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128 POSTER

Unraveling therapeutic bio-signatures through pathway mapping at the single cell level using an analysis platform for simplified interrogation of complex data sets

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Solid tumors comprise genetically heterogeneous cell populations whose growth and survival depends on the complex interplay of distinct, yet overlapping, signaling networks. A major challenge in developing a course of therapy is determining which signaling nodes to target for a specific malignancy. Profiles from siRNA gene silencing are integral to mapping disease-specific signaling cascade(s) and provide insight to key targets for therapeutic intervention. Successful siRNA screening relies not solely upon optimizing transfection, but also cell analysis systems capable of high content screening (HCS) at the single cell level, within overall populations (sample well), and across multiple data sets. The Guava EasyCyte™ Plus flow cytometer, with integrated Guava® Simplicity software, provides a revolutionary new platform for cell-based analysis. The software's intuitive architecture and ease of use facilitates the comparison of multiple experimental conditions or disease states through heat-map visualization. To demonstrate, a mini-drug screening was performed. Following exposure to a panel of 80 cytoactive compounds, cells were assayed for multiple parameters of apoptotic induction as well as monitoring alterations in mitotic state. Parallel to screening, siRNA silencing was performed to identify genes that impact Camptothecin (CPT)-induced apoptosis. Knockdown efficiency of each gene target was examined via intracellular staining and optimized for each cell line prior to functional screening. "Hit" compounds were further tested in combination with siRNA knockdown. For this study, changes in the phosphorylation state of multiple proteins were also measured. Briefly, apoptotic assays following gene silencing identified enhancers (PTEN) and inhibitors (GSK3a) of CAM-induced cell death as well as modulators of cell cycle progression (MDM2, CHK1). Moreover, it was evident that certain genes are functionally linked and thus part of the same or overlapping networks. "Hit" screening identified cytochalasin as a potential therapeutic with similar biological profiles as CPT. However, phospho-mapping suggests their specific mechanisms of action are quite different. In summary, this experimental methodology, when used in concert with Guava Technologies' cell analysis platform and Simplicity software, significantly expedites the drug discovery process by providing a means for extraction of key biological findings from complex experimental results.

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Design, synthesis and evaluation of bivalent conformationally constrained Smac mimetics as a new class of anticancer agents

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Second mitochondria-derived activator of caspase (Smac) is a protein released from mitochondria in response to apoptotic stimuli. Smac promotes apoptosis, at least in part, by effectively antagonizing several members of inhibitor of apoptotic proteins (IAPs), including XIAP, cIAP-1 and cIAP-2, by targeting one or more BIR domains in IAPs. We designed and synthesized a series of non-peptidic, cell-permeable, bivalent small-molecules which mimic the dimeric Smac protein for targeting IAPs (bivalent Smac mimetics). We performed extensive evaluations of these bivalent Smac mimetics for their interaction with IAP proteins and their activity and mechanism in cancer cells. Our studies show that these Smac mimetics bind to XIAP, cIAP-1 and cIAP-2 with low nano-molar affinities. They function as extremely potent XIAP antagonists by concurrently targeting both the BIR2 and BIR3 domains. Consistent with their potent binding affinities to cIAP-1, these bivalent Smac mimetics potently and effectively induce rapid degradation of cIAP-1 protein in cancer cells. Our data showed that the lengths of the linker in these bivalent Smac mimetics have a significant effect on their ability in induction of cIAP-1 degradation. These bivalent Smac mimetics potently inhibit cell growth with IC_{50} values between low nanomolar and sub-micromolar and effectively induce apoptosis in a subset of cancer cell lines. Their potencies in inhibition of cell growth and induction of apoptosis nicely correlate with their ability in induction of cIAP-1 degradation. The most potent bivalent Smac mimetic SM-164 is capable of inducing of robust apoptosis in cancer cells at concentrations as low as 1 nM and effectively inhibits tumor growth in the MDA-MB-231 xenograft model. Importantly, SM-164 shows a minimal toxicity to normal cells *in vitro* and to mouse tissues *in vivo*. Taken together, our data provide strong evidence that bivalent Smac mimetics may have a great therapeutic potential for the treatment of human cancer by induction of apoptosis through targeting multiple IAP proteins.

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Structure–activity relationships for a library of C2-aryl substituted monomeric pyrrolo[2,1-c][1,4]benzodiazepines (PBD) antitumour agents

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There is growing interest in pyrrolo[2,1-c][1,4]benzodiazepine (PBD) antitumour agents as one example of this class, the synthetic DNA-interactive sequence-selective PBD dimer SJG-136, is likely to move into Phase II clinical trials later this year. Unlike this synthetic agent which contains two PBD units and works by cross-linking DNA, the naturally-occurring PBDs isolated from various *Streptomyces* species are monomeric compounds that form single covalent bonds to the N2 of guanine in the DNA minor groove in a similar manner to the recently licensed marine-derived anticancer agent trabectidin. We have previously reported that insertion of an aryl group at the C2-position of the C-ring of monomeric PBDs can dramatically enhance their overall in vitro cytotoxicity and their selectivity towards particular cell lines (especially melanoma). Novel C2-aryl PBDs of this type have not been observed in nature, and the precise role of the C2-aryl substituent in enhancing their activity was not understood. We report here the use of combinatorial technologies to synthesize a library of over 110 C2-aryl substituted monomeric PBD analogues via palladium-catalyzed cross-coupling. Each library member retains the C2/C3-endo unsaturation observed in the naturally occurring compounds, and the C2-aryl substituents contain a diverse array of ring types (including mono-, bi- and tricyclic systems) and heteroatoms (O, N and S). In addition, for comparative purposes, some library members contain C2-aliphatic alkenyl substituents (as found in the most potent natural product sibiromycin) or a combination of aryl and alkenyl substituents (i.e., C2-styryl derivatives). Biological evaluation of library members has confirmed that introduction of a C2-aryl group significantly enhances overall in vitro cytotoxicity and DNA-binding affinity with a good correlation between the two. The most active library members include the C2-aryl analogue SG2897 which has remarkable DNA-binding affinity (i.e., ?Tm = 20.8ºC) and cytotoxicity (e.g., IC50 = 0.62 nM in SK-MEL-5 melanoma; 1.45 nM in K562 leukaemia) and is significantly more potent than the best natural product sibiromycin (i.e., ?Tm = 16.3ºC, IC50 = 10 nM in SK-MEL-5; 1.40 nM in K562) which lacked efficacy in clinical trials and, unlike SG2897, is problematic to synthesize. These data will be reported in full along with the results of preclinical studies on SG2897 presently underway.

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Specific induction of the p53 pathways by low dose cytotoxic drugs assessed by gene expression pattern analysis

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Background: Induction of the p53 pathway is seen as a potentially exciting new therapeutic approach in human tumor therapy. Recently small molecule activators of the pathway have been identified by both biochemical and cell-based screens. Nutlin, a MDM2 inhibitor, has shown pre-clinical efficacy. Using expression arrays as a powerful method to determine small molecule specificity, we noted potent p53 activation by several known cytotoxic drugs at very low doses. Actinomycin D (ActD, a known DNA-interacting transcription inhibitor) and Leptomycin B (LMB, an inhibitor of exportin I driving nuclear protein export) both showed p53 activation in our reporter assay. LMB is too toxic to be used clinically while ActD is one of the older chemotherapy drugs which have been used in treatment of a variety of cancers. Potentially beneficial therapeutic use could be achieved if these compounds were shown to activate p53 pathways without the corresponding toxic effect. Literature reviews have demonstrated that at

low dosages, these compounds could stimulate the expression of some p53 pathways genes. A comprehensive study showing the differential expressions of all possible p53 pathways genes has not been reported.
Materials and Methods: The drugs were added at low dosages to isogenic p53 knock-out HCT116 colon adenocarcinoma cell lines for 16 hours. Total RNA from the cells was extracted and the expression profiles of the treated cells were compared to untreated cells by hybridization to the Illumina microarrays. Flow cytometry was performed concurrently to demonstrate cell cycle arrest and apoptosis. Genotoxic effect was determined by measuring double-stranded DNA breaks using H2AX assay. Array results were analyzed using TIGR array analysis software and interactions of differentially expressed genes were mapped using Ingenuity program. Array results were confirmed by quantitative polymerase chain reactions on selected genes.
Results: By comparing the expression profiles of the p53 isogenic cell lines, microarray analysis revealed that ActD and LMB were able to activate p53-dependent pathways at dosage of not more than 10 nM. Reduction in the number of cells in S phase was also observed at these dosages. Higher dosages of these compounds led to accumulation of cells at sub G1 phase and differential expression of genes not related to p53 pathways. The activation of p53 pathways at low dosages is similar to treatment with Nutlin. No DNA fragmentation was observed at all dosages used.
Conclusions: ActD and LMB at low dosages are able to stimulate p53-dependent pathways without a general toxic effect.

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SRJ09, a lead compound in anticancer drug design: in vitro, in vivo and mechanistic studies

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Background: The search for more effective and selective anticancer drugs is currently being researched actively involving the various entities of the drug discovery programme. We have shown andrographolide (AGP), a compound isolated from a local herb, *Andrographis paniculata*, to have anticancer activity *in vitro* and *in vivo*. In order to improve the antitumour properties of AGP, semisynthetic derivatives of this compound were synthesised in our laboratory, with the aim of identifying the most promising anticancer compounds and to elucidate their mechanism(s) of action.
Materials and Methods: Cell viability assays (MTT and SRB) were used to determine the *in vitro* growth inhibitory properties of compounds. Nude mice were utilised for the *in vivo* antitumour study. Flow cytometry was performed to assess cell cycle arrest and apoptosis. Western blotting was used to determine the cellular protein levels.
Results: SRJ09 (3,19-(2-bromobenzylidene)andrographolide) displayed better antitumour activity when compared with AGP and other derivatives (SRJ11 and SRJ23). In the NCI *in vitro* anticancer screen the compound showed selectivity towards melanoma, colon, renal and breast cancers. The antitumour activity of AGP, SRJ09, SRJ11 and SRJ23 was shown to be not compromised by P-glycoprotein activities in MES-SADx5 multidrug resistant cell line. The *in vivo* antitumour study showed SRJ09 delayed quadruple tumour growth by 4 days in HCT-116 colon cancer xenografted mice treated with 400 mg/kg dose (q4dx3) when compared with control. SRJ09 induced a G_1 arrest in MCF-7 breast and HCT-116 colon cancer cells and the effect was attributed to decreased CDK-4 and increased of p21 expressions without affecting the expression of cyclin D1. Apoptosis was the main mode of cell death induced by SRJ09 and was p53 and bcl-2 independent.
Conclusions: In conclusion, SRJ09 emerged as the lead anticancer agent given its ability to induce G_1 specific cell cycle arrest and apoptosis and to have *in vivo* antitumour activity. Additionally, NCI's *in silico* SOM analysis indicated this compound might have a novel molecular target. Therefore, further studies in improving the anticancer properties of SRJ09 by chemical modification will be advantageous.

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Discovery of potent and selective focal adhesion kinase inhibitors

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Background: Focal Adhesion Kinase (FAK) is a non-receptor tyrosine kinase that regulates multiple cell functions and it is known as a key driver of tumor cell proliferation, migration, and survival. NVP-TAE226, known as a dual inhibitor of FAK and IGF-IR with 2-phenylamino-pyrimidin-4-ylamino-benzamide scaffold, demonstrated the inhibition of growth of 4T1 murine breast tumor cells and metastasis to the lung in an orthotopic model in a dose-dependent manner. However, a potential effect on the glucose metabolism through Insulin receptor (Ins-R) kinase inhibition was suspected because of the modest selectivity of NVP-TAE226 over Ins-R kinase (approx. 8-fold). Under a particular condition in C57BL6 mice, which exhibit high sensitivity to glucose metabolism interference, an increase of insulin and glucose levels was observed at a dose of 100 mg/kg p.o..
Material and Methods: To discover FAK inhibitors with higher selectivity over Ins-R kinase than NVP-TAE226, the scaffold was modified on the basis of the structural information of FAK and Ins-R kinase. With this approach, 2-phenylamino-pyrimidin-4-ylamino-2,3-dihydro-isoindol-1-one and 2-phenylamino-pyrimidin-4-ylamino-3,4-dihydro-2*H*-isoquinolin-1-one were found to be potential scaffolds to show high selectivity not only for Ins-R kinase but also other tyrosine kinases.
Results: Among the synthesized compounds, compound **1** showed higher selectivity over Ins-R kinase than NVP-TAE226 (more than 150-fold). As a result of further optimization studies of these series, compound **2** and **3**, which exhibited more than 780-fold selectivity over Ins-R, did not show any effect on the insulin and glucose levels in the sensitive model using C57BL6 mice. Furthermore, theyshowed equivalent or more potent anti-tumor activities compared with NVP-TAE226 in the *in vivo* studies.

	IC_{50} [μmol/L]				
	FAK	CDK1	IGF-1R	Ins-R	c-Src
NVP-TAE226	0.0053	0.56	0.12	0.044	2.3
1	0.0011	2.9	0.5	0.19	7.4
2	0.0042	>10	>10	>3.3	>10
3	0.0012	>10	1.9	1.8	2.3

Conclusions: These novel classes of selective and small molecule FAK inhibitors have potential clinical applications with potent *in vivo* anti-tumor activities and high tolerability.

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The rational design of inhibitors of the telomere-hnRNP A1 interaction

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The heterogeneous nuclear ribonucleoparticle (hnRNP) A1 and A2 proteins are multi-functional proteins that associate with telomeres, stimulate telomerase activity, participate in mRNA transport, and are involved in pre-mRNA splicing. They have sequence-specific RNA and single-stranded DNA binding activity, via tandem RNA recognition motifs (RRM). A1 and A2 are required for the viability of transformed human cells, but are dispensable for the growth of normal cells. We undertook a rational design approach to develop small molecules capable of inhibiting binding of A1 and A2 to telomeric single stranded DNA. Based on published x-ray structures, we chose to target the core of the RRM binding pocket that interacts with the nucleotides TAG within the TTAGGG telomeric repeat. Using Biacore (TM) analysis we determined that the TAG oligo retained good affinity for A1, and using x-ray crystallography confirmed that its binding to A1 was analogous to the full telomeric repeat. Using the TAG trinucleotide as a