CROWN BIOSCIENCE

3 Preclinical Strategies to Identify Predictive Cancer Biomarkers

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WHITE PAPER

V1.0

The Challenge: How to Discover Robust and Sensitive Biomarkers Early in Drug Development to Identify the Right Patient Populations and Cancer Indications

The majority of experimental anticancer agents fail during the late stages of clinical development, after substantial time and expense have been invested[1]. One strategy that could help overcome these setbacks is to develop and validate robust and sensitive biomarkers early in the drug development process[2]. Using a biomarker-guided approach offers the opportunity to identify the most promising candidate therapies at an early stage and tailor novel therapeutics for specific patient populations and cancer indications. This approach has been fueling precision medicine strategies that have shown improvements in drug efficacy and patient safety, while reducing the attrition rate of anticancer agents.

This White Paper presents biomarker discovery and validation strategies in early-stage drug development through a systems biology approach, and also explores the translation of preclinical biomarkers to the clinic to improve trial outcomes. Three case studies are presented highlighting how to identify and utilize biomarkers using a range of translational preclinical models. These strategies allow researchers to funnel therapeutics through *in silico*, *in vitro*, *ex vivo*, and *in vivo* model systems efficiently, reducing timelines and maximizing data utilization.

Biomarker Utility in Modern Oncology Drug Development

Cancer is an extremely heterogeneous disease, with individual patients showing widely differing responses to the same treatment[3]. This has led to the rise of precision medicine approaches that aim to target the right drug to the right patient at the right time. This relies on developing specific therapeutics to treat a particular tumor characteristic, and correctly identifying patients harboring this characteristic. With more than 6,000 targeted therapeutic agents currently in development, there is a significant need to identify predictive biomarkers to stratify the cancer patient population to receive the correct treatments based on their tumor type.

The use of cancer biomarkers has taken great steps forward in recent years. A major advancement in the field came in 2017 with the first FDA approval for an anticancer agent based on a biomarker regardless of the cancer's origin or location. The FDA granted accelerated approval to Keytruda® (Merck) to treat adults and children with unresectable solid tumors, specific genetic biomarkers (known as microsatellite instability-high or deficient DNA mismatch repair), and a lack of satisfactory treatment alternatives or tumor progression following prior therapy[4].

Soon after, the FDA approved the first next-generation sequencing oncology panel test to serve for multiple companion diagnostic indications[5]. More recently, the FDA approved a second treatment - Vitrakvi® (Loxo Oncology/Bayer) - based on a specific genetic biomarker (TRK gene fusion without a known acquired resistance mutation) rather than tumor type. Vitrakvi was the first anticancer agent to receive a tumoragnostic indication at the time of initial FDA approval[6].

These biomarker-based approvals represent major milestones in the field of precision medicine in general and precision oncology specifically. Many oncology trials continue to pursue biomarkers for patient selection, which is not surprising given that such trials have shown higher overall success probabilities than trials without biomarkers[7].

The Benefits of Early-Stage Biomarker Development

Target validation and biomarker identification in early-stage drug development has many benefits. Researchers can gain in-depth insight into agent mechanisms of action as well as pharmacodynamic responses. These efforts are critical to identify the most promising therapeutic candidates and then focus downstream efforts on those with the most favorable profiles and real potential. By embedding a systems biology approach early in drug development, optimal targets and models can be selected and utilized at the preclinical stage.

To do this effectively, researchers need access to a large suite of well-characterized models that adequately capture the diversity observed in patient populations. This allows agents to be funneled through database selection, computer modeling, *in vitro* screening, *ex vivo* models, and *in vivo* testing in an intelligent way, reducing costs and timelines and fully capitalizing on data value.

Early identification of biomarkers also allows data informed decisions on clinical trial design. By translating preclinical biomarkers into the clinic as companion diagnostics (CDx), relevant clinical populations can also be identified and stratified to optimize the chances of success.

Novel Strategies to Identify and Utilize Biomarkers

Novel strategies to discover and apply biomarkers are discussed below through three case studies. These strategies cover in vitro screening, *in vivo* corroboration of clinical data, and retrospective data analysis combined with mouse clinical trial (MCT) data to help optimize clinical biomarker selection.

Case Study 1

Identifying Biomarkers from *In Vitro* Screening

Case Study 1 aimed to identify a primary cancer indication and generate biomarker sets using an *in vitro* screening platform. The agent evaluated was BEZ235, a small molecule PI3K/mTOR dual inhibitor[8]. Deregulation of the PI3K/mTOR signaling pathway is common across multiple human cancer types, contributing to tumorigenesis, tumor progression, and treatment resistance[9].

The main objectives of Case Study 1 were to:

- Predict the response to BEZ235 for cancer cell lines across a range of indications
- Develop an understanding of the genetic signatures of the responsive indications
- Provide advice and guidance on future in vivo model selection

A multi-step approach was taken to meet these objectives, which consisted of an *in vitro* screen to assess the anti-proliferative activity of BEZ235, followed by biomarker discovery through correlating BEZ235 pharmacology data with genomic baseline information, including gene expression, gene mutation, copy number variation, and pathway/network activation.

Step 1: BEZ235 Cell Panel Screening

The anti-proliferative activity of BEZ235 was examined on a panel of 307 human cancer cell lines across a range of different cancer indications (Figure 1). Data analysis began with endpoint calculations (for IC_{50} and AUC) and comparison across the cancer types. A logarithmic relationship was observed between IC_{50} and AUC, with AUC being the preferred endpoint for further analysis.

Significant variance in response was observed across the different cancer types. If too much variation is found within screening results, correlation analyses are based on indication specific grouping. Otherwise, all cancer types can be analyzed in one group. From the results and variance seen in this screen, hematopoietic and lymphoid cell lines were selected to be taken forward for further interrogation.

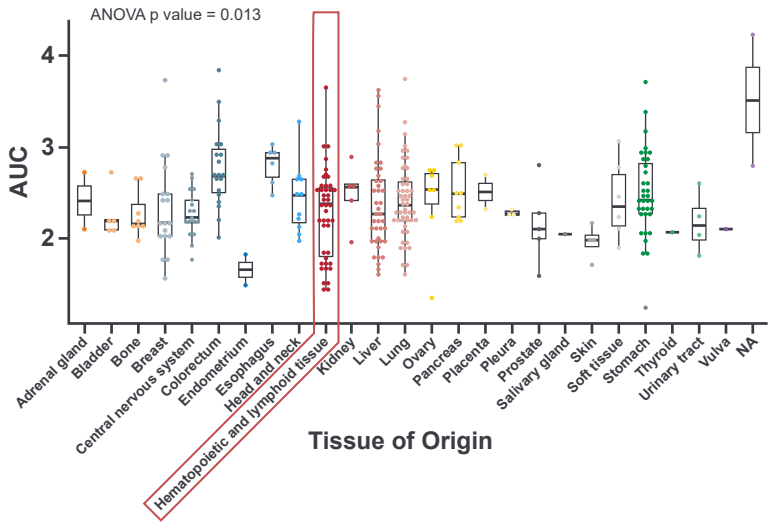
Step 2: Data Partitioning for Biomarker Discovery

To classify cell lines based on drug response, an AUC greater than 2.6 was defined as insensitive, while an AUC less than 2.2 was defined as sensitive. Cell lines with an AUC between these two values were not included in the analysis. In the hematopoietic and lymphoid cell line group, 14 cell lines were defined as sensitive to BEZ235 treatment, while 14 cell lines showed no response and were therefore determined to be insensitive.

Step 3: Single-Gene Analysis

A complete analysis of the genomic mutational landscape was performed as step 3, with candidate genes nominated and sorted according to statistical significance (from a pool of 20,000 genes). Correlations and inverse correlations were identified between response to BEZ235 and genetic mutations. From these analyses, 23 genes were identified whose mutational status strongly correlated with sensitivity to BEZ235.

Figure 1: *In Vitro* Screening of BEZ235 Indicates Significant Variance Across Cancer Types



Step 4: Composite Biomarker Construction and Evaluation

Next, a 13-gene biomarker set expression analysis was constructed. This composite biomarker correctly predicted sensitivity to BEZ235 for the panel of 24 hematopoietic cancer cell lines, with a prediction accuracy of 83% (Figure 2). Relevant genes in this biomarker panel included KANK1 which is regulated by phosphorylation through PI3K/Akt signaling, and SART1 which are proteins involved in the regulation of cell proliferation.

Moving beyond single-gene analysis, pathway and network analyses identified a signature composed of 21 gene sets/ pathways that significantly differentiated BEZ235 sensitive and insensitive cell lines (Figure 3).

For example within these pathways, HER2 is a tyrosine kinase receptor upstream of the PI3K-Akt-mTOR signaling pathway, which controls many cellular processes that are important for the formation and progression of cancer(10). As genetic alterations and biochemical activation of the PI3K-Akt-mTOR pathway are frequent events in preneoplastic lesions and advanced cancers (and also indicate a poor prognosis), inhibition of this pathway is an attractive concept for cancer prevention and/or therapy.

Case Study 1 Conclusions

Case Study 1 proposes genetic signatures that predict cell line sensitivity to BEZ235, including identifying genes whose expression levels correlate with drug sensitivity, and gene sets which differentiate sensitive/insensitive cell lines. The results provide information that can be harnessed to identify the most appropriate *in vivo* models to further investigate BEZ235.

Case Study 1 also confirms the utility of an *in vitro* screening approach for biomarker discovery. These screens provide multiple tracks of unbiased, data driven statistical analysis in mutation, expression, and pathway to derive potential candidate biomarker sets for correct indication and potential patient selection criteria. It should be noted that these screens are usually preliminary, acting as a prelude to more in-depth functional and mechanistic studies *in vivo* and for the clinical phase.

While cell lines were used for screening in this case study, other platforms are also available for initial *in vitro* biomarker discovery. This includes organoids models, providing a translational bridge between *in vitro* and *in vivo* studies.

Figure 2: Transcriptomic Biomarker Predicts Hematopoietic Cancer Cell Line Response to BEZ235

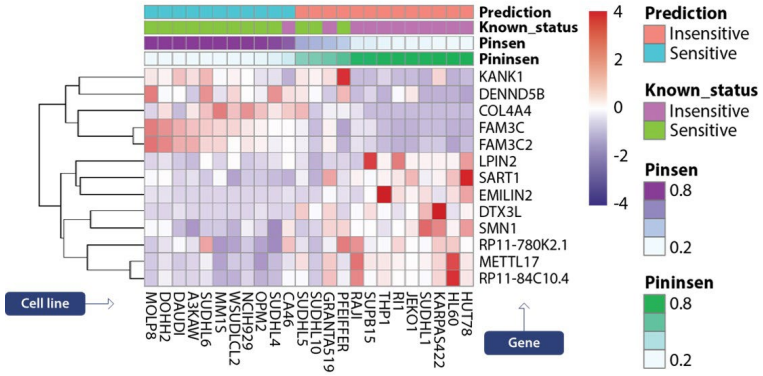
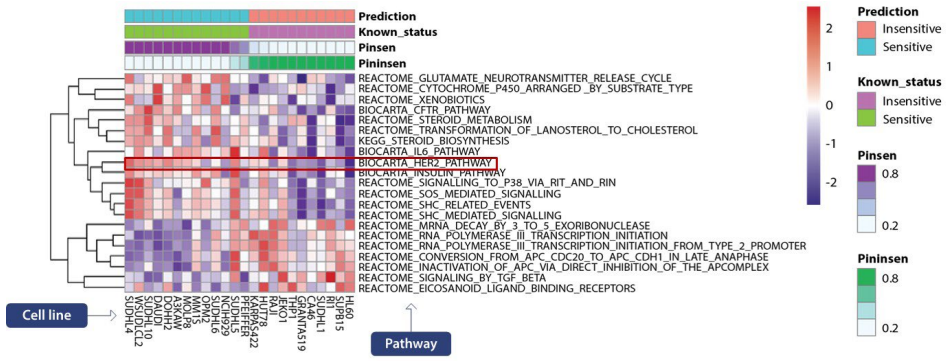


Figure 3: Biological Pathway Signature Significantly Differentiates BEZ235 Sensitive and Insensitive Cell Lines



Case Study 2

Exploring Drug Mechanism of Action using Unique PDX Models

This case study aimed to demonstrate how clinical observations can be corroborated using preclinical *in vivo* models. Specifically, RET fusion models were applied to study selpercatinib (LOXO-292, Loxo Oncology) which was designed to inhibit native RET signaling as well as potential acquired resistance mechanisms.

Genomic alterations in the RET kinase, including fusions and activating point mutations, lead to overactive RET signaling and uncontrolled cell growth. RET fusions have been identified in several cancer types, including non-small cell lung cancer (NSCLC) and thyroid cancer. RET-altered tumors often exhibit "oncogene addiction", a phenomenon that renders them dependent upon a single activated oncogene and therefore highly susceptible to therapies targeting the RET kinase.

Ponatinib is a small molecule inhibitor, approved for use in CML and Ph+ALL with broad activity against multiple tyrosine kinases, including RET. However, acquired resistance to ponatinib often limits its activity and utility. Therefore, more specific and selective RET inhibitors with increased potency, less off-target toxicities, and the ability to combat resistance to first-line therapies are needed.

The main objectives of Case Study 2 were to confirm the mechanism of action of LOXO-292 by:

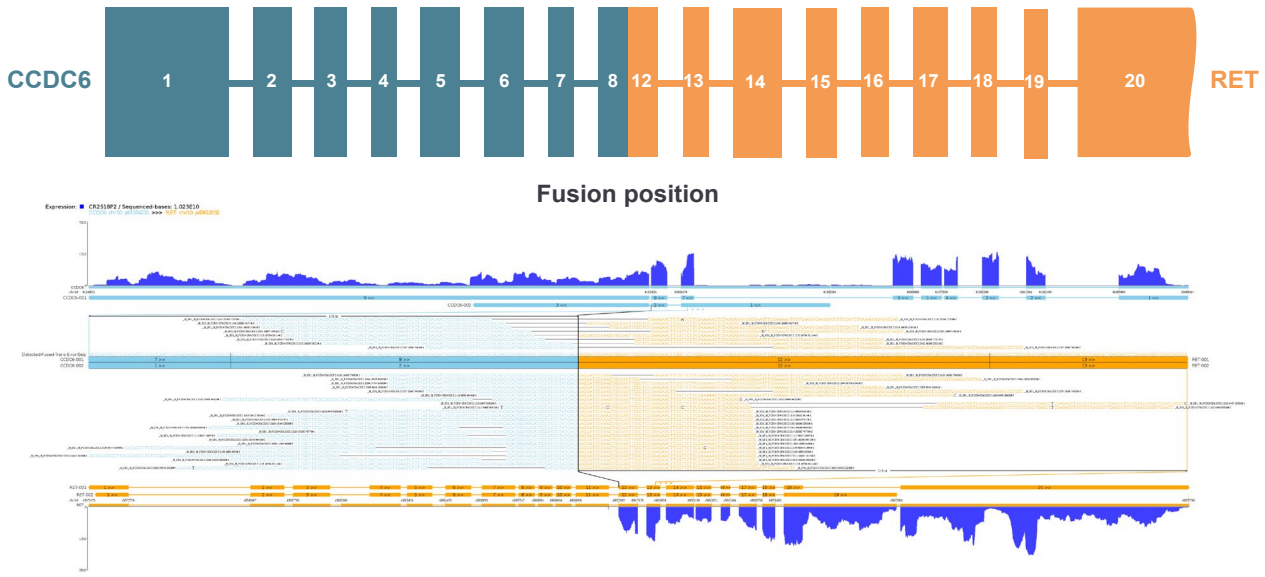
- Identifying appropriate mouse models recapitulating clinical RET events
- Conducting preclinical efficacy studies to compare LOXO-292 with relevant cancer therapies.

Recapitulating RET Fusions in Unique PDX Models

As a starting point for LOXO-292 assessment, appropriate *in vivo* models recapitulating clinical genotypes were needed. Patient-derived xenograft (PDX) models retain many features of human tumors due to their derivation process – generated directly from patient samples and never adapted to grow *in vitro* (on plastic). This means that PDX retain more of their parental tumor histopathology and genetics, without the genetic drift seen over many years of passage with conventional, cell line derived xenografts.

By searching collated databases of well-characterized and sequenced PDX, appropriate models featuring RET fusions were identified for further study. This included the colorectal cancer model CR2518, which harbors a CCDC6-RET fusion (Figure 4).

Figure 4: PDX Model CR2518 Harbors a CCDC6-RET Fusion



LOXO-292 was assessed vs ponatinib in an orthotopic CR2518 brain cancer model. The model was developed by implanting PDX CR2518 stereotactically in the brain to mimic brain metastasis. In this study, treatment with LOXO-292 showed superior efficacy, characterized by a marked improvement in survival, over ponatinib and vehicle control (Figure 5).

To further understand the mechanism of LOXO-292 action, PDX model CR2518 was used to develop a ponatinib resistant model (CR2545). This ponatinib resistance was induced with extended, repeated ponatinib treatment of CR2518 (Figure 6).

Given the clinical significance of ponatinib resistance, the molecular events that led to its emergence were further explored. Sequencing the CR2545 PDX model revealed a dominant valine 804 methionine (V804M) mutation in addition to the pre-existing RET gene fusion. Previous structural analysis has suggested that this mutation leads to a bulkier amino group and disruption of the pocket domain that may impede access to inhibitors, such as ponatinib(11,12). The V804M mutation has also been reported to confer a gain of function for the RET protein, resulting in increased kinase activity, cell transformation, and to act as a gatekeeper for response to some small molecule inhibitors(13).

Despite harboring multiple RET alterations, CR2545 remained sensitive to LOXO-292 treatment, with a dose-dependent inhibition of tumor growth and greater efficacy than ponatinib or cabozantinib (Figure 7).

Following these promising results, LOXO-292 moved into clinical trials. Clinical data showed that, regardless of indication, any patients carrying the RET fusion demonstrated a very high objective response rate, close to 70% (Table 1). In patients with the V804M mutation, the objective response rate was also high, at 60%. Consistent with the mechanism of action of LOXO-292, no response was observed in patients lacking the fusion or an activating RET mutation.

Figure 5: LOXO-292 Improves Survival over Ponatinib in Orthotopic Brain Model

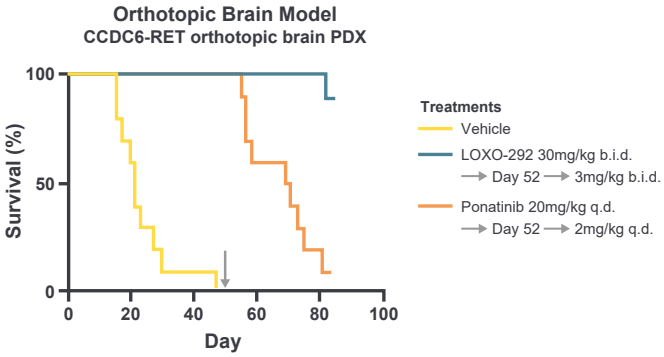


Figure 7. Efficacy of LOXO-292 in aPonatinib-Resistant PDX Model

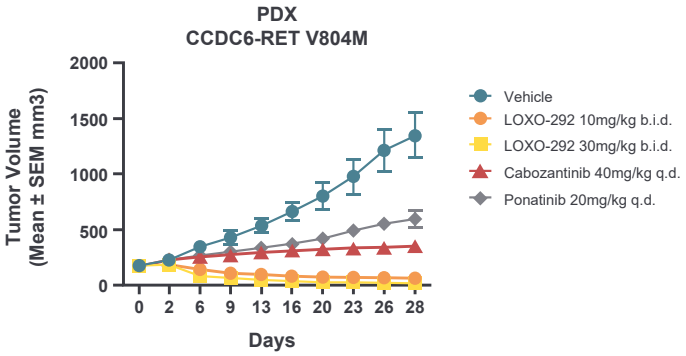
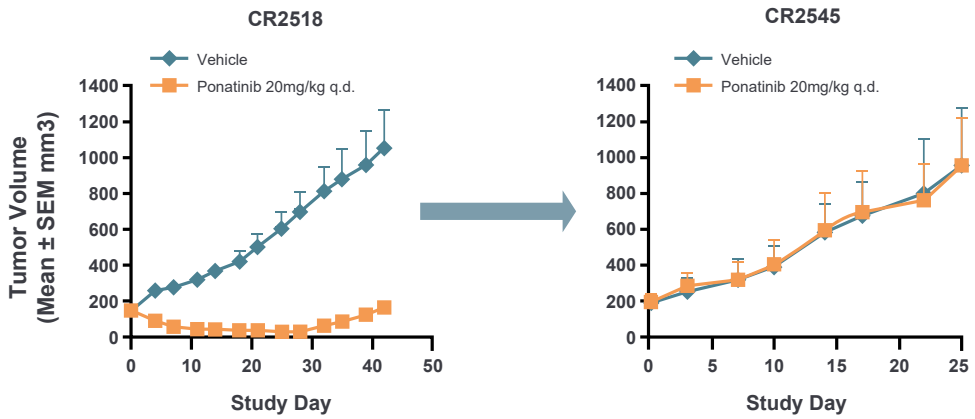


Figure 6: Development of a Drug-Induced Ponatinib Resistant PDX Model



Case Study 2 Conclusions

This case study confirmed the mechanism of action of LOXO-292 through the generation of a novel RET fusion and mutation *in vivo* model. The study also provided essential preclinical data showing the superior efficacy of LOXO-292 over SoC in a relevant PDX model. This demonstrates the benefit and importance of thoroughly understanding a true translational model and of its ability to help in corroborating clinical data and understanding clinical trial design.

The two key molecular events described in this study, the RET fusion and V804M mutation, have the potential to be used as prognostic biomarkers to predict response in the clinical setting. Of note, LOXO-292 is currently being studied in the global LIBRETTO-001 Phase 1/2 trial in patients with RET-altered tumors and has shown great promise for treating NSCLC[14].

Case Study 3

Optimizing Clinical Trial Design via Hypothesis-Free Biomarker Discovery

Case Study 3 presents data highlighting how hypothesis-free biomarker discovery can be implemented to identify criteria to improve the clinical success rate of a therapeutic agent. The main objective of this case study was to:

- Retrospectively analyze biomarker(s) for an agent already in Phase 1 trials with a specified indication, to evaluate whether trial selection criteria could be improved

This was performed without knowledge of the agent or its targets and mechanism of action.

The Hypothesis-Free Biomarker Discovery Approach

Unlike traditional biomarker analyses, a hypothesis-free approach is applicable without any knowledge of the drug or mechanism of action and is not limited by patient heterogeneity. A variety of tools are used in hypothesis-free biomarker discovery, through a systematic and empirical pipeline, including:

- Mouse clinical trial (MCT) design and implementation
- Sample collection and pathology analysis

Table 1: Clinical Efficacy of LOXO-292 in Patients with RET-Altered Cancers

	RET Fusion-Positive Cancers			RET-Mutated MTC	No Known Activating RET Alteration
	All	NSCLC	Other		
Enrolled	49	38	11	29	4
Eligible for Response Evaluation	47	38	9	29	3
ORR	70% (61%-89%)	68% (51%-83%)	78% (40%-97%)	59% (39%-77%)	0% (0%-71%)
Confirmed ORR	64%	66%	71%	56%	0%
CR	–	–	–	2	–
uCR	–	–	–	–	–
PR	30	25	5	13	–
uPR	3	1	2	2	–
SD	10	8	2	8	2
PD	2	2	–	2	1
Not Evaluable	2	2	–	2	–

Data presented at ASCO 2018. Data cut off: April 1 2018. Follow up as of July 19 2018 (TC + NSCLC).

CR: complete response; MTC: medullary thyroid cancer; NSCLC: non-small cell lung cancer; ORR: objective response rate; PD: progressive disease; PR: partial response; SD: stable disease; uCR: unconfirmed complete response; uPR: unconfirmed partial response

- NGS and bioinformatics analysis
- Genomic profiling
- Integrated analysis using proprietary and public big datasets
- Systematic analysis of genes and pathways

The overall goals of these analyses are to:

- Understand true mutations from variants
- Associate/determine the functional effects of genetic aberrations
- Understand deleterious effects at the DNA, RNA, and protein levels
- Support the identification of the most appropriate indications and most suitable target patient populations

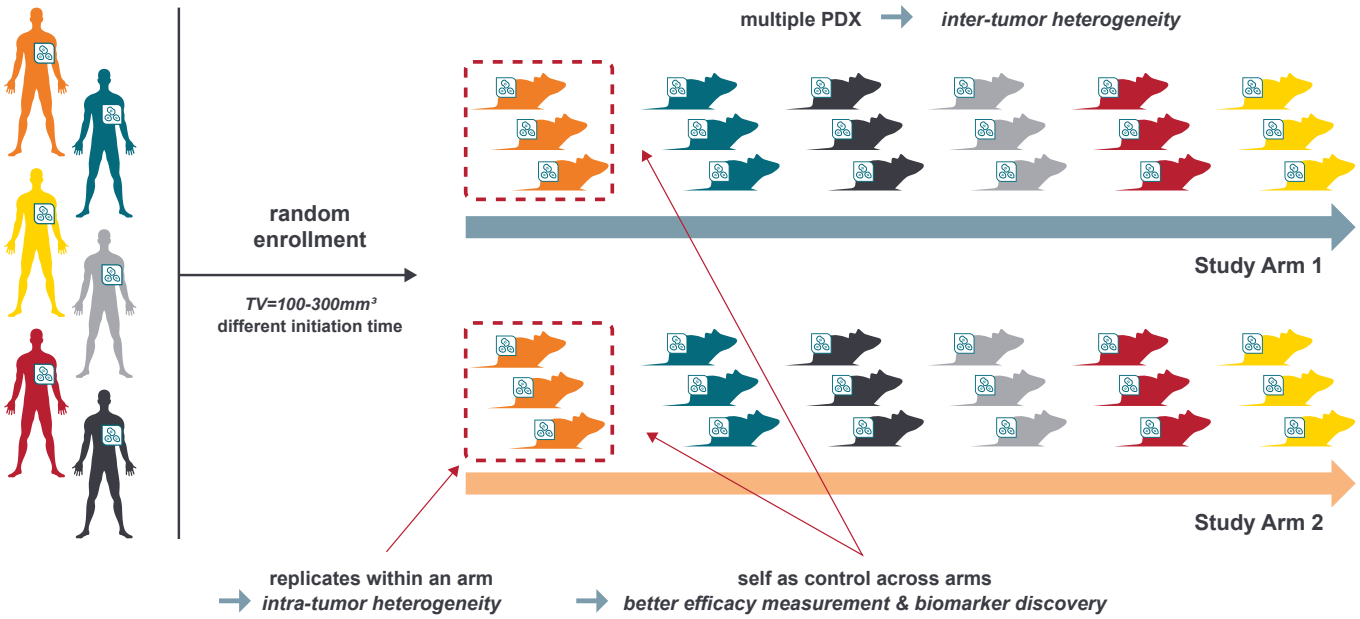
The Importance of Mouse Clinical Trials in Hypothesis-Free Biomarker Discovery

A key aspect of this study was to utilize an MCT. MCTs are population studies that serve as surrogate clinical trials but in a preclinical setting. They are designed to replicate a clinical trial, with the added advantage of including multiple replicates of the same patient in each arm, as opposed to $n = 1$ in the clinical situation (exemplified in Figure 8)[15].

The most relevant models are chosen based on target and biomarker data, or models can be used to screen and generate data for hypothesis-free biomarker discovery, such as in this study. An existing statistical framework that maximizes MCTs for efficacy evaluation and biomarker discovery was also applied in this study.

Six biomarker panels were identified from an MCT that included 77 PDX models treated with the compound already in Phase 1 trials (Figure 9). The first four biomarker panels (BCS Classes 1–4) were based on gene mutations identified from genomic sequencing.

Figure 8: Example 3:3 Mouse Clinical Trial Design



Specifically, the first biomarker (BCS Class 1) consisted of a panel of 20 hypermutated genes, while the second biomarker (BCS Class 2) was based on the inclusion criteria that were used to enroll subjects in the sponsor's Phase 1 trial. The fourth biomarker (BCS Class 4) represented gene mutations, and was subsequently added by the sponsor to their patient stratification (enrollment) criteria following this integrated analysis of MCT data. BCS Class 5 and 6 were based on gene expression data, and although no knowledge about the drug was shared at the time of the study, the fifth biomarker turned out to well characterize the mechanism of the drug.

Case Study 3 Conclusions

Case Study 3 showed that by combining an MCT with *in silico* analysis, original biomarker selection criteria could be confirmed for the test agent along with the newly identified biomarkers, which were better able to define responder and non-responder patient subsets. This improved the accuracy of patient stratification, and clinical trial enrollment criteria were updated, leading to expanding the drug indication and opening the trial to a larger group of relevant patients.

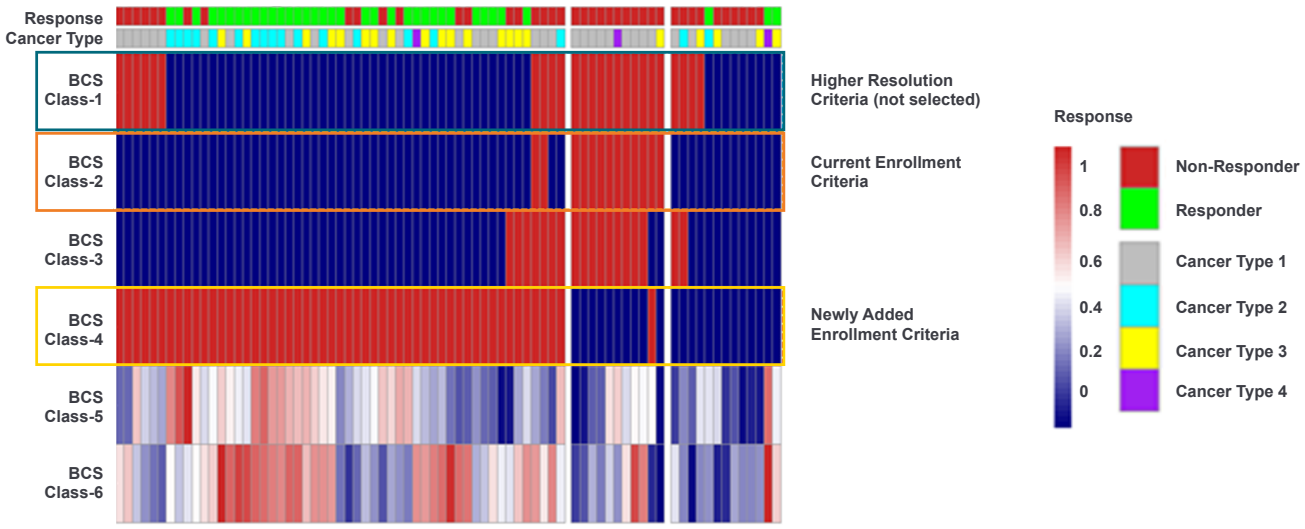
In addition to the advantages discussed above for hypothesis-free biomarker design and applications, other benefits of MCT/*in silico* analyses include the potential to rescue and repurpose previously "unsuccessful" investigational agents, and to increase success rates and trial accuracy through in depth, big data analysis.

Summary

Early identification of biomarkers during preclinical drug development provides a wealth of benefits - including facilitating biomarker selection and CDx clinical testing - which ultimately offers multiple opportunities to de-risk clinical trials.

By using a systems biology approach, leveraging and extensively characterizing a variety of experimental systems (cell lines, ex vivo models, preclinical animal models) with robust analytical techniques, an optimal platform for biomarker discovery can be established that helps accelerate drug development programs while enhancing the chances for success.

Figure 9: Biomarkers Identified from an MCT Evaluating a Phase 1 Drug Candidate



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