is the underlying mechanism of synergism between EGFR/HER2 TKI and 5-FU in HER2-positive cancer cells.

mTOR

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Pharmacodynamics and anti-tumour activity of KU-0063794, a potent and specific inhibitor of mTOR kinase

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Background: The mTOR kinase is a critical regulator of cell growth, receiving stimulatory signals from growth factors via the phosphatidylinositol-3-OH kinase/AKT pathway, and integrating nutrient inputs in the form of amino acid, glucose and oxygen availability. In mammals, there are two distinct and mutually exclusive mTOR complexes, the substrate-defining subunits of which are raptor (the mTORC1 complex) and rictor (mTORC2). mTORC1 complexes are strongly inhibited by rapamycin and its analogues, whereas mTORC2 complexes are not. In some tumours, inhibition of mTORC1 by rapalogues removes a negative feedback mechanism resulting in activation of AKT, and this has been correlated with resistance to these agents. Here we describe the in vivo properties of KU-0063794, a potent and selective ATP-competitive inhibitor that directly targets mTOR kinase.
Materials and Methods: Anti-tumour and pharmacodynamic studies were carried out in immunodeficient rodents bearing either U87-MG or MCF-7 xenografts. Compound was dosed once daily by oral gavage. Pharmacokinetics were measured in plasma and pharmacodynamics in ex vivo tumour tissue by western blotting or immunohistochemistry.
Results: KU-0063794 inhibits phosphorylation of S6ser235/6 and AKTser473, which respectively are downstream targets of mTORC1 and mTORC2, in U87-MG xenografts growing in nude mice, and MCF-7 xenografts growing in severe combined immunodeficient (SCID) mice. Target inhibition correlated with plasma exposure to the drug, and significant inhibition of both targets was sustained for at least 24 h in U87-MG xenografts following an acute 75 mg/kg oral dose. Pharmacokinetic studies showed that exposure to the drug was similar in both mouse strains following an acute 75 mg/kg dose (AUCs = 573.6 and 614.4 mM.h in nude and SCID mice respectively), and exposure in nude mice scaled well with increasing dose (AUCs = 157.6 and 573.6 mM.h following doses of 18.75 and 75 mg/kg respectively). Chronic dosing of up to 75 mg/kg qd KU-0063794 was well tolerated, and resulted in a dose-dependent inhibition of U87-MG geometric mean delta tumour volume of up to 88%. In MCF-7 xenografts, an acute dose of KU-0063794 inhibited AKT, PRAS40 and S6 phosphorylation, whereas an acute dose of rapamycin resulted in activation of AKT, inhibition of S6 and no change in PRAS40 phosphorylation; these data are consistent with inhibition of mTORC1 and mTORC2 by KU-0063794 and inhibition of mTORC1 only by rapamycin.
Conclusions: The data show that inhibition of TOR kinase by KU-0063794 is well tolerated, results in dose-dependent anti-tumour activity, and offers a differentiated, potentially advantageous in vivo pharmacology to rapamycin and its analogues.

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Cellular characterization of OXA-01, a potent and selective dual mTORC1 and mTORC2 kinase inhibitor

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Background: The PI3K/AKT/mTOR pathway is constitutively activated in many human cancers. mTOR is a clinically validated target in this pathway with rapamycin analogs such as CCI-779, RAD001 and AP23573 showing modest anti-tumor responses in patients. mTOR exists as a part of 2 complexes; mTORC1 (rapamycin-sensitive complex in vitro) and mTORC2 (rapamycin-insensitive complex in vitro). Rapamycin treatment of cells primarily inhibits mTORC1, resulting in inhibition of pS6K1 and p4E-BP1. However, in the majority (65%) of cell lines tested, rapamycin treatment also stimulates pAKT (mTORC2 activity), which may antagonize the anti-proliferative (mTORC1 mediated) effects of rapamycin in tumor cells.
Methods and Results: To identify compounds with greater antitumor efficacy than rapamycin analogs, we have generated a series of small molecule inhibitors that selectively inhibit the protein kinase activity of the mTOR catalytic subunit to directly inhibit both mTORC1 and mTORC2. One such selective compound, OXA-01 inhibits both mTORC1 and mTORC2 biochemical activities with IC50 values of 29 nM and 7 nM, respectively (100 μM ATP). In MDA-MB-231 breast cancer cells, this compound completely inhibited phosphorylation of 4E-BP1 and S6. AKT phosphorylation was also completely inhibited by OXA-01 in BT-474 breast cancer cells indicating inhibition of mTORC2 activity in cells. In addition, this compound inhibited proliferation of MDA-MB-231 and BT-474 breast cancer cell lines at concentrations that correlate with target inhibition. OXA-01 also induced apoptosis in a number of cell lines in the presence of 10% FCS as determined by DNA fragmentation ELISA and Caspase 3/7 assays. Oral dosing of OXA-01 at 50 mg/kg in mice resulted in sustained plasma levels significantly greater than the concentration required for cellular activity, and a good correlation was observed between inhibition of tumor phospho-4E-BP1 and tumor growth inhibition in the MDA-MB-231 xenograft model. Daily oral dosing at 75 mg/kg, twice a day for 14 consecutive days resulted in 100% mean tumor growth inhibition over the dosing period.
Conclusions: These data suggest that an mTOR kinase inhibitor such as OXA-01 may provide improved antitumor activity compared to rapamycin analogs.

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A phase I trial evaluating pharmacodynamics of deforolimus (AP23573, MK-8669) delivered orally on multiple dosing schedules

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Background: Deforolimus is a non-prodrug mTOR inhibitor that has shown promising anti-tumor activity when administered intravenously (IV) QDx5 every 2 wks. Rapid, potent and prolonged inhibition of mTOR activity in peripheral blood mononuclear cells (PBMCs) has been observed with this dosing regimen using a pharmacodynamic (PD) assay that measures phosphorylation of the mTOR target 4E-BP1. In a phase 1 trial (Trial 106), oral deforolimus delivered on various schedules demonstrated an activity and safety profile consistent with the IV form. Here, PD activity in this trial was assessed to determine the degree and duration of target inhibition and to compare to IV delivery.
Methods: Trial 106 was a dose escalation trial in adult patients (pts) with refractory or advanced solid tumors. Seven oral dosing regimens were investigated: QDx28, QDx21 Q28D, QDx6 QW, QDx5 QW and QDx4 QW. B.I.D. and loading dose schedules were also explored. Whole blood samples were collected prior to dosing and at up to 8 post-dose timepoints throughout the 28-day cycle, and protein extracts from PBMCs analyzed by Western blot. Levels of 4E-BP1 phosphorylated at Ser65/Thr70 (P-4E-BP1) were normalized to total levels and the median calculated for each dose group.
Results: Evaluable PD data were obtained from 141 of 147 treated pts. Rapid and potent mTOR inhibition was observed in PBMCs from all pts tested, with P-4E-BP1 levels decreasing by 83−93% within 24 hr at all dose levels (10–100 mg). Substantial mTOR inhibition was also maintained throughout the entire 28-day dosing cycle: in all schedules except QDx21 (7 day holiday), >70% inhibition was observed at most timepoints, even 4 days after dosing (QDx4). In the 24 patients dosed with the 40 mg QDx5 regimen, mTOR activity was inhibited by >90% within 24 hr after the first dose and inhibition was maintained at levels >75% throughout the 28-day cycle. These results are comparable to those obtained following IV dosing (3−28 mg).
Conclusions: Rapid, potent and prolonged inhibition of mTOR activity was observed in PBMCs following oral dosing with deforolimus. Based on these findings and previously reported activity and tolerability data, the 40 mg QDx5 regimen has been selected for further evaluation in SUCCEED, a global phase 3 trial of pts with metastatic soft-tissue and bone sarcoma in the maintenance setting.

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mTORC1/mTORC2 selective inhibitors: Identification and characterization of novel small molecules with anti-tumor activity

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Background: mTOR is a key regulator of signaling pathways that control cell growth, proliferation, survival, autophagy and angiogenesis,

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