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Novel mammalian chromatin reconstitution *in vivo*

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A recent breakthrough in cancer research is the discovery that various transcription factors (TFs) that are involved in cancer, such as p53 and pRB, BRCA-1, AML-1/ETO, PML-RAR-a, etc., actually regulate cancer by modifying chromatin. However, the exact mechanism of TF-mediated chromatin modification in mammalian cells *in vivo* is not known. For example, it is not known whether a specific TF modifies specific histones and specific amino acids within the histones during this process or whether all of the TFs modify the same histones. Because histone modification is one of the ultimate steps in the regulatory circuits of these TFs, such studies may yield a new approach to cancer therapy. For instance, small molecules can be synthesized that can either augment or block specific TF-histone interactions and thus, regulate cancer. The major road-block in determining such TF-histone interactions arises because mammalian cells *in vivo* are not amenable to introduction of mutant histones. By microinjecting plasmid-encoded genes and transcriptional regulatory sequences in early mouse embryos and examining the genes' expression, we have developed a novel system in which chromatin modulation can now be studied in mammals. Results from our and other laboratories suggested that expression from such microinjected plasmids reflects physiological regulation present in these embryos. Utilizing this system, we previously observed that the paternal pronuclei of one-cell embryos do not contain chromatin structure. In contrast, chromatin structure was observed in two-cell embryos. Our recent experiments further indicated that co-injection of purified histones and a plasmid-encoded reporter gene into the paternal pronuclei of one-cell embryos at a specific histone-DNA concentration could reconstitute the behavior observed in two-cell embryos, both structurally (chromatin assembly) and functionally (acquisition of promoter repression and subsequent relief of this repression by functional enhancers or by histone deacetylase inhibitors). Thus, injection of exogenous histones into one-cell embryos faithfully reproduced the chromatin-mediated transcription observed in two-cell embryos and provided the first *in vivo* mammalian model system in which the role of individual histones, and particular domains within the histones that are specifically targeted during a given TF-mediated enhancer function, can be examined using purified mutant histones.

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Novel peptidomimetic inhibitors of Stat3 signaling and oncogenesis

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One member of the family of Signal Transducer and Activator of Transcription (STAT) proteins, Stat3, participates in malignant transformation. The critical role of Stat3 in the growth and survival of human tumor cells provides a valid basis for targeting Stat3 for development of novel inhibitors. We previously identified a Stat3 SH2 domain-binding peptide, PY*LKTK and its tripeptide derivatives, PY*L or AY*L (where Y* represents phosphotyrosine), which were demonstrated to inhibit Stat3 signaling and oncogenesis. Here we report novel tripeptide mimics that have been developed for improved selectivity and efficacy with regard to inhibition of Stat3 activity. The presence of these peptidomimetic compounds in nuclear extracts results in a dose-dependent decrease in the level of Stat3 DNA-binding activity *in vitro*, with efficacies that are five- to ten-fold higher than previously obtained for tripeptides. In whole cells, a representative peptidomimetic identified as JSK 610 selectively suppresses constitutive Stat3 activation and transcriptional activity in Src-transformed fibroblasts, as well as in human breast and lung carcinoma cells that express constitutively-active Stat3. In a manner that reflects inhibition of aberrant Stat3 activity, JSK 610 inhibits proliferation and induces apoptosis of transformed cells. Altogether, we present evidence for novel peptidomimetic-based specific inhibition of Stat3 signaling, and demonstrate the profound biological outcome of growth inhibition and induction of apoptosis in relevant model human tumor cell lines.

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Insight into the mode of action of rViscumin through transcriptional profiling

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Transcriptional profiling is a suitable tool for the identification of new drug candidates for applications in oncology and immunotherapy. Once a novel drug is identified the technique can also be used to obtain detailed information on the mode-of-action. To this aim, a target-oriented cDNA array system (onco-set) was used for the analysis of drug induced transcriptional changes in various intra- and intercellular signal transduction pathways leading e.g. to apoptosis, cell-cycle regulation or cytokine production. We set up an array-based classification system for the molecular characterisation of the novel anti cancer drug rViscumin and compared it to profiles from known cytostatic or antibiotic drugs in a surrogate cell system. The ribosome inactivating protein rViscumin is a potent apoptosis inducing drug with immunomodulatory and anticancer activity *in vitro* and *in vivo*. rViscumin is a recombinant heterodimeric plant derived protein which is currently in clinical development. We applied this drug to a model cell system (THP-1 human monocytic cell line) and compared the expression profile changes induced by standard cytostatic drugs, i.e. Taxol, Hycamtin and Doxorubicin to that induced by rViscumin. In a time course analysis the transcriptional changes with a 24 h interval after the start of treatment were monitored. In rViscumin treated cells the eucaryotic initiation factor-2 (eIF-2, playing a role in regulation of translation) was downregulated by a factor of 5 whereas apoptosis related gene products as metallothionein, DNAse 1 precursor or Adducin-1 alpha were upregulated by at least a factor of 4 compared to the untreated control. Genes regulated in THP-1 cells treated with e.g. Taxol were different and included a downregulation of MAPK2 / Erk2 and G2 / mitotic cyclin B. rViscumin in this setup showed a unique transcription profile and its mode of action can be distinguished from the mode of action of any of the other anticancer drugs tested. These data are in good correlation with the results from a COMPARE analysis based on data from the NCI 60 cell line screening results. The results gave new insight into the molecular mechanism of rViscumin and lead to the identification of predictor gene classes, which can potentially be utilised for monitoring clinical responses. Array-based transcriptional profiling using the onco-set gene collection is a valuable tool for screening and characterisation of novel anti cancer drugs.

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Preclinical efficacy, toxicology and pharmacokinetics of NVP-LAQ824, a novel synthetic histone deacetylase inhibitor

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Reversible acetylation of nuclear histones is a major regulator of gene expression which may act by altering accessibility of transcription factors to DNA by changing higher order structures in the nucleosome. Cell specific patterns of gene expression result from a balance between the competing activity of histone acetyl transferases (HAT) and histone deacetylases (HDAC). Perturbations of this balance have been linked to cancer. Small molecule inhibitors of HDAC have been shown to have antiproliferative and apoptotic effects in human tumor cell lines *in vitro*. Additionally, several compounds have been reported to inhibit human tumor xenograft growth in nude mice. A subset, including suberoylanilide hydroxamic acid (SAHA), FK-228 and MS-275, are under evaluation in clinical trials as anticancer agents. Our efforts to discover novel small molecule HDAC inhibitors (HDAIs) led to the identification of the potent HDAI NVP-LAQ824, which is currently in Phase I clinical trials. To support the development of NVP-LAQ824, we investigated preclinical efficacy in the athymic nude mouse, pharmacokinetics in the mouse and rat and toxicity in the rat. We report here the dose-response of NVP-LAQ824 in the HCT116 human colon, A549 human lung and MDA-MB-435 human breast xenograft models in athymic nude mice. In each of these models, tumor stasis was observed at tolerated doses and schedules. Plasma and tumor concentrations of NVP-LAQ824 were obtained from athymic mice bearing HCT116 tumors at two doses and from athymic mice bearing MDA-MB-435 tumors at one dose. These data show that NVP-LAQ824 clears rapidly from plasma and persists at significant concentrations in tumor tissue up to 16 h post-dose. An 8-cycle toxicology study in rats was carried out at three doses, followed by a 4 week recovery. Toxicity includes a reversible dose-dependent reduction in body weight gain and food consumption, and reversible changes in the myelopoi-

etic/erythropoietic cell ratio. Pharmacokinetic parameters were obtained for each dose level after the first dose, first cycle and last dose, last cycle and indicate dose-proportional drug exposure of all treated animals.

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Lipid rafts as gateway for antitumor alkyl-lysophospholipids to induce apoptosis

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Synthetic alkyl-lysophospholipids (ALPs), such as 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine, are antitumor agents known to accumulate in cell membranes. The aim of this study was to understand the mechanism by which ALP enters the cell and induces apoptosis. We demonstrate that in murine lymphoma S49 cells, ALP inhibits de novo biosynthesis of phosphatidylcholine (PC) at the CTP:phosphocholine cytidylyltransferase (CT) step. Exogenous lysoPC providing an alternative route to generate PC (via acylation), rescued the cells from ALP-induced apoptosis. This indicates that a continuous rapid PC turnover is essential for cell survival. To reach CT, ALP needs to be internalized. This internalization did not involve receptor/clathrin-coated pit-mediated endocytosis, nor fluid phase endocytosis. Instead, intact lipid rafts in the plasma membrane were found essential, as ALP was found to accumulate in lipid rafts and artificial disruption of these microdomains resulted in dissociation of ALP from rafts. This led to a reduced ALP endocytosis, and inhibition of apoptosis. Interestingly, an ALP-resistant cell variant, S49AR, showed no impaired PC metabolism after ALP treatment and revealed reduced ALP internalization and reduced levels of sphingomyelin, an essential component of lipid rafts. Therefore, we argue that altered lipid composition of lipid rafts determine raft-mediated endocytosis. For the first time, lipid rafts are recognized as potential targets for anticancer therapy.

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NADPH oxidase 1 (NOX 1): a novel target for colon cancer therapy

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Recent studies have demonstrated the presence of several novel membrane oxidases in mammalian tissues that share homology with gp91phox, the catalytic moiety of the NADPH oxidase (NOX) found in phagocytic leukocytes. These flavoproteins catalyze the NADPH-dependent reduction of oxygen to superoxide and related reactive oxygen species (ROS). Because the mechanism of ROS generation after exposure of human tumor cells to a wide range of growth factors (including EGF, PDGF, insulin, bFGF, and GM-CSF) remains to be determined, we examined the expression of NOX 1 in a panel of cultured human cancer cells using real-time RT-PCR. NOX 1 mRNA was quantitated as the ratio of the levels of NOX1/18S mRNA using specific plasmids containing either NOX1 or 18S. NOX 1 expression ratios were very high (>130,000) in human colon cancer cell lines (CaCo2, LS174T, and HT-29) and barely detectable (ratios <200) in human MDA-MB468, BT474, and ZR-75 breast cancer cells or DU-145 and LNCap human prostate cancer lines. In a panel of twelve human colon cancers paired with adjacent normal tissues obtained from the City of Hope Frozen Tumor Bank, NOX 1 ratios ranged from 20,000 to 800,000 in 10/12 tumors, were undetectable in 2/12 tumor and normal samples, and were substantially greater in tumor than normal tissue in 7/10 samples. As was the case in cell lines, NOX 1 expression ratios were low (<500) in 12/12 human breast cancer specimens and 6/6 prostate cancers. To assess NOX 1 as a therapeutic target, growth inhibition by the NOX inhibitor diphenylene iodonium (DPI) was examined *in vitro*. The IC_{50}'s for DPI were 5, 20, and 40 nM for CaCo2, LS174T, and HT-29 cells that express high levels of NOX 1, and were >2000 nM for DU-145 and MDA-MB468 cells that demonstrate very low level expression of the oxidase. These experiments suggest that growth factor-related reactive oxygen production may play an important role in signal transduction and tumor cell proliferation; and that NOX 1 may be a new target for the development of novel treatments for colon cancer. (Supported by CA 62505)

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Molecular modes of action of antimalarial artemisinin derivatives as novel anticancer drugs

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Twenty-two chemically characterized compounds derived from Traditional Chinese Medicine were analyzed in drug-sensitive and multidrug-resistant tumor cell lines. The antimalarial artesunate (ART), a semisynthetic derivative of artemisinin from the Chinese plant *Artemisia annua L.*, was among the most active compounds. ART did not exhibit cross-resistance to multidrug-resistant tumor cells overexpressing either the resistance-conferring MDR1, MRP1, or BCRP genes. Isogenic p53 -/- knock out tumor cells were as sensitive as their p53+/+ counterparts indicating that ART was not subject to p53-mediated chemoresistance. The evaluation of ART's anticancer activity in 55 cell lines of the National Cancer Institute, U.S.A., showed that ART was most active against leukemia and colon cancer cell lines. We mined the N.C.I. database and correlated the IC_{50} values with microarray mRNA expression profile of 464 genes. By hierarchical cluster analysis we identified oncogenes and proliferation-regulating genes which were strongly downregulated in ART-sensitive leukemia and colon cancer cell lines. The role of proliferation for ART's response was corroborated using a panel of *Saccharomyces cerevisiae* strains with defined genetic knock out mutations. Furthermore, ART correlated significantly with proliferation parameters (cell doubling times, G0/G1 and S cell cycle phases). We extended our analyses to other artemisinin derivatives, arteether and artemether. Using hierarchical cluster analysis we found that one cluster of genes correlated with the IC_{50} values of all three derivatives, the majority of them being proliferation-associated genes. This speaks again for a general role of the proliferative state for the response of tumor cells towards artemisinin derivatives. Another cluster contained genes correlating specifically with one of the 3 drugs. The correlation to different genes may explain differing anticancer activities of artemisinins.

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Blockade of endothelin A receptor by ABT 627 suppresses tumor growth, neovascularization and potentiates cytotoxic paclitaxel activity in ovarian cancer cells *in vitro* and *in vivo*

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The endothelin-1 (ET-1)/ETA receptor (ETAR) autocrine pathway is overexpressed in many human tumors, including ovarian carcinoma, and may provide a new target for anticancer therapy. Engagement of ETAR by ET-1 triggers activation of tumor cell proliferation, survival, neoangiogenesis and invasion. In primary and metastatic ovarian carcinomas, ET-1 overexpression is associated with enhanced neovascularization as well as with vascular endothelial growth factor (VEGF) expression. ABT627 (Atrasentan) is a p.o.-active ETAR antagonist that selectively inhibits the ETAR activities and is under clinical develoment in cancer patients. We therefore tested whether ABT627 may potentially block ovarian tumor progression and may affect neovascularization and apoptosis. When tested in culture ABT627, inhibited tumor growth in both primary cultures (PMOV1 and PMOV2) and cell lines (OVCA 433 and HEY) of ovarian carcinoma. In contrast, the ETBR antagonist, BQ 788, does not display inhibitory effects. Furthermore ABT627 inhibited VEGF production and enhanced proapoptotic effect of paclitaxel. Extending these studies *in vivo*, we explored the therapeutic effects of ABT627 on HEY ovarian carcinoma xenografts. HEY cells produced high amount of ET-1, expressed high affinity ETAR (Kd=0.1 nM; 35,600 sites/cell) and developed rapidly growing solid tumors in nude mice. ABT627 (2mg/Kg/24h i. p. for 21 days) produced similar inhibition of tumor growth as paclitaxel (20mg/Kg i. v. Q4x3) with a reduction of 65% (p=0.005) and 67% (p=0.006), respectively, compared with control. Similar results were obtained with high dosage of ABT 627 (10mg/Kg/24h). Immunohistochemical evaluation of tumors revealed that the reduced size of ABT627-treated tumor xenografts coincided with reduced neovascularization and with enhanced ovarian cancer cell death. Administration of ABT627 and paclitaxel in HEY tumor xenografts caused a remarkable antitumor effect. Tumor regression was accompained by a significant inhibition of VEGF,