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Cellular accumulation of cisplatin is mediated by the ubiquitously expressed sodium-dependent human ASCT1-amino acid transporter

R. Kroning[1,4], E.A. Carter[2], E. Srivatsan[1,3], A.K. Lichtenstein[1,4], G.T. Nagami[1,5]. *[1]Dept. of Medicine, [2]Dept. of Chemistry and Biochemistry, University of California Los Angeles, Los Angeles, USA; [3]Dept. of Surgery, [4]Dept. of Hematology/Oncology, [5]Dept. of Nephrology, VA Greater Los Angeles Healthcare System, Los Angeles, USA*

Cellular accumulation of cisplatin (DDP) is not well understood, however, the involvement of an active transport system has long been suggested. We have shown previously that L-cysteine (Cys) strongly and competitively inhibited the uptake of DDP in renal tubule epithelial cells and that pre-complexation of DDP with Cys was not responsible for this effect (Cancer Chemother Pharmacol 45: 43, 2000). These data implicate competitive DDP transport through the ubiquitously expressed ASC-amino acid transport system (named after substrates Ala, Ser, Cys). Human ASCT1 is a sodium-dependent transporter of the ASC-system (J Biol Chem 268: 15329, 1993). To demonstrate the involvement of the ASC-system in DDP accumulation we micro-injected Xenopus laevis oocytes with *in vitro*-synthesized ASCT1-cRNA derived from a pOTV-ASCT1 clone and compared their subsequent DDP accumulation rates with those of control oocytes either injected with dH2O or ASCT1-antisense-cRNA. Functional expression of ASCT1 was verified by measuring the transport of marker amino acid L-[3H]-alanine. During a 20min exposure to 400microM DDP the expression of ASCT1 accelerated DDP accumulation by over 110% compared to basal DDP uptake in control groups. A 10-fold excess of Cys completely inhibited DDP transport in ASCT1-expressing oocytes but not in control oocytes. Due to the low endogenous expression of amino acid transporters in oocytes these data suggest that in ASCT1-expressing oocytes approx. 50% of DDP uptake occurs through passive diffusion and 50% through ASCT1-mediated transport and agree with existing data on DDP uptake in cancer cells. RT-PCR analysis established expression of ASCT1-RNA in a number of clinically important tumors including cervix, ovary, uterus, lung, breast, and renal cell lines, thus supporting the rationale of targeting the ASC-system for tumor treatment. A 30min pre-incubation with 100nM phorbol 12-myristate 13-acetate (PMA), a PKC-dependent activator of amino acid transport, significantly sensitized A2780 ovarian carcinoma cells to DDP. To further support the concept of competitive binding, we compared lowest-energy structures of DDP and Cys using 3-dimensional models with electron density overlay. The distances between the amino group present in each molecule and the electronegative centers Cl- in DDP and S- in Cys differ by only 0.59Å. This structural similarity suggests that DDP can bind to a Cys-binding site. This study demonstrates that expression of the human ASCT1 can modulate the transport of DDP and suggests a clinically relevant role of the ASC-amino acid transport system in the accumulation of DDP in human cancer cells.

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New hydrazones, a novel class of experimental antitumor agents

J. Hofmann[1], G. Scheran[1], J. Easmon[2], G. Puerstinger[2], G. Musumarra[3], D.F. Condorelli[3], S. Scire[3], G. Heinisch[2]. *[1]University of Innsbruck, Institute of Medical Chemistry and Biochemistry, Innsbruck, Austria; [2]University of Innsbruck, Pharmaceutical Chemistry Department, Innsbruck, Austria; [3]University of Catarnia, Department of Chemical Sciences, Cartarnia, Italy*

Bicyclic hydrazones are compounds exhibiting antitumor activity (Easmon et al., Int. J. Cancer, 94, 89-96, 2001). Several of the novel compounds exhibit above-average inhibition of leukemias, melanomas, colon, non-small cell lung and renal carcinoma cells in the NCI panel of cell lines. Compounds EPH101 (4-acetylpyrimidine 2'-benzothiazolylhydrazone), EPH136 (3-acetylisoquinoline 2'-bezoxazolylhydrazone) and showed positive results in the NCI hollow fiber assay. A COMPARE analysis showed that the mechanism of action of the novel compounds is not related to any of the standard antitumor drugs. A bioinformatical approach, based on the IC_{50} values and the levels of gene expression of 9700 genes in the NCI panel of cell lines showed that genes belonging to the following groups are important for the antiproliferative activity: (i) DNA-, ATP-, and GTP-binding proteins, ATPases and GTPases, (ii) genes involved in tumor proliferation, (iii) proteins involved in cell adhesion, cell migration and neuronal development, (iv) kinases and (v) calcium-binding proteins. Following treatment with 2-fold IC_{50} concentrations of EPH136 alterations in the expression of 5000 genes were determined by gene array. The genes found to be overexpressed more than 2-fold belong to the same groups as that found by the bioinformatical approach. In addition, RNA-binding proteins, transcription factors (belonging to the DNA-binding proteins), phosphatases, the GABA and benzodiazepine receptor, protein-degrading enzymes and protease inhibitors were overexpressed. In conclusion, the novel hydrazones exhibit antitumor activity *in vitro* and *in vivo* by a new mechanism of action which is unknown at present. Financial support was provided in part by the Austrian Science Foundation (FWF), project No. P12384-MOB

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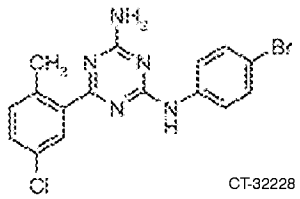
CT-32228: a lysophosphatidic acid acyltransferase-beta (LPAAT-b) inhibitor, induces apoptosis in a variety of solid tumor, leukemia and lymphoma cell lines, but not in normal cells

P. de Vries, L. Bonham, T. Martin, I. Stone, M. Anderson, J. Moses, J. Pound, D. Hollenback, M. Coon, J.W. Singer. *Cell Therapeutics, Inc., Seattle, USA*

LPAAT-b is a member of a family of 5 intrinsic membrane enzymes that catalyze the biosynthesis of phosphatidic acid (PA). PA is associated with the activation of raf and mTOR. LPAAT-b is highly expressed in a large proportion of lung, ovary, prostate, bladder, cervix, and brain tumors, while its expression in normal tissues is primarily limited to endothelial, smooth muscle and inflammatory cells. CT-32228 (N-(4-bromophenyl)-6-(5-chloro-2-methylphenyl)-[1,3,5]triazine-2,4-diamine), was derived following medicinal chemistry optimization of a diversity library screen to identify compounds that inhibited LPAAT-b, but not the related housekeeping enzyme LPAAT-alpha. CT-32228 had a Ki of 47 nM in a high through-put cell free screening assay against LPAAT-b expressed in SF-9 membranes. Subsequently, CT-32228 was tested in standard 72 h *in vitro* proliferation and cytotoxicity assays using a panel of 12 hematologic malignancy cell lines and 16 solid tumor cell lines. In the proliferation assays, CT-32228 had an average IC_{50} of <100 nM in the leukemia and lymphoma cell lines, without a clear correlation between disease type (i.e. genetic alteration(s)) or total LPAAT enzyme levels. In intact IM-9 cells, CT-32228 inhibited the acylation of lysophophatidylmethanol, an alternate substrate for LPAAT-b, with an IC_{50} of 22 nM. The anti-proliferative IC_{50} on the solid tumor cells on average was 1.5 times higher than that for the hematologic malignancies. Again, no correlation was found between the IC_{50} and genetic alterations.



CT-32228

CT-32228 also showed a broad spectrum of activity in the NCI's *in vitro* antitumor screen consisting of 60 human tumor cell lines and is currently being tested in the hollow fiber assay. The anti-proliferative effect of CT-32228 on tumor cells was irreversible after 72 h exposure and was due to induction of apoptosis, which was already detectable after 8 h. Cell cycle analysis showed that CT-32228 caused an arrest in G2/M. In contrast to the effect on tumor cells, CT-32228 at the same concentrations had no or very little effect on normal human bone marrow progenitors or murine T-lymphocytes. In the human HT-29 (colon) and DU-145 (prostate) xenograft tumor models in nude mice, repeated IV treatment (BID × 10) with CT-32228 showed antitumor efficacy in a dose dependent manner and was well tolerated. These data suggest that inhibition of LPAAT-b activity may represent a novel and selective target for cancer therapy

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Diverse and unique cellular effects of SK-7068, a novel histone deacetylase inhibitor

J.-H. Park[1], Y.J. Jung[1], J.W. Lee[1], H.-S. Jong[1], T.-Y. Kim[1,2], Y.-J. Bang[1,2]. *[1]Cancer Research Institute, Seoul National University College of Medicine, Seoul, Korea; [2]Department of Internal Medicine, Seoul National University College of Medicine, Seoul, Korea*

Epigenetic gene silencing is a common molecular alteration in cancer cells and recruitment of HDAC-containing complexes to promoter regions leads to the transcriptional silencing of various target genes such as tumor suppressor genes. Accordingly, inhibition of HDAC enzymatic activity is expected to induce re-expression of aberrantly repressed genes and might

be a promising cancer therapy. Recently, we have synthesized new HDAC inhibitors and among more than 100 lead compounds, SK-7068 was found to be the most effective in various cancer cells. The growth inhibition effect by SK-7068 was 4 times potent than that by MS-275, a benzamide-based HDAC inhibitor. As expected for a HDAC inhibitor, SK-7068 inhibited cellular HDAC enzymatic activity by 90% and induced the acetylation of H3 and H4. Although more than 9 isotypes of HDAC have been identified so far, it is unclear whether HDAC inhibitors specifically target specific isotypes of HDACs. To address this, we have tested whether the level of HDAC expression is correlated with sensitivity to SK-7068. SK-sensitive cells showed relatively higher level of HDAC1, suggesting that SK-7068 might selectively target HDAC1 isotype. SK-7068 treatment induced cell cycle arrest at G1-phase and also activated mitochondrial-caspase dependent apoptosis. Interestingly, SK-7068 treated cells were arrested at M-phase as well, which was demonstrated by increased mitotic index and dense chromatin formation. This is the first observation that a HDAC inhibitor might directly affect the mitotic check point regulation. Taken together, a novel HDAC inhibitor SK-7068 shows diverse and unique cellular effects in contrast with other HDAC inhibitors. Finally, cDNA microarray analysis revealed that transcriptional silencing of some novel genes was reversed after treatment with SK-7068. The experiments to understand the biologic significance of these target genes are now underway and final result will be presented in the meeting.

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Linking genotype to phenotype: production and large scale functional analysis of gene knockouts in human cancer cells

A. Lofquist, C. Hagios, R. Finney E.. *Pangenex Inc, Seattle, USA*

To fully realize the impact of genomics technologies such as expression profiling, proteomics, metabolic pathway modeling, or SNP analysis, sequence information from the human genome database needs to be directly tied to drug discovery. Since gene inactivation (knockout) is predictive for effects of drug candidates, genetic methods to inactivate human genes are key to linking genotype with phenotype and for discovering the therapeutic utility of novel and known genes. Here we describe a gene knockout technology designed to directly link genotype with phenotype on a large scale. We use a plasmid-based vector to gene-trap and recover genes expressed in human cancer cells. From these, a library of over 7,500 knockout vectors has been generated. Homologous recombination with these vectors is exceptionally high (up to 10-1) allowing for creation of arrays of cells with gene knockouts. The array of cells (either as single copy or multiple copy knockouts) can be subjected to multiple assays to determine which genes are essential for genesis or maintenance of various attributes of cancer cells or to determine which genes, when inactivated, sensitize cells to various chemotherapeutic agents and radiation. The gene-trap vectors contained secretory alkaline phosphatase (SEAP) that allowed for monitoring of endogenous trapped promoter activities. We created a clonal SEAP-reporter library in the pancreatic tumor cell line MiaPaCa-2, and screened for genes responsive to the activation of the erb-B-receptor family; erb-B1, erb-B2 and erb-B3 but not erb-B4 were found to be expressed in MiaPaCa-2 cells. Stimulation of the MiaPaCa-2 library with EGF, TGF-alpha, Amphiregulin, Betacellulin or Heregulin-alpha resulted in increased SEAP activity in 3% of the clones. 75% of these clones responded only to TGF-alpha, 10% were responsive to EGF, Amphiregulin and TGF-alpha, and 15% responded to both EGF and TGF-alpha. Further screening of the MiaPaCa-2 library for increased sensitivity to gemcitabine, 5-FU, cisplatin, camptothecin, and DNA damaging radiation yielded a number of clones representing potential drug targets. The described technologies allow us to establish a direct link between genes represented in the human genome database and drug target discovery. Furthermore, the ability to create the genetic lesion in alternative cancer cells using highly efficient homologous recombination vectors allows us to evaluate these genes in diverse disease backgrounds.

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Differential kinetic properties of monomeric and oligomeric phosphorylated c-met

J.L. Hays[1,2,3], S.J. Watowich[1,2,3]. [1]*Universtiy of Texas Medical Branch, Human Biological Chemistry and Genetics, Galveston, USA;* [2]*Sealy Center For Structural Biology,* [3]*W.M. Keck Center for Computational and Structural Biology, USA*

One mechanism for regulating receptor tyrosine kinases (RTKs) is for the active and inactive states of the enzyme to have different kinetic properties. An important aspect of this regulatory mechanism is how oligomerization-induced conformational changes modulate the kinetic properties of RTKs. Recombinant TPR-MET protein, a functionally active translocation oncoprotein derivative of c-MET, has been expressed and purified for enzymatic analysis. This naturally occurring oncoprotein contains the cytoplasmic domain of the cMET receptor fused to a coiled coil motif from the nuclear pore complex (TPR). CytoMET, a monomeric analog of TPR-MET, has also been expressed and purified for differential enzymatic analysis. ATP and peptide substrates corresponding to *in vivo* phosphorylation sites within the cytoplasmic domain of the receptor have been kinetically characterized for fully phosphorylated TPR-MET and fully phosphorylated CytoMET. Our kinetic data shows that the catalytic activity of TPR-MET is modulated by oligomerization, independent of activation loop phosphorylation, and thus suggests that oligomerization-induced conformational changes occur within the cytoplasmic domain of RTKs. These results have significant implications for structure-based design of RTK inhibitors and the development of a detailed mechanistic model of RTK activation.

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Correlation between NF-kB activity and IkB degradation in tumor cells using bioluminescent reporters

L. Sambucetti, P. Kwon, C. Yu, S. Naravula. *Xenogen Corporation, Drug Discovery, Alameda, CA, USA*

Recent evidence indicates that NF-kB and the signaling pathways that are involved in its activation play an important role in tumor development. Constitutively activated NF-kB transcription factors have been associated with tumorigenesis, cancer cell proliferation, preventing apoptosis and increasing the angiogenic and metastatic potential of a tumor. NF-kB is regulated in part by its association with its inhibitor, IkB. IkB kinase phosphorylates IkB in response to agents that activate NF-kB. Phosphorylated IkB is ubiquitinated and rapidly degraded, releasing NF-kB and allowing it to translocate to the nucleus and activate transcription of its target genes. In this study we used two bioluminescent reporters to track NF-kB activation in tumor cells in response to agents that activate NF-kB. The first construct consisted of five NF-kB response elements upstream of a basal promoter controlling transcription of the luciferase gene (NF-kB-RE-luc). A second reporter was designed to link luciferase activity to IkB degradation. This construct consisted of an IkB-luciferase fusion protein driven from the EF-1alpha promoter (EF-1alpha-IkB-luc). The luciferase reporters were introduced separately and stably into human prostate carcinoma cells, PC-3M. Bioluminescence was measured in intact cells using an IVIS™ Imaging System (Xenogen Corporation). TNF-alpha and PMA increased the luciferase expression from NF-kB-RE-luc and decreased EF-1alpha-IkB-luc activity, while no effect was noted on the activity of luciferase produced from an SV-40 promoter. In the EF-1alpha-IkB-luc cell line, reduced luciferase activity correlated with a decrease in the level of IkB-luciferase fusion protein, as determined by immunoblot blot with IkB and luciferase antibodies. These cell-based assays together may be used to monitor NF-kB activation and can specifically distinguish agents that alter the stability of IkB.

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Identification of tumor associated protease substrates using combinatorial chemistry

S. Janssen, S. Denmeade. *The Johns Hopkins University, Oncology, Baltimore, USA*

Prostate-specific antigen (PSA) and human kallikrein 2 (hK2) are closely related products of the human kallikrein genes KLK3 and KLK2, respectively. Both PSA and hK2 are produced and secreted by normal and malignant prostate cells. The only putative physiological substrate for hK2 include the gel-forming seminal proteins semenogelin I and II and the N-terminal propeptide activation sequence in pro PSA. Our lab is developing prodrugs and protoxins that are specifically activated by prostate tissue proteases. Although hK2 concentrations in the extracellular fluid are ~ 100-1000 times lower than PSA, hK2 possesses ~ 1000-10,0000 fold greater enzymatic activity. Therefore, hK2 represents an attractive target for targeted prodrug therapy. To identify hK2 substrates, we synthesized a fluorescently-quenched combinatorial peptide library containing 1.6 million different peptides (one bead-one peptide). Each bead contained a unique peptide consisting of 6 randomized amino acids, flanked by the fluorophore 2-amino benzoic acid and the fluorescent quencher 3-nitrotyrosine. Incubation of this library with hK2 identified approximately 40 positive peptide sequences (beads). The determination of the peptide sequence of these digested beads is currently under investigation.