and is frequently dysregulated in tumor cells. Two distinct mTOR signaling complexes have been identified: mTORC1 (mTOR-raptor) and mTORC2 (mTOR-rictor). Rapamycin, a non-ATP competitive inhibitor of mTORC1, effectively blocks phosphorylation of mTORC1 substrates, is anti-angiogenic, and inhibits cell proliferation. However, in some solid tumor types, phosphorylation of Akt (a substrate of the mTORC2 complex and key regulator of cell survival) is actually elevated post rapamycin treatment. Hence, inhibitors of both mTORC1 and mTORC2 may have distinct mechanistic and phenotypic effects in tumor cells compared to rapamycin.
Methods and Results: We identified a series of mTOR inhibitors via high-throughput screening and chemical optimization, using *in vitro* and cellular assays to monitor mTOR activity. These compounds are potent (*in vitro* IC50 < 10 nM), ATP-competitive and highly selective for mTOR compared to PI-3 kinase and a diverse panel of protein kinases. Cellular assays demonstrate that these compounds inhibit phosphorylation of the mTORC1 substrates p70 S6 kinase and eIF4E binding protein 1 (4E-BP1), and of the mTORC2 substrate Akt, leading to G1 cell cycle arrest and inhibition of tumor cell proliferation. Selected compounds from this series are orally bioavailable, and *in vivo* pharmacodynamic analysis following oral administration in mouse xenograft tumor models produces dose-dependent inhibition of mTOR substrates, induction of apoptosis and tumor growth inhibition at well-tolerated doses. In contrast, the effects of selective mTORC1 inhibition by rapamycin lead to potent cytostatic effects in xenograft tumors but little apoptotic cell death, consistent with the maintenance of mTORC2/Akt-mediated survival signals in these tumors.
Conclusions: mTOR-selective small molecule inhibitors were discovered that demonstrate potent *in vitro* and cellular activity, oral bioavailability and strong anti-tumor activity at well-tolerated doses. These data indicate that selective inhibition of both mTORC1 and mTORC2 may have significant utility in cancer therapy.

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Dependence on PI3K and RAS–RAF pathways drives the activity of the combination of RAD001 and RAF265, a novel inhibitor of the RAF-MAPK pathway

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Background: PI3K-Akt-mTOR and Ras-Raf activation can induce cellular immortalisation, proliferation and resistance to anticancer therapeutics such as EGFR inhibitors or chemotherapy. Our goal was to assess the consequences of the inhibition of these two pathways in tumor cells with activation of either Ras-Raf, PI3K-Akt, or both pathways. We investigated whether the combination of a novel Raf inhibitor, RAF265, with an mTOR inhibitor, RAD001, could lead to enhanced anti-proliferative and cytotoxic effects in vitro.
Material and Methods: We used A549 (Kras mutant, low pTEN), HCT116 (Kras, PI3KCA mutant), HT29 (Braf V599E mutant), and MDAMB231 (Kras, BRAF G463V mutant) cell lines. Immunobloting was performed to evaluate inhibitor activity and Akt phosphorylation. Cell proliferation was assessed by clonogenic assay. MTT assay and Bliss additivism model was used to classify the effect of the combination on cell viability.
Results: A decrease in pS6 and pMEK was found after treatment of all the cell lines with RAD001 and RAF265, respectively. Exposure to RAD001 was associated with an increase of pAkt in A549, HT29, and MDAMB231 cell lines. In HCT116, HT-29 and MDAMB231 cells, RAD001 alone (0.1−10 nM) failed to decrease cell viability while in A549 cells, RAD001 alone achieved a decrease of 30% in cell viability. In HT29 and MDAMB231 cells, RAF265 alone showed significant activity with IC50 values of 5 to 10 microM while in A549 and HCT116 cells, IC50 was not reached at concentrations of RAF265 up to 10 microM. However, in the presence of 1 nM RAD001, the IC50 for RAF265 was 5 microM in A549 cells, and 10 microM in HCT116 cells. The effect of the combination was found to be additive in A549 cells and synergistic in HCT116 cells. In HCT116 cells, RAF265 exposure led to a marked decrease in phosphorylation of mTOR downstream effectors, such as S6 and 4EBP1. The combination showed no additional activity in HT29 and HCT116 cells. In clonogenic assay, A549 and HCT116 colonies were smaller with the combination than with either drug alone, suggesting increased inhibition of cell proliferation.
Conclusion: Combination of the RAF inhibitor with mTOR inhibitor is an effective strategy to enhance cytotoxic and antiproliferative effects on cells with deregulation of both, Ras-Raf and PI3K-PTEN. The combination is additive in A549 cells and synergistic in HCT116 cells, possibly through a cross-inhibition of 4EBP1 and S6, mTOR downstream effectors.

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Synergistic activity of the mTOR inhibitor deforolimus (AP23573; MK-8669) and the anti-androgen bicalutamide in prostate cancer models

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Background: Deforolimus is a non-prodrug rapamycin analog which specifically and potently inhibits mTOR, a downstream effector of the PI3K/Akt and nutrient sensing pathways. Recently, cross-talk between the PI3K/Akt/mTOR and androgen receptor (AR) signaling pathways has been implicated in progression of prostate cancer from androgen-dependence (AD) to androgen-independence (AI). Androgen-deprivation therapy is often successful initially, but most patients progress to AI demonstrating the need for alternate or combination therapies. In this study deforolimus was evaluated in combination with the anti-androgen bicalutamide in prostate cancer models.
Results: Deforolimus alone was shown to inhibit proliferation of 7 prostate cell lines by 20−60% (maximal inhibition). Sensitivity was associated with loss of PTEN, a negative regulator of the mTOR pathway frequently mutated in prostate cancer. The anti-proliferative activity of deforolimus and bicalutamide, alone and in combination, was determined in 3 cell lines representing different stages of prostate cancer progression. The combination was strongly synergistic in both LNCaP (AD) and C4−2 (AI) cells but only additive in RWPE-1 (normal prostate epithelium) cells. Dramatic growth inhibition was also seen in C4−2 cells under anchorage-independent (soft agar) conditions with colony formation inhibited by ~75% in cells treated with the combination compared to control. To explore the molecular basis of synergistic activity, we compared the pharmacodynamic effects of deforolimus and bicalutamide. p-S6 (Ser235/236) levels were reduced by deforolimus and further reduced by the combination in LNCaP and C4−2 cells, consistent with the synergistic effect of both compounds on cell growth. Deforolimus does not interfere with the clinically relevant inhibition of AR expression by bicalutamide. In LNCaP and C4−2 cells, bicalutamide alone decreased levels of prostate specific antigen (PSA), an AR target gene. In agreement with reports on other mTOR inhibitors, deforolimus increased PSA expression, however treatment with both agents led to an overall decrease in PSA. This suggests that PSA levels are a potential marker of tumor growth in patients treated with the combination. In vivo studies using a prostate cancer xenograft model are ongoing.
Conclusions: These data provide support for the clinical testing of deforolimus in combination with bicalutamide to treat androgen-dependent and -independent prostate cancer.

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Erlotinib, an EGFR kinase inhibitor, sensitizes mesenchymal-like tumor cells to the actions of OXA-01, a selective non-macrolide inhibitor of mTORC1/mTORC2

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Background: Human cancers frequently rely on the cooperative interaction of the Ras-Raf-MAPK proliferative pathway and the PI3K-Akt-mTOR survival pathway to drive and maintain tumorigenesis. One key upstream activator of these pathways is EGFR, a protein frequently overexpressed in cancers of epithelial origin. Several recent reports have confirmed a correlation between expression of epithelial cell proteins such as E-cadherin and sensitivity to selective EGFR inhibitors, such as erlotinib, in multiple tumor types. We have proposed that cells which have undergone an epithelial-to-mesenchymal transition (EMT) and acquired characteristics of mesenchymal cells may become less dependent EGFR signaling pathways and more reliant on alternate signaling networks, and are therefore less sensitive to EGFR antagonists. In order to effectively target both epithelial and mesenchymal-like cells within a tumor, we hypothesized that a combination of targeted therapies may be most effective.
Methods and Results: We tested the combination of the selective, low molecular weight EGFR inhibitor, erlotinib, and OXA-01, a selective, non-macrolide inhibitor of mTORC1/mTORC2 in vitro. The combination synergistically inhibited proliferation in all mesenchymal-like NSCLC and pancreatic cell lines tested, while the effects were additive in epithelial cell lines. Analysis of the downstream effectors of the PI3K and MAPK pathways indicated that erlotinib sensitized cells to the effects of OXA-01, resulting in greater inhibition of phosphorylation of mTOR, Akt, 4E-BP1 and S6. Erlotinib enhanced phosphorylation of IGF-1R, potentially driving

cellular reliance on PI3K signaling. These data are consistent with the hypothesis that mesenchymal-like cells rely more heavily on the PI3K-Akt survival pathway, and that dual inhibition of the MAPK and PI3K signaling networks may drive cellular dependence on Akt-mediated survival signals. Mesenchymal-like cancer cells are further characterized by increased cellular motility which has been linked to the metastatic potential of these cells. OXA-01 inhibited migration of mesenchymal-like cells and inhibited OTC2-mediated regulation of the F-actin cytoskeleton.
Conclusions: These observations suggest that erlotinib may sensitize mesenchymal-like cancer cells to mTOR inhibition. The combination of erlotinib and OXA-01 may be an effective strategy to target heterogeneous tumors, and may inhibit the metastatic potential of mesenchymal tumor cells.

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Dose-finding study of pegylated liposomal doxorubicin (PLD) and the mTOR inhibitor RAD001 (R) in patients (pts) with advanced solid tumors

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Background: R is an orally available mTOR protein kinase inhibitor derivative of rapamycin that has direct effects on tumor cell growth combined with an antiangiogenic mechanism. R enhances the cytotoxicity of cisplatin, paclitaxel, gemcitabine and doxorubicin in preclinical studies.PLD is a pegylated liposomal formulation of doxorubicin with a better tolerability and reduced cardiologic and GI toxicities and alopecia. Aim of the present study is to assess the feasibility of combining R and PLD.
Materials and Methods: A phase Ib study is ongoing in pts with advanced solid tumors. Treatment consists of R given daily continuously at the starting dose of 2.5 mg daily and PLD given at 40 mg/m^2 on day 1 q28 days (1 cycle). The dose of R is escalated according to 3 + 3 cohort design depending on the observed toxicity. Treatment is planned until disease progression or unacceptable toxicity. The dose limiting toxicity (DLT) is assessed on pre-defined criteria during cycle 1. The plasma disposition of PLD is analyzed when given concomitantly (cycle 1) or 48 hrs before R (cycle 2). Tumor response is evaluated every 2 cycles by modified RECIST.
Results: 12 pts were recruited from 2 centers over 6 months, 6 pts in Cohort 1, and 3 in Cohorts 2 and 3, respectively. Median age was 51 (range 27−68 years), ECOG PS was 0−1, the main tumor type was ovarian ca. Preliminary safety data related to cycle 1 are available in the first 9 pts: no DLTs were observed in Cohort 1 (R 2.5 mg/day) but 3 of 6 pts temporarily required R discontinuation due to G2 mucositis (respectively for 5, 8 and 10 days). The administration of R was therefore changed from continuous to intermittent for 21 days q28. Also in Cohort 2 (R 5 mg/day) no DLT were observed and Cohort 3 (7.5 mg/day) is ongoing. The most frequent grade 1−2 treatment related toxicities observed during cycle 1 were: mucositis (78%), skin toxicity (33%), fatigue (22%). No >G1 hematological toxicity was reported. A confirmed partial response was observed in 1 pt with ovarian ca. in Cohort 1. The disposition of D was studied in 6 pts at cycles 1 and 2: total plasma exposure to PLD was unaffected by R.
Conclusions: Preliminary results suggest that the combination of R and PLD is feasible but that a discontinuation interval of R administration is needed to keep an adequate daily dose. No major pharmacokinetic interference of R on PLD disposition was observed.

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The serine 2481-autophosphorylated form of mTOR directly binds the mitotic apparatus to control breast cancer cell proliferation: A new role of mTOR as mitotic checkpoint in cell cycle progression

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Background: The widely accepted role of mTOR is primarily to sense and integrate nutrient and growth factor signals to regulate protein synthesis. Although mTOR is also recognized as a regulator of cell cycle progression and cell proliferation, the molecular mechanisms by which mTOR might mediate these events have been poorly defined. We here sought to analyze whether changes in the sub-cellular compartmentalization of mTOR and of its autophosphorylated form (*i.e.* mTOR^{Ser2481}) occur after acquisition of auto-resistance to the anti-HER2 antibody trastuzumab (Tzb) in breast cancer (BC) cells.
Materials and Methods: Two pools of Tzb-conditioned SKBR3 BC cells optimally growing in the presence of >100 ug/ml Tzb (SKBR3/TzbR POOL1 and POOL2) were obtained by continuously culturing HER2-dependent SKBR3 cells in the presence of high-doses of Tzb for more than 12 months. Changes in the sub-cellular compartmentalization of mTOR/mTOR^{Ser2481} were monitored using a high-resolution, automated confocal imaging system (BD Pathway™ Bioimager).
Results: A homogenous cytoplasmic/perinuclear distribution of total mTOR was observed in Tzb-sensitive BC cells. Perinuclear expression was somewhat increased in a dotted-manner in Tzb-resistant pools. Surprisingly, mTOR^{Ser2481} was found to be massively accumulated within nuclear dots displaying dynamic expression during the M phase. mTOR^{Ser2481} dots showed a close association near and between separating chromosomes and also decorating the contractile ring in BC cells undergoing cytokinesis. In the case of BC cells at the anaphase stage of mitosis, eye-catching mTOR^{Ser2481} dots could be seen symmetrically splitting in the region of the mitotic spindle. The number of immunopositive condensations of mTORSer^{2481} directly related with the percentage of mitotic cells in the absence of Tzb treatment (2-fold higher in Tzb-resistant BC cells). Moreover, the rate of mTOR^{Ser2481}-immunolabeled dividing cells was significantly decreased in Tzb-treated SKBR3 parental cells whereas it remained unaltered in Tzb-treated SKBR3/TzbR POOLs.
Conclusions: mTOR-dependent regulation of the rate of cell cycle progression has been considered a secondary consequence of the mTOR's primary function (*i.e.* to make cycle progression dependent on a sufficient level of cell growth). We now propose that mTOR^{Ser2481} is a novel mitotic checkpoint that directly controls BC cell proliferation through its previously unrecognized capacity to bind the mitotic apparatus.

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Phase II study of MTOR-inhibitor RAD001 and erlotinib for advanced, gemcitabine-refractory pancreatic cancer

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Background: PI3-kinase/Akt pathway is constitutively activated in pancreatic cancer. The sensitivity of pancreatic cancer cell lines to erlotinib may be dependent on inhibition of this pathway. RAD001 selectively inhibits mTOR, a key protein kinase that is activated via the PI3-kinase pathway. Erlotinib and the mTOR inhibitor rapamycin produced a synergistic anti-tumor effect in preclinical studies. Prior phase I study identified both a weekly and daily phase II RAD001 dose when administered with daily erlotinib.
Methods: Forty adult patients with previously treated stage IV pancreatic adenocarcinoma, ECOG PS 0−1 with adequate hematologic, hepatic and renal parameters and measurable disease will be enrolled. Each cycle lasts 28 days and consists of RAD001 30 mg weekly and erlotinib 150 mg once daily. Staging radiological studies are performed every 8 weeks. Pre-treatment tumor biopsy samples are assessed for PTEN, total and activated Erk, Akt, and mTOR expression. Primary study endpoint: 6-month survival. Secondary: Progression-free survival and correlation of biomarkers with outcome.
Results: 13 patients have been enrolled; 10 males, all received prior gemcitabine. A median of one cycle has been administered (range 1−2). There was one grade 5 toxicity, possibly related. Grade 3 toxicities: diarrhea (n = 1), cholangitis (n = 3), fatigue (n = 1). Grade 2 toxicities: pneumonia (n = 2), dehydration (n = 2), nausea (n = 2), mucositis (n = 2), rash (n = 2). Progressive disease occurred in 3, CA 19−9 improvement (<50%) in one. There were 4 hospitalizations, 3 for cholangitis and sepsis. Cholangitis occurred in presence of biliary stents; these patients are now receiving prophylactic quinolones. Interim analysis will be conducted after enrolling 16 patients.
Conclusions: The immunosuppressive properties of RAD001 may predispose patients with biliary stents to cholangitis. These patients can be considered for antibiotic prophylaxis. Updated clinical and correlative data will be presented at meeting.

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Vorinostat significantly enhances the antitumor activity of temsirolimus in renal cancer

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Background: Mammalian target of rapamycin (mTOR) is a critical kinase that is involved in the regulation of protein translation, nutrient uptake and autophagy. mTOR is frequently activated in cancer due to constitutive