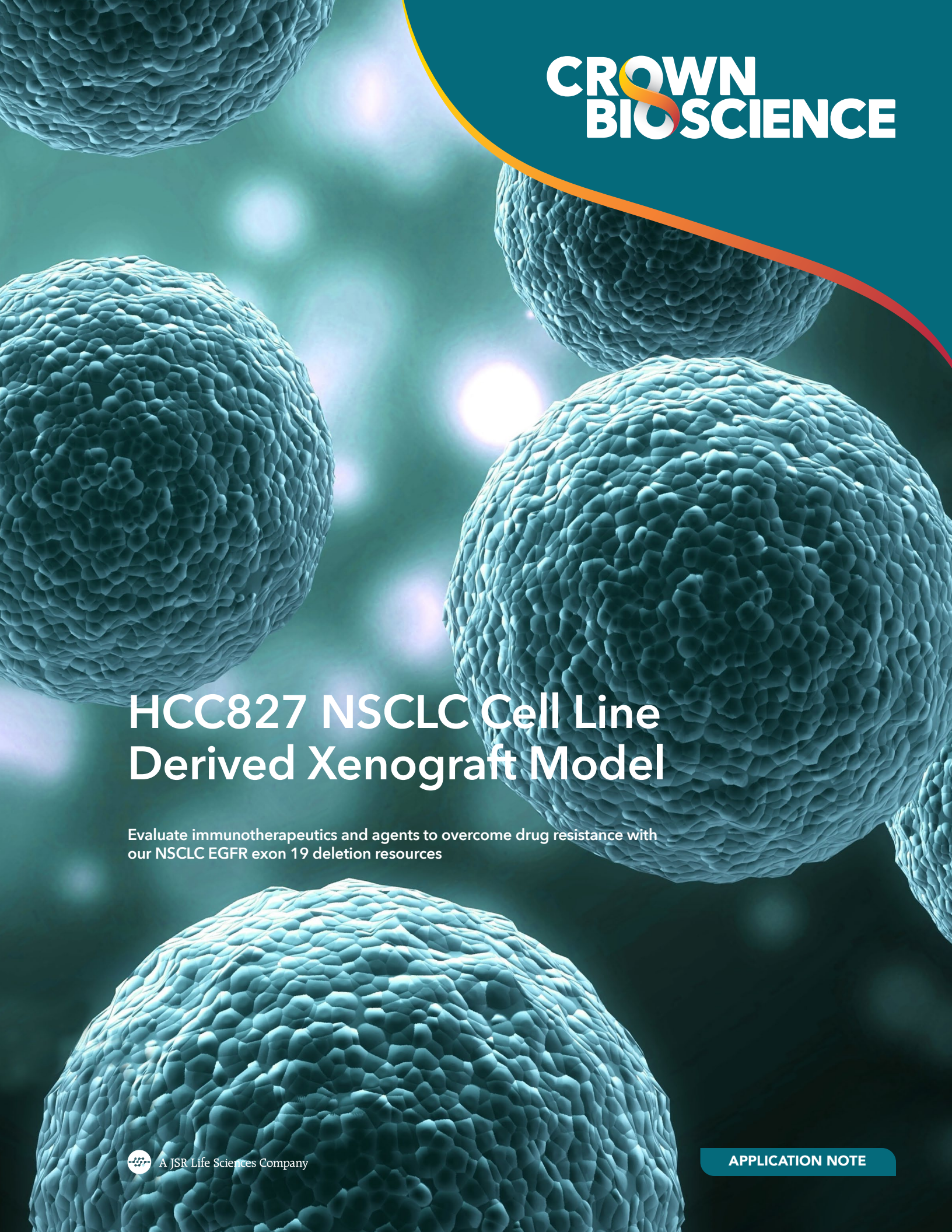


CROWN BIOSCIENCE

HCC827 NSCLC Cell Line Derived Xenograft Model

Evaluate immunotherapeutics and agents to overcome drug resistance with our NSCLC EGFR exon 19 deletion resources

A JSR Life Sciences Company

APPLICATION NOTE

Executive Summary

As personalized medicine research progresses in oncology, highly specific preclinical models are required to investigate individual patient-relevant mutations. These models can improve the understanding of tumor background, response, and resistance mechanisms.

Crown Bioscience provides the HCC827 NSCLC model to study the clinically relevant EGFR exon 19 E746-A750 deletion. Highly responsive to first generation EGFR TKIs, we have derived and characterized resistant cell lines and xenograft models. This allows preclinical evaluation of novel agents and combination regimens for both primary disease and to overcome resistance, mediated here by c-MET amplification.

The HCC827 model also presents high PD-L1 expression, and is suitable for immunotherapeutic studies. We have validated the model in our Mi**Xeno**™ platform of transient human immunity for use in anti-PD-1/PD-L1 evaluation.

NSCLC Response and Resistance is Determined by Key Molecular Features

Lung cancer is comprised of several defined histological and molecular subsets. These can be classified primarily by the key gene mutations, amplifications, and fusions that they harbor, and which determine the response seen to targeted agents.

Over the past decade, it has become evident that subsets of non-small cell lung cancer (NSCLC) can be defined at the molecular level by key 'driver' gene alterations that occur, including in the EGFR, KRAS, ALK, FGFR, BRAF, HER2, AKT1, MEK1, MET, NRAS, PIK-3CA, RET, and ROS1 genes. For example, EGFR exon 19 kinase domain deletions occur in approximately 48% of EGFR-mutated NSCLC cases. This mutation results in cellular oncogene-addiction and an increased sensitivty to first, second, and third generation EGFR tyrosine kinase inhibitors (TKIs), including erlotinib (Tarceva®), gefitinib (Iressa®), and afatinib (Giotrif®)[1].

However, despite these positive clinical responses to EGFR TKIs resistance is acquired, with disease progression typically occurring in patients around 10 to 16 months after treatment commenced. Genetic mechanisms of resistance found in tumor samples from these relapsed patients include secondary EGFR mutations (T790M in exon 20 which occurs in >50% of NSCLC patients), amplification of the gene encoding the MET kinase (5 to 10%), and mutations in the downstream signaling lipid kinase, PIK3CA (<5%)[2,3]. Different resistance mechanisms suggest different follow-on therapies e.g. MET inhibitors for treating patients with acquired MET amplifications. It is therefore critical to understand the mechanism at play in any given patient's tumor and to then identify the most appropriate follow-on therapy as part of a precision profiling and personalized medicine strategy.

Although there have been significant advances in the treatment of subsets of patients with molecularly defined NSCLC, the prognosis in the majority of patients has improved only modestly. Clearly, further treatment strategies and combination therapies are needed. The emerging role of immunotherapies in cancer is providing new hope and excitement. In 2015, the FDA approved the use of the PD-1 checkpoint inhibitors nivolumab (Opdivo®) and pembrolizumab (Keytruda®) for the treatment of NSCLC[4,5].

Approval of nivolumab in advanced squamous NSCLC with progression on or after platinum-based chemotherapy was based on a study in which patients receiving nivolumab had an average overall survival 3.2 months longer than patients who received docetaxel[4]. Further approval in non-squamous metastatic NSCLC followed studies showing that overall survival for subjects receiving nivolumab was 12.2 months, compared with only 9.4 months for docetaxel use[6]. A consistent observation in these studies was the durability of response (which was significantly longer following immunotherapy than chemotherapy treatment) and combined with toxicity profiles which are more favorable than chemotherapy, this makes immunotherapy a particularly attractive option for advanced NSCLC, dependent on correct patient stratification[7].

Crown Bioscience NSCLC Resources

Crown Bioscience has a longstanding track record in lung cancer research, with a range of NSCLC and EGFR deletion, insertion, amplification, and mutation resources available for all stages of drug development. This includes **HuPrime**® patient-derived xenograft (PDX) models and **ValidatedXeno**™ cell line derived xenograft models.

The focus of this Application Note is the HCC827 **ValidatedXeno** cell line derived xenograft model, which is an adenocarcinoma cell line and harbors an activating EGFR mutation in exon 19 (deletion E746-A750; also found in our NCI-H1650 cell line derived xenograft, and LU1235 NSCLC **HuPrime** PDX models). The **ValidatedXeno** collection is well-characterized and validated, with all available model data (including gene expression profiled externally using Affymetrix® Human Genome U133 Plus 2.0 Array Plate, and mutation analysis of up to 33 common oncogenes and tumor suppressor genes measured by OncoMap 3.0 core) stored in **XenoBase**® our online cell line/xenograft database. **XenoBase** can be accessed directly from the homepage of the Crown Bioscience website at **www.crownbio.com**.

The HCC827 model has also been validated in our **MiXeno** immunotherapy platform due to the high expression of PD-L1. Fighting cancer by taking advantage of a patient's own immune system is showing considerable success in oncology research. However, a lack of experimental immunotherapy models is a major obstacle toward developing better immunotherapeutic treatments and answering questions on why some patients and diseases benefit from these treatments while others do not.

Crown Bioscience has developed a range of immunotherapy research platforms available for preclinical drug development, including platforms of human, mouse, and chimeric immunity (covered in detail in our *In Vivo* Immunotherapy Drug Discovery Application Note). The **MiXeno** platform of transient human immunity in mice is created by mixing human peripheral blood mononucleated cells (PBMC) with xenograft models, including HCC827. The **MiXeno** model provides a simple alternative to the full stem cell reconstitution approach, and also allows immunotherapeutic antibody-dependent cellular cytotoxicity (ADCC) effects to be evaluated in xenograft models. The HCC827 **MiXeno** model has been utilized in the evaluation of anti-PD-1 and anti-PD-L1 checkpoint inhibitors.

HCC827 Model Background and EGFR Status

The model type, information, background, and EGFR mutation status for HCC827 are shown in **Table 1**.

Table 1: Summary of HCC827 Model Background

Cancer Type	Model	Model Type	Model Information	Background
NSCLC; adeno carcinoma	HCC827	Subcutaneous	A lung epithelial adenocarcinoma, derived from a Caucasian female aged 39 years[8]. HCC827 has an acquired mutation in the EGFR tyrosine kinase domain (E746-A750 deletion)[9].	MF-1 nude, BALB/c nude

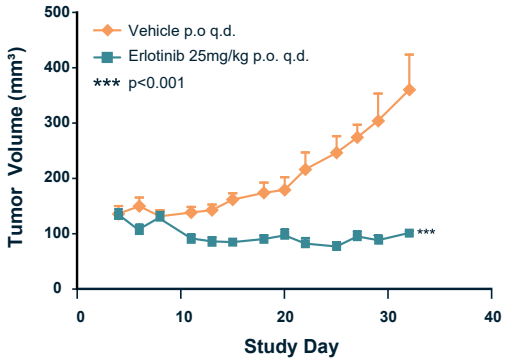
HCC827 Subcutaneous Model Standard of Care Data

EGFR exon 19 deletions confer increased sensitivity to first generation EGFR tyrosine kinase inhibitors such as erlotinib and gefitinib[1]. Treatment of the HCC827 model with erlotinib was well-tolerated, with tumor regression observed (**Figure 1**).

Generation of EGFR Tyrosine Kinase Inhibitor Resistant Models - *In Vitro* Generation of Resistant Cell Lines

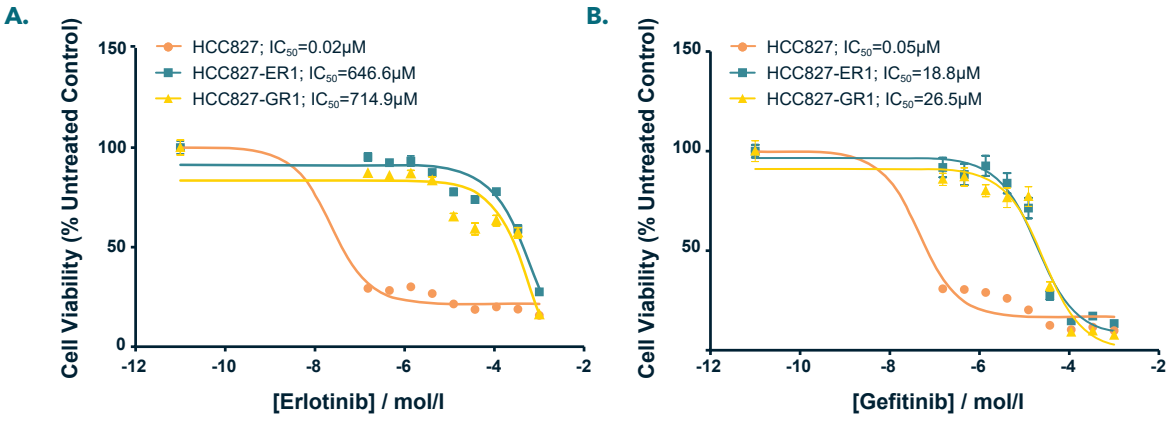
To understand the mechanisms of EGFR TKI resistance, erlotinib and gefitinib-resistant cell lines (HCC827-ER1 and HCC827-GR1, respectively) were generated via repeated *in vitro* exposure of the HCC827 wild type cell line to escalating concentrations of the respective inhibitors. Cross resistance was observed between the two resistant variants (**Figure 2**).

Figure 1: HCC827 Subcutaneous Model SoC Data Single Agent Erlotinib



n=5 animals per group.

Figure 2: Response of HCC827, HCC827-ER1, and HCC827-GR1 to Erlotinib and Gefitinib



A: Erlotinib IC_{50} values. B: Gefitinib IC_{50} values.
HCC827-ER1/GR1 >10,000-fold shift in IC_{50} for erlotinib
HCC827-ER1/GR1 >500-fold shift in IC_{50} for gefitinib

Genetic characterization was performed for all three cell lines (HCC827, HCC827-ER1, and HCC827-GR1) to establish possible mechanisms of resistance. It was confirmed that all three cell lines still harbored the EGFR exon 19 E746-A750 deletion as well as having a matched STR profile to the HCC827 wild type line; however, the EGFR exon 20 T790M 'gatekeeper' mutation was not detected in the wild type or resistant cell lines. Both c-MET copy number and Axl gene expression were statistically significantly increased ($p \leq 0.05$; ANOVA) for the resistant cell lines compared with wild type HCC827 (**Figure 3**).

In Vivo Translation of Resistance - Cell Line Derived Xenograft Model

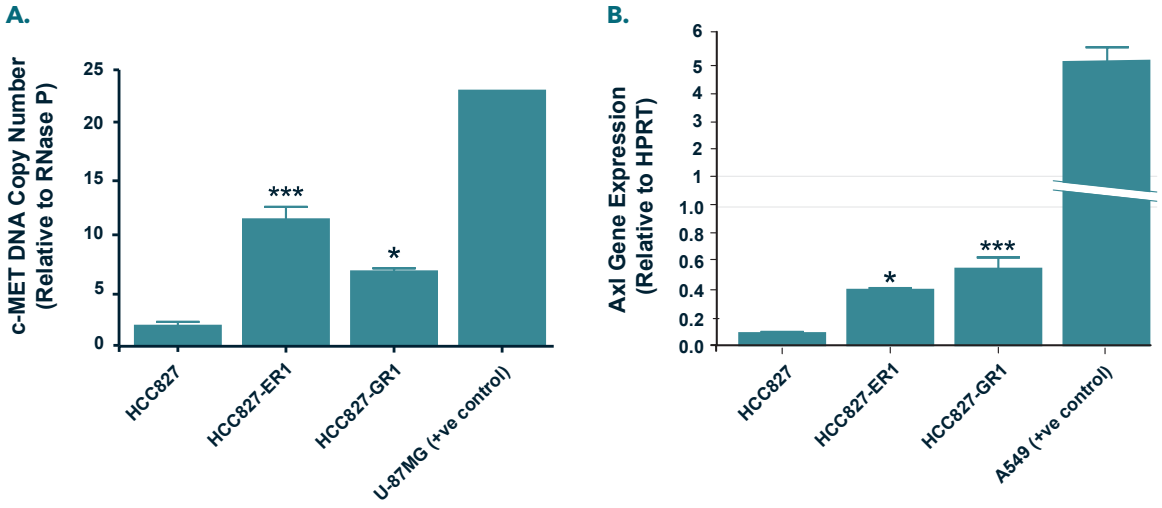
The HCC827-ER1 resistant cell line was implanted subcutaneously into MF-1 nu/nu mice. The model was treated with erlotinib, and tumor growth was compared with equivalent treatment of the wild type HCC827 model (**Figure 4**).

For the wild type model, treatment with erlotinib resulted in stable tumor regression up to Day 36 and beyond. For HCC827-ER1, although there was tumor growth inhibition when compared with vehicle control (T:C of approximately 0.4) there was clear tumor regrowth. In clinical terms, this denotes partial regression versus progressive disease, and is indicative of treatment escape/resistance.

In Vivo Refinement of the HCC827-ER1 EGFR TKI Resistant Cell Line

To further refine the HCC827-ER1 model, xenografts were outgrown under gefitinib dosing pressure (outgrowth shown in **Figure 5**).

Figure 3: HCC827-ER1 and HCC827-GR1 Genetic Characterization



A: c-MET genomic amplification characterized by qPCR relative to RNase P, U-87 MG cell line included as positive control. B: Axl gene expression characterized by RT-PCR relative to HPRT, A549 cell line included as positive control. Both panels: *p<0.05, ***p<0.01.

Figure 4: HCC827-ER1 Subcutaneous Model SoC Data Single Agent Erlotinib

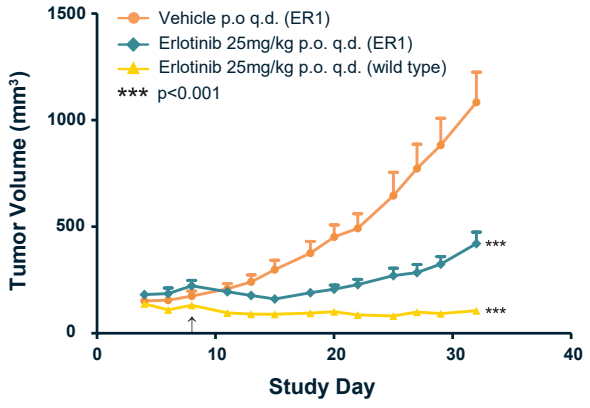
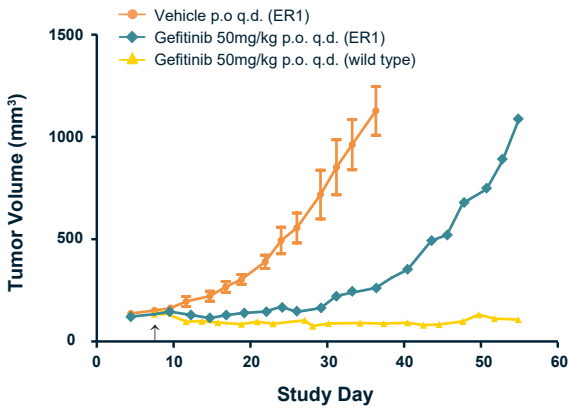


Figure 5: HCC827-ER1 Resistant Outgrowth and Generation of PCS030



The xenograft was excised, disaggregated, and purified *in vitro* to re-derive the cell line (denoted PCS030) which was genetically characterized via c-MET, Axl, and EGFR kinase sequencing. A significant increase in c-MET copy number was observed between the PCS030 and wild type HCC827 cell lines; however, no significant difference was observed for relative Axl gene expression (**Figure 6**). It was also confirmed that both pre- and post-resistance cell lines contained the EGFR exon 19 E746-A750 deletion, and did not contain the exon 20 T790M 'gatekeeper' mutation.

Single Cell Clone Generation

To generate a number of cell lines with a range of c-MET amplification, clones were generated *in vitro* from single-cell isolates of the resistant cell lines HCC827-ER1, HCC827-GR1, and PCS030. c-MET copy number was shown to be amplified across all clones when compared with their respective mixed clone cell lines (**Figure 7**).

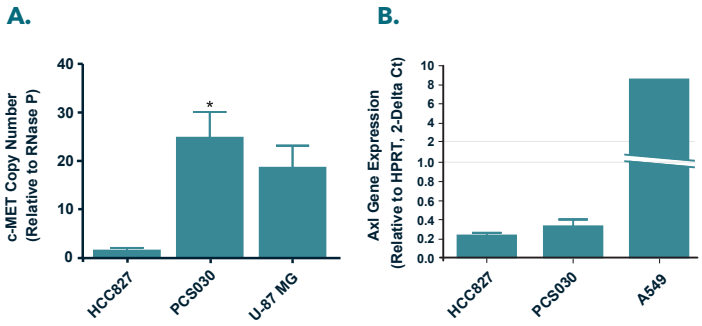
Combination Index *In Vitro*

Combination treatment was assessed *in vitro* in the HCC827, HCC827- ER1, and HCC827-GR1 clone 10 cell lines. Cells were treated with either erlotinib, crizotinib, or a combination of the agents (results shown in **Table 2**). An increasing synergistic response corresponded with increasing c-MET amplification from the HCC827 to the HCC827- GR1 clone 10 cell line. These clones can be used *in vivo* to evaluate combination strategies to overcome resistance.

In Vivo Combination Therapy Confirms the Role of c-MET Amplification in the Resistance Mechanism for HCC827 ER1[10]

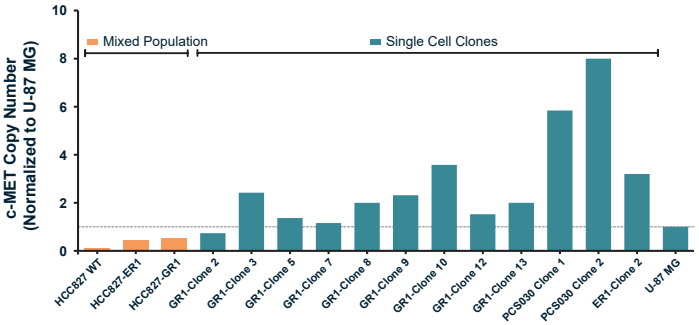
Subcutaneous HCC827 and HCC827-ER1 models were treated with either erlotinib, crizotinib (which is used in this context as a c-MET inhibitor to target the c-MET genomic amplification previously documented in **Figure 3**), or a combination of both agents concurrently. Treatment of HCC827 xenografts with erlotinib and the erlotinib/crizotinib combination resulted in tumor regression (without an additive effect); however, there was no effect of crizotinib alone (**Figure 8A**).

Figure 6: PCS030 Genetic Characterization



A: c-MET copy number characterized by qPCR relative to RNase P, U-87 MG cell line included as positive control; *p<0.05. B:Axl gene expression characterized by RT-PCR relative to HPRT, A549 cell line included as positive control.

Figure 7: HCC827-GR1 and PCS030 Single Cell Clones: c-MET Copy Number



c-MET copy number characterized by qPCR relative to RNAse P. c-MET copy number is shown normalized to U-87 MG (positive control).

Table 2: HCC827, HCC827-ER1, and HCC827-GR1 Clone 10 Combination Treatment Analysis[10]

Cell Line	Compound/Combination	Combination index (CI)				Weighted CI	Score
		ED50	ED75	ED90	ED95		
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 IC_{50})
	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

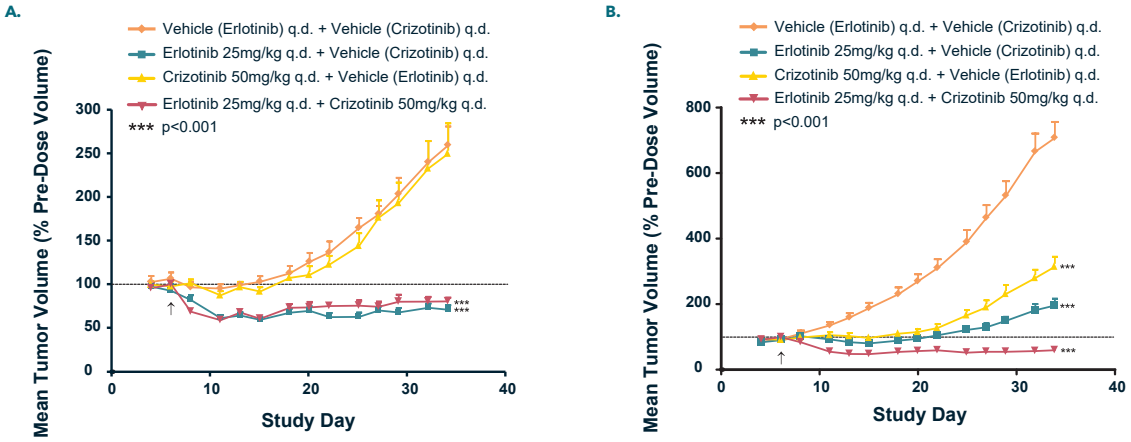
Combination ratios of 1:1 and 1:100 (erlotinib:crizotinib) used in the parental line, and 1:1 and 100:1 used in the resistant cell lines. Following treatment, cell viability assessed using CellTiter-Blue™. Synergistic effect calculated based on combination index values according to the Chou and Talalay method (CalcuSyn).

For the HCC827-ER1 model[11], erlotinib and crizotinib monotherapy resulted in tumor growth inhibition when compared with vehicle control, but no tumor regression (progressive disease). Combination treatment resulted in a statistically significant reduction in tumor growth compared with erlotinib or crizotinib monotherapy and restored tumor regression. This indicates an additive effect and confirms the role of c-MET in the resistance mechanism for HCC827-ER1 (**Figure 8B**).

Combination Therapy Strategies with the HCC827 Model

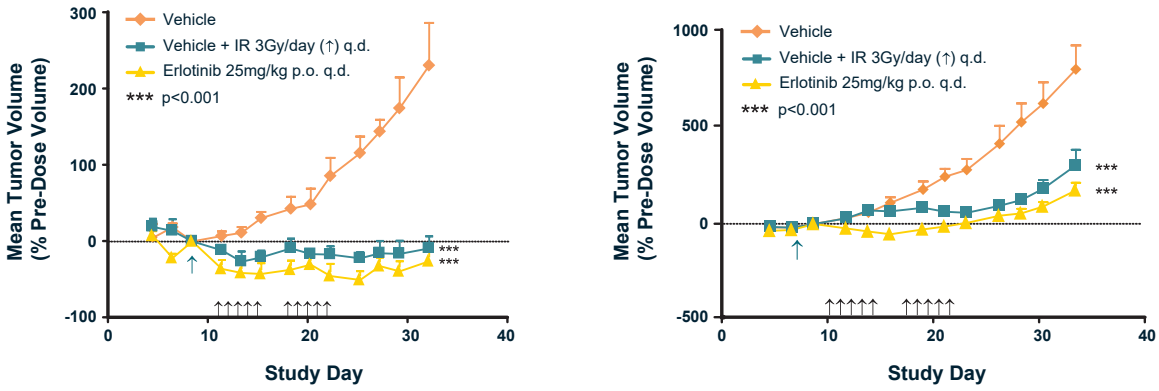
Overexpression of c-MET occurs in about half of NSCLCs and has been associated with resistance to EGFR TKIs, radiation therapy, and poor patient survival[12,13]. As c-MET amplification has been documented as the mechanism driving EGFRTKI resistance in the HCC827-ER1 cell line, crizotinib and erlotinib were evaluated in both the HCC827 and HCC827-ER1 models in combination with IR. Initially, HCC827 response to IR alone was evaluated. The subcutaneous HCC827 and HCC827-ER1 models were treated using the image-guided small animal radiation research platform (**Figure 9**). The wild type model showed high sensitivity to IR, resulting in tumor regression similar to that seen with erlotinib treatment (**Figure 9A**). The erlotinib resistant cell line showed a higher growth rate, and reduced sensitivity to radiation as well as to erlotinib (**Figure 9B**). Further evaluation of other resistant clones is ongoing.

Figure 8: HCC827 and HCC827-ER1 Combination Erlotinib and Crizotinib Treatment



Tumor volume expressed as % of initial dose volume. Dosing from Day 6 (↑). n=5 mice per group, error bars = SEM. A: HCC827 B: HCC827-ER1.

Figure 9: HCC827 and HCC827-ER1 IR Treatment Data



Tumor volume expressed as % of initial dose volume. Erlotinib dosing from Day 8 (↑), daily IR dosing (↑). n=5 mice per group, error bars = SEM. A: HCC827 B: HCC827-ER1.

Combination studies with erlotinib and crizotinib followed our initial IR investigations. IR treatment of HCC827 xenografts (**Figure 10A**) resulted in tumor regression equivalent to that seen with erlotinib monotherapy. Concurrent treatment of erlotinib or crizotinib with IR also resulted in tumor regression. However, as there was no statistically significant difference between IR monotherapy and any of the combination groups, it is likely that there was no additive combination effect in this model.

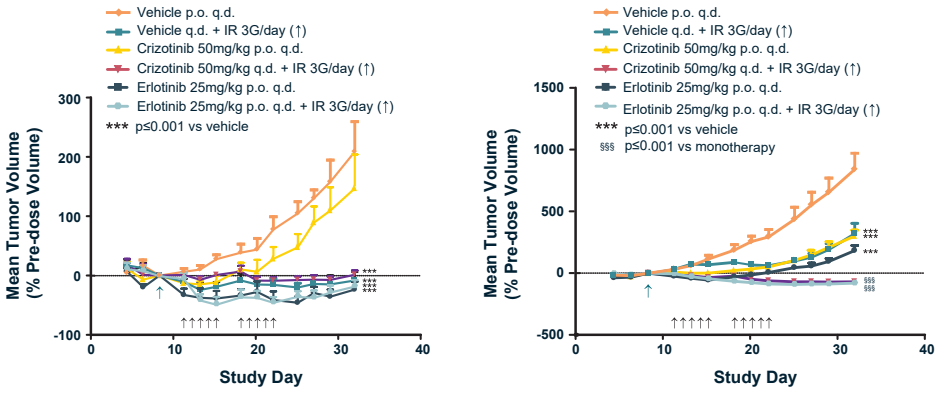
Unlike HCC827, treatment of c-MET-amplified HCC827-ER1 xenografts with IR monotherapy resulted in a statistically significant tumor growth inhibition (50% compared with vehicle control), but not tumor regression (**Figure 10B**). However, when IR was combined with crizotinib, tumor regression was restored, therefore supporting the role of c-MET signaling in the radiation-resistance of this model. Interestingly, combination treatment with erlotinib and IR also restores tumor regression and warrants further investigation as several studies have highlighted the role of EGFR inhibitors as potential radiosensitizers across a range of cancer types[14,15,16].

Outgrowth of *In Vivo* Combination Therapy

The regrowth of HCC827 and HCC827-ER1 tumors was followed from the cessation of dosing to study termination (**Figure 11**). In the HCC827 model, there was an equivalent tumor regression with erlotinib and IR monotherapy, and the combinations of erlotinib/IR and crizotinib/IR during the dosing phase. Following cessation of dosing, there was regrowth in treatment groups with the exception of combination treatment of erlotinib/IR, where tumor regression was sustained until the end of the study (**Figure 11A**). This suggests that EGFR TKI efficacy may be enhanced sufficiently by concurrent IR (or vice versa) to produce a stable curative response; further studies are required to confirm whether this is the case.

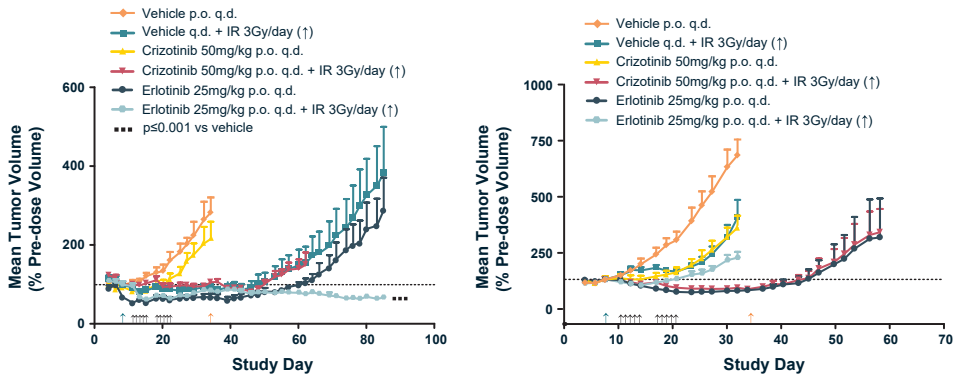
For the HCC827-ER1 model, tumor regression was not retained following cessation of dosing with erlotinib/IR or crizotinib/IR suggesting that neither combination results in a stable curative response over the period/ doses tested (**Figure 11B**). The efficacy would be enhanced through maintenance of dosing pressure and/or increased dose levels; however, resistance versus the combination treatment may still emerge at a latter point.

Figure 10: HCC827 and HCC827-ER1 Combination IR and Erlotinib or Crizotinib Treatment



Tumor volume expressed as % of initial dose volume. Erlotinib/crizotinib dosing from Day 8 (↑), daily IR dosing (↑). n=5 mice per group, error bars = SEM. A: HCC827 B: HCC827-ER1.

Figure 11: Outgrowth of HCC827 and HCC827-ER1 Models



Tumor volume expressed as % of initial dose volume. Dosing from Day 8 (↑) to Day 34 (↑), daily IR dosing (↑). n=5 mice per group, error bars = SEM. A: HCC827 B: HCC827-ER1.

Combining all three treatments would reduce this risk and may provide the degree of stable response seen in the non-resistant HCC827 model.

Immunotherapy HCC827 Research - Checkpoint Inhibitor Evaluation using the HCC827 MiXeno Model[17]

The HCC827 Mi**Xeno** model has been utilized as a platform for evaluating anti-PD-1 and anti-PD-L1 antibodies (detailed in our standalone Mi**Xeno** Factsheet). Using Xeno**Base**, 1036 cancer cell lines including skin, lung, kidney, large intestine, liver, and prostate lines were screened for expression levels of PD-L1 (CD274; **Figure 12**). From our results, we performed FACS analysis on 16 of the high scoring cell lines, to further determine the surface PD-L1 expression levels (results shown in **Table 3**). The HCC827 cell line was identified as highly expressing PD-L1, and was used to develop a Mi**Xeno** model for *in vivo* efficacy evaluation. PBMC were injected intravenously into the mouse, followed by tumor cell inoculation.

Figure 12: Screening of PD-L1 Expression Levels using XenoBase

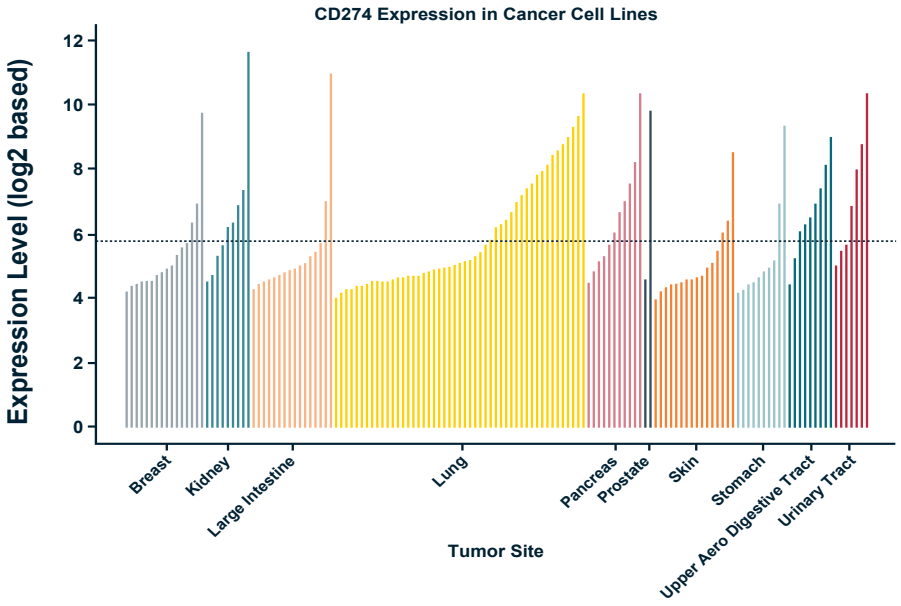


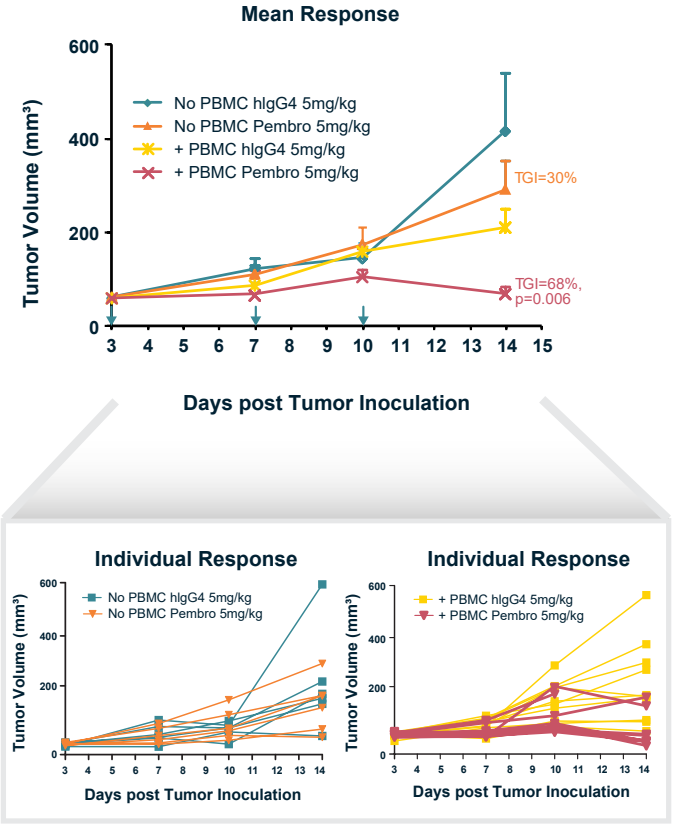
Table 3: MiXeno Immunotherapy Model HCC827 Validation: PD-L1 Expression Levels from XenoBase and by FACS Analysis

Cancer type	Cell line	PD-L1 Level (XenoBase)	PD-L1 level (MFI)
Melanoma	SK-MEL-28	5.5561	7
	A2058	5.0168	35.9
	A375	4.8704	13.4
NSCLC	NCI-H292	9.2971	136.8
	HCC827	**8.4718**	**95**
	NCI-H358	7.5277	150.9
	SK-MES-1	7.3042	157.8
	NCI-H1975	6.8753	134.8
	NCI-H2228	6.3227	131.2
	NCI-H1650	5.8356	31.4
TNBC	MDA-MB-231	8.1606	283.1
Kidney Cancer	786-O	7.2959	43.7
	A498	5.1619	19.5
H&N	SCC-4	7.3154	26.7
Bladder Cancer	5637	9.0472	NA
	RT-112	4.9792	NA

Treatment of the resulting immunocompetent model with an anti-PD-1 antibody (pembrolizumab) resulted in approximately 70% tumor growth inhibition (TGI), whilst treatment of the control model (lacking PBMCs) resulted in TGI of only 30%. Antitumor efficacy was observed to vary in mice implanted with different PBMC donors upon treatment with the same drug (**Figure 13**)[18].

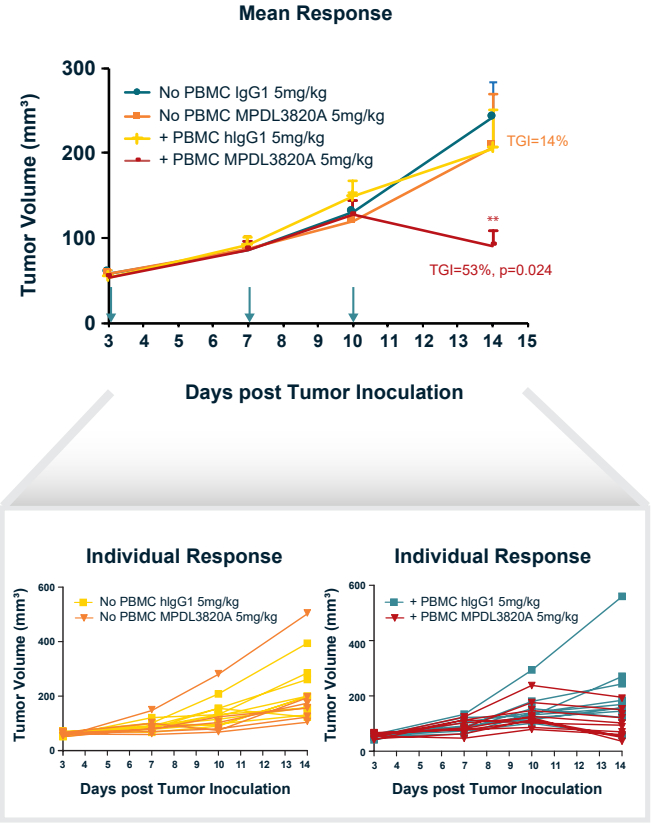
The HCC827 **MiXeno** model has also been used to demonstrate the significant antitumor activity of an anti-PD-L1 antibody (MPDL3820A). Treatment of the immunocompetent model with MPDL3820A resulted in approximately 50% TGI whilst treatment of the control model (lacking PBMCs) resulted in TGI of only 14% (**Figure 14**). Antitumor efficacy was observed to vary in mice implanted with different PBMC donors upon treatment with the same drug. Increased lymphocyte presence in the blood was also observed following anti-PD-L1 antibody treatment (**Figure 15**)[18].

Figure 13: MiXeno HCC827 NSCLC Model: Pembrolizumab Anti-PD-1 Evaluation[18]



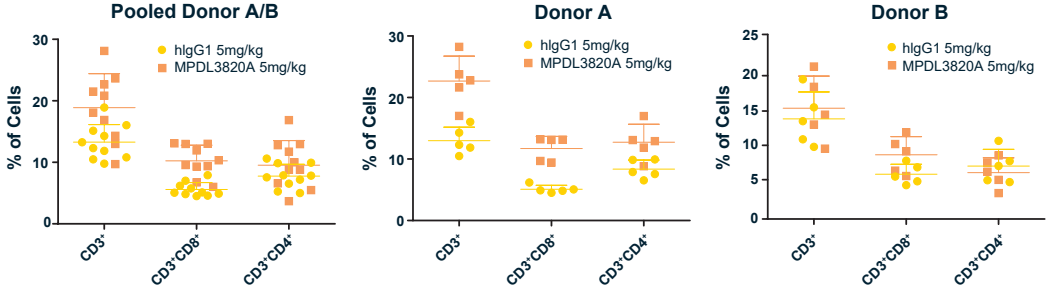
Day -3: PBMC implantation, i.v. Two donors: Donor A: mice #1-5/arm; Donor B: mice #6-10/arm. Day 0: Tumor cell inoculation, s.c. Arrows indicate the administration of antibodies. Graph shows mean tumor volume + SEM.

Figure 14: MiXeno HCC827 NSCLC Model: MPDL3820A Anti-PD-L1 Evaluation[18]



Day -3: PBMC implantation, i.v. Two donors: Donor A: mice #1-5/arm; Donor B: mice #6-10/arm. Day 0: Tumor cell inoculation, s.c. Arrows indicate the administration of antibodies. Graph shows mean tumor volume + SEM.

Figure 15: MiXeno HCC827 NSCLC Model: Increased Lymphocyte Presence Following MPDL3820A Treatment[18]



Blood samples analyzed by FACS on Day 14 after human PBMC implantation.

Summary

Crown Bioscience provides a range of NSCLC and EGFR cancer xenograft resources, including the HCC827 **Validated**Xeno cell line derived xenograft model, which harbors the clinically relevant EGFR exon 19 E746-A750 deletion which confers oncogene addiction and sensitivity to EGFR TKIs.

Treatment of the HCC827 model with erlotinib induced tumor regression; however, resistance to erlotinib and gefitinib could be generated *in vitro* via continuous exposure to the respective drug. Analysis showed that the resistant models have amplified c-MET copy number when compared with the wild type cell line.

Combination treatment of a c-MET inhibitor (crizotinib) and erlotinib overcomes resistance in these c-MET driven EGFR TKI resistant models both *in vitro* and *in vivo*; therefore, c-MET inhibition supports the hypothesis of c-MET driven resistant mechanisms in NSCLC harboring EGFR activating mutations. In addition, c-MET amplification confers resistance to IR which was reversed with a crizotinib combination approach. Further interrogation of combination effect could be evaluated by outgrowth approach to determine the longevity of response and emergence of resistance.

Gene and protein expression of a HCC827 wild type cell line showed high PD-L1 expression. A HCC827 **Mi**Xeno model of transient human immunity was therefore validated for the evaluation of human immunotherapeutics. The **Mi**Xeno platform is developed by mixing PBMCs with xenograft models and provides a simple alternative to a full stem cell reconstitution approach for immunotherapy research. Our validated **Mi**Xeno HCC827 model responds to anti-PD-1 and anti-PD-L1 treatment, with anti-PD-L1 response accompanied by an increased lymphocyte presence in the blood, confirming that this is a valid platform for immunotherapy research.

In summary, the HCC827 model represents a clinically relevant subset of NSCLC which can be used to interrogate targeted agents, resistance, and immunotherapeutics. In addition, Crown Bioscience's unique models offer opportunities to investigate combination strategies with targeted agents and immunotherapeutics.

Contact us at **busdev@crownbio.com** for any further questions or information required on our HCC827 model, our NSCLC and EGFR resources, our *In Vivo* Immunotherapy Drug Discovery Application Note, or for information on other Crown Bioscience products and services.

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