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New biological assay to evaluate the pharmacodynamic effects of rapamycin in cancer patients

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Rapamycin is a natural macrolide antibiotic with anti-tumor properties which inhibits the kinase activity of the mammalian target of rapamycin (mTOR) resulting in inhibition of the translational regulators p70S6 kinase and 4E-BP1. The clinical development of rapamycin and analog agents have been optimize by incorporating biological correlative endpoints to guide dose selection and, eventually, to predict patients outcome. Previous studies have shown that inhibition of p70S6 kinase in PBMCs can be used to determine the pharmacodynamic effects of rapamycin, and to establish a relationship between dose and plasma concentration of the agent that can be used as a predictor of treatment efficacy. In this study we have developed a new biological assay to determine the biological effects of rapamycin in patient's plasma.
Standard curve samples were prepared by diluting rapamycin in cell culture medium, and a linear dose related inhibition of the phosphorylation of the ribosomal p70S6 kinase by the agent was detected at a range between 0 to 700 pM, in presence of 1% normal human plasma, in the flasks containing Raji's B lymphoma cell culture. Patient plasma samples were equally diluted in cell culture media to maintain 1% plasma/flask. Protein extracts from cells were immunoprecipitated with p70S6 kinase antibody, 32P-labeled kinase assay was performed to the immunoprecipitated fractions, and kinase activity measurements of the samples were made by scintillation counting. We also performed a western blot analysis for the dose-response effect of rapamycin on Raji's protein extracts and looking for both phospho and total p70 S6 kinase to validate the accuracy of our bioassay.
In conclusion, we have developed a biological assay to measure biological effects of rapamycin in plasma that can be used to predict treatment efficacy in clinical trials.

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Dielectrophoresis (DEP) as sorting technology for microdiagnosis in breast cancer

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Background: Recent studies have demonstrated the existence of breast cancer subtypes as identified by gene expression profiling (Perou et al, Nature 2000) and postulated the existence of a breast cancer stem cell population (Al Hajj et al, PNAS, 2003). These data suggested a critical role for enrichment techniques that would allow for a detailed identification and analysis of these various breast cancer cell populations. Dielectrophoretic (DEP) forces are characteristic cell properties that occur when a cell is exposed to a non-uniform electrical field. We compared two different techniques, dielectrophoresis fluid-flow-fractionation (DEP-FFF) and superparamagnetic beads assay used to enrich breast cancer cells from specimens collected with a fine-needle aspiration biopsy (FNAB) in an *in vivo* model of breast cancer.
Methods: Three sets of animals (5 mice for each set) were evaluated in a total of eight experiments. Breast cancer cells (MDA-435) were grown, subsequently injected above the rear flank of female athymic nude mice and, approximately 3 weeks after the implant an FNAB of the implanted tumor was performed. A pathologist evaluated the cellularity of the specimen and if considered adequate two paired samples were distributed for a) DEP-FFF; b) Superparamagnetic beads assay. *DEP-FFF methods*: Cells were counted and then suspended in electrosmear buffer suspension at a known concentration. Between 0.5 to 1.2×10^6 total cells were loaded into the electrosmear chamber at the low frequency end and were allowed to relax for 5 min. Using a syringe pump, cells were then flowed over the electrosmear slide from the low frequency (20KHz) end at a flow rate of 100 μl/min towards the high frequency end (1 MHz).
Results: Eight different FNA samples were obtained and electrosmear and superparamagnetic experiments were run as soon as the samples arrived. Time between harvest and electrosmear analysis varied from 2 to 8 hours. Microscopic examination of the samples following electrosmear preparation and staining revealed that all samples comprised mixtures of tumor cells and RBC. A separation between cancer cells and RBCs was observed in all specimens processed with DEP/FFF (100%). Positive selection with Anti-HEA antibodies with superparamagnetic assay was observed in only 1 (12.5%) of the total cases.
Conclusions: The study demonstrated that DEP/FFF is superior to the superparamagnetic sorting technique when applied to microscopic specimens collected by FNAB. DEP/FFF represents an innovative technology with the potential of sorting and enriching human samples collected with minimally invasive techniques (e.g. FNAB and ductal lavage).

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Correlation between effects of PTK787/ZK 222584 on the interstitial fluid pressure of rat mammary tumours and their vasculature measured by contrast-enhanced MRI

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PTK787/ZK 222584 (PTK/ZK) is a novel oral angiogenesis inhibitor that targets all VEGFR tyrosine kinases (VEGF-R1, -R2, -R3) which contribute to tumour angiogenesis and metastasisis*. Anti-angiogenic activity has been confirmed in the clinic by dynamic contrast enhanced MRI (DCE-MRI). Compared to normal tissues, solid tumours have a raised interstitial fluid pressure (IFP) due to low lymphatic drainage, high vessel permeability and poor perfusion. We compared the effects of PTK/ZK on IFP and DCE-MRI measured parameters.
BN472 rat mammary carcinomas were grown orthotopically in syngeneic rats. Tumours of >0.5 g were studied by insertion of a needle (WIN method) to measure IFP and non-invasively by DCE-MRI using GdDOTA and Endorem to measure tumour vascular permeability (VP), interstitial leakage space (LS), blood inflow (BFI) and relative blood volume (rBV). Data are summarised as mean±SEM with significance set at $p<0.05$.
Daily treatment (30–200 mg/kg po) for 7 days inhibited tumour growth (max T/C=37%) and decreased the IFP (max: 37±12%) dose-dependently; these effects were significantly correlated (r=0.62, p=0.002). One dose of PTK/ZK (100 mg/kg po, daily) was used to compare changes in IFP with those in the DCE-MRI measured vascular parameters after 3 days treatment. PTK/ZK significantly inhibited tumour growth (T/C=53%), and decreased LS compared to baseline (22±4%, $p<0.001$). There was a trend for the rBV to decrease (11%), and this was significantly different to the change in rBV for vehicle-treated animals (+8%). Other DCE-MRI parameters were not affected. Vehicle treatment had no significant effects compared to baseline for DCE-MRI or IFP. The resting (pre-treatment) IFP correlated significantly with rBV (r=0.44) and BFI (r=0.46). After PTK/ZK treatment, the decrease in IFP (17±4%, p=0.006) showed a significant positive correlation with the decrease in rBV (r=0.71), but BFI and rBV were significantly *negatively* correlated ($r = -0.78$). VP and LS did not correlate significantly with IFP either pre- or post-treatment.
An IFP decrease may be a biomarker for tumour response to PTK/ZK. The decrease in IFP can be detected as a decrease in rBV which may, paradoxically, reflect improved tumour perfusion. Thus, an early effect of anti-angiogeneic therapy can be 'normalisation' of tumour vasculature which could aid combination with cytotoxic therapy by increasing drug uptake.
*PTK/ZK is co-developed by Novartis and Schering AG, Berlin.

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IC50 determination for receptor-targeted compounds and downstream signaling

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Receptor tyrosin kinase inhibitors (TKI) have been widely used for targeted therapy in cancer. These drugs inhibit the phosphorylation of tyrosin kinases at the receptor level as well as downstream signaling pathways such as MAP Kinase and PI3K-Akt pathways, therefore inhibiting cell proliferation and tumor growth. Further development of TKIs targeting the EGFR family is ongoing. We herein describe a streamlined approach to identify TKI by analyzing multiple signaling pathway components in multiplexed assays.
We used MCF7 cell line as a model to study the effect of 12 lead tyrosine kinase inhibitor candidates (A1-A12) on MAP Kinase and PI3K-Akt pathways. MCF-7 cells were treated with different doses (0.1 nM – 10 μM) of the drug for 1 hour followed by HRG stimulation for 5 min. Cells were immediately lysed and assayed for Her2 and Her3 phosphorylation, Her3-PI3K, Her3-Shc, Akt and Erk phosphorylation in multiplexed proximity-based eTag assays.
Among the 12 TKI candidates tested, four compounds exhibited IC50s for Her2 Phosphorylation in sub-nanomolar range, while others show

higher IC50s in the range of 5–200 nM. Three out of the four candidates inhibited Her3 phosphorylation with IC50s of 0.03, 5.3, and 12 nM. The most potent candidate showed similar IC50s (~0.03 nM) for Her2 and Her3 phosphorylation. Moreover, it inhibited both the Akt and Erk phosphorylation with IC50s at ~0.1 nM. The inhibition of Her3-PI3K complex was also detected with IC50 at 64 nM. This compound likely possesses functional efficacy in inhibiting cell proliferation and possibly tumor growth in appropriate human xenograft models provided that it has favorable pharmacokinetic properties. Another compound showed 2 logs higher IC50s for Her3 (5.3 nM), Akt (3.5 nM), and Erk (1.9 nM) phosphorylation compared to Her2. It therefore may not be as potent as compound 4 in inhibiting cellular growth. The other 2 compounds had IC50s of 0.097 nM and 0.086 nM, respectively, for Her2 phosphorylation.
In summary, we demonstrated that multiplexed eTag Assay system for receptor dimerization, phosphorylation, and signaling pathways could provide a unique robust tool for the screening of cancer drug candidates in a fast, reliable, and efficient manner as compared to other currently existing methods.

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Differential Her family receptor dimerization and downstream signaling in cancer cell lines

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Her receptors are validated targets for cancer therapies in solid tumors. The efficacies of targeted therapeutics vary in different types of cancer as well as from patient to patient. The downstream signaling mechanisms for these receptors have been well characterized. However, the cross-talking between the receptor signaling pathways warrants comprehensive analysis of downstream signaling in each cancer cell line or clinical sample to determine the activation status that leads to perpetual cell proliferation and survival. We developed multiplexed proximity-based eTag assays for Her family receptor dimerization and signaling phosphorylation to streamline analysis of in vitro or in vivo models of cancer.
Cancer cell lines with different expression levels of Her1, 2 and 3 receptors were analyzed for receptor dimerization and downstream signaling pathway activation. These cell lines were MCF7, SKBR3, MDA-MB-468, 22RV1, A431, BT474, Clau6, MDA-MB-231, A549, ZR-75–1 and BT-20. They were stimulated for 10 min with different doses (0–100 nM) of HRG or EGF, followed by immediate lysis. The lysates were analyzed with four proximity-based multiplexed eTag assays as follow: Multiplex I for Akt, Erk, JNK, P38; Mutiplex II for FAK, MEK, Stat3, BAD, and RSK; Multiplex III for Her3/2, Her3/1, Her3-PI3K, Her3-SHC, Her3 phosphorylation; and Multiplex IV for Her2/3, Her1/2, Her2-PI3K, Her2-SHC, and Her2 phosphorylation
The results showed that the MAP kinase pathway was activated in all cell lines stimulated with EGF or HRG. We detected the Her2-SHC and Her3-SHC complex formation concurrent with Mek, Erk, Rsk, and BAD phosphorylation, a linear cascade of MAP kinase activation. The induction levels vary from 2 to10 folds. Her3-PI3K and Akt activation varied in different cell lines; they were detected in MCF7 treated with HRG. The activation of Her3-PI3K pathway in HRG-stimulated MCF7 was related to Her2/3 heterodimerization. The Her2-PI3K complex detected in HRG-stimulated MCF-7 cells likely represents an indirect interaction via Her3 in the HRG-induced Her2/3 heterodimer. FAK is strongly down regulated by EGF in BT-20 and A431 cells that express high levels of Her1. Stat3 was strongly phosphorylated following EGF stimulation in cell lines expressing high levels of Her1, such as MDA-MB-468, A431, and BT-20.
In summary, we validated the multiplexed eTag assay system as unique robust tool for the receptor dimerization and signaling pathway profiling. The current analysis demonstrated unique signaling patterns that are related to different receptor expression of EGFR, Her2 and Her3 in various cancer cell lines.

Regulatory affairs

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The cancer therapy evaluation program, National Cancer Institute initiative to enhance combination investigational agent clinical trials

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The Cancer Therapy Evaluation Program (CTEP) of the Division of Cancer Treatment and Diagnosis, National Cancer Institute is committed to facilitating preclinical and clinical studies involving the combinations of anticancer investigational agents originating from more than one pharmaceutical collaborator. CTEP has 150 active Investigational New Drug applications (INDs); this puts CTEP in a unique position to facilitate combinations of biologics and drugs for multiple therapeutic target types. All of the collaborative clinical agreements between CTEP and pharmaceutical or biotechnology collaborators contain provisions to allow for mutually agreeable combination studies, both preclinical and clinical, sponsored by the NCI without additional agreements between the collaborators or CTEP. To expedite the initiation of such studies, a modification of the Intellectual Property Option to Collaborator (the Option) has been finalized which provides all collaborators contributing an agent for a combination study with a non-exclusive royalty free license to any invention that might arise using the combination. Furthermore, this same option applies to preclinical studies designed to provide data in support of a clinical trial. The provisions for the sharing of data between collaborators have also been updated. Thus, the need for collaborators to negotiate cumbersome intellectual property or data sharing agreements prior to approving such studies has been eliminated. Such arrangements have led to the initiation of approximately two dozen investigational agent combination protocols and there are currently an equal number in preparation or in review. More detailed information on this and on other initiatives to enhance the initiation of clinical trials will be presented.

Drug delivery

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Phase I and pharmacokinetic (PK) study of OSI-7904L in combination with Cisplatin (CDDP) in patients with advanced solid tumors

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OSI-7904L is a liposomal formulation of a potent non-competitive thymidylate synthase inhibitor (TSI) that does not require polyglutamation for activity; the parent drug was previously tested as 1843U89. This formulation increases plasma residence and offers superior preclinical antitumor activity compared to parent drug or 5-FU. The minimally overlapping toxicity profiles of OSI-7904L and CDDP and the additive antitumor activity seen when platinum analogues were combined with OSI-7904L in xenograft studies provided the rationale for this phase I study. This evaluation is designed to determine the maximum tolerated dose (MTD), dose limiting toxicities (DLT) and PK profile of the combination. The order of dosing is based on a suggestion of sequence-dependent efficacy observed in xenograft models. CDDP is administered via 2 h IV infusion followed by OSI-7904L given IV over 30 minutes; both given every 21 days. To date, 11 pts have been treated (6M/5F), median age 53 (range 39–84) and tumor types: cholangial (3), colorectal (2), pancreas (2), renal, head & neck, breast and mesothelioma (1 each). All except one pt received prior chemotherapy with a median of 2 regimens (range 0–6). OSI-7904L/CDDP doses in mg/m^2 (no. pts/cohort) were: 6/60(4), 9/60(3), 12/60(4). A total of 30 cycles have been given, median 3/pt (range 1–6). Mild to moderate toxicity was observed up to 9/60 mg/m^2 including fatigue, nausea, vomiting, anorexia, diarrhea, mucositis and rash. DLT was observed in 2 of 4 pts at 12/60 mg/m^2. One pt experienced grade (G) 3 rash, G3 hyperbilirubinemia, G3 anemia, G3 thrombocytopenia, G4 febrile neutropenia and G4 mucositis which proved to be fatal; the other reported G3 nausea and vomiting despite adequate treatment and G3 ileus. The MTD has been exceeded. Therefore, the 9/60 mg/m^2 cohort is being expanded. A PR has been confirmed in a pt in the 6/60 mg/m^2 cohort with refractory breast cancer who had prior TSI exposure (5-FU and capecitabine). One pt with cholangiocarcinoma has ongoing stable disease after 16 weeks. PK data indicate biphasic plasma clearance of total OSI-7904L with a median terminal half-life of 77.8 h. Cmax values increased linearly with dose. Substantial inter-patient variability was observed in AUC for each dose group. There was no apparent increase in the free platinum AUC with increasing OSI-7904L dose. 2′-dU levels as well as baseline homocysteine and TS genotype samples are being analysed. In conclusion, this schedule has a toxicity profile similar to other TSI/CDDP combinations. Accrual continues with further analyses of PK/PD information.

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Endothelin-1 antagonist selectively modulates tumor blood flow and potentiates responses to both chemotherapy and radiotherapy

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Although derived from the host tissue, the tumor vasculature is under the influence of the tumor microenvironment and needs to adapt to