brain tumors and are considered among the deadliest of human cancers. We postulate that NSC-mediated therapy of glioma will increase tumor-selectivity and decrease systemic toxicities, and thus potentially achieve therapeutic indices sufficient to eradicate invasive and residual tumor cells that are otherwise lethal. We generated a v-myc immortalized, clonal human NSC line, HB1.F3, which has been modified to stably express the cytosine deaminase (CD) therapeutic transgene (HB1.F3.CD). CD coverts 5-fluorocytosine (5-FC) prodrug to chemotherapeutic 5-fluorouracil (5-FU). Pre-clinical safety data in mice indicate that the HB1.F3.CD NSC line is non-toxic, non-immunogenic, non-tumorigenic, and chromosomally and functionally stable over at least 15 passages. Identification of a single copy and single insertion site for both v-myc and CD genes was determined by LAM-PCR and confirmed by Q-PCR. We believe that use of this stable, sustainable, and expandable NSC line will circumvent the problems associated with characterization, senescence, and replenishment sources of primary stem cell pools. Our pre-clinical therapeutic efficacy studies using the HB1.F3.CD NSCs in combination with 5-FC prodrug in laboratory animals demonstrated 70-90% of anti-tumor responses, as measured by decreased tumor burden or increased survival time in glioma-bearing mice (and in solid tumor metastases to brain, and medulloblastoma models). Our data also indicate that HB1.F3.CD cells can be labeled with iron oxide superparamagnetic nanoparticles, which allows in vivo MRI monitoring of NSC migration to intracranial glioma in tumor-bearing mice. We now propose the use of HB1.F3.CD NSCs in human patients with recurrent high-grade glioma. The NIH Recombinant Advisory Committee has approved the use HB1.F3.CD cells for recurrent glioma at a public hearing December, 2007. We are developing a pilot study in patients with recurrent high-grade glioma to assess the safety and feasibility of HB1.F3.CD NSCs injected directly into the brain parenchyma at the time of surgical tumor resection, in combination with oral 5-FC. We postulate that HB1.F3.CD

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Development of anti MUC1 DNA aptamers for the imaging of breast cancer

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Aptamers have shown great potential as novel targeted radiopharmaceutical entities for the diagnosis and imaging of various diseases. They offer reduced immunogenicity, good tumour penetration, rapid uptake and clearance compared to their monoclonal antibody counterparts. In previous work, we have reported on the modification of aptamers against breast cancer related biomarkers and their labelling with radionuclide ligands.

We have conjugated previously selected aptamers against the protein core or the tumour glycosylated MUC1 glycoprotein to different ligands (MAG2 or meso-2,3-Dimercaptosuccinic acid) and labelled them with 99mTc, for the diagnostic imaging of breast cancer. The conjugation is achieved using standard peptide coupling reactions between an amino modification on the aptamer and the carboxylic group on the ligands. An efficient and convenient labelling of the aptamer with short half-life radioisotopes was achieved as the last step of the synthesis (post-conjugation labelling). Labelling with 99mTc has taken place using tin chloride as reducing agent and analysed by HPLC, were both the UV and the gamma emission was monitored. The labelled aptamers were separated from free 99mTc using ultrafiltration and monitored by HPLC at all stages, before being injected and imaged for their pharmacokinetic properties. For the analysis of the pharmacokinetic properties of the aptamer-radionucleotide conjugate, we have used gamma-camera imaging in MCF-7 breast cancer tumour model systems.

The aptamer-chelator conjugates have strong 99mTc binding properties and the resulting complexes are highly stable in vivo, both in terms of nuclease degradation and leaching of the metal. We studied the effect of more than one molecules of aptamer per complex on the binding and pharmacokinetic properties of the radiolabeled products. We checked if different ligands affect the accumulation of the aptamer in different organs, as they alter the lipophilic properties of the conjugate.

These results aim to open new possibilities for the diagnostic imaging and potentially the targeted radiotherapy of breast cancer.

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Gene alteration profile and correlation to sensitivity to small molecule inhibitors in lung cancer cell lines

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The success of targeted therapies in the treatment of cancer relies on the identification of both the key components altered and the markers that can predict response to the treatment.

Here we present a detailed gene alteration profile of a panel of lung cancer cell lines and evaluate the correlation of the genotype with the sensitivity to selected small molecule inhibitors.

The genetic status of several cancer genes was analyzed by direct sequencing and/or real time quantitative PCR in a panel of 13 lung cancer cell lines. Then the effect on kinase activity and cell viability of several drugs was tested.

The genes analyzed were those most commonly altered in lung cancer, namely TP53, P16, KRAS, RB, CMET, EGFR, ERBB2, LKB1, MYC, PTEN, NRAS and PIK3CA. Next, the sensitivity of the cells to treatment with inhibitors of PI3K (LY294002), mTOR (Rapamycin), C-MET (PHA665752) or EGFR (erlotinib) was tested. We used levels of phosphorylation at downstream targets as surrogate markers of the inhibitory ability of the drug in a given cell line and calculated IC50 from dose-response curves. In most cases, an increased sensitivity to a given compound was correlated to a higher ability to reduce the levels of phosphorylation at their downstream targets. Sensitivity to EGFR (erlotinib), PI3K (LY294002) and CMET (PHA665752) inhibitors was evidenced in cells carrying single alterations at ERBB2, PTEN and CMET, respectively as compared to cells carrying simultaneous activation of these pathways.

In summary, we showed the effectiveness of drugs specifically designed against molecules genetically altered in tumour cell lines.

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Expression profiling reveals different targeted genes of the oral platinum(IV) prodrug oxoplatin and its putative activation product cisplatin

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Oxoplatin (cis,trans,cis-diammine-dihydroxo-dichloro-platinum(IV)), presently under investigation as an oral antitumor agent, was screened in vitro using MTT proliferation assays in a panel of 38 diverse human tumor cell lines. With exception of renal cell and ovarian cancer, cell lines sensitive to oxoplatin were detected for all other tumor entities. Using flow cytometry, the drug was shown to induce cell cycle arrest (propidium iodide staining), reactive oxygen species (ROS; dihydroethidium fluorescence) and cell death (annexinV / propidium iodide staining). Generally, the mean IC50 value for oxoplatin (sensitive cell lines: 7.6 ± 5.8 µg/ml; n = 27) was ~ 2.5-fold higher than for cisplatin. However, the small cell lung cancer (SCLC) cell line H526 showed similar sensitivity to oxoplatin and cisplatin and was used for genome wide expression profiling (Applied Biosystems Human Genome Survey Microarray V2.0) following application of the two compounds. Only 35 % of the downregulated and 10 % of the upregulated genes were identical in response to cisplatin or oxoplatin, respectively. Diametrically regulated genes included ribosomal proteins, DEAD box polypeptide 1, tubulins, pyruvate kinase, stathmin and high mobility group nucleosomal BD2 (HMGN2), indicating that oxoplatin is not a simple prodrug of cisplatin and that these two platinum compounds comprise distinct mechanisms of action. Since untreated H526 cells exhibited marked acidification of the medium to pH = 6.5 rapidly, effects of acids on oxoplatin were investigated. While addition of 50 – 800 µg/ml lactate had no effect on the cytotoxic activity of oxoplatin against COLO 205 cells, pretreatment of the compound with 5 mM ascorbic acid yielded enhanced activity (~ 2.5-fold), and thiols activated oxoplatin to a minor extent (< 10%). Following a 15 min exposure of oxoplatin to 0.1 M HCl representing gastric acid, the resulting platinum species cis-diammine-tetrachloro-platinum(IV) revealed a more than twofold increase in cytotoxicity against various cell lines. In conclusion, oxoplatin constitutes an effective oral anticancer agent that can be converted from its relatively inert native form into more active platinum intermediates different from cisplatin by acidic conditions in the stomach or tumor regions, depending on its pharmaceutical formulation. Platinum(IV) compounds are expected to have a higher probability than platinum(II) drugs to reach the malignant tissue intact and to be activated eventually at the intended target site.

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Increasing doxorubicin antitumor activity through COX-2 inhibition without increasing cardiotoxicity

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The clinical use of doxorubicin (DXR) and other anticancer anthracyclines is limited by a severe cardiotoxicity upon chronic administration. DXR induces cardiotoxicity by acting unchanged or after reductive metabolisation to toxic species. We recently discovered that anthracyclines can also undergo an oxidative degradation to non-toxic compounds. In the heart, anthracycline degradation is mediated by myoglobin and diminishes cardiotoxicity. In tumors, anthracycline degradation should be mediated by peroxidases with a consequent loss of antitumor activity. We analyzed the effect of COX-2 as an important catalyst of DXR oxidative degradation in tumors and we investigated if by impairing COX-2 activity it is possible to increase DXR activity in the tumor without increasing its toxicity in the heart.

COX-2 over-expressing (MDA-MB-231, SkOv3) and low-expressing human cancer cell lines (MDA-MB-468, T47D) were treated with DXR in the absence or presence of celecoxib, a specific COX-2 inhibitor. In both types of COX-2 over-expressing cells DXR and celecoxib had greater than addictive effects, whereas in COX-2 low-expressing cells celecoxib did not synergize with DXR. We next sought to establish whether celecoxib synergized with DXR through suppressing its degradation by the peroxidase component of COX-2. To this end we measured the cellular levels of DXR, its reduced metabolites, and the products of partial or complete DXR degradation. Celecoxib increased the levels of DXR and its reduced active metabolites in SkOv3 cells, but not in MDA-MB-468 cells, as one would expect if celecoxib inhibited DXR degradation that was specifically mediated by COX-2. Conversely, but consistently, DXR oxidative degradation products were detected only in SkOv3, and their levels decreased upon co-treatment with celecoxib. The effect of COX-2 was quite specific to DXR as Celecoxib did not synergize with non-anthracycline test compounds. Studies with isolated cardiomyocytes also showed that celecoxib had marginal or no effects on DXR toxicity; this latter finding was in keeping with the notion that in cardiomyocytes DXR would be degraded primarily by myoglobin.

Collectively, these experiments show that celecoxib increased the antitumor activity of DXR, but not its cardiotoxicity, by blocking its COX-2 dependent degradation in tumors. Improving the therapeutic index of DXR through the co-administration of coxibs is an hypothesis worth of testing in preclinical translation models.