106 POSTER

A highly efficient approach to the anticancer drug discovery of potent small molecule kinase inhibitors

J. Mendoza, J. Dickson, I. Popa-Burke, R. Mohney, J. Murray, C. Toole, L. Cheatham, P. Bernasconi, K. Williams, N. Hodge. *Amphora Discovery Corporation, Research, Research Triangle Park, NC, USA*

With the increasing number of novel proteins being identified from genomics, proteomics, and traditional biochemical approaches there is a need to develop more efficient methods for the discovery and optimization of small molecule ligands that can be used both to elucidate the function of these proteins and as starting points for drug design.
We have developed a highly efficient approach to the anticancer drug discovery of small molecule kinase inhibitors which show high selectivity for individual targets, sets of targets (dual inhibitors, etc), or target families. We have screened over 20 protein kinases of current therapeutic interest in the cancer area (e.g. AKT1, FLT3, ABL1, PDGF-α, HCK, SRC, GSK3-β, etc) against a diverse, purified and quantitated chemical library of over 120,000 drug-like molecules using high-throughput microfluidic-based enzyme technology to identify small molecule kinase inhibitors. The high precision and reproducibility of the data allowed us to evaluate structure-activity relationships for potency and selectivity directly from the primary screen. All compounds were screened against a collection of more than 60 enzymes (kinases, proteases and phosphatases), allowing for the rapid removal of promiscuous compounds very early in the discovery process. Enzymology studies on the most promising hits, including measurement of concentration of compound in buffer, gave us accurate determinations of K_i and IC_{50} values, as well as mechanisms of action.
This broad, accurate screening approach has led to the rapid identification of multiple series of drug leads for several cancer kinase targets with unique, patentable structures possessing nanomolar enzyme potency. Included are series with a variety of selectivity profiles, as well as those that show good to excellent selectivity (>100-fold) against all other targets tested from the kinase, phosphatase and protease enzyme families.
To demonstrate this method, we will present our early discovery efforts around AKT1 and FLT3, two kinase targets of current cancer therapeutic interest.

107 POSTER

Screening the DTP Diversity Set for compounds that inhibit the growth of human melanoma cells with defined mutations

A.J. Sakko, V.J.N. Bykov, K.G. Wiman, J. Hansson. *Karolinska Institute, Oncology-Pathology, Stockholm, Sweden*

Background: The majority of human melanomas harbour activating mutations in either the *NRAS* or *BRAF* gene. To date, no reported therapies have been directed at suppressing specifically oncogenic *NRAS* or *BRAF* in human melanomas. The aim of this study, therefore, was to screen compounds held by the DTP (Developmental Therapeutics Program, NCI/NIH) in an attempt to identify new compounds that inhibit the growth of melanoma cell lines with defined mutations.
Materials and Methods: Using a panel of nine human melanoma cell lines with defined mutations in either codon 61 of *NRAS* or codon 600 of *BRAF*, the DTP Diversity Set (a library consisting of 1 990 representative compounds selected from a larger main library) was screened to identify compounds that specifically inhibit the growth of melanoma cell lines with either *NRAS* or *BRAF* mutations. TUNEL analysis was employed to determine if any growth suppression identified was due to induction of apoptosis, and the expression of proteins in the RAS/RAF signal transduction pathways potentially affected by candidate compounds were investigated by immunoblot analysis.
Results: A number of compounds were identified that specifically inhibited the growth, and induced apoptosis, of melanoma cell lines that harbour either codon 61 *NRAS* or codon 600 *BRAF* mutations. Immunoblot analysis revealed that treatment of melanoma cells with these compounds resulted in decreased NRAS or BRAF expression and decreased phosphorylation of ERK.
Conclusions: This data suggests that the DTP Diversity Set may comprise lead compounds with anticipated action in a specific subset of melanoma patients.

108 POSTER

Profiling in vitro and in vivo of MS275, SAHA, LAQ824 and VPA as HDAC inhibitors currently tested in clinical trials

T. Beckers[1], C. Burkhardt[1], H. Wieland[1], J. Braunger[2], P. Gimmnich[2], T. Maier[3]. [1]*Altana Pharma AG, RDR-B4/Therapeutic Area Oncology, Konstanz, Germany;* [2]*Altana Pharma AG, RDR-P3/Therapeutic Area Oncology, Konstanz, Germany;* [3]*Altana Pharma AG, RDR-C4/Therapeutic Area Oncology, Konstanz, Germany*

Class I and II histone deacetylases (HDAC) comprise a family of 11 isoenzymes potently inhibited by trichostatin A, a Zn^{2+} complexing hydroxamate. There is now clear evidence from preclinical and clinical studies, that inhibition of histone (protein) deacetylation by targeting HDACs is a new effective approach for cancer therapy. Currently compounds from four different chemical classes, namely benzamides, hydroxamates, butyrates and a cyclic peptolid are in clinical phase I and II trials: MS275 (Schering/Mitsui), SAHA (Aton Pharma/Merck), PXD101 (Prolifix/Topotarget), LAQ824 (Novartis), Phenylbutyrate (Elan Pharmaceuticals/NCI), Pivaloyloxymethylbutyrate (Titan Pharmaceuticals), Valproic acid (VPA/G2M Cancer Drugs) and Depsipeptide/FK228 (Fujisawa/NCI). We selected MS275, SAHA, LAQ824 and VPA for in depth profiling studies, namely (i) HDAC inhibition in a biochemical assay, (ii) cytotoxicity/apoptosis induction, (iii) histone hyperacetylation in cells, (iv) spi1 –LUC reporter gene activation, (v) cell cycle distribution, (vi) differentiation induction and finally (vii) anti-tumor efficacy in-vivo. Trichostatin A, Apicidin and HC-Toxin were included as tool substances.
In the biochemical assay using HDAC activity isolated from HeLa cell nuclear extract, the hydroxamate analogs SAHA and LAQ824 were highly active with IC_{50} = 57.6 nM and 3.2 nM, respectively. The benzamide MS275 and VPA depicted much weaker activity with IC_{50} = 10,8 μM and 225 μM, respectively. All compounds induced histone H3 K^{23} hyperacetylation in HeLa cervical carcinoma cells in a Cellomics ArrayScan HCS assay at relevant concentrations, proving the target inhibition in cells. In contrast to LAQ824 or SAHA, induction of a sp1 (p21) – LUC reporter gene in HeLa cells by MS275 was weak. In the Alamar Blue proliferation as well as cell death detection ELISA assays, all HDAC inhibitors were active with LAQ824 as superior agent (inhibition of A549 NSCLC proliferation IC_{50}=53.4nM, apoptosis induction ≈ 250 cisplatin units at 1 μM). The HDAC inhibitors were combined with standard chemotherapeutic agents, showing additive or synergistic effects in the MDA-MB468 breast carcinoma model. Cell cycle analysis was done in A549 NSCLC, HeLa cervical carcinoma and normal human bronchial epithelial cells. After 24h treatment with 10 μM SAHA or MS275, a depletion of cancer cells in S-phase became apparent with apoptotic cells in subG1 after 48h. Finally, the activity of LAQ824, MS275 and SAHA was evaluated in-vivo using A549 NSCLC xenografts. At well tolerated doses of 120mg/kg (SAHA, po/qdx21), 25mg/kg (MS275, po/qdx21) and 50mg/kg (LAQ824, iv/qdx18), best T/C values were between 0.23 and 0.6.
In summary, the HDAC inhibitors as studied are potent, cell cycle independent inducers of apoptosis with good anti-tumor activity in xenograft studies. From the in-vitro data we conclude, that benzamide and hydroxamate based inhibitors behave differently in certain assays. This might be explained by a distinct target inhibition profile.

109 POSTER

Identification of small molecule inhibitors of PKB/AKT in a high throughput screen

W. Aherne[1], S. Burns[1], J. Travers[1], Y. Newbatt[1], M. Rowlands[1], D. Barford[1], I. Collins[1], N. Thompson[2], M. Garrett[1], P. Workman[1]. [1]*Institute of Cancer Research, Centre for Cancer Therapeutics, Surrey, UK;* [2]*Astex Technology, Cambridge*

The serine/threonine protein kinase PKB/AKT is an important cancer therapeutic target acting downstream of PI3kinase. The cellular substrates of PKB/AKT include the pro-apoptotic proteins BAD and caspase 9, the Forkhead family of transcription factors and GSK-3. In spite of intense interest in PKB/AKT as a therapeutic target, small molecule inhibitors have not yet progressed to clinical trial.
We have run a high throughput screen to identify PKB/AKT inhibitors using an AlphaScreen format. Two antibody-substrate pairs were evaluated and superior S/N and overall signal stability were obtained using Crosstide (KGSGSGRPRTSSFAEG) and its corresponding antibody (CST9331) rather than AKTide (ARKRERAYSFGHHA) and a corresponding affinity purified antibody prepared in house. Recombinant human PKB enzyme (Piftide) (0.2nM), ATP (30M), biotinylated peptide substrate (20nM) and compound (30M) or DMSO control (0.2%) were incubated in the presence of antibody (0.15nM) for 70 mins and the reaction stopped by the addition of a mixture of streptavidin-coated donor and protein A acceptor beads in

an EDTA buffer. After overnight incubation, the Alphascreen counts were measured on a Fusion reader. Approximately 65,000 compounds were screened in 384-well format in 17 batches. The kinase inhibitor H8 gave an IC50 of ~30M and was used as a positive control on each plate (43.3 13.1% inhibition at 30M). Across the whole screen Z' factors were 0.830.07. Hits were identified as compounds inhibiting activity by >3SD of the compound wells on each plate. The activity of hits (707) was confirmed on Alphascreen (50) followed by a filter-binding assay. Overall confirmed hit rate was ~0.1%. AlphaScreen false positives included compounds with biotin-like moieties, a surfactant-like compound and two highly coloured azo-dyes, consistent with expected mechanisms of interference with t his assay format. Several hit series were identified including a number of toxoflavins (0.78–15M) and a rhodanine analogue (IC50 26M) that have similarity to previously disclosed PKB/AKT inhibitors and thus confirm the ability of the screen to identify valid hits.

110 POSTER

In vitro screening of anticancer drug penetration into tumour tissue

A.H. Kyle, L.A. Huxham, A.I. Minchinton. *BC Cancer Research Centre, Medical Biophysics, Vancouver, Canada*

The failure of many anticancer drugs to control the growth of solid cancers may stem in part from inadequate delivery to tumour regions distant from vasculature. Unfortunately, extricating the effect of tissue penetration from the many other factors that affect a drug's efficacy in tumours with distance from vasculature is generally not possible. In this study we use a novel, effect-based assay that employs multilayered cell culture (MCC), a tissue-engineered disc grown from tumour cells on a permeable membrane. MCCs are similar to multicellular spheroids but their planar nature permits flux through the cultures to be more easily measured. Like spheroids they exhibit a gradient in proliferation but since MCCs grow as discs, this gradient forms as a mirror image from either side towards the middle. In this study, we exploit this symmetry by exposing MCCs to drugs from one side and then comparing their effect on the exposed side versus the far side of the cultures. This approach circumvents issues that normally arise from the biochemical gradients that occur with distance into tissue (e.g. changing intrinsic sensitivity of cells to drugs with depth into tissue). In this study we have examined representative drugs from a selection of anticancer drug classes, including platinum based agents (cisplatin), microtubule agents (vincristine, vinblastine, vinorelbine and paclitaxel) and topoisomerase II inhibitors (etoposide). The distribution of drug activity within the cultures was assessed 1 to 3 days after exposure via immunodetection of S-phase cells using bromodeoxyuridine. Using an automated computer analysis routine, the effect of the drugs in the first three cell layers located on either edge of the cultures relative to controls was then assessed. Results for penetration of the agents through the MCCs were grouped into three classes: uniform tissue distribution (cisplatin), 1–3 fold decrease in drug exposure to cells on the far side versus the exposed side of the cultures (vincristine, vinblastine and paclitaxel) and greater than 3-fold difference (vinorelbine and etoposide). This model could be applied as a screening system for the discovery of biologically active drugs, which exhibit desirable penetration properties. This research is supported by the National Cancer Institute of Canada with funds from the Canadian Cancer Society, the Michael Smith Foundation For Health Research and the Canadian Institutes for Health Research.

111 POSTER

Development and validation of a mass spectrometric assay to evaluate drug–drug interaction in human liver microsomes. Application to anticancer agents

F.I. Raynaud, J. Moreno-Farre, P. Workman. *The Institute of Cancer Research, Cancer Research UK Centre for Cancer Therapeutics, Sutton, UK*

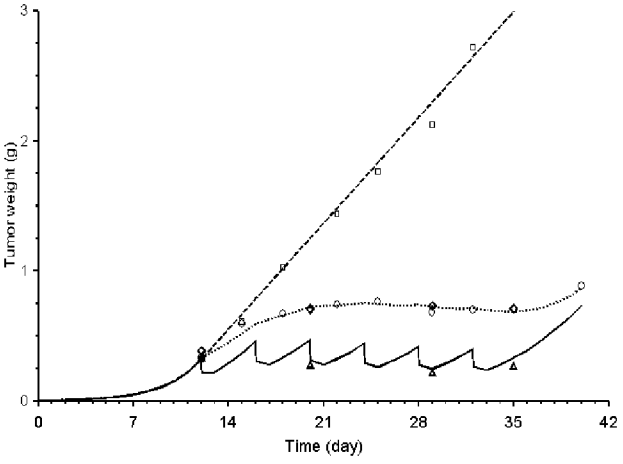
Most anticancer agents are administered in combination with other anticancer drugs or alternative therapies. New drugs that affect molecular targets are likely to be prescribed chronically i.e orally thereby increasing the risk of interactions with other drugs. This can potentially lead to toxicity and it is therefore important to predict possible drug-drug interactions with novel compounds. We present an in vitro assay in human liver microsomes, which screens for potential inhibition of CYP1A2, 2A6, 2C9, 2C19, 2D6, 2E1 and 3A4 in a single microsomal incubation. A mixture of probe substrates (phenacetin at 10 μM, coumarin at 5 μM, tolbutamide at 60 μM, amitriptyline at 5 μM, cloroxazone at 50 μM, bufarolol at 5 μM and midazolam at 3 μM) are incubated in human microsomes (0.4 mg of protein in 200 μL) for 30 min followed by protein precipitation with methanol. The metabolites are measured by LCMSMS. 6-hydroxychloroxazone is detected by selected reaction monitoring in negative ionisation mode following separation on a Synergi hydro-RP (50mmx4.6mm, 4 μm) column with a gradient of 10mM ammonium acetate and methanol whilst all other metabolites are monitored in positive mode following elution from the same column with 0.1% formic acid and methanol. The total run time is 11 minutes. Validation of the assay, performed by measurement of the K_m for each substrate showed values similar to those reported in the literature. Studies with known inhibitors 7-ethoxyresorufin (10 μM), 8-methoxypsoralen (10 μM), omeprazole (50 μM), sulfaphenazole (5 μM), quinidine (2 μM), diethyl-dithiocarbamate (50 μM), testosterone (50 μM), ketoconazole (1 μM), confirmed the ability of this method to identify specific inhibitors of all the enzymes probed for. Currently used drugs such as doxorubicin, daunorubicin, carboplatin, 5-fluorouracil, mitomycin C, etoposide, tamoxifen, vincristine, vinblastin have also been evaluated in this drug-drug interaction screen. Novel agents such as CYC202, SR4554, 17-AAG have also been tested. This validated technique is currently routinely used in our drug development programs.
This work was supported by Cancer Research UK.

112 POSTER

Pharmacokinetic-pharmacodynamic (PK-PD) modeling of tumor growth inhibition in mice: a magnetic resonance imaging study

M. Rocchetti[1], A. De Grassi[1], M. Simeoni[2], E. Pesenti[1], M. Russo[1], I. Poggesi[1]. [1]*Nerviano Medical Science, Pharmacokinetics, Dynamics and Metabolism, Nerviano, Italy;* [2]*University of Pavia, Dipartimento di Informatica e Sistemistica, Pavia, Italy*

A PK-PD model, based on a system of differential equations, has been developed linking the dosing regimen to the tumor growth in xenograft models (Simeoni et al, Cancer Res. 64:1094; 2004). In this model it is assumed that tumor cells hit by an anticancer drug stop proliferating and go to death through a transit compartmental system, which represents the hypothetical progressive degrees of damage of tumor cells. The model is therefore able to predict the mass of proliferating and damaged cells fitting the total tumor mass vs. time curves. This communication describes the results of a study in which total and viable tumor mass was monitored using magnetic resonance imaging (MRI).



Ø: controls measured by caliper, dashed line is model-fitted controls; ○: total tumor mass measured by caliper, ♦: total tumor mass measured by MRI, dotted line is model-fitted total tumor mass; Δ: viable tumor mass measured by MRI, solid line is model-predicted mass of proliferating cells.

HCT 116 tumor bearing nude mice were given placebo or irinotecan (50 mg/kg, q4dx6). MRI assessment was performed using T2-weighted images on a Bruker Pharmascan equipment. Total tumor mass and viable tumor mass were evaluated using the multislice procedure. Total tumor mass were also evaluated using timely standard caliper measurements. Plasma pharmacokinetics of irinotecan were obtained from a previous assessment. Simultaneous fitting of the average total tumor mass in control and treated animals was performed using non-linear regression (Winnonlin 3.1, Pharsight).
Model fittings were good and provided reliable parameter estimates. Pharmacodynamic parameters were consistent with those previously published for irinotecan. The MRI-viable tumor cells were in agreement with the mass of cycling cells (i.e., not damaged) predicted by the model. This reinforces the biological relevance of this PK-PD model.