308 POSTER

Antitumor activity of the new aureolic acid derivatives mithramycin SDK and SK in human ovarian cancer xenografts

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Aureolic acid antibiotics, like mithramycin (MTM), are natural polyketides that interact with DNA at GC-rich sites and inhibit binding of GC-rich DNA binding proteins, like Sp1 transcription factors. Targeted gene inactivation in the *S. argillaceus* producer strain has recently yielded two new analogues, MTM SDK (SDK) and SK (SK), with improved activity as transcriptional repressors and anticancer agents compared to MTM (*NAR 34:1721, 2006*). SDK inhibited transcription of multiple Sp1-regulated genes controlling cell proliferation, apoptosis, migration, invasion and angiogenesis, consistent with the pleiotropic role of Sp1 transcription factors in cancer development and progression. SDK inhibited proliferation and induced apoptosis of several human cancer cell lines, including ovarian cancer cells, with minimal effects in normal cells. To extend these observations to an *in vivo* model, we established human ovarian cancer xenografts by intraperitoneal (i.p., orthotopic model) or subcutaneous injection of A2780 cells in female CD-1 nude mice. In the orthotopic model, SDK, given by i.p. injections for 11 consecutive days at the daily dose of 400 μg/Kg, prolonged survival compared to vehicle-treated control mice. Median survival for SDK was 80.5 versus 55 days for the control group (P = 0.00368). There was also a significant delay in the appearance of ascites in SDK treated mice versus control mice (P = 0.0135). SDK was more effective than MTM that gave a median survival of 58 days (P = 0.28 vs. control and P = 0.0474 vs. SDK), confirming the greater activity of the new analogue seen *in vitro*. SK gave marginal benefits in survival compared to the control group (median survival 68 days and P = 0.0657 vs. control mice). Both SDK and SK were well tolerated in mice without signs of toxicity. In the subcutaneous tumor model, SDK and SK, given by i.p. injections q2d × 10 at a dose of 600 g/kg, induced similar tumor growth delay. These results suggest that SDK and SK could be effective agents for treatment of ovarian cancer, blocking expression of genes involved in cancer cell proliferation and intraperitoneal dissemination.

309 POSTER

A systems biology approach to the analysis of DNA-interactive antitumour agents

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Background: We are developing a systems biology approach to the analysis and integration of the varied biological data from investigations of the molecular pharmacology of DNA-interactive agents. A computational model of the tumour cell is being developed that models the relationship between drug treatment, cell cycle distribution, growth inhibition, and senescence. The parameterisation of this model requires robust data from a well-characterised cell line. Here we describe the approach to the selection of this cell line, and initial data from it. The process involves optimisation against a variety of goals, including cell growth characteristics, sensitivity to DNA-interactive agents, tractability in a variety of assays, and not least clinical relevance.
Materials and Methods: Four colorectal cell lines have been chosen for initial study: HCT116, HT-29, HCC 2998 and KM12. Flow cytometry combining propidium iodide, BrdU incorporation and immunofluorescence for Ki-67 and histone H3 are used for cell cycle analysis alongside MTT assays and x-Gal senescence staining. Our trial DNA-interactive agent is 3,11-difluoro-6,8,13-trimethyl-8H-quino[4,3,2-kl]acridinium methosulphate (RHPS4), a potent inhibitor of telomerase linked to its selectivity for G-quadruplex DNA. However, recent studies suggest a more complex mechanism of antitumour action at the telomere (Leonetti et al. Mol. Pharmacol., 2004, 66, 1138−46).
Results: MTT assays reveal that GI_{50} values can be time dependent: HT-29 cells become 8-fold more sensitive to RHPS4 between 24 and 72h, while KM12 become 4-fold resistant. Cell cycle analysis shows a dose- and time-dependent effect in all cell lines, which computational modelling analyses as dose- and time-dependent blocks at two transitions: a) either the G1->S transition, or the G1/G0 equilibrium, plus b) the G2->M transition. The model predicts that analysis of senescence data will allow these two possibilities to be differentiated.
Conclusions: This data is being used to develop a computational model of the cell of sufficient complexity to reproduce these effects, yet of sufficient simplicity to yield useful predictive information and biological insights. Already it has proven useful in guiding optimal experimental design.

Drug screening

310 POSTER

New assays for histone methyltransferases

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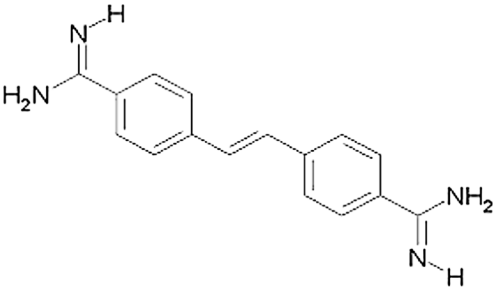
Background: Transcriptional regulation is maintained by posttranslational modifications of histone proteins such as methylation of lysines and arginines. Whereas inhibitors of histone deacetylases are already in clinical trials as anticancer agents very little is known about inhibitors of histone methyltransferases. We wanted to combine virtual screening and enzyme assays in the search for new inhibitors of arginine methyltransferase PRMT1.
Material and Methods: Biotinylated histone peptide fragments that are immobilized on streptavidine microplates are able to serve as substrates for fungal arginine methyltransferase PRMT1 and human PRMT1. The methylation is detected by a primary anti-dimethyl arginine antibody. The readout is performed with a europium labelled secondary antibody and subsequent time-resolved fluorescence analysis. We created a homology model of PRMT1 using the COMPOSER module of the program SYBYL 7.0. The virtual screening was carried out with the NCI Diversity SET using the program GOLD.
Recombinant aspergillus nidulans PRMT1 was used as the source of enzyme. The validation of the most potent compounds was carried out with commercial available human PRMT1. Furthermore, the hits were checked for their cellular activity in an estrogen-reportergen assay and in an antibody based cellular assay for the methylation level on histone H4 arginine3.
Results: The stilbene derivative stilbamidine showed an inhibition of PRMT1 with an IC_{50} of 60 μM and did not inhibit the lysine methyltransferase SET7/9. Additionally, a strong inhibition of the receptor activation by estradiol at 150 μM and a dose dependent inhibition of methylation between 50 and 150 μM in the cellular assay were discovered.



Stilbamidine.

Conclusions: A new inhibitor of PRMT1 was discovered that shows potential for the treatment of hormone dependent cancers.

311 POSTER

Screening for mitotic kinesin KSP inhibitors: implication of the microtubule binding regions of KSP motor domain as drug target

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Small chemical molecules that cause cell cycle arrest in mitosis might be useful for treatment of malignant tumors, since most cells in human body are not actively dividing in comparison to tumor cells. Discovery of monastrol, the first small chemical inhibitor against human kinesin protein KSP, reveals that KSP is one of attractive targets as anticancer drugs to perturb mitotic progression, to activate the spindle checkpoint and to trigger apoptosis. Until now, a number of KSP inhibitors have been identified, including HR22C16, CK0106023, terpendole E, K and S-trityl-L-cysteine.

These KSP inhibitors function as allosteric inhibitors of KSP ATPase activity, which cause the rate of ADP release extremely slower, but do not interfere with the KSP-microtubule interaction. To discovery another types of cell-permeable KSP inhibitors, we have been screening synthetic chemical library through *in vitro* ATPase assays followed by cell-based analyses. 28,000 small chemical molecules were examined by measuring inhibition ability of microtubule-induced ATPase activity of KSP motor domain *in vitro*. And then, the positive chemical molecules were treated with synchronized and asynchronized HeLa cells to check their ability to perturb mitotic progression. As a result of our screening procedure for KSP inhibitors, we identified two cell-permeable small chemical molecules. Their treatment partially accumulated mitotic HeLa cells with characteristic mono-astral spindle phenotype that was same as a typical phenotype induced by KSP inhibition with monastrol. Although they could inhibit microtubule-induced ATPase activity of KSP motor domains *in vitro* (IC50=5 uM), they could not affect KSP ATPase activity in the absence of microtubules, suggesting that they might interfere with KSP-microtubule interaction. They could affect neither dynamics of microtubules, nor ATPase activity of other kinesin members we tested (CENP-E, MKLP1, Kid, KIF4) *in vitro*. Together, we found a new type of KSP inhibitors that affect KSP ATPase activity only in the presence of microtubules, which might serve as starting points for the development of new anticancer drugs with improved efficacy. And also this study suggests that the microtubule binding surface of kinesin KSP motor domain might be an attractive candidate for anticancer drug target.

312 POSTER

Preclinical evaluation of a rationally designed novel class of non-hydroxamate Histone Deacetylase inhibitors (HDACi)

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Inhibitors of Histone Deacetylases (HDACs) are promising anti-cancer agents which are showing signs of activity in early clinical trials. Current HDACi are however of limited potency and many are hydroxamic acids with unattractive pharmacological properties. Furthermore, although current evidence strongly suggest class I (nuclear) HDACs, particularly HDAC1, as the key targets for anticancer activity, the majority of HDACi do not demonstrate class selectivity.

We have designed and synthesised a series of non-hydroxamate candidate molecules using novel cascade combinatorial synthesis. Molecular modelling/docking studies demonstrated localisation of our compounds within the HDAC active site, suggesting a potentially novel binding mode.

Compounds were compared against the hydroxamate HDACi, SAHA and the non-hydroxamate HDACi, MS-275, currently in phase II clinical trials. Our compounds demonstrate *in vitro* tumour cell cytotoxicity comparable to MS-275, with IC50 values of <1 μM. Inhibition of HDAC enzymatic activity measured using a cell-free assay identified several agents exhibiting IC50 values of <10 μM (compared to 3 μM for MS-275). In order to address HDAC activity inhibition in situ and activity against class I HDAC, we measured levels of hyperacetylated histone-H4 following in vitro drug treatment using flow cytometric analysis. MS-275 demonstrated a 2.8±0.7 fold increase in acetylation compared to control whereas our lead compound demonstrated a much greater 7.2±1.0 fold increase. In vivo administration of our lead compound resulted in a significant 3.7 day delay in growth of an ovarian xenograft tumour model.

These 'proof of principle' studies support these non-hydroxamate based agents as novel HDACi and suggest class selectivity, improved pharmacokinetics and potential as valid anticancer therapeutics.

313 POSTER

Pediatric preclinical testing program (PPTP) evaluation of the KSP inhibitor Ispinesib (SB-715992)

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Background: Ispinesib is a novel small molecule inhibitor of kinesin spindle protein (Eg5), a mitotic kinesin required for separation of the spindle poles. Ispinesib inhibits growth of a broad range of cancer cell lines at low nanomolar concentrations and induces regressions or tumor growth delay against adult cancer xenografts. The COG Phase 1 Consortium is initiating a phase 1 trial of ispinesib for children with refractory solid tumors.

Methods: The PPTP includes an *in vitro* panel (23 lines) as well as panels of xenografts (n = 61) representing most of the common types of childhood solid tumors and childhood ALL. Ispinesib was administered IP (10 mg/kg) to a representative subset of xenografts on a q 4d × 3 schedule repeated once at day 21. Three measures of antitumor activity were used: 1) response criteria modeled after the clinical setting [e.g., partial response (PR), complete response (CR), etc.]; 2) treated to control (T/C) tumor volume at day 21; and 3) a time to event measure based on the median EFS of treated and control lines (intermediate activity required EFS T/C > 2, and high activity additionally required a net reduction in median tumor volume at the end of the experiment).

Results: Ispinesib induced significant tumor growth delay in 82% (14/17) of evaluable solid tumor xenografts. Using a time to event measure of efficacy, ispinesib had intermediate and high levels of activity against 6 (35%) and 3 (18%) of the 17 evaluable solid tumor xenografts, respectively. Intermediate or high activity for the EFS measure was observed for most diagnoses [e.g., Wilms tumor (WT), rhabdoid tumor (RT), Ewing sarcoma (ES), rhabdomyosarcoma (RMS), and GBM], but not for neuroblastoma. Ispinesib induced maintained CRs in 3 xenografts: 1 of 2 WT, 1 ES, and 1 of 2 RT, and it induced a CR in 1 of 4 GBM. Preliminary analysis of results from the ALL panel suggests substantial activity against several xenografts. Ispinesib induced excessive toxicity in mice bearing osteosarcoma xenografts, and excessive toxicity precluded analysis of 6 xenografts for other diagnoses.

Conclusions: Ispinesib demonstrated broad activity against the PPTP's solid tumor xenografts. Antitumor activity manifested primarily as tumor growth delay, though tumor regressions were also observed. Further preclinical work with ispinesib will include an evaluation against the PPTP *in vitro* panel and further testing against the ALL panel.

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314 POSTER

Extra-vascular penetration of taxanes may limit their efficacy

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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. We compared the tissue penetration of tritium labelled paclitaxel and docetaxel in tumour xenografts and in multilayered cell culture (MCC), a tissue-engineered model of the tumour extra-vascular compartment. Drug distributions in the xenografts were mapped relative to blood vessels to obtain profiles of drug as a function of distance from vasculature. In the MCCs, drug levels were determined as a function of distance in from the exposed edge of tissue. Results were compared with predictions from an *in vitro* effect-based assay.

Experiments were carried out in tumour xenografts and MCCs using human HCT-116 colon carcinoma cells. For paclitaxel the effect of the vehicles Cremophor EL versus Tween 80 was examined. Paclitaxel (10 mg/kg) and docetaxel (5 mg/kg, Tween 80) were administered by tail vein injection. Tumours were removed and frozen 2 and 8 hours after drug injection. For MCC experiments, cultures were exposed to 0.3 and 3 μM of each drug for 1 and 2 hours and then frozen. Following cryosectioning, slides were clamped against tritium sensitive film and exposed for a period of 3 months. Results from xenograft and MCC-based autoradiography studies found that both drugs penetrated poorly. Of the two, paclitaxel exhibited approximately 2-fold greater tissue penetration than docetaxel, with drug falling by half 55 μm ± 5 μm away from vessels for paclitaxel versus 28 μm ± 5 μm for docetaxel (2 hour time point). The effect of vehicle on paclitaxel distribution was surprising in that at the 2 hour time point paclitaxel in Tween 80 showed significantly higher peak tissue levels relative to Cremophor EL (55% increase) but by 8 hours both vehicles produced similar drug distributions. In MCCs, drug levels fell to half max by 28 μm ± 5 μm into the tissue for paclitaxel versus 17 μm ± 5 μm for docetaxel (0.3 μM drug, 1h). Results were consistent with previous data obtained using an *in vitro* screening assay in which paclitaxel showed significantly better tissue penetration than docetaxel.

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