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PTEN target therapy for glioblastoma

H. Kanno[1], S. Yamada[1], N. Kawahara[1]. [1]*Yokohama City University, Neurosurgery, Yokohama, Japan*

Background: Glioblastoma (GBM) is a highly lethal brain tumour present as one of two subtypes with distinct clinical histories and molecular profiles. The primary GBM subtype presents acutely as a high grade disease that typically harbors mutations in EGFR, PTEN and INK4A/ARF, and PTEN plays acritical role in a PI3 kinase/Akt signaling pathway. The potential role of the PTEN-PDZ binding domain in tumor suppression has been reported previously. Here, we synthesized a peptide consisted of PDZ binding domain in PETN conjugated with a protein transduction domain, and examined the potential of the peptide as a tumor suppressor agent targeting PTEN.
Material and Methods: Using a peptide synthesizer, we synthesized a peptide composed of PDZ binding domain in PTEN conjugated with TAT protein as a protein transduction domain. We used 4 glioma cell lines (YKG1, U251, U87, and T98) for this study and then examined PTEN/Akt expressions with western blotting and RT-PCR. The intracellular delivery of the peptide was evaluated using the fluorescence-labeling peptide. Growth inhibition, cell death, metabolic changes, and cell cycle inhibition of the synthesized peptide derived from PTEN morphological changes were examined with cell counting, soft agar colony formation assay, TUNEL assay, BrdU labeling assay, and immunocytohemistry.
Results: The synthesized peptide composed of PDZ binding domain in PTEN conjugated with TAT showed a significant growth inhibitory effect, cell cycle inhibition, induction of apoptosis in glioma cells at 1 to 10 μM in concentration in culture medium. PTEN/Akt expressions in glioma cells were correlated with growth inhibitory effects.
Conclusions: The intracellular delivery of PTEN-PDZ binding domain is a possible useful method as a glioma therapy when PTEN/Akt in cells does not function normally. The peptide composed of PTEN-PDZ binding domain conjugated with TAT is promising as a agent of a molecular target therapy for glioblastoma.

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Chemical proteomics profiling of erlotinib in NSCLC cell lines suggests novel mode of action

A. Augustin[1], J. Lamerz[1], S. Golling[1], H. Meistermann[1], M. Tzouros[1], H. Burtscher[2], O. Spleiss[1], L. Essioux[1], H. Langen[1], B. Klughammer[3], on behalf of Tarceva Clinical Biomarker Subteam. [1]*F. Hoffmann − LaRoche AG, Prlpp, Basel, Switzerland;* [2]*Roche Diagnostics GmbH, Tr-D2, Penzberg, Germany;* [3]*F. Hoffmann − LaRoche AG, Pdeob, Basel, Switzerland*

Recently, it has been shown that NSCLC patients carrying an activating EGFR mutation in their tumour respond especially well to treatment with tyrosine kinase inhibitors like erlotinib and gefitinib (Paz-Ares et al. 2010). Whereas gefitinib does not seem to confer clinical benefit to an unselected patient population (Thatcher et al. Lancet 2005) erlotinib does (Shepherd et al. NEJM 2005; Cappuzzo et al 2010). In order to find out whether the differences seen in the clinical outcome can be attributed to differences in binding capacities of the two molecules to other enzymes than EGFR, a chemical proteomic study was carried out. Erlotinib was first chemically modified and conjugated to Sepharose in order to generate an affinity matrix. Non-small cell lung cancer cell lysates were pre-incubated with erlotinib before fractionation on the immobilized compound. Captured proteins were eluted, separated by SDS-PAGE and identified by nanoLC-MS/MS and protein database mining. Specific interactors were isolated by statistical analysis of the mass spectrometry signals. Besides EGFR, other enzymes were specifically displaced from the matrix by erlotinib. *In vitro* assays showed a differential inhibition of these enzymes between erlotinib and gefitinib. Currently the effect of erlotinib on selected cell lines in general (using e.g. expression profiling among other techniques) and on the downstream signaling of these proteins in particular is under investigation. In addition the cell lines are screened for mutations in the genes coding for these enzymes. Results of these experiments will be reported. In conclusion, this study may lead to the identification of additional tumor markers, which would allow better patient selection for treatment with erlotinib.

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Computational modeling and molecular optimization of stabilized alpha-helical peptides targeting NOTCH-CSL transcriptional complexes

R.E. Moellering[1], Y. Shen[2], O.G. Wiest[2], J.E. Bradner[3], G.L. Verdine[1]. [1]*Harvard University, Chemistry and Chemical Biology, Cambridge, USA;* [2]*University of Notre Dame, Chemistry and Biochemistry, South Bend, USA;* [3]*Dana-Farber Cancer Institute, Medical Oncology, Boston, USA*

Aberrant transcription factor activity is a hallmark of cellular transformation and cancer progression. A notable example is found in the Notch signaling pathway, which is altered in more than 50% of T-cell acute lymphoblastic leukemia (T-ALL) cases as well as numerous other cancers through gain-of-function mutations in *NOTCH1*. Despite the obvious therapeutic potential of directly targeting transcription factor complexes, this class of proteins has been generally regarded as intractable or "undruggable." Employing a novel chemical approach that combined the surface recognition of a protein therapeutic with the synthetic accessibility of a small molecule, we previously reported the design, synthesis and characterization of a "hydrocarbon stapled" peptide inhibitor of the MAML1-NOTCH1-CSL transcription factor complex {Moellering et al., 2009, Nature, 462, 182−8}. As multiple NOTCH isoforms (NOTCH1−4) play non-redundant roles in an array of basal and pathogenic cellular processes, potent and specific inhibition of unique isoforms would be therapeutically desirable.
Toward this goal we have employed a complement of computational molecular modeling, structure-based design and iterative medicinal chemistry strategies to identify more potent and specific inhibitors of NOTCH-CSL complexes. Molecular dynamic MM-GBSA modeling was first used to quantify the contribution of individual MAML1 contact residues as a framework to select optimized binding interactions. The resulting model agreed with the conclusions of our original study and identified numerous hydrophobic residues present in SAHM1 that might be modified to optimize polar and Van der Waals contacts. With these positions in mind we designed a library of stapled peptide analogues with variable natural and non-natural amino acids at these positions. Compounds were assayed for biochemical inhibition of NOTCH complex formation by a novel nanobead-based competition assay in parallel with a NOTCH1-driven reporter gene assay to determine cellular efficacy. From this effort several optimized positions were found to confer increased biochemical and cell-based activity in analogue compounds. Homology modeling of individual NOTCH isoforms identified sites for differential targeting by SAHM peptides as well. Incorporation of optimized residues into larger SAHM analogues increased potency by more than one order of magnitude. Subsequent analysis of target gene repression, cell proliferation and pathway specificity in multiple cancer models supports the development of more potent and specific inhibitors of NOTCH function.

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Use of anti-VEGF therapy and antiestrogens in breast cancer cells: molecular mechanisms involved in response

M.A. Berciano-Guerrero[1], J.R. de la Haba-Rodríguez[1], L. López-Sánchez[1], V. Hernández[1], I. Porras[1], G. Pulido[1], A. Cañas[1], R. López-Pedrera[2], A. Rodríguez-Ariza[1], E. Aranda[1]. [1]*Hospital Provincial de Cordoba, Servicio Oncología Médica. IMIBIC., Cordoba, Spain;* [2]*Hospital Provincial de Cordoba, IMIBIC., Cordoba, Spain*

Background: Much is known about the mechanisms involved in the response to anti-hormonal treatment or those involved in the response to antiangiogenic therapy. It is also known the association between angiogenesis and hormonal status, both in physiological and pathological settings. However, the molecular and cellular mechanisms contributing to the efficacy of combined antiangiogenic-antihormonal therapy in breast cancer are still unknown. This combination is currently in clinical trials, but unfortunately there are scarce preclinical studies contributing to the rationale of combining antiangiogenic and antihormonal therapies.
Aims: To define the mechanisms involved in the response to combined antiangiogenic-antihormonal treatments in breast cancer cells.
Methods: Breast cancer cell lines with different estrogen dependence (MCF-7, BT-474, MDA-MB-231) were subjected to an estrogen gradient (estradiol), and treated with antiestrogens (fulvestrant or tamoxifen) plus bevacizumab (anti-angiogenic). Cellular proliferation and apoptosis were determined using the corresponding kits. Proliferation and survival intracellular signaling pathways, estrogen alpha and VEGF receptors activation and COX-2 expression were analyzed by western-blot using specific antibodies. VEGF-A concentration in culture medium was determined by ELISA.
Results: In estrogen-dependent breast cancer cells (MCF-7 and BT-474) the pro-proliferative effect of estradiol decreased after bevacizumab treatment (table 1).

Table 1

Breast cancer cell line	Treatment[a]			
	E	E+B	E+F	E+F+B
MCF-7 *(ERa+, ERb+)*	100.0±2.9[b]	19.8±1.0	87.6±1.7	20.1±0.2
BT-474 *(ERa+)*	100.0±16.3	36.9±0.6	82.9±6.2	29.2±0.2
MDA-MB-231 *(ERb+)*	100.0±7.7	106.2±2.4	105.1±2.8	98.5±3.6

[a] 44E = Estradiol 100 nM, B = Bevacizumab 200 ug/mL, F = Fulvestrant 100 nM, T = Tamoxifen 100 nM [b] Proliferation 48 hours (%) ±SD.

Furthermore, the combination of the antiangiogenic with an antiestrogen enhanced this antiproliferative effect, that was also related to the reduction in the levels of VEGF-A in the culture medium and to diminished ER alpha phosphorylation. The combined treatment also altered the phosphorylation of Akt and Erk1/2 signaling kinases.

Conclusions: Our results suggest that in estrogen dependent breast cancer cells the anti-proliferative effect of bevacizumab depends on estradiol concentration, that in turn affects VEGF production levels, using a different mechanism to apoptosis. The combination of bevacizumab with antiestrogens enhances this antitumoral effect, altering intracellular signaling pathways of proliferation and survival.

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Radiosensitization by B-Raf inhibitor PLX4720 correlates with genotype status in anaplastic and differentiated thyroid carcinomas

T. Dasgupta[1], S.S. Yom[1], T. Sottero[1], X. Yang[1], L.A. Orloff[2], T.M. Jahan[3], D.A. Haas-Kogan[1]. *[1]University of California San Francisco, Department of Radiation Oncology, San Francisco CA, USA; [2]University of California San Francisco, Department of Otolaryngology, San Francisco CA, USA; [3]University of California San Francisco, Department of Medicine, San Francisco CA, USA*

Background: Undifferentiated or anaplastic thyroid cancers (ATC) comprise only 5% of all thyroid carcinomas in the US per year but account for one third of annual thyroid cancer-related mortality. Treatment is surgical resection with post-operative radiation and chemotherapy, or palliation for patients with unresectable tumors. These modalities prolong survival by a matter of months. B-Raf and its downstream effector MEK are critical to the molecular pathogenesis of ATCs and 24% of ATCs express the B-Raf mutation V600E. The aim of this study was to determine whether the specific B-Raf V600E inhibitor, PLX4720, is a radiosensitizer in ATCs *in vitro*.

Materials and Methods: Cell lines ARO 81−1 (ATC), WRO 82−1 (follicular TC), NPA 87 (papillary TC) and SW 579 (poorly differentiated TC) were sequenced for the B-Raf V600E mutation. IC50 values of PLX4720 with and without varying doses of radiation were determined using luminescent cell viability and clonogenic assays. PLX4720 concentration-dependent expression of MEK and phospho-MEK were determined by western blotting of total cell lysates.

Results: ARO and WRO were heterozygous for B-Raf V600E; NPA 87 was homozygous for B-Raf V600E; and SW 579 was homozygous for wild type (wt) B-Raf genotype. The IC50 of PLX4720 in SW (wt B-Raf) was significantly higher than for the cell lines encoding B-Raf V600E mutations. In the ARO and NPA cell lines, 8 Gy radiation combined with PLX4720 led to a significant decrease in cell viability, an effect not observed in SW or WRO cells. Preliminary clonogenic studies corroborate these luminescent cell viability data. In ARO, WRO and NPA, a concentration-dependent decrease in phospho-MEK expression was observed, though total levels of MEK remained unchanged. In wt B-Raf cell line SW, there was no drug-dependent decrease in phospho-MEK expression.

Conclusions: Inhibitory effects of the B-Raf specific inhibitor PLX4720 in ATC *in vitro* are enhanced by radiation. Inhibitory effects of PLX4720 correlate with B-Raf V600E genotype of various TC cell lines. Concentration-dependent inhibition of MEK phosphorylation by PLX4720 in TC is limited only to cells encoding B-Raf V600E.

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Semi-rational design of β-catenin targeting peptides for the inhibition of Wnt-dependent signaling

T.N. Grossmann[1], J.T.H. Yeh[1], G.L. Verdine[1]. *[1]Harvard University, Chemistry and Chemical Biology, Cambridge, USA*

Wnt-dependent signaling is a conserved signal transduction pathway involved in embryonic development and tissue self-renewal. Deregulation of this pathway is associated with several forms of cancer such as colon cancer, hair follicle tumors and several leukemias.[1] The carcinogenic activation of Wnt-signaling is commonly triggered by the reduced ability of a cell to degrade β catenin, a key component of the signal transduction cascade. Restoring this ability therapeutically would impact cancers that depend on deregulated Wnt-signaling to grow. The manipulation of requisite protein-protein interactions is one approach to interfere with the pathway. Due to large interaction areas however, the precise manipulation is complicated. In principle, an isolated peptide of a protein could be used to inhibit protein-protein interactions. However, such small peptides usually exhibit little or no secondary structure when excised from the stabilizing protein context. The “stapled peptide” strategy in which an all-hydrocarbon cross-link is generated by olefin metathesis of nonproteogenic amino acids is an efficient approach to increase the helical character of peptides to target α-helical binding motifs.[2 4] Unlike their unstapled analogues these hydrocarbon-stapled peptides have shown to be helical, protease resistant, and cell permeable.

Here we report the semi-rational design of stapled α-helical peptides targeting β-catenin. The peptide sequences are derived from α-helical segments of multiple proteins binding to β-catenin. The usage of phage display-based sequence optimization and the incorporation of an all-hydrocarbon cross-link facilitated an increase in affinity to β-catenin by more than two orders of magnitude. In addition, it was possible to render the peptides cell permeable, therefore omitting the use of transfection agents. These investigations were carried out with fluorescein-labeled peptides. In an established Wnt-driven reporter system active peptides have shown robust inhibition of Wnt-mediated transcription.

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Design of a novel covalent EGFR mutant-selective inhibitor

R. Tjin Tham Sjin[1], K. Lee[1], M. Sheets[1], T.St. Martin[1], D. Niu[1], P. Chaturvedi[1], W. Westlin[1], R. Petter[1], M. Nacht[1], J. Singh[1]. *[1]Avila Therapeutics, Cellular and Molecular Biology, Boston MA, USA*

Background: Erlotinib and gefitinib are effective first-line therapeutics for NSCLC patients who harbor activating mutations in Egfr. Unfortunately, most patients relapse due to the emergence and/or acquisition of secondary mutations or amplification of potential escape pathways. A T790M mutation in Egfr is detected in approximately 50% of patients with drug-resistant tumors, which renders erlotinib/gefitinib ineffective. Although irreversible inhibitors in current clinical studies, such as PF299804, BIBW2992 and HKI-272 demonstrate anti- $Egfr^{T790M}$ activity *in vitro*, they have higher affinity for wild-type (WT) Egfr, resulting in dose-limiting toxicity such as diarrhea and skin rash. We have developed a novel covalent irreversible inhibitor that selectively and potently inhibits both $Egfr^{T790M}$ and the initial activating Egfr mutations but, importantly, is WT-sparing. Such a drug has the potential to effectively treat first- and second-line NSCLC patients with Egfr mutations. Further, covalent inhibitors provide many advantages including improvements in potency, selectivity, prolonged duration of action and translational biomarker opportunities.

Material and Methods: Cell lines expressing $Egfr^{WT}$, $Egfr^{D746-750}$ and $Egfr^{T790M/L858R}$ were used to evaluate the activity of Egfr inhibitors in cell proliferation assays and in pEgfr signaling. An Egfr-specific biotinylated irreversible covalent probe was used to determine the correlation between target-site occupancy and inhibition of pEgfr signaling. Washout experiments were performed to assess prolonged duration of action in cells by the covalent inhibitor.

Results: In cell proliferation assays CNX-419 showed a >50-fold selectivity for the $Egfr^{T790M/L858R}$ and activating Egfr mutations over WT and a >10-fold selectivity in pEgfr signaling. The biotinylated covalent probe confirmed a direct correlation between target-site occupancy by the small molecule and inhibition of pEgfr signaling. Washout experiments demonstrated that pEgfr activity continued to be inhibited after compound removal, confirming the compound’s prolonged duration of action.

Conclusions: CNX-419 is a potent irreversible small molecule that is selective for the activating and drug-resistant mutants of Egfr while sparing of WT-Egfr. Development of such a drug should increase the therapeutic window for NSCLC therapy, allowing adequate dosing to inhibit mutant Egfr while avoiding side effects of current therapies caused by WT-Egfr inhibition.