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Enzastaurin (LY317615.HCl) suppresses signaling through the PKC and AKT pathways, inducing apoptosis, suppressing tumor-induced angiogenesis and reducing growth of human cancer xenografts

J. Graff[1], A. McNulty[1], S. Parsons[1], J. Lewis[1], B. Konicek[1], C. Banks[1], A. Capen[1], L. Douglass[2], J. Carter[2], J. Starling[1]. [1]*Eli Lilly and Company, Cancer Growth and Translational Genetics, Indianapolis, USA;* [2]*Wood Hudson Cancer Labs, Newport, KY, USA*

PKC and PI3 Kinase/AKT pathway activation have been broadly implicated in human cancer development, motility, cell cycle progression, apoptosis, angiogenesis and chemoresistance. The bisindolylmaleimide, Enzastaurin (LY317615.HCl), was developed as an ATP-competitive inhibitor of PKCβ and was advanced to the clinic as an anti-angiogenic. In addition to its robust anti-angiogenic activity, we recently showed that Enzastaurin suppresses signaling through the PKC and AKT pathways, blocking phosphorylation of GSK3β, ribosomal protein S6, AKT and CREB and inducing tumor cell apoptosis. Accordingly, Enzastaurin induces apoptosis in a wide array of human cancer cells and suppresses growth of human cancer xenografts. As in cultured cells, GSK3βser9 phosphorylation is reduced in xenograft tissues and parallels a marked, time-dependent reduction in peripheral blood mononuclear cells harvested from these xenograft-bearing mice.
Enzastaurin has recently advanced to Phase III human clinical trials for the treatment of recurrent Glioblastoma Multiforme and Diffuse Large B Cell Lymphoma. To continue to support the progress of Enzastaurin in clinical trials, we have focused our pre-clinical studies on defining biomarkers for possible patient stratification, refining the mechanism of action and exploring the utility of Enzastaurin in combination with standard oncolytics and newer, targeted anti-cancer agents. Our data now show that Enzastaurin induces apoptosis through both intrinsic and extrinsic cell death pathways. Further, we show that pGSK3βser9 may provide a rational, mechanism-based biomarker for Enzastaurin activity and, possibly, patient selection. Finally, our preliminary data have revealed that Enzastaurin may be effective in combination with standard oncolytics as well as targeted therapies.

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Discovery of a novel anti-tumor agent targeting NF-kB pathway

H. Ochi, T. Yanagi, M. Nakajo, A. Takemiya, M. Mochizuki, H. Oshima, W. Watanabe, T. Mishina, H. Nakamura. *Mitsubishi Pharma Corporation, Pharmaceuticals Research Division, Yokohama, Japan*

NF-κB is a major transcription factor for angiogenesis-related genes (MMP-9 and IL-8) and anti-apoptotic genes. Recent studies suggest that inhibition of NF-κB function could be an attractive target for anticancer drugs, and then some NF-κB inhibitors are currently in development as a new molecular therapeutics. We have synthesized a group of indazole derivatives and evaluated their potentials as anticancer agents. Here we report the discovery and characterization of a novel anti-tumor agent DYA-686, a small molecular weight inhibitor targeting NF-κB pathway.
DYA-686 was found through a screening where inhibition of PMA-stimulated MMP-9 production in immortalized human endothelial cells was assayed by using gelatin zymography. The compound dose-dependently inhibited TNF-α-induced IL-8 production in EBC-1 (a human NSCLC cell line) and pancreatic HPAC cells and suppressed NF-κB-dependent transcriptional activity in HPAC cells by luciferase-reporter assay. The compound also showed direct inhibitory effects on proliferation of various cancer cell lines including EBC-1, HPAC and rat glioma C6 cells. Furthermore, oral daily dosing of DYA-686 to nude mice resulted in inhibition of C6-induced intradermal neovascularization as determined by counting the number of blood vessels by light microscopy and also potently inhibited in vivo sc growth of EBC-1 and C6 tumor xenografts. Whereas DYA-686 showed no inhibitory effects on nuclear translocation of p65 of NF-κB and on the activities of 156 kinases including IKKs in the KinaseProfiler screening, the compound suppressed the expression of p65 in EBC-1 cells on immunoblot analysis.
These results suggest that DYA-686 is an inhibitor of NF-κB pathway with a unique mechanism of action, exerting anti-angiogenic and anti-tumor activities.

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Analysis of small molecule Ras/Raf interaction inhibitors in C. elegans identifies both on-target and off-target activities

A.D. Cox[1], V. Gonzalez-Perez[1], D.J. Reiner[1], C.J. Der[1], V. Khazak[2]. [1]*University of North Carolina at Chapel Hill, Chapel Hill, NC, USA;* [2]*NexusPharma, Inc., Langhorne, PA, USA*

A key goal of anticancer drug discovery is to rapidly uncover and identify both on-target and off-target activities of potential new drugs. To evaluate compounds targeting oncogenic forms of the small GTPase Ras and a major downstream effector, the serine/threonine kinase Raf, we have used the nematode C. elegans to report on the activity and selectivity of putative Ras/Raf interaction inhibitors in vivo. The conserved Ras/Raf/MAPK signaling pathway regulates vulval formation; a gain-of-function mutation in the Ras homolog LET-60 (let-60 gf), similar to the G12V mutation in oncogenic Ras, causes a quantifiable multivulva (Muv) phenotype. Gf mutations in LIN-45 (Raf homolog) can substitute for LET-60. The Muv phenotype can thus be used as a readout for excessive activity of Ras/Raf signaling, and genetic epistasis experiments can determine the level of inhibition for pharmacologically active agents.
Small molecule Ras/Raf interaction inhibitors were identified in a yeast two-hybrid screen, confirmed in vitro, and shown to reverse Ras transformation in cell-based assays. We tested if the inhibitors disrupted the Muv phenotype in let-60-gf worms (activated Ras) but not in lin-45-gf worms (activated Raf). Three different Ras/Raf inhibitors, MCP-110, MCP-116 or MCP-146, caused significant and dose-dependent reductions in the Muv phenotype of let-60-gf worms, whereas the negative control (MCP122) did not. In addition, lin-31(n301) (tissue-specific MAPK pathway transcription factor) worms were resistant.
Interestingly, two MCP compounds also caused a switch from solitary to social feeding behavior that is also seen in loss-of-function mutations in NPR-1, a G-protein coupled receptor (GPCR) that is the worm homolog of neuropeptide Y receptor. These two MCP compounds may thus have additional off-target activity that results in blocking NPR-1, a supposition that is currently being tested. Our results support the notion that off-target activities that would not be uncovered in cell-based assays can be identified using simple in vivo preclinical models. Desirable off-target activity might be developed separately for future use. These studies thus contribute to identifying which MCP compounds to pursue for future development, and further support the use of C. elegans as a tool to demonstrate the potency and selectivity of drugs designed to specifically target the Ras/Raf interaction or other molecularly targeted anticancer candidates.

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mTORC1 inhibition with rapamycin leads to activation of PI3K/AKT signalling via an mTORC2 dependent mechanism in melanoma cells

J. Werzowa[1], B. Pratscher[1,2], D. Cejka[2], H. Pehamberger[1], V. Wacheck[2]. [1]*Medical University of Vienna, Department of Dermatology, Vienna, Austria;* [2]*Medical University of Vienna, Department of Clinical Pharmacology, Vienna, Austria*

Background: The mTOR inhibitor rapamycin has been clinically studied as a novel approach for the treatment of advanced malignant melanoma with only limited success. In cancer cells, inhibition of mTORC1 may lead to activation of AKT. Therefore, we studied the signalling pathways modulated by rapamycin in malignant melanoma and evaluated compounds for combination therapy with rapamycin.
Methods: Mel-Juso and 518A2 melanoma cell lines were assessed for cell viability, cell death, and modulation of the cell cycle. Expression and phosphorylation status of mTOR pathway components were quantified using Western blotting. For combination therapy, rapamycin was combined with the PI3K inhibitor LY294002 and a siRNA directed against rictor.
Results: Rapamycin demonstrated limited anti-tumor activity in the two melanoma cell lines. On a molecular level, rapamycin inhibited phosphorylation of the well-established mTOR targets S6K1 and 4E-BP1, but also led to massive phosphorylation of AKT suggesting the activation of a feedback loop. Interestingly, LY294002 alone also led to enhanced AKT phosphorylation after prolonged treatment. Inhibition of rictor via siRNA transfection led to reduced p-AKT levels in cells that have been stimulated with rapamycin or LY294002 pointing towards a role of mTORC2 in the feedback activation of AKT. Combination of rapamycin and LY294002 resulted in synergistic reduction of cell viability, G1/G0 cell cycle arrest and downstream target phosphorylation. Surprisingly, combination of LY294002 at high concentrations with rapamycin interrupted the feedback activation, whereas combination of LY294002 at lower concentrations did not reduce AKT phosphorylation. PTEN status of the examined cell lines did not influence the AKT feedback loop significantly.
Conclusion: Our data suggest that inhibition of mTORC1 leads to activation of PI3K/AKT in melanoma cells. The mTORC2 protein is

clearly involved in this feedback loop. Combination of rapamycin with the PI3K inhibitor LY294002 showed synergistic activity, which appears to be independent of the feedback activation of AKT. These findings might be of relevance for future therapeutic strategies with mTOR inhibitors in melanoma.

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Histone deacetylase inhibitors abrogate the levels and activity of estrogen receptor (ER) α and histone deacetylase (HDAC) 6 in human breast cancer cells (BCCs)

W. Fiskus[1], A. Mohapatra[1], P. Bali[1], M. Balasis[1], R. Rao[1], P. Atadja[2], J. Wu[1], K. Bhalla[1]. [1]*H. Lee Moffitt Cancer Center, Tampa, FL, USA;* [2]*Novartis Pharmaceutical Corp, Cambridge, MA, USA*

Background: Since estrogen (E2) is mitogenic, depletion of the levels and/or activity of ERα is an effective therapy of ERα+ breast cancers. The heat shock protein (hsp) 90-based super chaperone machine binds and maintains ERα in an active conformation, allowing it to bind E2 and affect transcription of genes, e.g., progesterone receptor (PR) β and the class IIB HDAC6. Hydroxamic acid analogue pan-histone deacetylase inhibitors (HA-HDIs), e.g., LAQ824 and vorinostat, inhibit HDAC6 and induce hyper-acetylation of α-tubulin and hsp90, two known substrates of HDAC6. Hyper-acetylation was shown to inhibit ATP binding and chaperone function of hsp90 toward its client proteins, e.g., Her-2, AKT and c-Raf. Inhibition of hsp90 by HA-HDI led to depletion of hsp90 client proteins. Here, we determined the effect of HA-HDIs on ERα and HDAC6 levels and activity, and on the survival of ERα+ BCCs MCF-7 and BT-474.
Material and Methods: Cells were exposed to LAQ824 (25 to 250 nM) or vorinostat (0.5 to 2.0 μM) for 8 to 48 hours. ERα, HDAC6, p21, PRβ, p-AKT, AKT, c-Raf and p-ERK1/2 levels were determined by immunoblot (IB) analyses. Hsp90 acetylation and binding to ERα was determined by immunoprecipitation (IP) and IB analyses. ERα transactivation was determined by utilizing ER elements bound to luciferase cDNA reporter construct.
Results: Treatment of the ERα+ BCCs MCF-7 and BT-474 with HA-HDI for 8 to 24 hours induced histone acetylation and upregulated the levels of p21, and induced acetylation of α-tubulin and hsp90. Hsp90 acetylation led to decreased binding of ERα to hsp90 and polyubiquitylation of ERα. Depletion of ERα levels by HA-HDI treatment was restored by co-treatment with the 20S proteasome inhibitor bortezomib, indicating proteasomal degradation of ERα. Depletion of ERα levels was associated with abrogation of E2-induced luciferase expression and decline in PRβ and HDAC6 levels. Treatment with HA-HDI also depleted the levels of p-AKT, AKT, c-Raf and p-ERK1/2 levels in MCF-7 and BT-474 cells. This was associated with the accumulation of MCF-7 and BT-474 cells in the G2/M phase of the cells cycle, as well as induced the poly-ADP ribose polymerase (PARP) cleavage activity of caspases and loss of cell survival of MCF-7 and BT-474 cells.
Conclusions: These findings demonstrate that treatment with HA-HDIs abrogates ERα levels and activity, suggesting a potential role in the therapy of ERα positive breast cancers that are refractory to aromatase inhibitors.

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The dual specific Src/Abl kinase inhibitor AZD0530 inhibits in vitro growth and induces apoptosis in non-small cell lung cancer lines

B. Helfrich, B. Frederick, D. Raben, P.A. Bunn. *University of Colorado Cancer Center, Denver, CO, USA*

Background: Overexpression and activation of the non-receptor tyrosine kinase Src occurs in 50–80% of lung cancers. Src is activated by association with EGFR and in turn activates EGFR in a synergistic manner. A relationship between activated Src, cancer progression and metastatic disease has been demonstrated and provides a rationale for evaluating Src targeting agents in non-small cell lung cancer (NSCLC).
Methods: The efficacy of AZD0530, an orally available dual specific Src/Abl kinase inhibitor, was evaluated in a panel of 18 NSCLC cell lines characterized for EGFR mutation status. *In vitro* analyses included MTT assays in comparison with gefitinib, FACS analysis, western immunoblotting, and migration assays.
Results: AZD0530 IC50s were sub-micromolar, indicating physiologically relevant growth inhibition, in 11 cell lines, including 1 wild-type (wt) EGFR line insensitive to gefitinib (Table). Src gene and protein expression (Affymetrix arrays and western, respectively) did not predict sensitivity.
To date, 6 lines with sub-micromolar AZD0530 IC50 have been further analyzed: 4 EGFR mutant lines including H1975, and 2 EGFR wt lines including the line H1703 with gefitinib IC50 > 10 μM. 24-hour treatment with 1 μM AZD0530 induced G1 arrest (increases of 21–32% and 13–23% in EGFR mutant and wt lines, respectively), and western analysis showed reduced p-Src, cyclin D, p-EGFR (Tyr845), p-STAT3, and p-ERK1/2. Boyden chamber migration (1 μM AZD0530, 24 hours) was inhibited 50–60% in EGFR mutant lines H3255 and H1975, and in EGFR wt line H1703. Migration was not inhibited in EGFR wt line H358. FACS analysis demonstrated apoptosis of 33–48% after 48 hours' treatment with 1 μM AZD0530 in lines with activating EGFR mutations.

Cell lines	IC50 (μM)	
	AZD0530	Gefitinib
Activating EGFR mutations (5 lines)	0.036−0.177	0.004−0.018
H1975 (harboring T790M EGFR-TKI resistance mutation)	0.939	8
EGFR wt, gefitinib IC50 < 0.3 μM (4 lines)	0.177−0.740	<0.3
EGFR wt, gefitinib IC50 ⩾ 10 μM (8 lines)	0.72 (1 line)	
	1–3 (4 lines)	⩾10
	7–10 (3 lines)	

Conclusions: In mutant and wt EGFR NSCLC lines, AZD0530 inhibited growth, induced G1 arrest and apoptosis, inhibited downstream signaling through STAT3 and ERK1/2, and reduced migration. These data support a rationale for Src-targeting agents in the treatment of patients with NSCLC. Further assays are planned to investigate invasion inhibition by AZD0530 in this panel of cell lines.

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Inhibition of mTOR and targeting insulin-like growth factor I synergistically enhance taxol-induced cytotoxicity in Her-2 overexpressing breast cancer cells

N. Alami[1], Z. Li[1], L. Shiry[2], B. Leyland-Jones[1]. [1]*McGill University, Oncology, Montreal, Canada;* [2]*Insmed Inc., Richmond, VA, USA*

Background: The ErbB2 (Her-2/Neu) receptor tyrosine kinase is over-expressed in approximately one-third of human breast tumors, and this phenotype correlates with a poor clinical prognosis. ErbB2-overexpression confers resistance to taxol-induced apoptotic cell death through inhibition of p34(Cdc2) activation via ErbB2-mediated upregulation of p21(Cip1). In addition, crosstalk between Her-2 signaling and the insulin-like growth factor-I receptor (IGF-IR) pathway activates the phosphatidylinositol 3′-kinase (PI3K)/Akt and ERK, and the downstream mammalian target of rapamycin (mTOR). The IGF binding protein-3 (IGFBP-3) is an antagonist of IGF-IR signaling. IGF-IR blockade prevents rapamycin (a specific inhibitor of mTOR)-induced Akt activation and sensitizes tumor cells to inhibition of mTOR.
This study was designed to explore the ability of rhIGFBP-3 and rapamycin to enhance paclitaxel efficacy in Her-2 overexpressing breast cancer cells.
Material and Methods: MCF-7/Her2−18 cells were cultured with taxol (1−50 nM) alone or in combination with rhIGFBP-3 (20 μg/ml) and rapamycin (5 & 10 nM) for 72h. Cell survival was measured using the MTT assay. For Western blot analysis, MCF-7/Her-2 18 cells were cultured in serum-free medium for 24h and subsequently treated with IGF-I (50 ng/ml), rhIGFBP-3 (20 μg/ml), rapamycin (10 nM) alone or in combination with taxol (25 nM) for 30min.
Results: Treatment with taxol, rhIGFBP-3 and rapamycin, as single agents, inhibited the growth of Her-2 overexpressing cells in a dose-dependent manner. The triple combination of various concentrations of taxol, with constant doses of rhIGFBP-3 (20 μg/ml) and rapamycin (5 & 10 nM) exhibited greater dose-dependent inhibition of cell proliferation than each agent alone or the respective double combinations. Synergistic interactions were obtained with all the double or triple combinations as evaluated by the isobologram method (CI < 1). Western blot analysis revealed that the combination of taxol, rhIGFBP-3 and rapamycin caused a further decrease of p-Her-2 and the downstream p-Akt and p-MAPK.
Conclusions: The different combinations showed strong synergistic interactions in the Her-2 overexpressing breast cancer cells. Furthermore, we demonstrate that, rhIGFBP-3 abrogated the rapamycin and taxol-induced MAPK activation. Further analysis of the effects of these combinations on mTOR downstream components, 4E-BP1 and S6K1, apoptosis, such as caspase activation, and cell cycle progression are ongoing.