body weight-based formula (calculated from a patient's gender, height and weight) that significantly reduced the inter-individual variability in C_{max} of tasisulam compared to flat dosing. Key observations during the first human dose study JZAA were that the dose limiting toxicity of tasisulam was bone marrow suppression, particularly thrombocytopenia, and that tasisulam had a long terminal half life (approximately 11 days), consistent with high albumin binding (99.7% to 99.9%). Based on the pharmacokinetic exposures of cancer patients in Phase 2 studies who achieved RECIST-defined response, an area under the curve (AUC) above a certain threshold was calculated for all patients and used to define a therapeutic range that minimized the risk of Grade 4 (G4) hematological toxicity (defined as developing either G4 thrombocytopenia or neutropenia in either Cycle 1 or Cycle 2).
Results: The risk in patients above this therapeutic range was ~50% whereas the incidence in patients within the hypothesized therapeutic range was ~15% or less, which was considered acceptable in the metastatic cancer setting. These findings have led to refinement of the current dosing algorithm to include patient predose albumin and lean body weight.
Conclusions: The goal of this tailored dosing paradigm for tasisulam is reducing the individual risk of Grade 4 hematological toxicity, while preserving the hypothesized therapeutic range. This dosing regimen is novel in cancer medicine to our knowledge and may be more widely applicable to other highly protein-bound cancer drugs. A dose and schedule using this approach have been defined and will be implemented in ongoing studies, including a Phase 3 study in second-line metastatic melanoma.

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Development and validation of a real-time multiplex PCR assay for the simultaneous quantification of *CK-19, MAGE-A3, HER-2* and *PBGD* in circulating tumor cells of breast cancer patients

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Introduction: Circulating tumor cells (CTCs) represent an important biological link in the spread of breast cancer from primary to metastatic disease. CTCs have already been established as strong predictors of prognosis in patients with metastatic breast cancer. The aim of our work was to develop a quantitative real-time multiplex PCR assay for *CK-19, MAGE-A3, HER-2* and *PBGD* and validate its performance in CTCs of early and metastatic breast cancer patients.
Materials and Methods: A tetraplex quantitative real time assay for *CK-19, MAGE-A3, HER-2* and *PBGD* was developed in the LightCycler 2.0 platform (Roche, Diagnostics). Specificity and sensitivity experiments were performed using the SKBR-3 cancer cell line. The method was applied in 66 patients with early breast cancer before the administration of adjuvant chemotherapy, 26 patients with verified metastasis and 16 female healthy volunteers. Peripheral blood (20 mL in EDTA) was obtained and after density gradient centrifugation, immunomagnetic Ber-EP4 coated capture beads were used to enrich for epithelial cells, keeping for each sample two fractions: the CTC and corresponding PBMC fraction. Messenger RNA was isolated from enriched epithelial cells using oligo $(dT)_{25}$ coated magnetic beads. After cDNA synthesis the expression of *CK-19, MAGE-A3, HER-2* and *PBGD* was tested, in both fractions.
Results: The analytical performance of the method was evaluated in SKBR-3 tumor cell line in respect to analytical sensitivity and specificity. Cross reaction studies, performed for each gene target in the presence of all other targets have shown a very high specificity for each analyte. RNA quality in all samples was evaluated by *PBGD* gene expression. We found 28/66 (42.4%) patients with early breast cancer positive for *CK-19*, 14/66 (21.2%) for *MAGE-A3* and 9/66 positive for *HER-2* (13.6%). In patients with verified metastasis we found 14/26 patients positive for *CK-19* (53.8%), 4/26 for *MAGE-A3* (15.4%), 5/26 patients positive for *HER-2* (19.2%). All healthy volunteers were found negative in their CTCs fractions for *CK-19* (0%), for *MAGE A3* (0%) and *HER-2* (0%).
Conclusions: We report for the first time a highly specific, reproducible and sensitive quantitative multiplex real-time PCR assay for the simultaneous detection of *CK-19, MAGE-A3, HER-2* and *PBGD*. The expression of these genes in CTCs will be further examined in a larger number of patients and results will be correlated with their clinical outcome.

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Pharmacokinetics and pharmacodynamics of the novel proteasome inhibitor CEP-18770 during a phase I trial in patients with solid tumor, non-Hodgkin lymphoma or multiple myeloma

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Background: CEP-18770 is a new proteasome inhibitor with boronic acid based structure under evaluation in phase I study in patients with solid tumors, non- Hodgkin lymphoma (NHL), or multiple myeloma (MM). It is an active inhibitor of the chymotrypsin-like activity of mammalian proteasome with IC_{50} of 3.0 nM. Its antitumor activity was demonstrated in mouse models of several tumors, particularly against the human multiple myeloma RPMI 8226 after repeated i.v. or oral administrations. It has also shown potent induction of apoptosis in human MM cell lines and in patient-derived cells.
Aims: to assess the pharmacokinetics (PK) and pharmacodynamics (PD) of CEP-18770 in patients with solid tumors or NHL participating to a phase I, dose-escalating study designed to determine the Maximum Tolerated Dose (MTD) of CEP-18770.
Patients and Methods: CEP-18770, supplied by Cephalon, was administered as i.v. bolus on days 1, 4, 8 and 11 of a 21-day cycle. The dose escalation followed a modified Fibonacci sequence starting from 0.1 mg/m^2. Blood samples were collected at pre-dose, after the administration on day 1 (from 5 min to 48 h), at pre-dose on days 4, 8, 11 and pre-dose on day 1 of the 2nd cycle. The plasma concentration of CEP-18770 was measured by a validated method based on liquid-chromatography coupled with tandem mass spectrometry; the method is highly sensitive (LOQ 0.2 ng/mL) with a precision CV% $\leqslant$8.3% and an accuracy range 93.8–107.7%. The % inhibition of the chymotryptic activity of 20S proteasome was assessed in blood by a fluorogenic kinetic assay.
Results: Dose was escalated up to 1.8 mg/m^2 through 8 dose levels in a total of 40 patients, with MTD defined at 1.5 mg/m^2. PK evaluation performed on day 1, showed linear PK of CEP-18770 over the dose range evaluated and acceptable inter-patient variability. In 16 patients at the MTD, mean$\pm$sd values of Cmax, AUCexp and terminal half-life were 366.0$\pm$117.4 ng/mL, 1048.8$\pm$536.5 ng/mL*h and 60.3$\pm$28.0 h, respectively. CEP-18770 was detectable up to 72 h at levels twice the LOQ. CEP-18770 inhibited 20S proteasome activity with effect increasing with dose and achieving maximal inhibition of 55$\pm$9% in patients at 1.8 mg/m^2 and of 45$\pm$12% in patients at MTD.
Conclusions: A PK and PD evaluation performed during the phase I clinical trial of the novel proteasome inhibitor CEP-18770 indicates that the drug achieves plasma levels that are able to inhibit proteasome activity.

Combinatorial chemistry, drug screening and synthesis

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3D culture systems for cancer drug evaluation

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Background: 2-dimensional (2D) monolayer cultures are generally used for testing anti cancer agents in vitro. However, 3D cultures (e.g. multicellular tumour spheroids; MTS) are of increasing interest in such applications. These systems mimic more faithfully the in vivo microenvironment in terms of cell–cell interactions, matrix deposition, nutrient and oxygen gradients and thus better recapitulate features of micrometastases or intervascular tumour regions. The aim of this study was to establish MTS-based growth and functional assays for target validation and drug screening.
Methods: A variety of techniques were explored to generate MTS and a standardised method established. MTS were characterised in terms of: (i) growth kinetics, (ii) cell viability, (iii) protein expression, (iv) migration on extracellular matrix proteins and endothelial monolayers, (v) invasion into MatrigelTM and (vi) co-culture with embryoid bodies to model invasion/angiogenesis. All assays were in microplate format (96 well) except migration (48 well). Highly malignant human glioblastoma (U87MG) and breast carcinoma (MDA MB 231) cells were selected and treated with the HSP90 inhibitor 17-AAG to exemplify assays utility.
Results: Our standardised MTS microplate method generates a single spheroid per well. MTS are highly reproducible in size and easy to handle. Acquisition of sequential images of MTS cultured for up to 14 days for

growth kinetics and 48−72 hours for functional assays, allows analysis of several parameters simultaneously. U87MG and MDA MB 231 show normal growth kinetics which are inhibited in a dose-dependent manner using 17-AAG. Both models also showed significant migration and invasion mirroring their in-vivo behaviour. Furthermore, MDA MB 231 MTS migration and invasion was inhibited with sub GI50 doses of 17-AAG. Direct comparison of cell viability (CellTiterGlo assay) following drug treatment in 2D and 3D showed that both tumour models are more resistant to 17-AAG in 3D. A method to analyse protein expression was optimised using the MDA MB 231 MTS and characteristic client depletion following HSP90 inhibition was demonstrated.
Conclusions: We provide evidence that the MTS model and derived functional assays are modifiable for a relatively high-throughput format. The reproducibility and simplicity of the assays make them attractive options for drug discovery projects potentially increasing the strength of prediction of in vivo activity.

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Novel Mcl-1 inhibitors for pancreatic cancer therapy

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The anti-apoptotic myeloid cell leukemia protein Mcl-1, a member of the Bcl-2 family proteins, has emerged as a promising therapeutic target. It was demonstrated that Mcl-1 is an important survival factor for pancreatic cancer cells; its down-regulation with siRNA for example, enhances the induction of apoptosis, chemosensitivity and radiosensitivity of pancreatic cancer cells. Therefore targeting Mcl-1 to overcome apoptosis resistance is an important strategy for the development of new drugs to treat pancreatic cancer.
Through high throughput screening approach we have identified several promising lead compounds which bind to the BH3 binding site in Mcl-1 selectively over Bcl-2 and Bcl-xL, and disrupt interactions between Mcl-1/Bid BH3 peptide and Mcl-1/Bax protein. We have synthesized several analogues and established initial structure–activity relationships. The novel synthetic analogue E288 is the most potent compound with K_i = 400 nM, 10 times more potent than the identified hits. NMR spectroscopy demonstrates that E288 binds to the same BH3 domain of Mcl-1 as the Bim BH3 peptide and antagonizes Mcl-1, inhibiting cell growth and inducing apoptosis in pancreatic cancer cells with high Mcl-1 levels (BxPC-3 and Panc-1) in a time and dose-dependent manner. By using murine embryonic fibroblasts (MEFs), wild type and deficient in both Bax and Bak (double knock out), it was demonstrated that the cytotoxic activity and induction of apoptosis by several analogues, depend on Bax and/or Bak, suggesting that they function as BH3 mimetics. Furthermore, the observed induction of apoptosis was Mcl-1 dependent demonstrated through applying siRNA approach, where the transient suppression of Mcl-1 abrogated E288 mediated apoptosis in both BxPC-3 and Panc-1 cell lines.
Collectively, these findings provide good promise for further chemical modifications of this compound and further optimization toward developing a new class of anticancer drugs, Mcl-1 inhibitors.

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Combination drug screening at the NCI

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A hurdle in selection of combinations to test in clinical trials is the complexity of signaling networks, feedback loops, and incomplete knowledge about how these are affected by the myriad genetic and epigenetic variations present in cancer. As a pragmatic approach to overcoming the challenges in choosing drug combinations, we have recently initiated an *in vitro* combination drug screen that accomodates testing of rationally designed choices, but also allows for serendipity. The screen utilizes 3 cell lines, chosen from the NCI-60 panel for diversity of their molecular characteristics. The non-small cell lung cancer cell line A549 is near triploid, with mutations in CDKN2A, KRAS and STK11. HCT-116, a colon cancer cell line has a nearly normal 2N karyotype, with microsatellite instability, and mutations in BRCA2, CDKN2A, CTNNB1, FGFR2, KRAS, MLH1 and PIK3CA. The final line in the screen is the prostate cancer cell line PC-3, which is a near tetraploid with many chromosomal rearrangements, and is mutant for TP53 and PTEN. Agents being considered for combination trials (test agents) are assayed in each of these 3 cell lines against a panel of well characterized agents (modifier agents), including recently approved kinase inhibitors and conventional cytotoxic agents. Cells are exposed to drugs for 3 days at 3 concentrations of both the “test” agent and each modifier agent, yielding a 3×3 concentration matrix. A Wilcoxon statistic is used to test the hypothesis that the growth inhibitory activity of the combination is better than that expected if the 2 single agents are independent and additive. The screen has identified a number of promising combinations, including some that would not have been predicted.

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Role of the epithelial phenotype in the sensitivity of pancreatic and breast cancer cell lines to Irvalec; in vitro synergism of the combination with gemcitabine

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Background: Irvalec is a novel marine-derived cyclic peptide belonging to the Kahalalide family of compounds, currently in phase II clinical trials. Epithelial–mesenchymal transition (EMT) is a biological process defining progression from polarised epithelial phenotype to a mesenchymal phenotype, which is distinguished by fibroblast-like features. We have studied the role of EMT markers in the sensitivity to Irvalec in a panel of pancreatic and breast cancer cell lines. Furthermore, we analyzed the combination of Irvalec with gemcitabine, the most widely used chemotherapeutic drug in pancreatic cancer, in the pancreatic cancer cell lines.
Material and Methods: Six pancreatic (BxPC-3, HPAC, AsPC-1, CFPAC-1, PANC-1, MIAPaCa-2) and five breast cancer cell lines (SK-BR-3, BT-474, MDA-MB-468, MCF7, MDA-MB-231) were obtained from the ATCC. Cell viability was measured by a cristal violet assay after treatment for 72 h. Protein expression levels of different EMT markers (E-cadherin, β-catenin, snail, twist-1, slug and vimentin) were analyzed by immunohistochemistry, immunocytochemistry and western blot. The combination of Irvalec and gemcitabine was analyzed using the median effect method of Chou and Thalalay using Calcusyn software program.
Results: All cell lines were tested with Irvalec. IC50 ranges were 0.06−8.7 uM and 0.1−6.5 uM for the pancreatic and breast cancer cell lines, respectively. The most sensitive Irvalec cell lines exhibited an epithelial phenotype (high E-cadherin, low vimentin and high twist-1 expression), whereas the mesenchymal phenotype was observed in the least sensitive cell lines. The potential synergism of the combination of Irvalec with gemcitabine was also evaluated in the panel of six pancreatic cancer cell lines, after treatment for 72 h with the different drugs, as single agents or in combination. The combination of Irvalec and gemcitabine had a synergistic effect at high doses (IC90 concentrations) in all pancreatic cancer cell lines tested, whereas at IC70 concentrations synergism was observed in the three most sensitive cell lines (CI values = 0.79, 081 and 0.85 for BxPC-3, CFPAC-1 and AsPC-1, respectively).
Conclusions: Sensitivity of pancreatic and breast cancer cell lines to Irvalec positively correlates with an epithelial phenotype. The *in vitro* synergism of the combination of Irvalec and gemcitabine provide a rationale for further development of this combination.

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Lithocholic acid competitively inhibits EphA2–ephrinA1 binding: pharmacological and structural considerations

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Background: Eph–ephrin system plays a central role during multiple morphogenetic processes and recent data suggest that, in a large variety of human cancers, up-regulated expression and/or de-regulated function of Eph–ephrin system may promote tumorigenesis and the development of a more aggressive and metastatic tumour phenotype.
In particular EphA2 upregulation is correlated with tumour stage and progression and expression of EphA2 in non-transformed cells induces malignant transformation and confers tumorigenic potential. Based on these evidences our aim is to develop small molecules able to modulate EphA2–ephrinA1 activity.