suggesting a distinct biologic role for this family member. Medicinal chemistry efforts have identified pyrrolo[2,1-f][1,2,4]triazine-based inhibitors of Aurora kinases. Structure–activity relationships were developed by introducing substituents at C4 and C6 positions of the pyrrolotriazine to optimize the potency of kinase inhibition. The lead Aurora kinase inhibitors caused dose dependent apoptosis and cell cycle effects that are consistent with the effects of Aurora A or B depletion. These results suggest that targeting the Aurora kinases present a novel opportunity for anti-mitotic cancer therapy.

Cyclins and CDK's

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Discovery of selective CDK9 small molecule inhibitors: CDK9 inhibition in tumor cells is associated with inhibition of proliferation and induction of apoptosis

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Background: CDK9 (in complex with Cyclin T or K) phosphorylates the C-terminal domain of RNA polymerase II (RNAPII CTD) as a required step for mRNA transcript elongation. CDK9 inhibition causes the rapid depletion of short-lived mRNA transcripts and their associated protein products. Since many genes encoding proteins involved in cell growth, proliferation, and survival are characterized by short-lived mRNAs and proteins, the consequences of CDK9 inhibition are expected to include anti-proliferative and pro-apoptotic effects through loss of function at many cellular pathways. Proteins with well-established roles in tumor development and growth that are depleted following CDK9 inhibition include Myc, Cyclin D1, and Mcl-1.
Methods: CDK9-selective small molecule inhibitors were discovered by high-throughput screens and chemical optimization using biochemical and in vitro cell-based assays, as well as x-ray crystallographic information from CDK9-inhibitor co-complexes.
Results: Identified CDK9 inhibitors demonstrate excellent activity in CDK9 biochemical and cell-based assays, with IC50 values <10 nM and 100 nM respectively. Representatives of this series show excellent selectivity for CDK9 vs other CDK family members. IC50 values in cellular proliferation and apoptosis assays were found to correlate well with inhibition of cellular RNAPII CTD Ser2 phosphorylation, indicating that CDK9 inhibition is associated with strong anti-growth and pro-apoptotic effects on tumor cells in vitro. Selected CDK9 inhibitors exhibit oral bioavailability and dose-dependent plasma exposure. In vivo pharmacodynamic analysis following oral administration of these inhibitors in mouse xenograft tumor models demonstrates potent and dose-dependent inhibition of RNAPII CTD SER2 phosphorylation, as well as reductions in Myc, Cyclin D1, and MCL-1 protein levels.
Conclusions: CDK9-selective small molecule inhibitors were discovered that demonstrate potent CDK9 inhibitory activity in both in vitro and in vivo studies, and show anti-proliferative and pro-apoptotic activity in multiple tumor cell lines. Further chemical optimization and biological characterization is proceeding, with the goal of identifying a CDK9-selective inhibitor candidate for clinical development as a cancer therapeutic.

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AZD5597, a novel CDK inhibitor causes inhibition of RNA polymerase II mediated signaling and stimulates apoptosis in vitro and in vivo

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Background: AZD5597 is a CDK inhibitor displaying potency against both the cell cycle CDKs 1 and 2 and the transcriptional CDKs 7 and 9. This profile could result in both cell cycle block and induction of apoptosis and therefore may be particularly efficacious as a novel anti-tumour therapy.
Materials and Methods: The *in vitro* effects of AZD5597 on CDK inhibition, cell cycle progression and apoptosis were investigated by incubating the compound with MCF-7 cells. Cells were fixed, then labeled immunocytochemically for intracellular phosphorylation of RNA polymerase II, DNA intensity, and Bak conformational changes respectively and quantified on a Cellomics ArrayScan. We subsequently investigated the effects of AZD5597 *in vivo* using a variety of tumour xenografts (BT474c; CoLo 205, A2780 and U87MG). Mice bearing established sub-cutaneous tumours (mean tumour volume 0.4–0.6 cm^3) were given a single dose of AZD5597 (25 mg/kg, ip). Tumours were taken 6 h (4 h for U87MG) post dose, halved and either frozen or fixed in formalin for subsequent analysis. The effects of AZD5597 on cell cycle were assessed by scoring mitosis; pRb IHC and phH3 IHC. Transcriptional effects of AZD5597 were assessed by scoring RNA polymerase II IHC. Assessments of apoptosis were based on examination of both cellular phenotype and cleaved caspase 3 IHC. AZD5597 was dosed (15 mg/kg, ip; Mon, Wed and Friday for 3 weeks) to mice bearing SW620 xenograft tumours to assess the effects on tumour growth.
Results: AZD5597 treatment of MCF-7 cells *in vitro* resulted in a cell cycle block (at G2/M), reduced the phosphorylation of RNA polymerase II, and increased initiation of cell apoptosis (as indicated by conformational changes in Bak). Consistent with these data, *in vivo* a single dose of AZD5597 resulted in cell cycle block (reduced mitosis, pRb and phH3 staining), a reduction in phospho RNA polymerase II staining, and induction of apoptosis. Similar results were seen in all four tumour types examined. In chronic dosing, AZD5597 resulted in a 55% inhibition of SW620 tumour growth *in vivo*.
Conclusions: AZD5597 results in cell cycle block and causes the inhibition of survival pathways that leads to induction of apoptosis *in vitro* and *in vivo*. These effects were observed in histologically distinct tumour models (breast, colorectal, ovarian and glioma).

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The complexity of cell cycle dynamic of anticancer drugs unraveled by the use of mathematical models suitable for a quantitative assessment of G1, S and G2M checkpoint activities

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Background: Current methods to assess cell cycle effects of drugs in cell populations are still limited to score the macroscopic variations on specific measurable quantities like percentages of cells in G1, S and G2M. These quantities result from the superimposition of the effects of cell cycle block and cell loss, their dynamics in the times before the measure, and the proliferation of surviving cells. They depend on but do not provide a direct measure of the activities of the molecular networks regulating G1, S and G2M checkpoints. A mathematical model was built based on the kinetics of cell cycle transit times of an ovarian cancer cell line and on parameters that directly describe the activity of each checkpoint, and giving as output cell cycle percentages and other macroscopic measurable quantities.
Material and Methods: Experiments were performed with a standardized protocol using the IGROV1 cell line with 5 clinically used anticancer drugs (cisplatin, taxol, doxorubicin, melphalan, topotecan) characterized by different molecular mechanisms of action. We measured cell cycle percentages and absolute cell number at four times after treatments (6, 24, 48, 72 h) with a wide range of drug concentrations. DNA-BrdUrd histograms and apoptosis were also collected at specific times.
Results: After obtaining a robust set of data of the concentration-dependent effects of all drugs by using different techniques we have analyzed the complexity of events (delayed cell cycle progression, short or long term blockades in different cell cycle phases, cell death) by the application of a mathematical model based on parameters that directly describe the activity of each checkpoint. By deciphering cellular data in terms of the global activities of G1, S and G2M checkpoints, each governed by specific molecular network, it became evident that there are differences in the complex cell response to each drug that have never been observed before by standard methodologies.
Conclusions: We found that the overall response of each drug is the result of the combination of a few types of checkpoint response, which operate with different strengths, and with specific drug concentration thresholds. The application of the simulation mathematical model to anticancer drug effects allows a quantitative knowledge of the dynamics of critical cellular events, that should be taken into account when using an anticancer drug alone or in combination in a less empirical and more rational way.

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Biological characterization of the dual CDK2/TRKA inhibitor PHA-848125

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Among the small molecule cyclin-dependent kinase modulators that are nowadays in the clinical arena the pyrazolo quinazoline PHA-848125 emerges as a potent inhibitor of the CDK2/cyclinA complex (IC50=45 nM) with the novelty of being particularly active also against TRKA (IC50=53 nM) proposing in this way to be a "selective dual inhibitor". It also maintains some activity against other cell cycle CDKs, even if

with a potency 3 to 10 fold lower than CDK2, and presents some cross-reactivity towards kinases known to be overexpressed in different human malignancies, such as LCK, KIT, ABL and PDGFr. The compound has potent antiproliferative activity against a wide panel of tumoral cell lines, for the majority of which the IC50 values range between 0.1 and 1 μM as measured at 72 hours.
By flow cytometry PHA-848125 treated cells show cell cycle arrest in G1phase and reduction of DNA synthesis. Western blot analysis reveals the consequent hypophosphorylation of the Retinoblastoma protein (pRb) and the decrease of cyclinA expression, all typical indicators of CDK2/cyclinA inhibition, and in parallel, in those cells that express TRKA, the reduced phosphorylation of this kinase and of its effector proteins.
PHA-848125, indeed, orally administered has significant antitumor activity in various human xenograft, transgenic, carcinogen-induced tumor animal models as well as in disseminated human leukemia models. The mechanism of action of this inhibitor is confirmed also "in vivo" by immunohistochemical and gene expression analysis conducted on tumor biopsies of treated mice.
The same analysis have been preclinically validated also in a normal tissue, the skin of animals treated with PHA-848125, to be transposed on clinical available samples. PK/PD modelling allowed prediction of active doses and selction of schedules that are currently used in clinical trials.
Target modulation studies using tissue samples of the patients enrolled in these trials are ongoing and will add value to the results obtained with the phase I studies and produce deeper knowledge of the compound to go on with its clinical development.

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Growth inhibition and induction of apoptosis in anaplastic large cell lymphoma cells treated with the CDK inhibitor flavopiridol

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The loss of cell cycle regulation due to abnormal function of cyclin-dependent kinases (CDKs) occurs in various tumours and leads to increasing genetic instability and selection of chemotherapy-resistant cells. ATP-competitive substrates have been designed to interfere with CDKs, and Flavopiridol (FP), a potent pan-CDK inhibitor tested in multiple phase I and II clinical trials, has shown anti-tumour activity in many cancer cell types. We investigated the effect of FP in Anaplastic Large Cell Lymphoma (ALCL), a high-grade non-Hodgkin lymphoma characterized by high mitotic index and overexpression of cell cycle-related proteins.
The efficacy of FP on ALCL cell lines was evaluated in vitro using survival, cell cycle and apoptosis assays. Immunoblotting and immunofluorescence analyses were used to assess expression and localization of signal transduction and cell cycle-related proteins, as well as effectors of apoptosis. Phosphorylation status of RB and RNA Pol II was used to test CDK activity, whereas QRT-PCR assessed drug-induced changes in transcription.
Here we show that ALCL cells are highly sensitive to the antiproliferative activity of FP, with average IC50 of 140 nM at 24 h and ⩽70 nM at 72 h. Despite growth inhibition, cell cycle analysis ruled out any significant blockade in G1 or G2/M phase, while revealed a sustained accumulation of subG1 cells concomitant to the disappearance of the S-phase cell population. Regulators of S-phase traversal (cyclin E, A, CDK2, E2F1) were proteolytically cleaved in FP-treated cells, whereas G1 and G2/M-related proteins (CDK4, cyclin D3 and B1) were downregulated independently of caspase activation. Consistent with the inhibition of CDK activity, hypophosphorylation of retinoblastoma protein, as well as reduction in CDK9-dependent phosphorylation of RNA Pol II, were observed in ALCL cells. This correlated with dramatic induction of cell death, occurring through rapid perturbation of mitochondria permeability and sustained downregulation of Mcl-1 protein and mRNA. Notably, few viable lymphoma cells were arrested in G1-phase at 24 h, despite continuous inhibition of CDK activity. Further sensitization was achieved when cells were synchronized at G1-S boundary before FP addition, indicating that treatment during S-phase progression is selectively toxic to transformed cells.
These data provide evidence that Flavopiridol is effective in inducing cytotoxicity, and represents a novel chemotherapeutic agent for ALCL lymphomas.

Gene therapy and antisense approaches

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Human glioblastoma organotypic slice culture: A novel model for the study of tumor biology and therapeutic response

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Background: The available models for assessment of treatment effects against malignancies have well recognized limitations that undermine their utility as surrogates for effect in humans. In order to bridge the gap between the preclinical models and the first human trials, we developed an ex-vivo organotypic tumor tissue culture model using glioma tissue from resection in patients with newly diagnosed or recurrent glioblastoma multiforme.
Methods: Freshly resected tumor tissue was subject to sectioning with a VibratomeTM to obtain 300 um thick slices which were equilibrated in artificial cerebrospinal and subsequently cultured in DMEM-F12 medium. The slices were tested in various treatment paradigms.
Results: To test their viability, we transduced the slices with an adenovirus expressing EGFP (Ad-EGFP) and assessed EGFP expression by immunofluorescent microscopy. Slices, obtained from non necrotic portions of the tumor exhibited robust expression of EGFP for 5 days to upto 1 month post transduction and maintained their 3-dimensional structure. Time-lapse imaging of the slices demonstrated a mixture of static and migratory populations of cells. Tumor slices were also amenable to paraffin fixation and generation of slides for immunostaining and downstream studies. Next, we tested the utility of the tumor slices in assessing the response of the tumor to therapeutic and investigational agents. Slices treated with soluble TRAIL, a death receptor ligand, exhibited induction of apoptosis which was detectable both by typical morphologic changes and by in situ TUNEL assay. Treatment of slices with vorinostat, a histone deacetylase inhibitor, expected in typical increase in p21 levels suggesting that this model can potentially be used to measure responses to therapeutic agents in human glioma tissue. Lastly, we studied the interaction between DiI-labeled human mesenchymal stem cells (hMSC) when overlaid upon human glioblastoma tissue slices; the model permitted observation of hMSC migrating into the tumor slices and surviving in the tumor microenvironment.
Conclusions: The human glioma organotypic slice model thus represents a novel method to study tumor biology and therapeutic responses which, because of its human syngeneity, close relationship to the patient's tumor and the lack of genetic instability leading to cumulative genetic alterations seen in cell cultures, bears promising potential in the study of cancer.

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In vivo efficacy and replication dynamics of intravenously administered oncolytic reovirus in nude mice bearing human melanoma xenografts

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Background: Reovirus type 3 Dearing strain (ReoT3D) is a dsRNA virus known to preferentially infect and kill cancer cells. We have previously examined the oncolytic spectrum of ReoT3D in the NCI-60 cancer cell line panel, and discovered that human melanoma cell lines were exceptionally permissive to ReoT3D-induced oncolysis in vitro. In the current study, we investigated the in vivo efficacy and replication dynamics of ReoT3D administered intravenously to nude mice bearing human melanoma.
Materials and Methods: UACC-62 xenografts were established subcutaneously in the right hind flank of female athymic nude mice. Once tumors reached an average weight of 236 mg (±49), animals were randomized to receive a single intravenous (IV) bolus of vehicle (n = 15) or ReoT3D at 5E07, 1.5E08 or 5E08 PFU (n = 15 for each dose group) and followed for 2 weeks, after which plasma, tumor and spleen tissues were harvested. Plasma samples were also collected at 2, 6, and 24 hrs postinjection (p.i.) and tumors were biopsied 2, 4, and 7 days p.i. in selected animals from each group. The amounts of ReoT3D dsRNA in the specimens were determined by real-time quantitative RT-PCR.
Results: Dose-dependent tumor growth retardation was observed in ReoT3D-treated animals ($P < 0.001$) with the effect most pronounced for the first 7 days. Although infused ReoT3D was rapidly cleared from plasma with estimated $T_{1/2}$ of 7.8 9.2 min (one phase exponential decay), its early plasma concentrations correlated with the administered doses ($P < 0.0001$). ReoT3D dsRNA was demonstrated in all biopsied tumors and the level consistently increased from day 2 through day 7 p.i. in all dose groups ($P < 0.0001$). ReoT3D dsRNA could still be detected 14 days p.i. in tumor, spleen and plasma, but without any significant differences among the