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

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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.

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

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**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.

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

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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.

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

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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