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Gene expression profiles in rat liver slices after treatment with different hepatic tumour promotors

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Promoting agents in the liver can be classified according to their effect on gene expression profiles in rat liver after *in vivo* treatment. For high-throughput drug screening there is a need for *in vitro* assays which should reflect the *in vivo* conditions. Using DNA microarrays containing 1600 genes of the rat genome, the utility of rat liver slices in gene expression studies was investigated. Precision-cut rat liver slices were prepared and exposed to phenobarbital (PB), alpha-hexachlorocyclohexane (HCH), dehydroepiandrosterone (DHEA) and ethinylestradiol (EE) for 24h. For comparison, livers of animals treated *in vivo* with these agents were analysed. The responses *in vitro* observed in drug metabolising genes, including cytochrome P-450 genes (CYPs), epoxide hydrolases, UDP-glucuronosyl transferases (UGTs), glutathione transferases (GSTs) and peroxisomal genes corresponded well with published data. Expression of UGTs, GSTs and several CYP genes, like members of the CYP2 and CYP3 families were increased in rat liver slices treated with enzyme inducers like PB and HCH. After treatment with the peroxisome proliferator DHEA, the expressed genes were those predominantly involved in lipid metabolism and β-oxidation; genes of the CYP4A subfamily were also upregulated. After exposure of rat liver slices to EE, the expression of only a few genes, like steroid 3-alpha-dehydrogenase, which is involved in the steroid metabolism and the Bcl-2 apoptosis related gene was changed. To establish gene expression profiles, a two-dimensional hierarchical clustering analysis was performed. The clusters for PB and HCH, either generated in the liver of treated rats and in exposed rat liver slices were similar. The expression profiles of DHEA-treated rat liver slices and those obtained in rat liver after *in vivo* treatment with DHEA or the peroxisome proliferator WY-14,643 were similar. However the expression profiles observed in liver after *in vitro* and *in vivo* exposure by EE was distinctly different from those produced by the other chemicals. In conclusion we could demonstrate that gene expression profiles for compounds that act via similar mechanisms show common effects on transcription *in vivo* and in rat liver slices. Our results support the use of rat liver slices as tool in predictive toxicology, i.e. for the development and screening of new drugs. Supported in part by the Bio-Regio-Project 0311942

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PX-478, a potent inhibitor of hypoxia-inducible factor-1 (HIF-1) and antitumor agent

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Hypoxic cancer cells are found in all solid tumors and are the most difficult cancer cells to kill. HIF-1 is a transcriptional activator that plays a critical role in mediating the growing tumors response to hypoxia. Downstream genes activated by HIF-1 lead to increased glycolysis, resistance to apoptosis and increased tumor angiogenesis. HIF-1 is a heterodimer consisting of a HIF-1alpha subunit whose rapid breakdown under aerobic conditions is mediated by the Von Hippel Lindau protein (pVHL), and an oxygen-insensitive HIF-1β (ARNT) subunit. HIF-1alpha protein is elevated many fold in cells under conditions of hypoxia (<1-5% O2) and has been found to be increased in many human tumors but absent in normal tissue. HIF-1alpha is, thus, an attractive target for cancer drug discovery with the potential to decrease cancer cell survival and inhibit angiogenesis. We have identified PX-478 as a novel small molecule inhibitor of HIF-1alpha (IC_{50} of 5 and 7 μM in MCF-7 human breast and HT-29 human colon cancer cells, respectively). PX-478 inhibits HIF transactivating activity in the same cells (IC_{50} 7-9 μM) possibly by inhibiting thioredoxin reductase which is required for the oxygen dependent degradation of HIF-1alpha. PX-478 also inhibits hypoxia-induced vascular endothelial growth factor formation by MCF-7 and HT-29 colon cancer cells (IC_{50} 7-9 μM). PX-478 shows a selective growth inhibition of RCC4/pVHL- human renal cancer cells which have constitutively elevated HIF-1 alpha, compared to RCC4/pVHL+ cells. PX-478 administered as a single course, ip daily at 60 to 120 mg/kg for 4 days, to scid mice with established (0.2 to 0.5 g) human tumor xenografts gave tumor regressions and excellent dose-dependent tumor growth inhibition of MCF-7 breast cancer (T/C 92%, growth delay 42 days), HT-29 colon cancer (T/C 85%, growth delay 27 days) and PC3 prostate cancer (T/C 95%, growth delay >35 days). Peak plasma levels of PX-478 after an ip dose of 150 mg/kg were 550 μg/ml and t1/2 alpha 23 min and t1/2β 93 min. Immunohistochemical measurement showed almost complete depletion of HIF-1 alpha in MCF-7 tumor xenografts in scid mice administered PX-478 60 mg/kg ip for 4 days. Thus, PX-478 is the first of a novel class of HIF-1 inhibitors that causes depletion of HIF-1 alpha in tumors and inhibition of HIF activity. PX-478 has excellent activity against established human tumor xenografts causing tumor regressions and prolonged growth delay after only a single course of treatment.

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Inhibition of lysophosphatidic acid acyltransferase-beta (LPAAT-b) by CT-32228 inhibits activation of RAS-RAF-Erk and PI3K/AKT/m-TOR pathways and selectively induces tumor cell apoptosis

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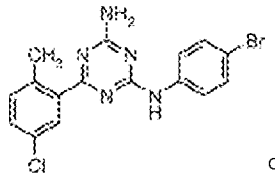
Lysophosphatidic acid acyltransferase-beta (LPAAT-b), an intrinsic membrane protein that catalyzes the synthesis of phosphatidic acid (PA) from lysoPA, has been implicated in cellular transformation. Elevated cellular PA has been documented in models of ras-transformation and it has been shown to affect tyrosine kinase signaling cascades, vesicle trafficking and cell motility through direct interactions with specific proteins or in a more generalized manner by altering locally the physical properties of membrane bilayers. Previously we reported that LPAAT-b cooperated with RAS or RAF in the activation of ERK1/2 and enhanced germinal vesicle breakdown in Xenopus oocytes. We show here that overexpression of LPAAT-b in NIH/3T3 cells is associated with proliferation in low serum and tumorigenicity in nude mice. Removal of the overexpressed gene reversed these parameters of transformation. RNAi knockdown of LPAAT-b in DU145 prostate cancer cells was potently antiproliferative and induced apoptosis in IM-9 myeloma cells. Isoform-specific nanomolar inhibitors of LPAAT-b activity typified by CT-32228 [N-(4-bromophenyl)-6-(5-chloro-2-methylphenyl)-[1,3,5]triazine-2,4-diamine] blocked translocation of RAF and activation of Erk and PI3K/Akt pathways in normal activated endothelial and vascular smooth muscle cells (VSMC).



CT-32228

Treatment of Angiotensin II or VEGF treated VSMC or human microvessel endothelial cells with CT-32228 induces profound, dose dependent decreases in the phosphorylation states of effectors of both pathways including Erk1/2, Akt, mTOR, GSK3beta, p70S6k and p90RSK. Both of these proliferative and cell survival signaling cascades involve PA-regulated processes, which could explain the anti-LPAAT-b effects. LPAAT-b inhibitors are potently antiproliferative and cytotoxic in all tumor cell lines tested and induce Type I and Type II apoptosis pathways as shown by the expression and activation of TRAIL/Apo-2L receptosome complexes and subsequent activation of Caspase 9 and Bax. In contrast, normal human bone marrow progenitors and activated naive murine T cells are resistant to both the antiproliferative and apoptosis effects of CT-32228. These data indicate that LPAAT-b plays an important role in tumor cell survival and that LPAAT-b represents a novel therapeutic target for cancer treatment.

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G-quadruplex interaction of 3,6,9-trisubstituted acridines leads to specific telomerase inhibition, induction of senescence and telomere shortening

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We have previously shown that acridines with appropriate aminoalkylamido disubstitution can bind to G-quadruplexes formed from human telomeric DNA sequences, and that this interaction also inhibits telomerase en-

zyme activity. Structure-based design methods have been used to significantly enhance selectivity and potency for both, quadruplex specificity and telomerase inhibition, by addition of a third substituent at the 9-position of the acridine ring. We report here on biological studies of trisubstituted compounds, including two with extended substituents at the 9-position (BRACO19, JCACI9). Enhanced quadruplex affinity shown by molecular dynamics based on the G-quadruplex crystal structure was paralleled by an increased differential between concentrations needed to inhibit telomerase activity in the TRAP reaction versus those which result in overt cytotoxicity in a 4 d SRB assay. JCACI9, the compound with the more extended 9-amino substituent, inhibited the TRAP reaction of MCF-7 breast cancer cell lysates at 2 μM, but had an IC_{50} for growth inhibition in MCF-7 cells of 45 μM. BRACO19 had an IC_{50} of 6 μM and produced telomerase inhibition at 1 μM. SBACI11 however, an agent with a short 9-substituent, lacked differential of growth and enzyme inhibition in this cell line. Specific telomerase inhibitory effects were accompanied by induction of cellular senescence as determined by β-galactosidase staining in long term MCF-7 cultures treated with BRACO19 and JCACI9 concentrations below acute cytotoxicity. A 2- to 5-fold increase in β-gal positive cells was found starting at 7 days of continuous treatment (6 population doublings, PD). In contrast, telomere shortening in the order of $\sim$ 1 kb measured by Southern blotting was seen after 33 PDs. We suggest therefore that these two events are not necessarily related, and that this behavior might be indicative of G-quadruplex inhibitors producing senescence by direct interference with telomere maintenance/capping rather than successive shortening. Thus, G-quadruplex interactive agents seem not to require the extended time lag characteristic of pure inhibitors of the catalytic activity of telomerase.

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The unfolded protein response pathway plays a key role in anti-cancer drug sensitivity

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All cells possess signaling pathways that allow them to detect environmental changes that adversely affect cellular processes and activate protective responses. An important stress protection pathway is the unfolded protein response (UPR), which is activated by conditions that interfere with protein folding in the endoplasmic reticulum (ER). Protein folding in the ER can be affected by environmental stresses, such as glucose deprivation or hypoxia, and pharmacological agents, such as tunicamycin and thapsigargin. UPR activation triggers signaling cascades that alter transcription, cell cycle progression, protein synthesis and stability, and cell survival. It has also been shown that agents that induce the UPR lead to dramatic changes in sensitivity to several classes of anti-cancer agents, including topoisomerase II (topo II) inhibitors, and DNA alkylating agents. For example, agents such as tunicamycin lead to a rapid depletion of topo II and resistance to etoposide. Conversely, the induction of the UPR increases sensitivity to cisplatin by an unknown mechanism. A key regulator of the UPR is BiP (GRP78), a resident ER chaperone. BiP binds to several effectors of the UPR and blocks their activation. When unfolded proteins accumulate in the ER, BiP releases UPR effectors to bind the unfolded proteins, thus allowing activation of the pathway. Over-expression of BiP prevents UPR induction, providing a tool for directly assessing how this pathway affects anti-cancer drug targets. We used CHO cells that over-express BiP to assess the contribution of the UPR in altered sensitivity of cells to topo II targeting drugs and alkylating agents. In the absence of stress, cells over-expressing BiP have the same levels of topo II and sensitivity to etoposide as the parental cells. However, unlike the parental cells, in the presence of UPR inducing agents, the level of topo II in BiP over-expressing cells remained unchanged and resistance to etoposide was not seen. This result demonstrates that induction of the UPR pathway is directly responsible for resistance to topo II targeting agents. Interestingly, CHO cells over-expressing BiP are highly resistant to cisplatin, even in the absence of UPR inducing conditions. This result indicates that BiP regulates proteins that play an essential role in determining cellular sensitivity to cisplatin. Experiments are underway to identify proteins regulated by BiP that are important for sensitivity to cisplatin.

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Identification of a new drug target using RNAi-based functional genomics

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A Genome-wide RNAi screen was performed to obtain functional information on genes involved in cell division in C. elegans and furthermore to identify similar gene functions in other organisms. We inhibited the expression of 91% of the 19,500 predicted open reading frames of the entire genome using RNA-mediated interference (RNAi). By using an *in vivo* time-lapse differential interference contrast microscopy assay we so far identified more than 750 genes required for the first two cell divisions in C. elegans embryos. One of these genes is H38K22.2, which plays an important role in these early divisions. Inhibition of the gene product of H38K22.2 by RNAi results in a severe cell division phenotype, the characteristics of which are formation of multiple female pronuclei, wrong spindle positioning and delayed P1 division, resulting in an early death of the embryo. We characterized the human homologue RP42, which shows 36% identities and 56% positives to the C. elegans gene. The RP42 protein shows a specific localization on the centrosomes during the cell cycle in human cells. Expression of the RP42 transcript in human tissues is generally very low, with the highest levels found in proliferating tissues. Importantly, expression is also up regulated in various tumours, namely lung, colon and pancreas. Expression of RP42 can be inhibited to 90% in human cells using an RNAi based assay. Functional results will be presented.

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PDGF receptor inhibition in tumor stroma, with STI571 or PDGF B-chain aptamers, enhances the effects of chemotherapy in experimental solid tumors by increasing tumor drug uptake

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Most solid tumors display interstitial hypertension. The increased interstitial fluid pressure (IFP) in tumors has been proposed to act as a barrier for tumor drug uptake. Many solid tumors also display PDGF receptor expression in tumor stroma cells. Furthermore, PDGF receptor activation in loose normal connective tissue increases IFP. Based on these observations we investigated the effects of PDGF receptor inhibition on tumor IFP, tumor drug uptake, and on the anti-tumor effects of chemotherapy. Studies were performed on subcutaneous PROb colon adenocarcinomas and KAT-4 thyroid carcinomas grown in syngeneic rats and SCID mice, respectively. Both tumor models display extensive tumor stroma formation and PDGF receptor expression restricted to stroma cells. PDGF receptor inhibition was achieved by systemic treatment with STI571 or PDGF B-chain aptamers. Inhibition of PDGF receptor signaling reduced tumor IFP in both models by approximately 30%. PDGF receptor inhibition increased tumor transvascular transport in the PROb tumor model and increased the uptake of Taxol in the KAT-4 tumor model. Similarly, an increased uptake of EPO906 (epothiloneB, a novel non-taxoid microtubuli stabilizing agent) occurred in KAT-4 tumors, but not in liver and kidney. Most interestingly, combination treatment of PROb tumors with STI571 and 5-FU, or of KAT-4 tumors with STI571 and Taxol or EPO906, dramatically enhanced the anti-tumor effects of the cytotoxic drugs. Treatment with only STI571 did not produce anti-tumor effects. The synergistic interactions were not observed *in vitro*, and co-treatment with STI571 did not lead to anti-angiogenic effects. Different STI571 treatment schedules, together with weekly EPO906B administration, confirmed the association between the beneficial therapeutic effects of co-treatment, and the reduction of IFP and enhanced tumor drug uptake induced by STI571 pre-treatment. In conclusion, our study demonstrates that PDGF receptor inhibition in tumor stroma cells reduces tumor IFP, increases the tumor uptake of chemotherapy drugs, and enhances their therapeutic effects. The study thus identifies inhibition of PDGF receptor signaling in tumor stroma as a novel, possibly general, combination strategy for enhancement of the therapeutic effects of standard chemotherapeutics. (Parts of the study were performed with financial support from Novartis, Basel, Switzerland)