Role of epithelial-mesenchymal transition (EMT) in sensitivity to CNX-2006,a novel mutant-selective EGFR inhibitor which overcomes *in vitro* T790M-mediated resistance in NSCLC

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Background

- EGFR is an established target in advanced NSCLC, and the EGFR tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib have been approved for the treatment of patients harbouring activating-EGFR mutations (1)
- Unfortunately, their efficacy is limited by acquired resistance, caused in ≈50% of patients by the T790M secondary point-mutation (2)
- Other mechanisms of resistance include the EMT (3)
- Novel EGFR inhibitors have been developed with the aim to overcome such resistance (1)
- Emergence of *in vitro* resistance due to T790M amplification has been reported for second-generation EGFR-TKIs (4)
- Therefore we evaluated the efficacy of CNX-2006, a prototype of the novel mutant-selective EGFR-TKI CO-1686, which is currently in a phase I clinical trial in previously treated mutant EGFR NSCLCs

Aim of the study

To characterize the activity of the T790M-mutant selective EGFR-TKI CNX2006 in preclinical models and evaluate the role of EMT in the sensitivity of NSCLC cells to the prolonged treatment with this drug

Methods

- Anti-proliferative activity of gefitinib or CNX-2006 in NSCLC cells was determined by sulforhondamine B assay (5)
- Genetic characterization of cancer cells was performed by PCR amplification of EGFR and K-Ras followed by sequencing of PCR products (6)
- EGFR phosphorylation was measured by Western blot analysis (7)
- Apoptosis was evaluated by Annexin V–FITC and PI staining, assessed by flow cytometry (5)
- Inhibition of *in vitro* cellular migration by CNX-2006 was measured by wound healing assay (5)
- Expression of EMT markers was measured by quantitative real-time reverse transcription–PCR (5)
- PC9GR4 and PC9DR1 were kindly provided by Dr. Jänne, Harvard University, Boston, USA (4)
- *In vivo* experiments were performed in *nu/nu* female mice inoculated subcutaneously with NCI-H1975 cells (5). After tumor establishment, mice were randomized into 3 groups receiving CNX-2006 at 25 or 50 mg/kg or the vehicle (5% DMSO:15% Solutol HS15 in PBS) daily via intra peritoneal injection

References

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4. **Ercan D, et al. Oncogene 2010;29:2346-56**
5. **Galvani E, et al. Neoplasia 2013; 15:61-72**
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Conclusions

- CNX-2006, a potent mutant-selective EGFR inhibitor both *in vitro* and *in vivo*
- *in vitro,* CNX-2006 is active in cells with activating EGFR mutations, T790M mutation, but is less active in $EGFR^{wt}$ cells
- EMT involved in the resistance to EGFR-TKIs in specific clones and further investigation on its mechanisms are warranted
- Modulation of EMT factors (MMP9) may prevent CNX-2006 resistance

Results

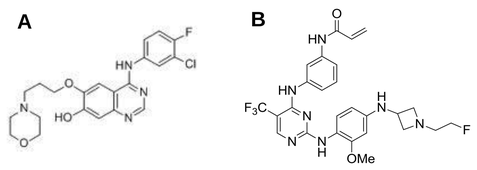

Figure 1. Chemical structure of gefitinib (A) and CNX-2006 (B)

Table 1. Anti-proliferative effect of gefitinib or CNX-2006 in NSCLC cell lines characterized for EGFR and K-Ras status

	EGFR status	K-Ras status	Gefitinib IC_{50} μM	CNX-2006 IC_{50} μM
Calu-6	Wt	Mut (Q61K)	15.3±0.3	3.1±0.2
H522	Wt	Wt	13.7±0.5	1.5±0.1
H1703	Wt	Wt	8.2±0.3	4.3±0.2
SW1573	Wt	Mut (G12C)	5.2±0.2	1.9±0.1
Calu-1	Wt	Mut (G12C)	19.0±0.8	8.0±0.3
A549	Wt	Mut (G12S)	8.0±0.2	2.7±0.1
HCC-827	delE746-A750	Wt	0.010±0.002	0.003±0.002
H3255	L858R	Wt	0.020±0.002	0.006±0.002
PC9	delE746-A750	Wt	0.040±0.003	0.009±0.001
H1975	L858R, T790M	Wt	10.0±0.8	0.072±0.002
PC9GR4	delE746-A750, T790M	Wt	2.1±0.2	0.008±0.002
PC9DR1	delE746-A750, T790M (ampl)	Wt	7.4±0.8	0.006±0.001

Wt, wild-type; Mut, mutant; del, deletion; ampl, amplification

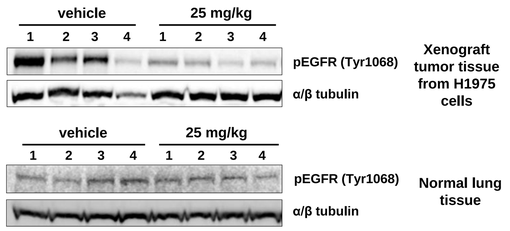

Figure 5. Modulation of EGFR phosphorylation *in vivo*. Twelve mice (n=4 per group) were used to establish H1975 xenografts. When tumors reached an average size of 300–500 mm^3 animals were dosed qd for 3 days and tumor and lung tissue were harvested at 1 hour post-last. The phosphorylation of both $EGFR^{L858R/T790M}$ and $EGFR^{wt}$ was evaluated by Western blot. α/β tubulin was included as a loading control

H1975 cells

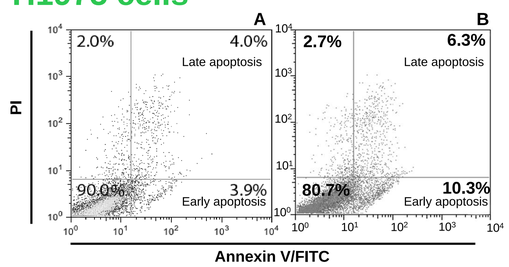

Figure 2. Apoptosis analysis. H1975 cells were treated for 24 hours with CNX-2006 at its IC_{50} (B). Cells were then stained with Annexin V–FITC and PI, and samples were analyzed by flow cytometry. Early and late apoptosis were then detected in comparison to H1975 control cells (A)

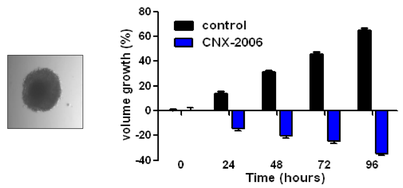

Figure 3. Spheroids growth inhibition. Spheroids of about 300 μm in diameter derived from H1975 cells were treated with CNX-2006 and the volume was measured after 24, 48 and 72 hours

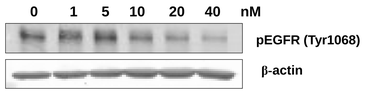

Figure 4. Modulation of EGFR-T790M phosphorylation *in vitro*. EGFR activation was measured in H1975 cells after 1 hour exposure to CNX-2006 as determined by Western blot. β-actin was included as a loading control

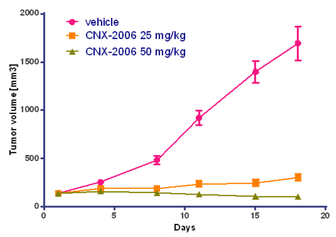

Figure 6. Tumor growth inhibition. Thirty mice (n=10 per group) were used to establish H1975 xenografts. When tumors reached an average size of 100–150 mm^3. animals were dosed qd for 17 days and tumor size was measured twice per week. The resulting tumor volume data was plotted as mean ± SEM

H1975 cells resistant to CNX-2006

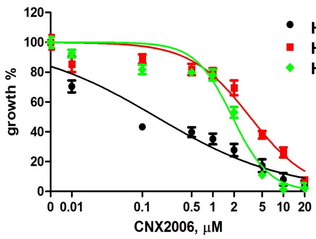

Figure 7, CNX-2006 resistant clones. Clones resistant to CNX-2006 were generated starting from both H1975 and PC9GR4 cells (data not shown), by exposing the cells to increasing drug concentrations. 30-fold resistant clones, growing in CNX-2006 concentrations 16-20 times the initial IC_{50}s were obtained. This resistance was retained for at least 3 months after drug removal.

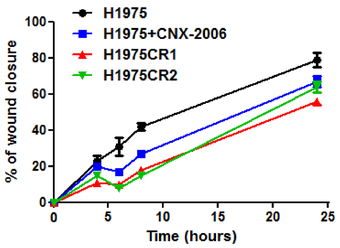

Figure 8. *In vitro* migration. Inhibition of cell migration was detected by the wound healing assay after treatment with CNX-2006 at its IC_{50}. The reduction of cell migration was observed in the resistant clones also in the absence of the drug.

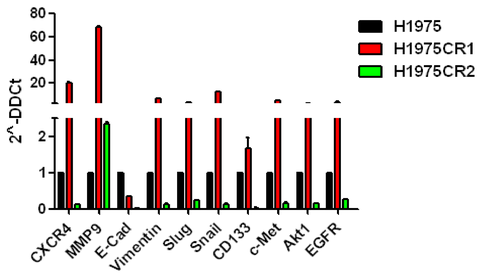

Figure 9. Gene expression. Fold change in the genetic expression of EMT markers as detected by quantitative RT-PCR. The results from the resistant clones were compared to the results in H1975 cells.