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In vivo and *in vitro* generation and characterisation of EGFR-TKI resistance in patient-derived xenograft (PDX) and cell line-derived xenograft (CDX) models of NSCLC with activating EGFR mutations.

Andrew Mckenzie[1*], Nektaria Papadopoulou[1], Lucy Page[1], Stephanie Cooper[1], Simon Jiang[1], Jane Wrigley[1], Jason King[1], Henry Li[2], Martin Page[1] and Rajendra Kumari[1]
[1]PRECOS Ltd., a Crown Bioscience Company, Loughborough, UK; [2]Crown Bioscience Inc. Beijing, China

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Background

Most non-small cell lung cancer (NSCLC) patients that have activating mutations in the EGFR gene will respond to treatment with epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) e.g. Erlotinib (Tarceva®) and Gefitinib (Iressa®).

However, following months of dosing, in about 50% of these cases a secondary mutation in EGFR (T790M) subsequently occurs, which results in resistance to treatment.

Other mechanisms of clinical resistance can also occur such as amplification of c-MET, HER2 and epithelial to mesenchymal transition (EMT). However, additional routes of resistance also exist and are poorly defined.

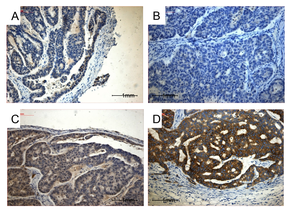
Using a novel Caucasian NSCLC patient-derived xenograft (PDX) model and a cell line-derived model, driven by the L858R EGFR mutation or exon 19 EGFR deletion respectively, we set out to recapitulate the reported clinical routes of resistance to EGFR inhibitors and to evaluate if additional mechanisms could also be identified.

Methods

HCC827 is an NSCLC adenocarcinoma cell line with an activating EGFR mutation (del E746-A750).

LU6422 is a Caucasian NSCLC adenocarcinoma PDX model with an activating EGFR mutation (L858R) which is maintained subcutaneously *in vivo* in nude mice (HsdOla:MF1-*Foxn1^{nu}*) admixed with a human stromal cell component.

A panel of IHC markers were used to confirm the histopathology of LU6422 as NSCLC adenocarcinoma (+ve TTF-1 (panel A); -ve for CK5/6 (panel B) & P63 (panel C)) and to confirm EGFR expression (panel D); scale bar = 1mm



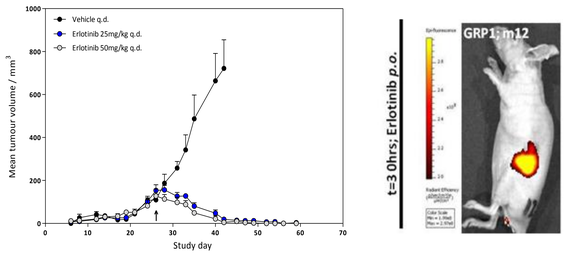
Resistant models of HCC827 and LU6422 were generated *in vitro or in vivo,* respectively, through repeated dosing or exposure to EGFR TKIs. Further models of HCC827 resistance were generated from the 'parental' lines following single-cell cloning of resistant HCC827 variants. Resistant material was characterised for further mutations in the EGFR gene by direct sequencing as well as for c-MET over-expression and genomic amplification by quantitative PCR.

Mechanism of resistance was confirmed by *in vitro* and *in vivo* combination dosing using inhibitors targeted to c-MET or EGFR.

LU6422 response to Erlotinib *in vivo*

Dosing was initiated on day 27 (arrowed) and partial regression of all tumours was observed at day 40 with complete regression by day 60 (50mg/kg and 25mg/kg erlotinib p.o. q.d.); error bars represent SEM. Caspase DyLight® (Thermo Scientific) gives an early read-out (30h post-treatment 25mg/kg p.o.q.d) of efficacy *in vivo* via Spectrum CT (PerkinElmer Corp).

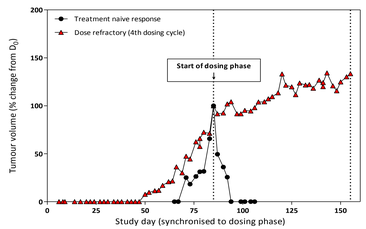
LU6422 (Adc, Het^{L858R}) *in vivo* response to Erlotinib



In vivo generation of EGFRi resistance (PDX)

Mice were dosed with Erlotinib or Gefitinib (12.5 or 25mg/kg) p.o. q.d. in treatment cycles of up to 10 weeks followed by outgrowth & re-passage. Graphs show the original treatment-naïve growth profile in two example mice (data expressed as % of the initial dosing volume) overlaid with the growth profile following 3 cycles of dosing pressure (equivalent to up to 4 months of q.d. dosing).

LU6422 *in vivo* dosing outgrowth: Erlotinib q.d.



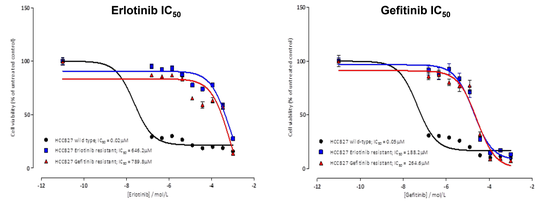
Treatment naive and post-treatment resistance phase tumour material was also characterised for mutations in EGFR by direct sequencing of exons 21 (L858R) and 20 (T790M; gatekeeper).

- Exon 21 L858R mutation was confirmed in all samples.
- No exon 20 T790M gatekeeper mutations were detected in any of the test samples.

Treatment naïve and post-treatment resistance phase LU6422 tumour material was characterised for *c-MET* genomic amplification by qPCR and *AXL* gene expression via RT-PCR. No increase in c-Met amplification or AXL gene expression was detected.

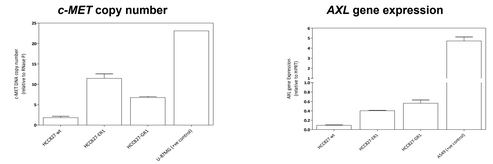
Generation of EGFRi resistance in HCC827 *in vitro*

EGFR-TKI resistant variants of HCC827 (ER1 and GR1) were generated *in vitro* following sub-culture with escalating doses of Erlotinib or Gefitinib respectively. The resultant cell lines exhibited a >1000 fold shift in IC_{50} compared with the parental line and exhibited cross-resistance with the alternate EGFR-TKI.



Treatment naive and post-treatment resistance phase tumour material was also characterised for mutations in EGFR by direct sequencing of exons 19 (del E746-A750) and 20 (T790M; gatekeeper).

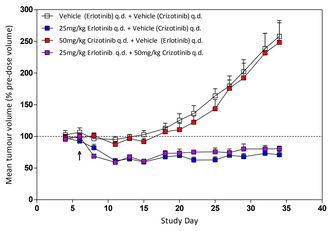
- Exon 19 deletion was confirmed in all samples.
- No exon 20 T790M gatekeeper mutations were detected in any of the test samples.
- Treatment naïve and post-treatment resistance phase HCC827 wild-type/resistant variants were characterised for *c-MET* genomic amplification by qPCR and *AXL* gene expression via RT-PCR.



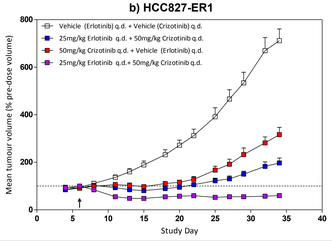
Combination dosing overcomes EGFR-TKI resistance

Mice bearing subcutaneous (s.c.) HCC827 or Erlotinib resistant HCC827-ER1 tumours were dosed with 25mg/kg Erlotinib (EGFR-TKI), 50mg/kg Crizotinib (c-Met inhibitor) or a combination of both concurrently. The graphs show the growth profiles for a) HCC827, or b) HCC827-ER1; tumour volume is expressed as a % of the initial dose volume, error bars represent SEM.

a) HCC827



b) HCC827-ER1



a) When nude mice (HsdOla:MF1-*Foxn1^{nu}*) bearing s.c. HCC827 tumours (n=5/group) were dosed with Erlotinib or a combination of Erlotinib + Crizotinib the tumours regressed; however, there was no effect with Crizotinib alone. Furthermore, there was no increased efficacy with Erlotinib + Crizotinib in combination when compared with Erlotinib alone, suggesting no additive effect in this model.

b) When nude mice (HsdOla:MF1-*Foxn1^{nu}*) bearing s.c. HCC827-ER1 tumours (n=5/group) were dosed with Erlotinib, there was a significant reduction in tumour growth compared with vehicle (Two-way ANOVA, p<0.001; ~70% tumour growth inhibition) but no tumour regression. In clinical terms, this denotes progressive disease versus tumour regression and indicates treatment escape or emergence of resistance.

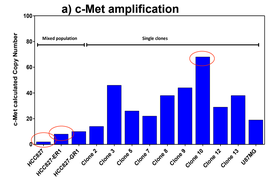
When HCC827-ER1 tumours were dosed with Crizotinib there was a y significant reduction in tumour growth compared with control (p<0.001; ~60% tumour growth inhibition) but no tumour regression. Combination treatment restored tumour regression indicating an additive effect and confirming the role of c-Met amplification in the resistance mechanism for HCC827-ER1.

Combination index *in vitro*

a) Single cell clones from EGFR-TKI resistant variants of HCC827 were generated and characterised for c-Met genomic amplification by qPCR.

b) Three of the cell lines, exhibiting an increasing scale of c-Met amplification (HCC827, HCC827-ER1 and HCC827-GR1 clone 10) were assessed *in vitro* to determine their response to combination treatment.

a) c-Met amplification



Cells were treated with Crizotinib, Erlotinib or combinations of both for 72h and cell viability were assessed by using CellTiter Blue™ (Promega). Combination ratios are 1:1 and 1:100 (Erlotinib:Crizotinib) in the parental line and 1:1 and 100:1 were tested in resistant lines.

b) Combination (CI) analysis

Cell line	Compound / combination	Combination Index (CI) ED50	ED75	ED90	ED95	Weighted CI	Score
HCC827	Erlotinib:Crizotinib; 1:1	340.40	1.37	6.98	21.57	45.04	No effect
	Erlotinib:Crizotinib; 1:100	64.75	0.08	0.12	0.17	6.60	No effect
HCC827-ER1	Erlotinib:Crizotinib; 1:1	0.33	0.79	1.89	3.42	2.12	Antagonism (synergism near IC50)
	Erlotinib:Crizotinib; 100:1	0.21	0.14	0.10	0.07	0.11	Strong synergism
HCC827-GR1 (clone 10)	Erlotinib:Crizotinib; 1:1	0.57	0.46	0.37	0.32	0.39	Synergism
	Erlotinib:Crizotinib; 100:1	0.12	0.04	0.02	0.01	0.03	Very strong synergism

Synergistic effect was calculated based on combination index values according to the Chou and Talalay method (CalcuSyn). The increasing scale of synergistic response corresponds with c-Met amplification. Results could be used to guide further *in vivo* efficacy testing and dose optimisation.

Summary

EGFR TKI resistant subtypes were generated *in vivo* from a proprietary Caucasian NSCLC PDX model (LU6422) and characterised for their resistance mechanisms (*programme on-going*).

EGFR-TKI resistant variants were generated *in vitro* from the HCC827 NSCLC cell line and resistance to EGFRi translates to the *in vivo* setting.

Cross-resistance to EGFR TKIs was observed in resistant HCC827 variants along with an elevation in the *c-MET* gene copy number which correlated with combination efficacy *in vitro*.

Combination treatment (c-Meti and EGFR-TKI) overcomes *in vivo* resistance in c-Met driven EGFR-TKI resistance.

Conclusions

These acquired resistance models will be invaluable in assessing novel agents targeting the EGFR pathway.

These models also open up opportunities for the assessment of new combination strategies which seek to prevent or overcome the emergence of resistance to EGFR TKIs.

Offer proof of concept for generation other resistant lines/models for current or new treatment strategies for MAbs or small molecules..

*Contact information: Andrew McKenzie, Andrew.Mckenzie@precos.co.uk