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Mechanism-based high-throughput screening (HTS)for the discovery of novel anticancer drugs

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Mechanism-based drug discovery complemented by structural and molecular modelling has become the paradigm for finding new anticancer drugs. HTS in which compound collections are screened for activity against a validated molecular drug target plays a pivotal role in the process. A HTS laboratory has been established in our Centre and the progress made will be reviewed. Our compound collection stands at >65000 compounds and acquisition continues as resources and on-going synthesis allow. Targets are brought to us by our own target identification and validation teams, other groups in the Institute and through collaboration with Cancer Research UK and other external organisations and are assessed against a number of validation criteria. Once a target has been accepted a 3-6 month period is required for assay development and optimisation. It is our intention to screen up to 6 cancer-related targets per year. Depending on the type of assay, throughputs of 8,000 compounds per day have been achieved. Hits from primary screening are then cherrypicked, activity confirmed and potency determined. Selectivity of the compounds is also assessed using appropriate counterscreens. HTS assays are characterised by miniaturisation and automation. If possible assays are run in 384-well plates and steps requiring manual handling avoided. The types of assays already utilised include scintillation proximity assays using FlashPlates® (kinases, histone acetyltransferases, phospholipase Cgamma), colorimetric assays (e.g. the ATPase activity of Hsp90), ELISA (histone deacetylases and kinases) and time-resolved fluorescence energy transfer assays (kinases). Cell-based assays have also been established including viability assays using the luminescent measurement of ATP, gene reporter assays, and cell-based ELISAs that report changes in the expression of specific proteins (Hsp70 as a marker of Hsp90 inhibition), or post-translational modifications (phosphorylation and acetylation) events. Many of these screens are on-going but hit rates have varied from 0.2-2.0% and some projects have rapidly progressed to hit to lead evaluation. These include 2 chemical series of histone acetyltransferase inhibitors and 1 series of novel compounds that inhibit the ATPase activity of Hsp90. Several series have been identified as inhibitors of PLC gamma. Tractable hits have also been identified in the cell-based screens. We thank our many collaborators who have been closely associated with this work.

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A high throughput screening for p21Waf1/Cip1 protein expression using Fluorometric Microvolume Assay Technology (FMAT)

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p21Waf1/Cip1 is a major regulator of Cyclin-CDK activities and cell cycle. Expression of p21 protein is regulated through p53-responsive elements and at post-transcriptional level. In cancer cells, p53 is frequently mutated leading to reduced induction of p21 expression and thus resistance to treatment by DNA-damaging agents. In order to discover compounds capable of restoring p21 protein level, we have developed a high throughput screening using Applied Biosystems Fluorometric Microvolume Assay Technology (FMAT) macro-confocal system. MCF-7 cells were seeded in 96 multi-well plates, treated with compounds, fixed and incubated with p21 monoclonal antibody and goat anti-mouse-Cy5. Each well is scanned across an area of one square millimetre within a depth of 100 mm using a 633 nm red laser beam focused on the bottom of the wells. FMAT provides a set of raw images. Fluorescence concentrated in a cell will be detected as specific signal and separated from background signal. Thus, the mean fluorescence of a population of cells can be calculated independently of the number of cells detected. This is of particular interest for anti-cancer drug screenings for which the number of cells may impact on the read out. Usually, 50 to 200 cells can be detected per well giving reasonable statistical significance. We have treated MCF7 cells with reference compounds known to increase p21 protein expression such as the HDAC inhibitor Trichostatin A and several DNA damaging agents. As expected, p21 protein expression measured by the FMAT is increased. We also compared the results obtained using either western blot detection of p21 protein in cell lysates or a commercial ELISA. The consistency of the results emphasizes the value of the FMAT assay for robust high throughput screening. New compounds have been successfully identified by FMAT screening. Real-time PCR or p21 promoter coupled to luciferase or fluorescent protein enables us to discriminate between compounds acting through different mode of action. Because the fluorescent signal is determined in each single cell, as would do a FACS profiling, the FMAT can provide reliable measurement regardless the homogeneity of cell seeding or anti-proliferative effect of screened drugs.

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The Role of Chk1 Signaling Pathways in Response to the Topoisomerase I Poison SN-38

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Human head and neck squamous carcinoma cell lines, A253 and FaDu, were utilized to identify mediators associated with response to topoisomerase I poison, SN-38, a metabolite of irinotecan. The drug sensitivity of FaDu cells to SN-38 was significantly higher than that of the A253 cells. To investigate molecular markers associated with response to SN-38, DNA fragmentation and altered expression of molecular markers associated with specific phases of the cell cycle were detected at 24 h post drug treatment (0.35 μM SN-38, 2 h exposure). In A253 cells, G2/M arrest following drug treatment was accompanied by DNA fragmentation in the 50-300 kb range, but SN-38-sensitive FaDu cells accumulated in S-phase concurrently with induction of smaller DNA fragmentation in the 4-80 kb range by pulsed-field gel electrophoresis analysis. Because the critical regulatory step in activating cdc2 during progression into mitosis appears to be dephosphorylation of Tyrosine 15 (Tyr15), we examined the Tyr15 phosphorylation status of cdc2 and total cdc2 protein expression in both cell lines. Slightly increased levels of cdc2 phosphorylation was observed in the A253 cells, while reduced levels of cdc2 phosphorylation was noted in the FaDu cells, corresponding to the abrogation of the G2-phase arrest. Inhibition of the human checkpoint kinase, chk1, abrogates G2 arrest in response to DNA damage. We, therefore, evaluated whether G2-phase arrest is associated with altered chk1 phosphorylation at Ser345. Increased chk1 phosphorylation induced by SN-38 was accompanied by the observed G2 phase arrest in the A253 cell line, while significant downregulation of chk1 and cdc25C phosphorylation, which resulted in the abrogation of G2/M checkpoint arrest, was noted in FaDu cells at this timepoint. These results suggest that alterations of chk1 signaling are associated with the response to topoisomerase I poison SN-38. Furthermore, the role of the two major mismatch repair proteins, hMLH1 and hMSH2, in the response to SN-38 was also evaluated. A253 cells possess higher levels of endogenous hMLH1, compared to FaDu cells. A deficiency in G2 arrest was observed in FaDu cells, suggesting endogenous hMLH1 protein expression is associated with the abrogation of G2/M arrest, subsequently with the response to topoisomerase I poison SN-38.

Structure activity relationships

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In silico and flexible docking screening using bioavailability, similarity and energetic filters: application to human thymidine phosphorylase

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Introduction: Thymidine phosphorylase (TP) is over-expressed in several tumour types (breast, colon, gastric) and expression has been correlated with increased microvessel density (angiogenesis). Therefore inhibition of TP may be of use in cancer chemotherapy. Currently only a few potent (nM) thymidine phosphorylase inhibitors (TPIs) exist. We haved employed an *in silico* screening strategy of the NCI 3D database using our human TP homology model (Cole 1999) in the search of new leads.
Objective: To screen the NCI 3D database using rigid and flexible docking in conjunction with a bioavailability screen and a similarity screen of known TPIs, to identify potential new lead candidates as TPIs.
Methodology: The NCI 3D database (250,521 compounds) was subjected to an in-house bioavailability filter based upon on Lipinski's rule of 5. The remaining 209,457 ligands together with six TPIs, and the substrate, thymidine, were docked into TP using DOCK 4.0 and AMBER united atom force field. Ligands were retained from rigid body docking if they bound more favourably than the substrate (-22 kcal/mol). The resulting 53,248 com-

pounds were redocked more exhaustively and then subjected to a modified similarity Tanimoto screen based upon the six TPIs. From the resulting ranked similarity and interaction energy scores, 535 ligands were selected for fully flexible ligand docking. The 535 docked solutions were then scrutinized for appealing interactions between the ligand and the active site residues, particularly hydrogen bonds, and packing efficiency in the active site.

Results: The top 40 ranked ligands were then ordered from the NCI repository. 13 were available and are currently undergoing biological evaluation.

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Structural consequences of trans-membrane association and mechanism of molecular recognition at the active site of human estrone sulfatase, a potential target for hormonal breast cancer therapy

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Human estrone (E1)/DHEA sulfatase (ES), along with cytochrome P450 aromatase and 17beta-hydroxysteroid dehydrogenase1 (17HSD1), is responsible for maintaining high levels of the active estrogen, 17beta-estradiol (E2), in tumor cells. ES catalyzes the hydrolysis of E1-sulfate, which is subsequently reduced to E2 by 17HSD1. The presence of ES in breast carcinomas and ES-dependent proliferation of breast cancer cells have been demonstrated. Selective estrogen enzyme modulators that inhibit these enzymes have shown promise as anti-proliferative agents. Rational design of specific ligands requires detailed understanding of molecular structure of the active site. Although the precise sub-cellular localization of the functional ES is not clear, this membrane-bound enzyme is distributed in the rough endoplasmic reticulum (ER). The full-length enzyme has been purified from the microsomal fraction of human placenta in the active form and crystallized. The three-dimensional structure of the enzyme has been determined by X-ray crystallography at 2.6 angstrom resolution. The structure shows a trans-membrane domain consisting of two anti-parallel alpha-helices that protrude from the roughly spherical molecule, thereby giving it a "mushroom"-like shape. These highly hydrophobic helices, each roughly 40 angstrom long and situated between residues 179 and 235, are capable of traversing the membrane, thus presumably anchoring the functional domain to the membrane surface facing the ER lumen. The location of the trans-membrane domain is such that the opening to the active site, buried deep in a cavity in the "gill" of the "mushroom", rests near the membrane surface. Furthermore, a spatially proximal polypeptide segment between residues 468 and 500, consisting of several hydrophobic sidechains and displaying high thermal motion, also presumably associate with the lipid bilayer. The residues from the membrane-associating regions line the entry path leading to the active site. The catalytic amino acid hydroxyl formylglycine 75 is found to be covalently linked to a sulfate moiety. While D35, D36, D342 and Q343 are involved in coordination of the Mg2+ ion, H290, H136, K134 and K368 play important roles in catalysis. Residues V101, F178, V177, L74 and F488 could participate in substrate recognition. Details of steroid-protein and lipid-protein interactions will be presented. This work is partially supported by the grants GM59450 and GM62794 from the NIH (to DG).

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Synthesis, structure and anticancer activity of a novel platinum(II) coordination compound of 4-aminosalicylic acid

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We have previously synthesized binuclear complexes of Pt(II)involving the naphthoquinone bridging ligand,attempting to explore the possible synergistic effect of anthracyclins and cis-platin. The results were encouraging and therefore we extended our research to Pt(II) complexes involving various hydroxyquinonic ligands. Considering that hydroxyquinones are slightly soluble, the selection of smaller ligands exhibiting coordination ability towards Pt(II) metal centers, is a good choice. Along this line, we present preliminary results on a new mixed-ligand Pt(II) complex of 4-amino-salicylic acid with imidazole. The complex was prepared by reacting of sodium 4-aminosalicylate with K2PtCl4 in aqueous medium in a 1:2 molar ratio. To the mixture, excess of saturated imidazole was added. The final mixture was stirred for 24h at RT. The dark yellow precipitate (MT: C10H8O3N3PtCl, MW: 448.5) was filtrated,washed with water and methanol and air-dried. It is soluble in DMSO.

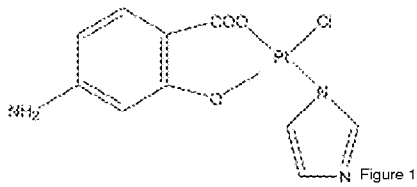

Figure 1

4ASA is coordinated to the Pt(II) central atom in a bidentating way, via the carboxylic and phenolic oxygen donor atoms forming a 6-atom ring. The imidazole and chloride ligands complete the square planar coordination environment of Pt(II). The IR spectrum confirms the above as the band at 3139 cm^{-1} is characteristic for non-coordinated –NH2 groups.Moreover,the ν(C=O) stretching vibration at 1627 cm^{-1} is shifted to lower frequencies compared to the uncoordinated ASA(C=O) group (1638 cm^{-1}).Accordingly,the ν(C-O) vibration is shifted to higher frequencies (1307 cm^{-1}) relative to that of 4ASA (1302 cm^{-1}). Finally,the ν(Pt-O) stretching vibration was found at 334 cm^{-1}. Human cancer cell lines of lung (A549), colon HCT-15), melanoma (SK-MEL-2) and ovaries (A2780) were used for the cytotoxicity test *in vitro* with the SRB assay. The cytotoxic activity was evaluated by measuring the concentration of the complex required to inhibit the protein synthesis by 50% (IC_{50}) compared to cis-platin. Each value is the result of triplicate experiments.

Table 1

Cell	cis-platin	complex
A549	0,275	0,075
HTC-15	1,490	0,650
SK-MEL-2	0,155	0,140
A-2780	0,240	0,148

The present results illustrate that the complex exhibits cytotoxicity against all cell lines tested,with higher rate of activity than that of cis-platin. Nevertheless,the solubility effects are of key importance to the improvement of the complex's toxicity. Therefore, research on the synthesis and study of new, mixed-ligand Pt(II) compounds of 4ASA and a variety of other N-donor ligands is in progress aiming to improve the solubility and bioactivity of the compounds.

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A molecular overlap tool for investigating potential binding mode similarity in sets of compounds

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The Developmental Therapeutics Program (DTP) currently offers web accessible tools such as COMPARE that use biological similarity comparison algorithms to mine the compound databases provided by DTP. By applying a method that focuses on structural aspects of these compounds, we provide an independent measurement that identifies structural commonalities that may help distinguish particular sets of compounds that are more likely to have the same underlying biochemical mechanisms of action. We present a new tool where sets of compounds can be aligned based on maximizing their molecular volume when hydrogen bond donors and acceptors are superimposed. The algorithm employs an evolutionary programming method that overlays structures based on a substitution matrix of atom types. Examples of the utility of this new application will be presented along with details about its accessibility through our web pages and integration with COMPARE.

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Development of an Mdm2/p53 fluorescence polarization high throughput inhibitor screening assay

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Mdm2 regulates p53 tumor suppressor function by three mechanisms: binding to the transactivation domain of p53, exporting p53 out of the nucleus, and ubiquitinating p53 for degradation. Mdm2 hyperactivity, due to amplification/overexpression of Mdm2 or mutational inactivation of ARF locus, is undesirable because it inhibits the function of wild type p53 and can lead