In vitro acquired resistance to the mutant selective EGFR inhibitor CO-1686 is associated with epithelial-mesenchymal transition (EMT)

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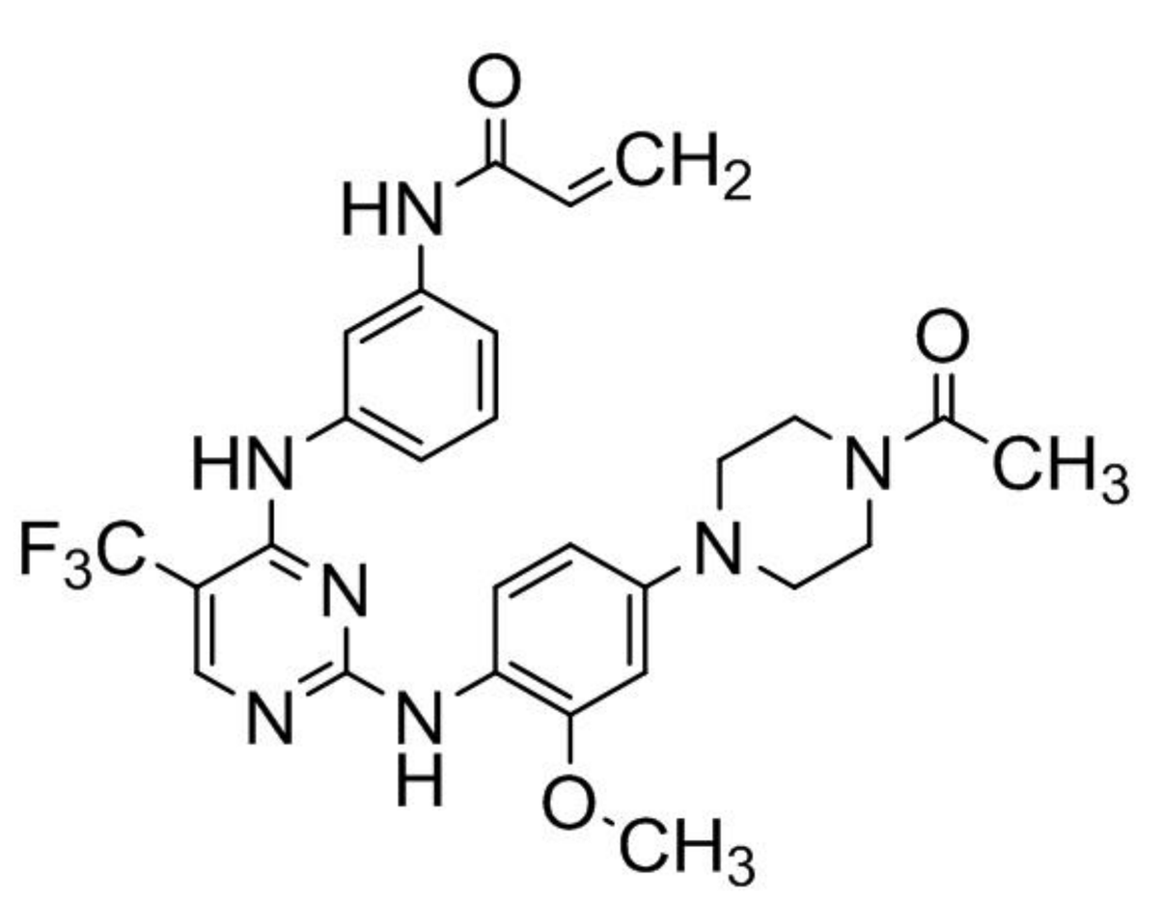
ABSTRACT

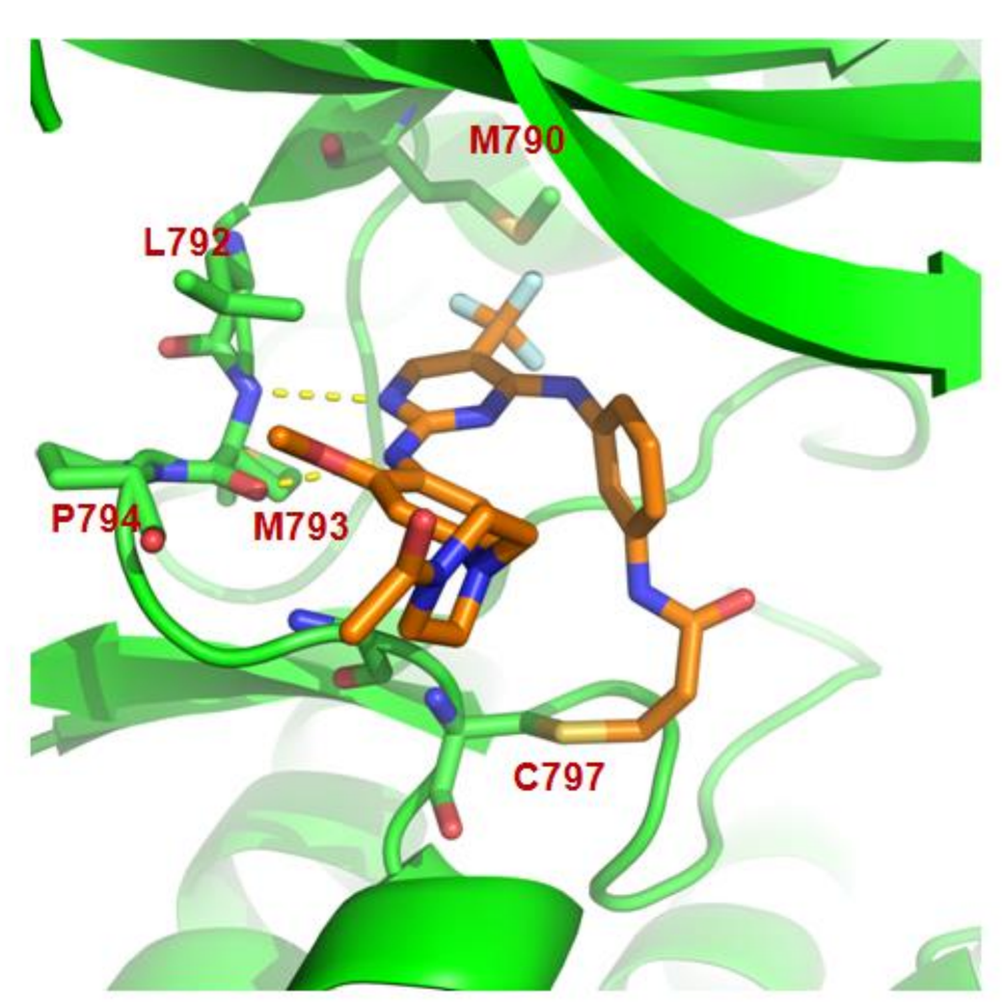
Non-small cell lung cancer (NSCLC) patients with activating epidermal growth factor receptor (EGFR) mutations initially respond well to first generation reversible EGFR tyrosine kinase inhibitors. However, clinical efficacy is limited by acquired resistance driven by the primary drug-resistance T790M mutation in EGFR. CO-1686 is a novel, irreversible and orally delivered kinase inhibitor that specifically targets the most common primary and acquired mutant forms of EGFR while exhibiting minimal activity towards the wild-type (WT) receptor, and has shown promising activity in on-going phase I/II clinical trials in NSCLC. To assess the mechanisms of acquired resistance to CO-1686, we continuously exposed the NSCLC cell line NCI-H1975 (EGFR L858R/T790M) to several months of increasing doses of CO-1686 until resistance (IC_{50} >100x over parental) developed. Drug resistance in the CO-1686 resistant (COR) clones extended to additional EGFR TKIs including Erlotinib and Afatinib. To determine if CO-1686 resistance in the COR cell clones was dependent on EGFR signaling, we examined the functional effects of *EGFR* siRNA knockdown in NCI-H1975 parental cells and CO-1686 resistant clones. Compared to the parental NCI-H1975 cell line, the resistant clones demonstrated a reduced dependence on EGFR expression for viability. Analysis of genes differentially expressed in the COR clones compared to the parental cell line demonstrated a significant enrichment of genes associated with EMT. Further genetic mutation or copy number alteration in the *EGFR* gene were not observed in COR clones. Consistent with a mesenchymal cell signature in the COR clones, vimentin expression was up-regulated and E-Cadherin down-regulated in the CO-1686 resistant clones at both the protein and RNA level. qRT-PCR analysis of additional markers further supported EMT including the up-regulation of *AXL, ZEB1, CDH5, FN1* and the down-regulation of the epithelial markers *MIR200B, CLDN4, EPCAM* and *CLDN7*. Higher basal levels of phospho AKT were also observed in the COR cell clones as compared to the parental NCI-H1975 cell line. Although not effective when used as a single agent, AKT inhibitors restored partial drug sensitivity to the COR clones when used in combination with CO-1686. Taken together, these preclinical data suggest that CO-1686 resistance is associated with an EMT and unlike first and second generation EGFR TKIs, is not mediated by further mutation of *EGFR*.

CO-1686 MECHANISM OF ACTION

CO-1686 covalently modifies Cys797 in the ATP binding pocket of the EGFR kinase domain



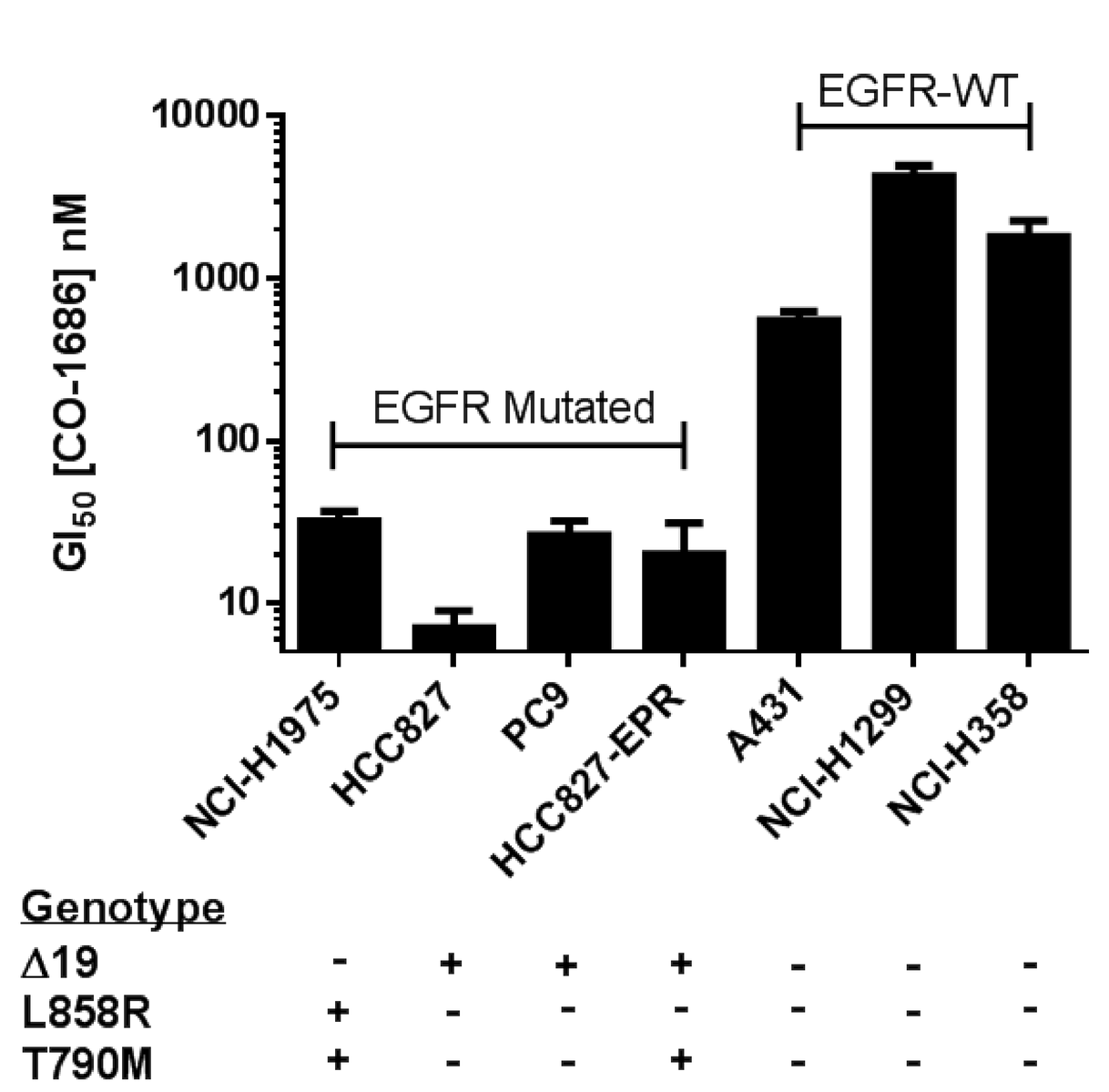
CO-1686

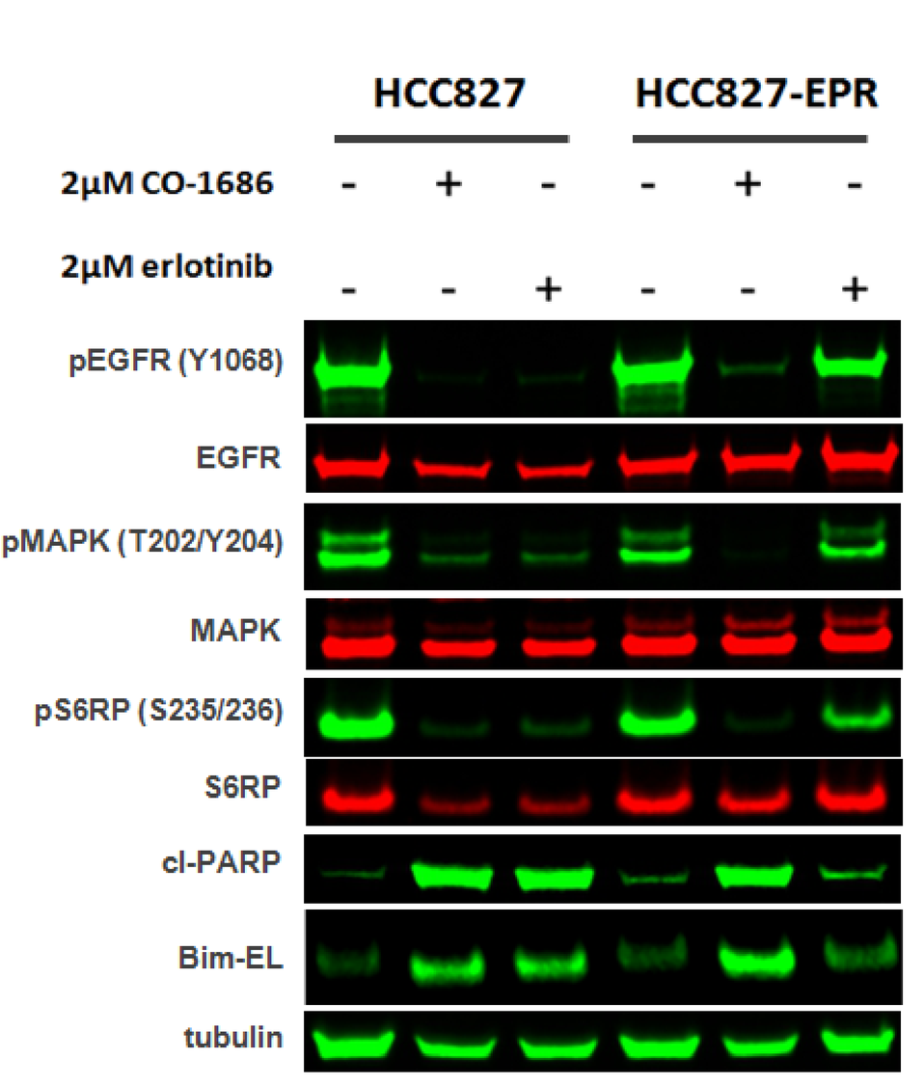


Covalent modification of EGFR

IN VITRO EFFICACY OF CO-1686

CO-1686 potently and selectively inhibits growth of NSCLC cells expressing mutant EGFR and induces apoptosis





ACQUIRED RESISTANCE IN NCI-H1975 CELLS FOLLOWING CHRONIC EXPOSURE TO CO-1686

CO-1686 resistant NCI-H1975 cells(CORs) are resistant to 1st, 2nd, and 3rd generation EGFR TKIs

Cell line	CO-1686	Afatinib	Erlotinib
NCI-H1975	9	36	2227
COR 1-1	3237	3114	>5000
COR 1-2	1700	1817	>5000
COR 10-1	2964	3197	>5000
COR 10-2	>4582	3866	>5000
COR 10-3	3815	2588	>5000

Resistance in CO-1686 resistant NCI-H1975 cells extends to other EGFR TKIs. NCI-H1975 cells were treated with increasing doses of CO-1686 in order to generate a population of drug-resistant cells. Once the population developed a CO-1686 IC_{50} ~100-fold higher than the parental cell line, five individual clones were isolated and characterized for sensitivity to CO-1686, Afatinib, and Erlotinib. Cell proliferation assays were performed with increasing concentrations of compounds for 72 hours. Cellular ATP content was measured using CellTiter Glo and IC_{50} [nM] values were calculated using GraphPad Prism.

CO-1686 resistant NCI-H1975 cells are not driven by mutations in common oncogenes

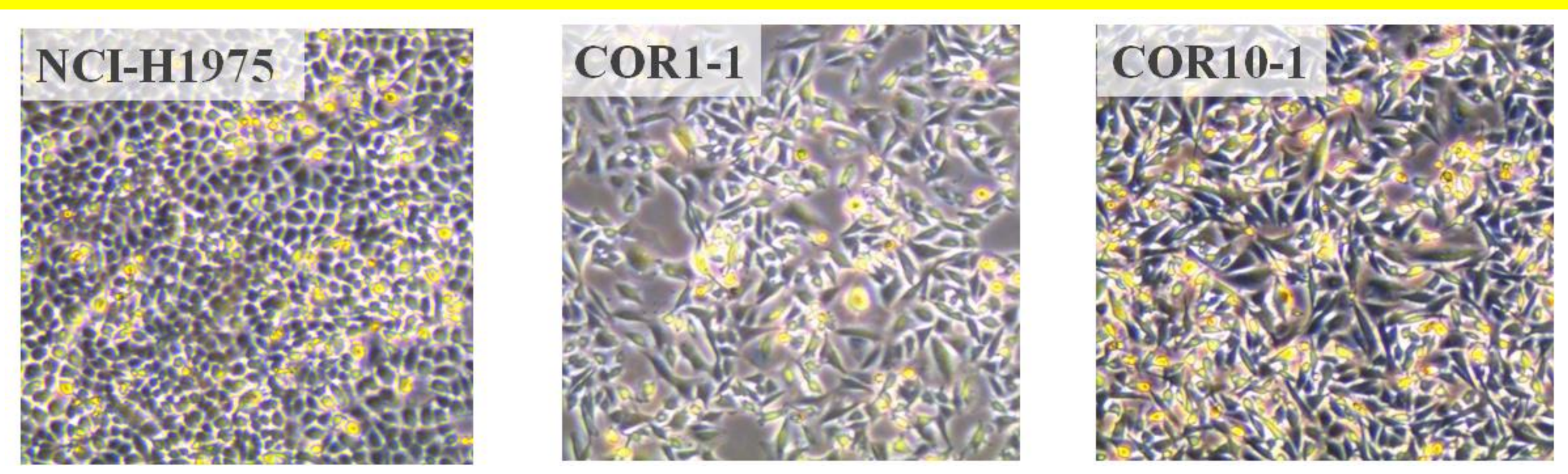
OncoCARTA V1.0 Panel

Gene	# mutations covered	Gene	# mutations covered
ABL1	14	*JAK2*	1
AKT1	7	*KIT*	27
AKT2	2	*MET*	5
BRAF	24	*HRAS*	6
CDK	2	*KRAS*	12
EGFR	43	*NRAS*	8
ERBB2	7	*PDGFR*	11
FGFR1	2	*PIK3CA*	13
FGFR3	5	*RET*	6
FLT3	2		

Sample	Gene	Mutation	WT Frequency	Mutation Frequency
NCI-H1975	EGFR	T790M	24%	76%
COR1-1	EGFR	T790M	18%	82%
COR1-2	EGFR	T790M	22%	78%
COR10-1	EGFR	T790M	0%	100%
COR10-2	EGFR	T790M	26%	74%
COR10-3	EGFR	T790M	19%	81%
NCI-H1975	EGFR	L858R	42%	58%
COR1-1	EGFR	L858R	34%	66%
COR1-2	EGFR	L858R	39%	61%
COR10-1	EGFR	L858R	0%	100%
COR10-2	EGFR	L858R	47%	53%
COR10-3	EGFR	L858R	39%	61%

CO-1686 resistant NCI-H1975 cells are not driven by mutations in common oncogenes. No novel mutations were identified when NCI-H1975 CORs were genotyped against 238 mutations spread across 19 genes using the OncoCARTA V1.0 panel. Loss of T790M allele was not observed in NCI-H1975 CORs.

CO-1686 resistant NCI-H1975 cells exhibit a spindle-like morphology commonly associated with an epithelial-mesenchymal transition (EMT)



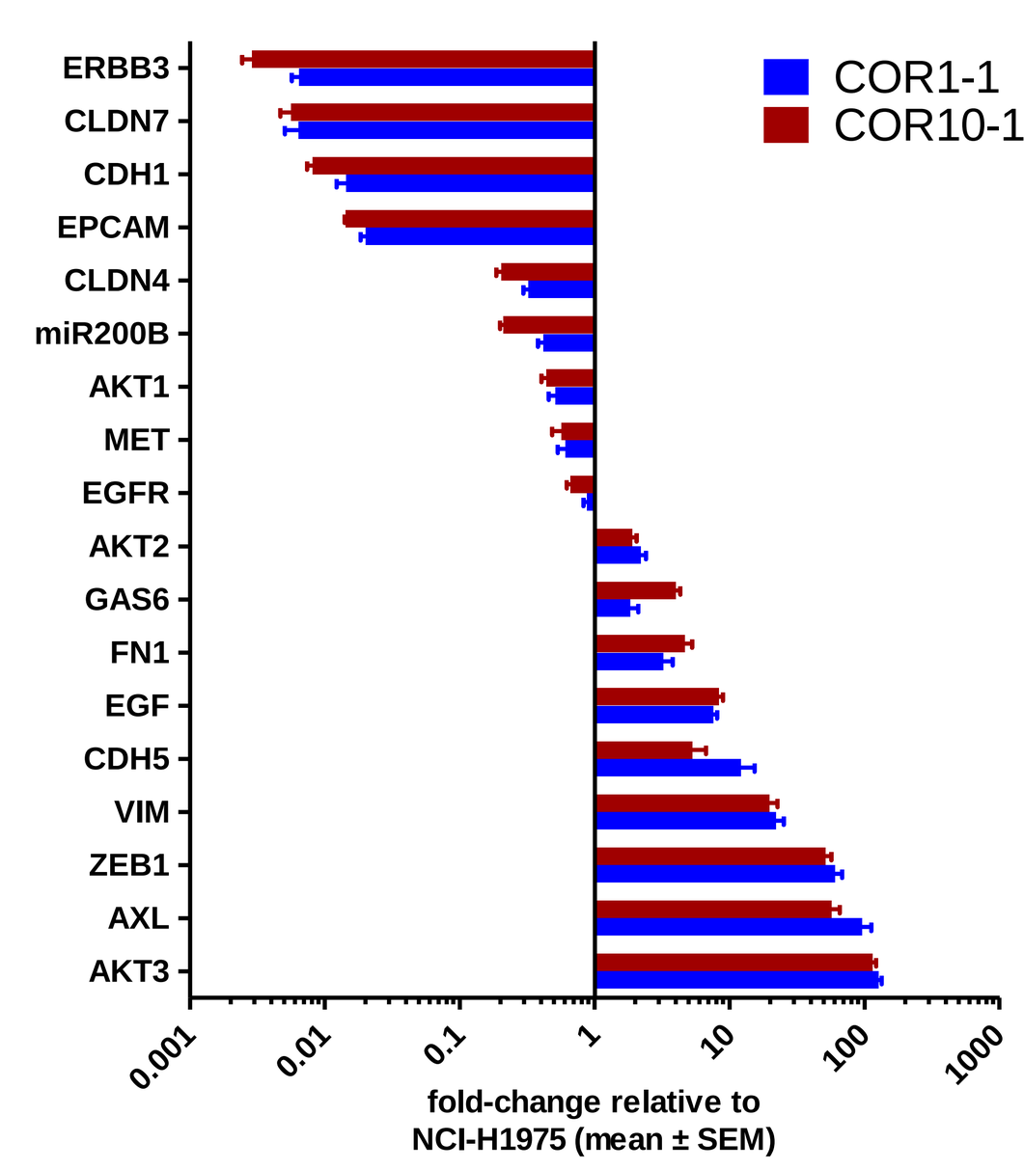
CO-1686 resistant NCI-H1975 cells are morphologically distinct from parental NCI-H1975 cells. NCI-H1975, COR1-1, and COR10-1 cells were seeded at equal density and allowed to grow to confluency. Cells were imaged using phase contrast microscopy at 5X magnification. Similar morphologies were observed for COR1-2, COR10-2, and COR10-3 (data not shown).

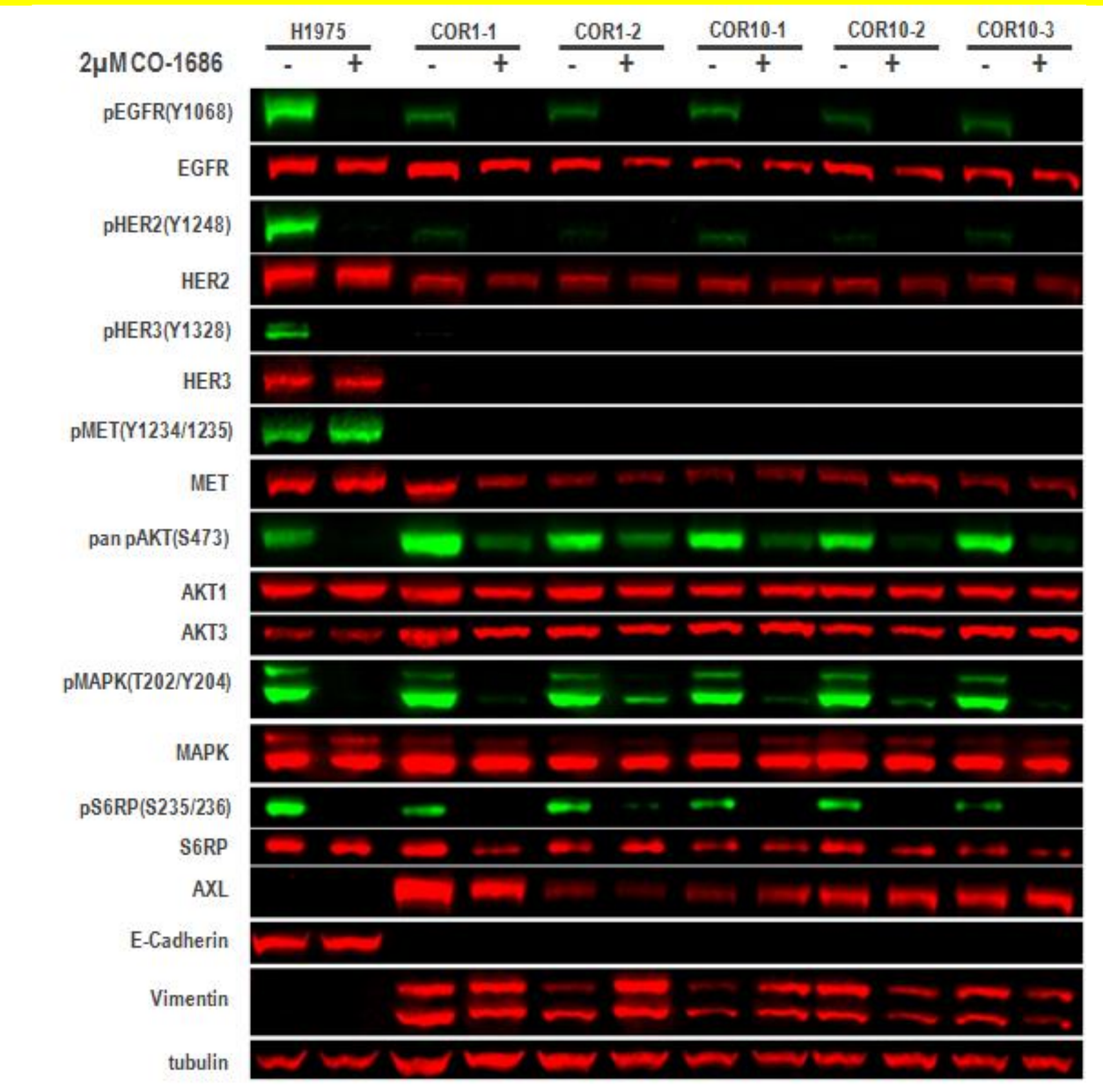
CO-1686 resistant NCI-H1975 cells do not activate alternative receptor tyrosine kinases (RTKs)

CO-1686 resistant NCI-H1975 cells downregulate ERRB-family members and c-MET. Total protein lysate from exponentially growing NCI-H1975 and COR1-1 was used to detect the relative phosphorylation of 49 different RTKs via R&D System's phospho RTK Array Kit. COR1-1 results demonstrate downregulation of EGFR, HER2, HER3, and c-MET as compared to parental NCI-H1975 cells. Similar RTK results were observed in another COR cell line (data not shown).

MOLECULAR CHARACTERIZATION OF RESISTANCE IN CO-1686 RESISTANT NCI-H1975 CELLS

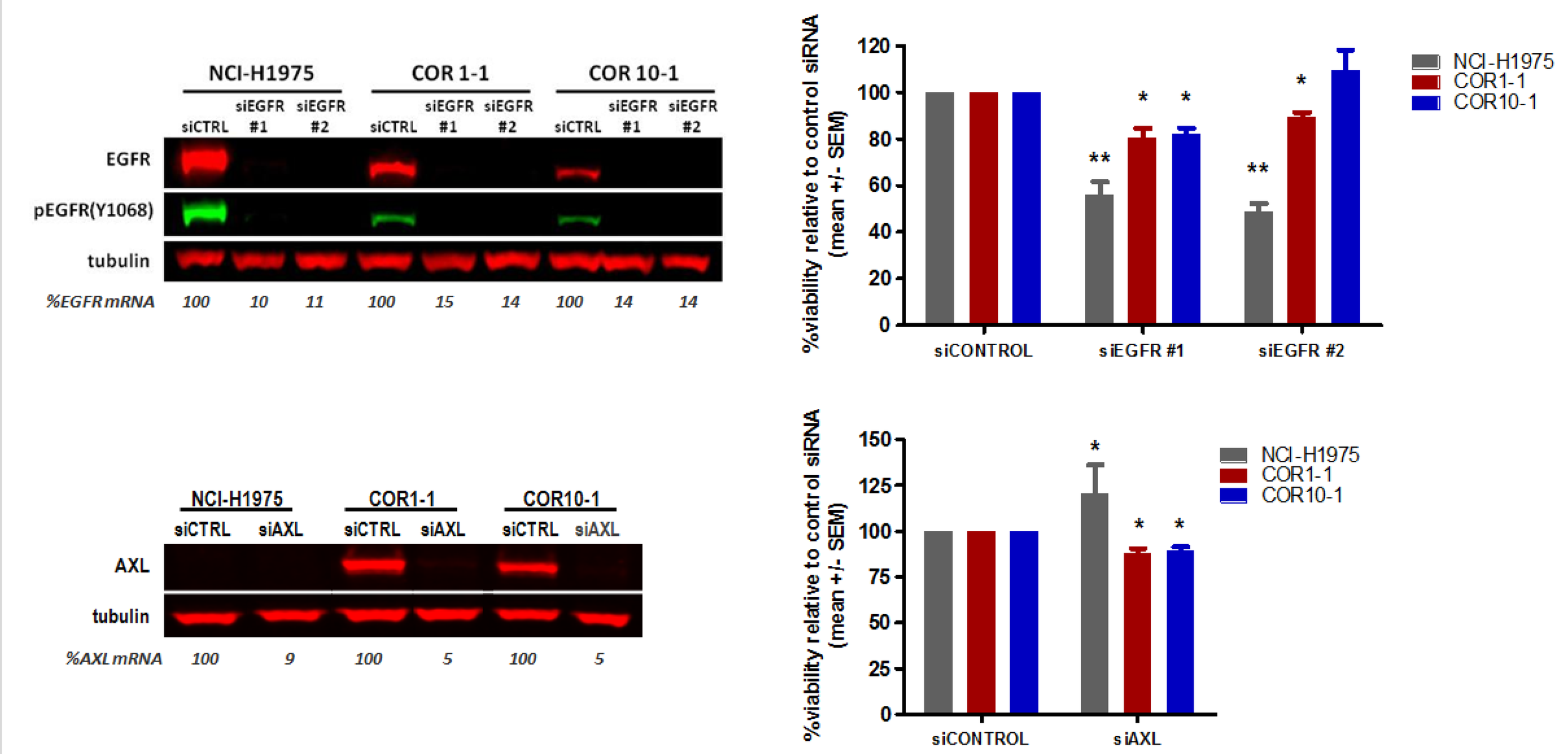
CO-1686 resistant NCI-H1975 cells harbor EMT-related biomarkers





Expression of EMT-related biomarkers in CO-1686 resistant NCI-H1975 cells at the RNA and protein levels.
Left: RNA extracted from exponentially growing cells was used to analyze mRNA expression via qRT-PCR. As compared to the parental cell line, the COR cells showed a decrease in the expression of genes associated with an epithelial phenotype (*CDH1, EPCAM, CLDN4/7*) and an increase in the expression of genes associated with a mesenchymal phenotype (*VIM, FN1, ZEB1*).
Right: Cells were treated with DMSO or 2μM CO-1686 for 60 minutes and protein levels were analyzed via Western blot. As compared to the parental cell line, COR cells showed decreased expression of ERBB family members and MET, but increased levels of AXL. Importantly, phospho AKT signaling was abolished in the parental, but persisted in the CORs cells following treatment with CO-1686. Finally, loss of E-Cadherin and gain of Vimentin expression (a hallmark characteristic of EMT) is evident in the CORs.

CO-1686 resistant NCI-H1975 cells are less dependent on EGFR signaling as compared to parental cells and do not depend on AXL signaling for viability



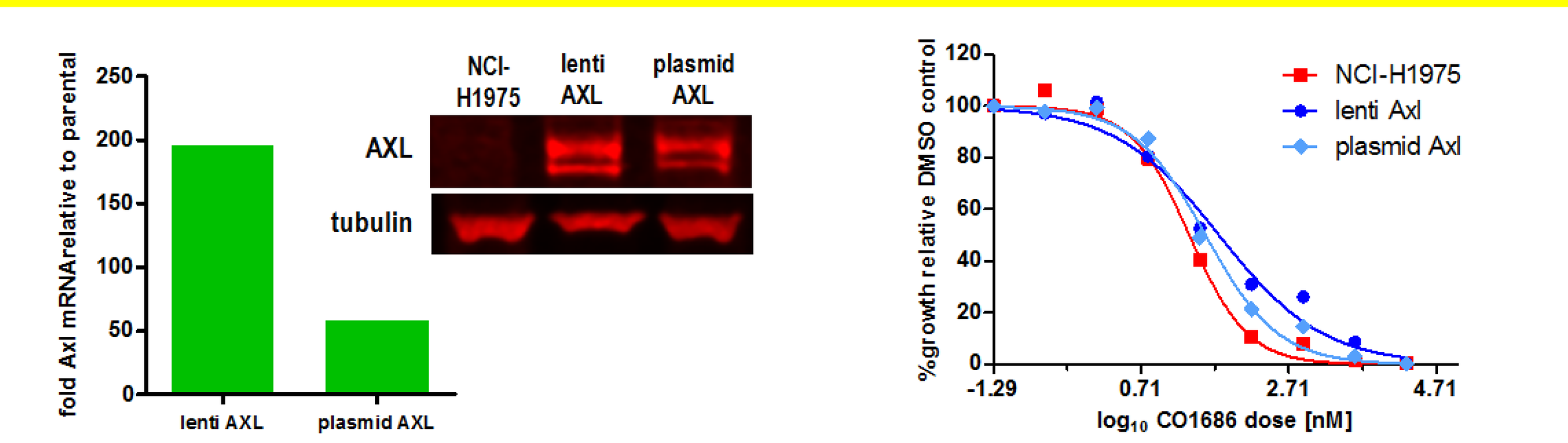
Top: **A small impact on CO-1686 resistant NCI-H1975 cell viability is observed following *EGFR* mRNA knockdown.** EGFR/AXL knockdown was confirmed at the mRNA and protein levels (top left). Resulting cell viability demonstrates the minor impact EGFR knockdown has on COR viability as compared to parental cells (top right).

Bottom: **Minimum impact on CO-1686 resistant NCI-H1975 cell viability is observed following *AXL* mRNA knockdown.**
AXL knockdown was confirmed at the mRNA and protein levels (bottom left). Resulting cell viability demonstrates the minor impact AXL knockdown has on COR viability.

*mRNA knockdown was measured via qRT-PCR at 24 hours post-transfection. Protein knockdown was measured via Western blot at 48 hours post-transfection. Cell viability was assessed via CellTiter Glo at 72 hours post-transfection. * indicates P < 0.05 and ** P < 0.005 comparing siEGFR or siAXL to siCONTROL*

AXL OVEREXPRESSION HAS LIMITED IMPACT IN CO-1686 RESISTANT NCI-H1975 CELLS

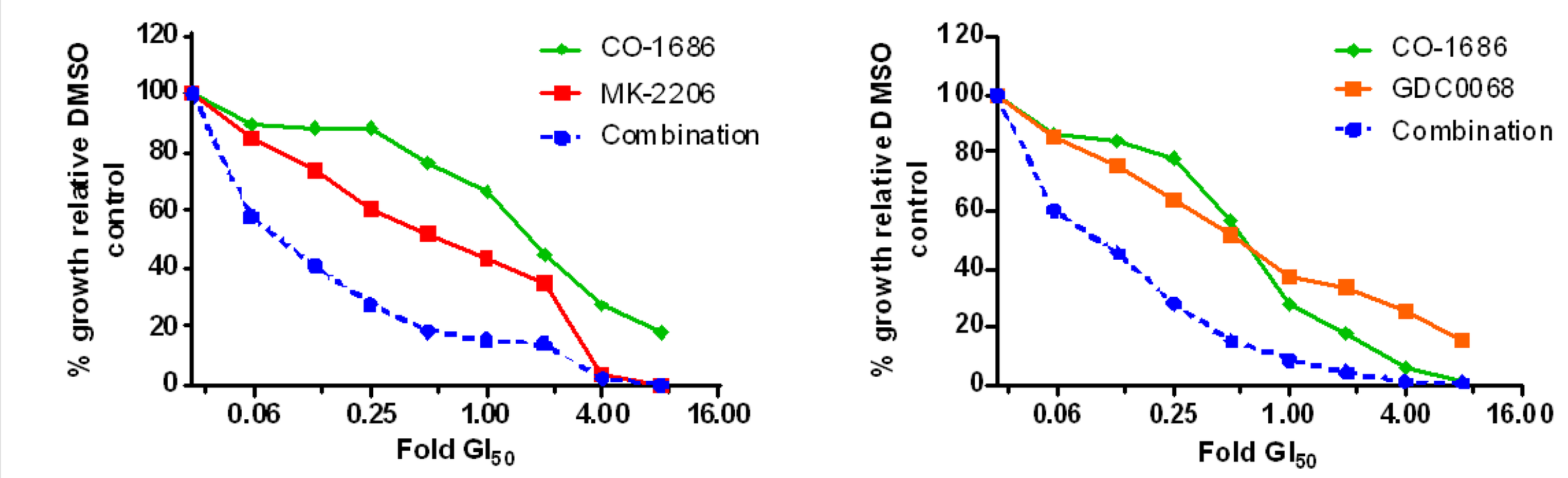
Overexpression of AXL alone does not confer resistance to CO-1686 in NCI-H1975 cells



Stable overexpression of AXL in NCI-H1975 via lentiviral (200-fold) or plasmid (60-fold).
Left: AXL expression was confirmed at the RNA level via qRT-PCR and protein level by Western blot.
Right: Following treatment with CO-1686, the viability of NCI-H1975 cells stably overexpressing AXL is similar to that of parental cells. Cell viability was determined at 72 hours post-treatment. Lenti-mediated *AXL* overexpression in NCI-H1975 cells is comparable to that observed in COR cells (data not shown).

EXPLORING DRUG COMBINATIONS IN CO-1686 RESISTANT NCI-H1975 CELLS

CO-1686 resistant NCI-H1975 cells are sensitive to CO-1686 + AKT inhibitor combinations

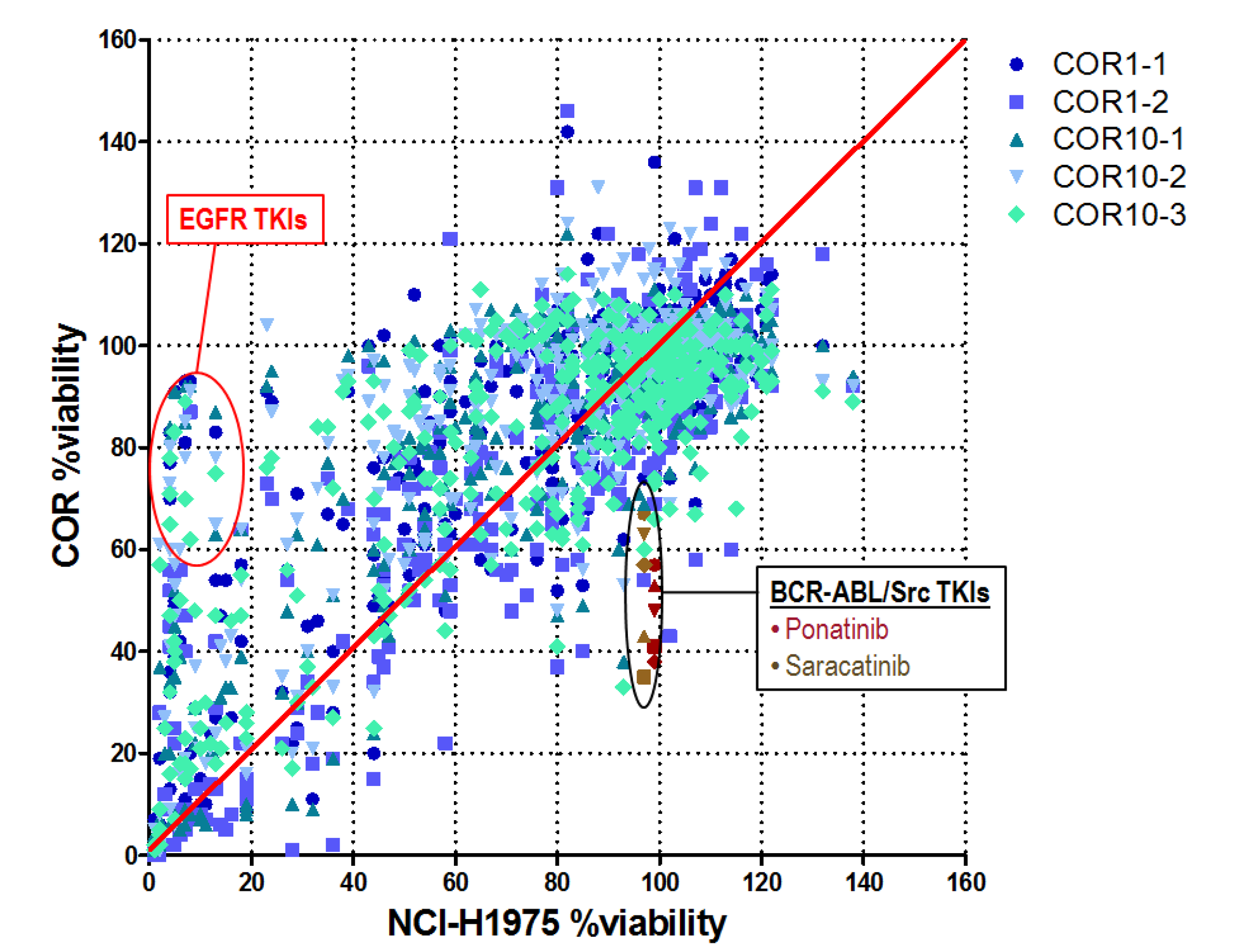


	CO-1686	MK2206
IC_{50} [nM]	1664	1620
CI @ ED_{50}	0.2636	

	CO-1686	GDC0068
IC_{50} [nM]	1117	1312
CI @ ED_{50}	0.3773	

Combining CO-1686 with AKT inhibitors has a synergistic effect on CO-1686 resistant NCI-H1975 cell viability. Due to persistent phospho AKT in NCI-H1975 CORs following treatment with CO-1686, two AKT inhibitors were used in combination against COR10-1 and cell viability was determined at 72 hours. Biosoft CalcuSyn software was used to determine CI values for synergy. Results were confirmed in another COR cell line (data not shown).

Discovering compounds to reduce CO-1686 resistant NCI-H1975 cell viability



Screening a 375 compound library against CO-1686 resistant NCI-H1975 cells. A library consisting of common kinase inhibitors and chemotherapeutic agents was screened at 200nM for activity against NCI-H1975 and CORs. Cell viability was determined 72 hour post-treatment. Data points falling below the diagonal red line indicate better activity in CORs vs. parental, while points above the line indicate better activity in parental vs. CORs.

CONCLUSIONS

CO-1686 is a novel, irreversible and orally delivered TKI that specifically targets the most common primary and acquired mutant forms of EGFR while exhibiting minimal activity towards the wild-type receptor

NCI-H1975 cell clones resistant to CO-1686 (CORs) were generated and exhibit features of an epithelial-mesenchymal transition (EMT)

NCI-H1975 COR cells do not have additional oncogenic driver mutations

NCI-H1975 COR cells are less dependent on EGFR expression for viability as compared to parental cells, and insensitive to other EGFR TKIs

Knockdown of AXL has limited impact on NCI-H1975 COR cell viability, and over-expression of AXL in the NCI-H1975 cell line has limited impact on CO-1686 sensitivity

When used in combination with CO-1686, NCI-H1975 COR cells are sensitive to AKT inhibitors suggesting a potential targeted therapy to explore in the clinic

NCI-H1975 COR cells may be sensitive to BCR-ABL/Src inhibitors as compared to parental NCI-H1975 cells

CO-1686 has shown promising activity in on-going phase I/II clinical trials in NSCLC, and is currently being evaluated in a phase I/II clinical trial in NSCLC patients with an activating mutation in EGFR who have failed treatment with a front-line EGFR inhibitor
(ClinicalTrials.gov identifier: NCT01626928)